Physical and Biochemical Energy Balance during an Isometric Tetanus and Steady State Recovery in Frog Sartorius at 0°C

RICHARD J. PAUL

From the Department of Physiology, University College, London, England

ABSTRACT Frog sartorius muscle stimulated isometrically for 3 s every 256 s to attain a steady state in which initial heat (Q_I), recovery heat (Q_R), rate of O_2 consumption (J_{O_2}), and isometric force (P_o) generated are constant for each cycle. For a 3-s tetanus given every 256 s, J