A Temporal Dissociation of Energy Liberation and High Energy Phosphate Splitting during Shortening in Frog Skeletal Muscles

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ABSTRACT Measurements of the time course of high energy phosphate splitting and energy liberation were performed on rapidly shortening *Rana pipiens* skeletal muscles. In muscles contracting 30 times against small loads ($<0.02\,P_o$), the ratio of explained heat + work (H + W) (calculated from the measured high energy phosphate splitting) to observed H + W (from myothermal and mechanical measurements) was 0.68 ± 0.08 and is in agreement with results obtained in isometric tetani of *R. pipiens* skeletal muscle. In lightly afterloaded muscles which were tetanized for 0.6 s and whose metabolism was arrested at 3.0 s after the beginning of stimulation, a similar ratio of explained H + W to observed H + W was obtained. However, in identical contractions in which metabolism was arrested at 0.5–0.75 s after the beginning of stimulation, the ratio of explained H + W to observed H + W declined significantly to values ranging from 0.15 to 0.40. These results suggest that rapid shortening at the beginning of contraction induces a delay between energy production and measurable high energy phosphate splitting. This interpretation was tested and confirmed in experiments in which one muscle of a pair contracted isometrically while the other contracted against a small afterload. The afterload and stimulus pattern were arranged so that at the time metabolism was arrested, 0.5 s after the beginning of stimulation, the total energy production by both muscles was the same. Chemical analysis revealed that the isotonically contracting muscle split only 25% as much high energy phosphate as did the isometrically contracting muscle.

Identification of the chemical sources of the energy liberated during contraction is important in understanding the molecular mechanism of muscle contraction. In frog muscle contracting at 0°C, the primary energy-yielding reaction is the splitting of phosphocreatine (PC), which occurs subsequent to ATP hydrolysis (Carlson et al., 1963, 1967; Marechal and Mommaerts, 1963; Wilkie, 1968). Studies of high energy phosphate splitting and energy, heat + work (H + W), liberation in repeated contraction-relaxation cycles indicate that energy is pro-

duced in proportion to PC hydrolysis and that the ratio of H + W to ΔPC is approximately −11 kcal/mol (Carlson et al., 1963, 1967; Wilkie, 1968). This result is independent of the type of contraction-relaxation cycle. Isometric and isotonic twitches and tetani lead to the same conclusion. Since the molar enthalpy change, ΔH, for PC splitting is −8.1 kcal/mol (Woledge, 1972), the above experiments suggest that only a fraction, approximately 0.74, of the observed H + W during contraction can be explained by PC splitting. This fraction is called the explained enthalpy fraction.

To further elucidate the relation of metabolism to energy production it is necessary to extend the above experiments to the time course of single isometric and isotonic contractions. As a muscle presumably returns to its precontraction state after a contraction-relaxation cycle, the above results do not imply that during a single cycle the same relation of energy liberation to metabolism will be observed. Homsher et al. (1975) have found that *during* a single isometric tetanus ranging from 0.6 to 5.0 s, PC is split in proportion to the energy liberation with an explained enthalpy fraction of 0.80 ± 0.09. This result suggests that an unknown reaction(s) occurs in proportion to PC splitting. Similar experiments on *Rana temporaria* (Gilbert et al. [1971] and Curtin and Woledge [1974, 1975]) also indicate the presence of an exothermic reaction(s) occurring during tetanic contractions, in addition to PC splitting, although the value of the explained enthalpy fraction is less than that in *Rana pipiens*. During rapid muscle shortening the rate of energy liberation is increased above that in an isometric tetanus. If the shortening muscle liberates energy at a faster rate because of a more rapid turnover of cross bridges, then, assuming the constancy of noncross-bridge energy liberation, the time course of high energy phosphate splitting during and after shortening should be proportional to the energy liberation and the explained enthalpy fraction should be 0.74. Results of Chaplain and Frommelt (1972) indicate that after the first 0.6 s of an isotonic tetanus energy is liberated in proportion to PC splitting with an explained enthalpy fraction of 0.74. In contrast, data of Kushmerick and Davies (1969) and Curtin et al. (1974) suggest that during rapid muscle shortening energy is not liberated in proportion to PC hydrolysis indicating the presence of a second type of unknown reaction unmasked during shortening. To resolve this uncertainty experiments were designed to examine high energy phosphate splitting and energy liberation over a series of contraction-relaxation cycles including shortening and during a single cycle in which shortening occurred. A preliminary account of this work has appeared (Rall et al., 1975).

METHODS AND MATERIALS

General

Specimens of *R. pipiens* of both sexes were obtained from the Steinhilber Co. (Oshkosh, Wis.) and kept in moist tanks at 6°C. The tanks were flushed several times each day with cold water. Animals were kept in this environment for 2 wk before being used in the experiment. On the day before an experiment, animals were killed by decapitation and semitendinosus or sartorius muscles were dissected. The ventral heal of each semitendinosus was carefully cut away from the tendon shared with the dorsal head. The dorsal

heads, attached to the pelvic bone, were then dissected free of the body. The muscles were aerated overnight with 95% O_2, 5% CO_2, at 4°C in a Ringer solution of 95.0 mM NaCl, 20.0 mM $NaHCO_3$, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, at a pH of 7.0. On the day of chemical experiments the pelvic bones of the sartorius muscle pairs were split under a dissecting microscope. At this point the muscles were also examined under the dissecting microscope and any muscle pairs exhibiting damaged fibers were rejected. The muscles were returned to the 4°C Ringer solution and allowed to recover for 3–4 h.

Mechanical Measurements

The isometric force transducers, isotonic transducers, and speaker coil ergometer used in these experiments have previously been described (Homsher and Rall, 1973).

Myothermal Measurements

Myothermal measurements were made on the same batch of frogs from which the chemical data were obtained and within a week of or during the course of the chemical experiments. Heat production by contracting muscles was determined using integrating thermopiles and fast Hill-type thermopiles. In experiments measuring the energy liberation in 30 repeated contraction-relaxation cycles, integrating thermopiles W1 and W2 were used. The thermopiles' construction, operating characteristics, absolute calibration, and use have been described (Homsher and Rall, 1973). In experiments measuring the time course of energy liberation in a single contraction-relaxation cycle, Hill-type thermopiles E4 and E5 were used. The dimensions, sensitivity, and equivalent half-thickness of E4 have been published (Homsher and Rall, 1973). Thermopile E5, of the same design as E4, had the following characteristics: number of active junctions, 312; thermopile resistance, 3,480 Ω; length of protective region, 10.8 mm; length of active region, 16.2 mm; sensitivity, 9.82 mV/°C; equivalent half-thickness, 26.5 μm.

Amplification and display of the thermopile output, correction of temperature recordings for heat loss, calculation of absolute energy liberation, and correction for conduction of heat from the muscle to the thermopile were as previously described (Homsher et al., 1975).

After making the heat measurements some or all of the muscles in a given series were exposed to a Ringer solution containing 10 mM procaine to block electrical activity, thus allowing measurement of the stimulus heat. The correction, averaging 2–8% of the observed heat, was then applied to the heat records. All muscles were then frozen in a liquid nitrogen-isopentane slush and analyzed for the free and total creatine content. When the time course of energy liberation was measured, the external work performed (determined from the load and displacement record of the isotonic transducer), the series elastic work (determined from the force development and compliance of the muscle-recording system), and the calculated thermoelastic heat (a thermoelastic heat coefficient of 0.01 was assumed [Gilbert et al., 1971]) were added to the heat record to obtain the total energy (in millicalories). The total energy output was then divided by the muscles' total creatine content (in micromoles).

Chemical Measurements

Two types of experiments were performed: *(a)* those in which the splitting of PC was measured in muscles which had shortened 30 times and *(b)* those in which the splitting of PC and ATP was measured in muscles frozen *during* or shortly after a single contraction. In the former experiments, pairs of semitendinosus muscles were exposed to a Ringer solution (0°C) containing 1 mM 3-iodoacetic acid (IAA) (gassed with 95% N_2, 5% CO_2) for 45 min (Mommaerts and Wallner 1967). The muscles were then mounted in the

immersion apparatus (Mommaerts and Schilling, 1964) which permits mechanical recording while muscles are held at 0°C, gassed with 98% N_2–2%CO_2, stimulated, and frozen by rapid immersion in liquid N_2-isopentane at −160°C. The muscles were removed from the immersion device and stored under liquid N_2 until chemical analyses were performed. In the latter experiments paired, oxygenated, unpoisoned sartorius muscles were mounted in a hammer device which permits complete freezing of a muscle within 90 ms (Homsher et al., 1975). The muscles were immersed in a Ringer solution thermostatically maintained at 0°C and allowed to incubate for 5 min, after which time the fluid was drained, by gravity, away from the muscles and precooled gas (95% O_2–5% CO_2) allowed to enter the chamber. Within 15 s muscle stimulation began and within 3 s thereafter the muscles were frozen. The flattened muscles were removed from the hammer device under liquid N_2 and placed in precooled stainless steel cartridges of the type described by Seraydarian et al., (1961).

Frozen muscles were extracted and analyzed for free creatine (C_F), total creatine (C_T), inorganic phosphate (P_i), ATP, and ADP as previously reported (Homsher et al., 1972; Mommaerts and Wallner, 1967). All chemical values were referenced to the muscle total creatine content. To estimate the extent of the reactions occurring in the muscle it was assumed that before stimulation the ratios of C_F/C_T, P_i/C_T, ATP/C_T, and ADP/C_T in paired muscles were identical. In muscles treated with IAA the amount of PC split was estimated from the difference between C_F/C_T ratio in the control and experimental muscles. In pairs of oxygenated, unpoisoned sartorius muscles, PC splitting was determined assuming that the increase in P_i and C_F are both indicators of the extent of PC breakdown and the following formula was employed:

$$\frac{\Delta PC}{C_T} = \frac{(\Delta C_F/C_T + \Delta P_i/C_T)}{2}.$$

Evidence for this assumption includes the following observations: *(a)* ATP hydrolysis was not significantly different from zero during (at 0.5 and 0.75 s) and at the completion (at 3.0 s) of the 0.6-s isotonic tetani investigated in this study (see Table II). Also in isometric tetani of 5 s or less, oxygenated, unpoisoned muscles do not hydrolyze significant amounts of ATP (Gilbert et al., 1971; Homsher et al., 1975; Curtin and Woledge, 1974). *(b)* For pooled data the difference between the measured extent of the reactions as estimated by ΔC_F and ΔP_i ($\Delta P/C_T - (\Delta C_F/C_T)$) is not significantly different from zero during rapid muscle shortening (at 0.5 and 0.75 s) and after relaxation (at 3.0 s) (see Table II). Also there is no significant difference in $(\Delta P_i/C_T) - (\Delta C_F/C_T)$ in experiments where an isotonic tetanus was compared to an isometric tetanus at 0.5 s (see Table IV). Thus both $\Delta C_F/C_T$ and $\Delta P_i/C_T$ appear to be measures of the same phenomenon; i.e., the splitting of phosphocreatine. Estimating $\Delta PC/C_T$ by the average of $\Delta P_i/C_T$ and $\Delta C_F/C_T$ has the advantage that the SE's are reduced and thus comparison among the data are sharper. But it should be noted that the conclusions are not changed when $\Delta C_F/C_T$ alone is used as the estimator of $\Delta PC/C_T$ but their strength is weakened by the larger errors. (See Tables II and IV.)

In all, 122 pairs of muscles were frozen and analyzed. Of this number eight pairs were rejected on the criterion that the average change in C_F, P_i, or ATP was more than 2 standard deviations away from the mean of their group, computed with the suspected deviant value included. If one of the measured parameters was rejected, all data from that muscle pair was rejected since there was no way of determining whether the deviant value was a result of measurement error or mismatching of the control and experimental muscle. The significance of the difference between sample means was tested using the Student's *t* test. A 5% level of significance was assumed throughout.

Calculation of Explained H + W and Explained Enthalpy Fraction

To compute the explained H + W, the extent (ξ_{PC}) of PC splitting, $\Delta PC/C_T$ was multiplied by the molar enthalpy change for PC splitting, ΔH_{PC}, (taken as −8.1 kcal/mol [Woledge, 1972]) yielding $\xi_{PC}\Delta H_{PC}$. The contribution due to ATP hydrolysis was calculated by multiplying the extent (ξ_{ATP}) of ATP hydrolysis, $\Delta ATP/C_T$, by its molar enthalpy change ΔH_{ATP} (taken as −11.46 kcal/mol [Woledge, 1972]), yielding $\xi_{ATP}\Delta H_{ATP}$. The sum, $(\xi_{PC}\Delta H_{PC} + \xi_{ATP}\Delta H_{ATP})$, is the explained H + W. The explained enthalpy fraction is the ratio of the explained H + W to the observed H + W.

RESULTS

Energy Liberation and PC Splitting in Repeated Lightly Loaded Twitches

IAA-treated semitendinosus muscle pairs were mounted on the immersion apparatus. After 15 min in the muscle chamber the muscles $'\ell_o$ and the maximal twitch tension were determined by giving both muscles several twitch stimuli. During the next 30–60 s, the experimental muscle was attached to an isotonic lever having zero afterload while the control muscle remained unstimulated and attached to the isometric transducer. The experimental muscle was stimulated 30 times at 3.2-s intervals and manually reextended to ℓ_o at 2–2.5 s after each stimulus. Five seconds after the 30th stimulus both muscles were frozen. Myothermal experiments were performed similarly except that both muscles of the pair were stimulated. The amount of work done against the equivalent mass of the lever was less than 2% of the total energy production. Table I, line E, shows that the explained H + W is significantly ($P < 0.01$) less than the observed H + W (line C). However, the explained enthalpy fraction (line F) is 0.68 ± 0.08 and is not different from the value seen in isometric contractions, i.e., 0.74. Thus the energy liberation by freely shortening muscles from a series of repeated contraction-relaxation cycles has the same metabolic cost as other types of contractions.

Time Course of Energy Liberation and High Energy Phosphate Splitting during Shortening

The above experiments say nothing about the relation of the time course of PC splitting to energy production during shortening. Our earlier work (Homsher et

TABLE I

ENERGY PRODUCTION AND PC SPLITTING FOR 30 LIGHTLY LOADED ISOTONIC TWITCHES

	Chemical measurements (mean ± SEM)		Myothermal measurements (mean ± SEM)
(A) Number of experiments	10		6
(B) Total distance shortened *(cm)*	12.88±0.71		11.65±0.38
(C) Observed total energy produced *(mcal/g)*	–		45.0±2.34
(D) Total creatine phosphate split *(μmol/g)*	3.75±0.34		–
(E) Explained H + W *(D × −8.1 kcal/mol, mcal/g)*		30.38±2.75	
(F) Explained enthalpy fraction *(E/C)*		0.68±0.08	

al., 1975) indicated that during an isometric tetanus, as early as 0.6 s after the beginning of stimulation, the time course of the energy liberation was proportional to PC splitting with a ratio of observed H + W/ΔPC of −11 kcal/mol. To determine whether similar behavior occurred during shortening the following experiments were performed. Pairs of unpoisoned sartorius muscles were mounted at ℓ_o on the hammer apparatus. Experimental muscles were attached to an isotonic lever which was afterloaded with 1 g, while control muscles were attached to an isometric transducer. The experimental muscles were then tetanically stimulated for 0.35 or 0.6 s and frozen at 0.5, 0.75, or 3.0 s after the beginning of stimulation. Comparable myothermal experiments were performed on pairs of muscles which were afterloaded with 2 g, one of which is shown in Fig. 1. The upper record represents muscle shortening and the lower heat production in a 0.6-s tetanus. Shortly after the beginning of stimulation the muscle shortens and heat is produced at a rapid rate. By 0.5 s shortening is 80%

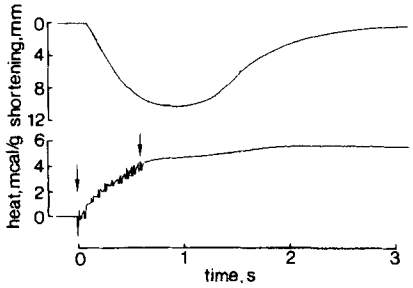

FIGURE 1. The time course of displacement and heat production by a pair of sartorius muscles afterloaded with 2 g at the muscles ℓ_o. The upper record is of displacement and the lower record heat production. The downward pointing arrows indicate the beginning and end of tetanic stimulation (10 Hz) for 600 ms. See text for further details.

of its maximal value and at 0.75 s it is 97% complete. From 0.7 to 1.2 s heat is produced at low rate. During relaxation the rate of heat production increased slightly but this is due to the dissipation of energy, stored in the afterload, produced during shortening. Relaxation is complete by 3 s. If the relationship between energy liberation and PC splitting by the shortening muscle is similar to that in the isometric tetanus, PC splitting should parallel this pattern of energy liberation.

In our first experiments with muscles frozen at 0.5 and 0.75 s (lines A and B, Table II) a significant PC splitting was found. However, at both 0.5 and 0.75 s the amount of PC split is insufficient to account for the observed energy production (lines A and B, Table III). In fact at both 0.5 and 0.75 s the explained enthalpy fraction is significantly less ($P < 0.001$) than the value (0.68) observed in repeated isotonic contraction-relaxation cycles. This result suggests the presence of a second type of energy imbalance. These experiments could be criticized because $\Delta ATP/C_T$ was not measured and perhaps ATP was being hydrolyzed. Whereas the fact that the $\Delta P_i/C_T$ is not significantly different from $\Delta C_F/C_T$ renders this possibility unlikely, these experiments were performed

again. In addition ATP hydrolysis was measured, and the H + W and high energy phosphate splitting at the end of a contraction-relaxation cycle was examined to determine whether the imbalance of energy liberation and PC splitting seen in the shortening phase persisted. The results (lines C–E, Table II)

TABLE II
ENERGY LIBERATION AND CHEMICAL CHANGE DURING RAPID MUSCLE SHORTENING

Experimental conditions*					Δ Content, E-C					
Batch of frogs	Stimulus duration	Freeze	*N*	PC/C_T	$\Delta C_P/C_T$	$\Delta P_i/C_T$	$\Delta PC/C_T$	$\Delta ATP/C_T$	Total energy (H + W)	*N*
	s	*s*			*nmol/μmol*				*mcal/μmol* C_T	
(A) October	0.35	0.50	15	0.809±0.009	5.9±2.8	3.5±5.4‡	−5.6±2.7	–	0.181±0.006	6
(B) January	0.60	0.75	28	0.751±0.004	6.3±2.5	8.0±2.2	−7.2±1.8	–	0.233±0.005	4
(C) September	0.35	0.50	11	0.846±0.010	6.1±2.5	−2.7±3.1	−1.7±2.1	−0.5±2.1	0.135±0.008	9
(D) September	0.60	0.75	13	0.845±0.006	10.6±2.0	4.1±2.5	−7.3±1.8	−0.6±1.1	0.167±0.014	7
(E) September	0.60	3.0	16	0.809±0.004	11.7±3.4	12.7±2.9	−12.2±1.9	−2.1±1.4	0.182±0.015	7

All values given as mean ± SEM.
* All experiments performed in 95% O_2, 5% CO_2 Ringer at 0°C with the muscles stimulated at 15–20 Hz and contracting against a 1-g afterload. Batch parameters: (Oct.) C_T/g = 35.17 ± 0.35 μmol/g (mean ± SEM), (*N* = 12), (Jan.) C_T/g = 32.45 ± 0.59 μmol/g (*N* = 8), (Sept.) C_T/g = 31.86 ± 1.44 μmol/g (*N* = 18).
‡ RESULTS FROM EIGHT PAIRS OF MUSCLES.

TABLE III
CALCULATIONS OF EXPLAINED ENTHALPY FRACTION DURING RAPID SHORTENING

Batch of frogs	Contraction arrested at	Observed H + W	Explained H + W (ΔH_{PC} = −8.1 kcal/mol)	Explained enthalpy fraction
	s	*mcal/μmol* C_T	*mcal/μmol* C_T	
(A) October	0.50	0.181±0.006*	0.045±0.022* ($P \ll 0.001$)‡	0.25±0.12*
(B) January	0.75	0.233±0.005	0.053±0.015 ($P \ll 0.001$)	0.23±0.06
(C) September	0.50	0.135±0.008	0.020±0.030 ($P < 0.005$)	0.15±0.22
(D) September	0.75	0.167±0.014	0.066±0.019 ($P < 0.001$)	0.40±0.12
(E) September	3.00	0.182±0.015	0.123±0.022 ($P < 0.05$)	0.68±0.15

* Values given are mean ± SEM.
‡ The probability that (explained H + W − observed H + W) = 0.

of these experiments, carried out on a single batch of frogs, show three important features. *(a)* Only a small amount of PC is split at 0.50 and 0.75 s. In fact, at 0.50 s the PC splitting is not significantly different from zero, while that at 0.75 s is. *(b)* In no instance is there a significant amount of ATP hydrolyzed. *(c)* At 3.0 s (line E, Table III) the explained enthalpy fraction is not significantly less than the expected value of 0.74.

The results of these experiments indicate that during muscle shortening the

amount of PC and ATP split can account for only 15–40% of the observed energy liberation. However, after relaxation about 70% of the energy liberation can be accounted for by the high energy phosphate splitting.

Comparison of the High Energy Phosphate Splitting in Single Isotonic and Isometric Contractions

Results of the previous section suggest that shortening produces a delay between energy liberation and high energy phosphate splitting, since 0.6 s into an isometric tetanus the explained enthalpy fraction is about 0.74 (Homsher et al., 1975). To be certain that shortening per se was responsible for the delay between H + W liberation and PC splitting, experiments directly testing this point were conducted. Muscles were arranged so that one muscle of a pair contracted against an isotonic afterload of 1 g while the other contracted isometrically. Stimulus duration was adjusted with respect to the time of freezing so that both muscles produced the same amount of H + W. If the H + W produced by both muscles was the same, then a delay in PC splitting produced by shortening would result in the isotonically contracting muscle splitting less PC than the isometrically contracting muscle. In pilot myothermal experiments it was found that if muscles were stimulated tetanically for 300 ms, at 500 ms the H + W produced by both muscles was the same, even though the heat produced by the shortening muscle exceeded that of the isometrically contracting muscle. This behavior is shown in Fig. 2 where the shortening muscle liberates heat at a rate greater than that of an isometrically contracting muscle. However, by 300 ms the isotonically contracting muscle is shortening slowly and consequently the rate of heat production declines markedly, and from 460 to 800 ms the rate of heat production is practically zero. The isometrically contracting muscle while producing heat at a lower rate continues to do so, and, in fact, the heat produced by the two types of contraction is identical at 640 ms. To obtain the total energy produced, the external work done by the shortening muscle is added to its heat production, and in this case at 500 ms the external work was 0.39 mcal/g. The total energy produced by the isometric contraction is given by heat production plus the thermoelastic heat absorbed (0.42 mcal/g) and the internal work done in stretching the series elastic element (0.31 mcal/g). The total energy produced at 500 ms by the isotonic contraction was 5.99 mcal/g and 5.79 mcal/g by the isometric. Table IV A contains the results of the myothermal experiments in which the total energy (H + W) produced by isotonic and isometric contractions at 500 ms was measured. There was no significant difference in either the total energy produced nor the work done by either muscle. The biochemical counterpart to the myothermal experiments was conducted in the same fashion. Pairs of muscles were mounted at ℓ_0 on the hammer device, one attached to an isotonic lever with a 1-g afterload, the other to an isometric transducer. The muscles were both tetanized for 300 ms and frozen at 500 ms.

A comparison of the distance shortened and the maximum velocity of shortening in the isotonic contractions indicates that the muscles behaved similarly in the myothermal and biochemical experiments. The results of chemical analyses (Table IV B) reveal no significant difference in the ATP or ADP

content of the isotonic and isometric muscles. However, the isometrically contracting muscle contained significantly more C_F and P_i than the isotonic muscle. From these measurements it is estimated that the isometrically contracting muscle split 10.6 ± 1.5 nmol/μmol C_T more PC than did the isotonically contracting muscle ($P < 0.001$) even though the total energy liberation was the same.

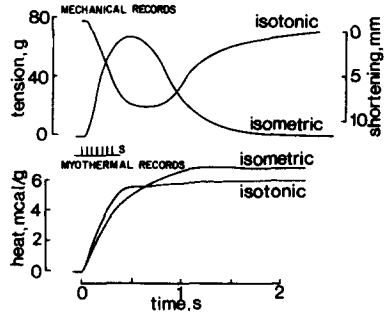

FIGURE 2. A comparison of the mechanical and myothermal behavior of a 300-ms isometric or isotonic tetanus. In this experiment a muscle pair was brought to ℓ_o (30 mm) afterloaded with 2 g, stimulated (at 20 Hz for 300 ms, as shown by the trace labeled *s*), and allowed to shorten. The muscles were then set to record isometrically at ℓ_o, stimulated as before, and allowed to generate tension. See text for further details.

TABLE IV

COMPARISON OF ISOMETRIC VS. ISOTONIC CONTRACTIONS

	Mean±SEM
(A) Myothermal measurements (N = 6)	
Distance shortened (in muscle lengths)	0.33±0.01
Velocity of shortening (muscle lengths/*s*)	0.96±0.03
Total isotonic energy (*mcal/μmol* C_T)	0.155±0.014
Isotonic external work (*mcal/μmol* C_T)	0.0106±0.0004
Total isometric energy (*mcal/μmol* C_T)	0.149±0.012
Isometric internal work (*mcal/μmol* C_T)	0.0093±0.0006
$P_o \ell_o/M$ (*kg/cm²*)	1.87±0.09
(Total isotonic energy − total isometric energy) (*mcal/μmol* C_T)	+0.0062±0.0041
(B) Biochemical measurements (N = 20)	
Distance shortened (in muscle lengths)	0.31±0.01
Velocity of shortening (muscle lengths/*s*)	1.01±0.03
Chemical change (isotonic − isometric, *nmol/μmol*)	
$\Delta C_F/C_T$	−13.2±2.1
$\Delta P_i/C_T$	−7.9±2.2
$\Delta PC/C_T$	+10.6±1.5
$\Delta ATP/C_T$	+1.1±1.0
$\Delta ADP/C_T$	−0.8±0.8
Calculated energy difference (($\Delta PC/C_T$) × −8.1 kcal/mol)	
Isotonic − isometric (*mcal/μmol*)	−0.086±0.012

These results support the idea that shortening induces a delay between heat production and PC splitting. From results in Table III (lines A and C) it was found that the average explained H + W by isotonic contractions at 500 ms was 0.033 ± 0.019 mcal/μmol C_T. Addition of this value to the explained energy difference from Table IV, 0.086 ± 0.012 mcal/μmol C_T, should give the explained H + W by the isometrically contracting muscle, 0.119 ± 0.022 mcal/μmol C_T. The explained enthalpy fraction for the isometrically contracting muscle at 500 ms is thus 0.79 ± 0.16 and is in excellent agreement with our previous data on isometric tetani (Homsher et al., 1975).

DISCUSSION

Three primary experimental observations are described in this paper. *(a)* In a series of contraction-relaxation cycles by rapidly shortening muscles, approximately 70% of the total energy liberation can be attributed to PC splitting. This value, which corresponds to a ratio of observed H + W/ΔPC of −11.9 ± 1.1 kcal/mol, is not significantly different from results in isotonic afterloaded twitches (Carlson et al., 1963; Wilkie, 1968), isometric twitches and tetani (Carlson et al., 1967; Wilkie, 1968; Homsher et al., 1972), and muscles which are stretched during a tetanus (Wilkie, 1968). Taken together these results indicate that at the end of repeated contraction-relaxation cycles, the ratio of observed H + W/ΔPC is independent of the type of contraction.

(b) During rapid shortening, energy is produced without equivalent PC or ATP splitting and during relaxation high energy phosphate is split with little heat production. This result is shown in Table III which indicates that in muscles frozen at 0.50 and 0.75 s only 15–40% of the observed energy production can be accounted for by high energy phosphate splitting. Furthermore comparing line C to E of the explained H + W in Table III, it can be seen that in a time interval (0.5–3.0 s) when only 0.047 ± 0.017 mcal/μmol C_T of energy is liberated, enough high energy phosphate is split to produce 0.103 ± 0.037 mcal/μmol C_T. This result is in agreement with those of Infante and Davies (1962) in which dinitrofluorobenzene (DNFB)-treated sartorius muscles, shortening against an afterload of ~0.1 P_o in a twitch, split 0.22 μmol/g of ATP by the peak of shortening and an additional 0.21 μmol/g during relaxation. Since little active heat production occurs after the peak of shortening in lightly afterloaded muscles (see Homsher and Rall, 1973, Fig. 3) these results suggest a thermoneutral ATP splitting subsequent to shortening. Further, evidence from Kushmerick and Davies (1969) suggests that the splitting of ATP by DNFB-poisoned muscles does not parallel the evolution of energy during shortening. This inference stems from their finding that in isovelocity contractions the rate of ATP splitting was ~0.93 μmol/g · s as compared to a value of 0.6–0.7 μmol/g · s in the early portions of an isometric tetanus. As Hill's (1964) work indicates that the rate of energy liberation by a muscle shortening near V_{max} ought to be four to five times that of the isometric contraction, Kushmerick and Davies' results suggest the presence of an additional source of heat production during shortening.

(c) The rate of high energy phosphate splitting during rapid shortening is less than that during an isometric contraction, even though the shortening muscle

liberates energy at a faster rate. Kushmerick et al. (1969) have found that lightly loaded muscles split 0.13 μmol/g less ATP than isometrically contracting muscles when contraction was stopped at a time when the isotonically contracting muscle was calculated (Hill, 1966) to have consumed 0.11 μmol/g more ATP than the isometric contraction. This observation suggests what the experiments in Table IV prove; namely, that at the time of its production the energy produced by shortening muscles has no measurable metabolic equivalent.

These results in conjunction with earlier experiments indicate that the explained enthalpy fraction *during* contraction varies depending upon the type of contraction. In single isometric tetani Homsher et al. (1975) found that energy was liberated in proportion to PC splitting with an explained enthalpy fraction of ~0.79. While similar experiments on *R. temporaria* yielded an explained enthalpy fraction of ~0.50 (Gilbert et al., 1971; Curtin and Woledge, 1974, 1975), the data are not inconsistent with a proportionality between the time course of energy liberation and PC splitting. It was therefore possible to consider the time course of energy liberation as being directly proportional to the time course of ATP or PC splitting. However, the results contained in this paper, and those from Curtin et al. (1974), indicating that energy liberation and high energy phosphate splitting do not parallel one another and that the relationship between the two depends on the mechanical behavior of the muscle, render this hypothesis untenable. The important conclusion derived from these results is that the time course of energy liberation by shortening muscles cannot be viewed as an acceleration of the same reaction sequence occurring in the isometric case. The presence of shortening produces a fundamental change in the relationship existing between isometric energy liberation and ATP or PC splitting.

The summarized results suggest the presence of two kinds of energy imbalances in contracting muscle. The first type involves an unknown reaction which is linked to PC splitting and which accounts for the difference in the observed H + W/ΔPC ratio between −8.1 and −11 kcal/mol. The second type of energy imbalance is one appearing during rapid shortening but vanishing by the end of relaxation. This imbalance is characterized by energy liberation during shortening with little high energy phosphate splitting followed by high energy phosphate splitting during relaxation with little energy liberation. From the data presented above it is impossible to specify whether this imbalance with shortening involves only the myofibrils, only the calcium releasing and pumping activity of the sarcoplasmic reticulum (activation processes), or both. However, during an isometric tetanus the observed H + W is produced in proportion to PC splitting suggesting that the sarcoplasmic reticulum also liberates energy in proportion to PC splitting. For the sarcoplasmic reticulum to be the source of the large energy imbalance observed in rapidly shortening muscles, it would be necessary to assume that shortening: *(a)* increases the rate of and doubles the net amount of energy liberation by activation processes, and *(b)* uncouples the energy production of activation from PC splitting. The former necessity implies a doubling of the amount of Ca^{++} release, against which Edman (1975) has produced evidence.

Assuming that activation processes are not altered by shortening then the

shortening-induced phase shift between energy liberation and PC splitting may occur in the myofibrils and thus presumably at the cross bridges. There are several possible explanations for this type of behavior.

(a) During shortening an unknown reaction occurs whose enthalpy change (ΔH_x) is negative (because heat is produced) and whose free energy change (ΔG_x) is also negative (since the reaction occurs spontaneously). Woledge (1971) has considered the implications of such a reaction and has pointed out that if the free energy change for the reversal of the unknown reaction ($\Delta G_{PC} - \Delta G_x$) is small, as would be the case for the greatest economy of the muscle, then the absence of heat production during PC splitting (i.e., during the reversal of the unknown reaction) together with an observed H + W/ΔPC ratio of −11 kcal/mol over a complete contraction relaxation cycle would indicate that ΔH_{PC} is approximately equal to ΔH_x. Since it appears that these conditions exist in a shortening muscle, one could tentatively conclude that the enthalpy change for the unknown reaction is about −8 to −11 kcal/mol. The presence of an unknown reaction occurring during shortening and reversed during relaxation is further suggested by the results of Dickenson and Woledge (1973) who found that apparently some of the heat produced during shortening is reabsorbed during relaxation.

(b) The main energy-releasing portion of a cross-bridge cycle occurs in a step which is separate from that in which ATP is split and shortening further separates these steps in time. This idea is similar to that suggested by Tawada et al. (1974). From structural and biophysical evidence, cross bridges are presumed to execute a cycle which includes: *(a)* attachment to actin, *(b)* power stroke in which work is done, *(c)* detachment, and *(d)* return of the free cross bridge to a position where attachment can occur again. Lymn and Taylor (1971) have developed a kinetic model for ATP splitting by the actomyosin system which fits in a natural way with the above cycle. Their work suggests that ATP is cleaved on the free myosin or cross bridge to produce a long-lived intermediate complex ($M \cdot ADP \cdot P_i$) which can subsequently interact with an actin site. This intermediate complex would be detectable by our methods of analysis as ADP and P_i. Calorimetric studies by Yamada et al. (1973) have shown that energy stored in the terminal bond of ATP is *not* released in the ATP cleavage step ($M \cdot ATP \rightleftharpoons M \cdot ADP \cdot P_i$) but is released in the product dissociation step *(s)* ($M \cdot ADP \cdot P_i \rightleftharpoons M + ADP + P_i$). Thus in resting muscle the cross bridges may exist predominantly in the form of a high energy $M \cdot ADP \cdot P_i$ complex which stores the energy of ATP hydrolysis. Therefore evidence exists that the energy releasing step in the cross-bridge cycle is separate from the ATP cleavage step. During contraction these $M \cdot ADP \cdot P_i$ complexes or cross bridges release their stored energy after binding to actin. Thus energy would be produced without apparent ATP or PC splitting. According to the Lymn and Taylor (1971) scheme before ATP can recharge the cross bridge the hydrolysis products of the previous cycle must dissociate from the actomyosin complex and this seems to be the rate-limiting step. Thus a lag or temporal dissociation of energy liberation and ATP splitting develops. The fact that no lag is measured during an isometric tetanus suggests that its magnitude is less than the freezing time of our measurements, 50–80 ms.

If rapid shortening were to decrease the rate of product dissociation from the cross bridge, the lag would be accentuated and we would observe: *(a)* the release of stored energy without PC splitting early in contraction, *(b)* PC splitting without energy production during relaxation when cross bridges are being recharged and energy stored, and *(c)* a balance over the whole contraction-relaxation cycle. Thus rapid shortening may alter the cross-bridge reaction sequence for ATP hydrolysis resulting in an amplified temporal dissociation between energy liberation and PC splitting. The implication of this hypothesis is that cross-bridge dissociation from actin may occur before product dissociation (e.g., caused by force exerted by other cross bridges attached to the same thin filament). If such a mechanism were occurring in the muscle the cross bridges must be capable of storing an amount of energy between 0.06–0.12 mcal/μmol C_T to account for the data in Table III. Assuming there are 0.14 μmol of myosin/g of muscle (Ebashi et al., 1969), there is about 0.28 μmol of S-1 subunits/g of muscle, each of which can exist as $M \cdot ADP \cdot P_i$. Consequently, the maximum amount of energy available from these cross bridges is (0.28 μmol/g $\times$ [11.5 kcal/mol] $\div$ [30 μmol C_T/g]) = 0.11 mcal/μmol C_T, in agreement with the necessary amount. While a variety of other models could be devised to account for the observed behavior, the important point remains that rapid shortening causes energy to be liberated before a measurable equivalent of high energy phosphate splitting.

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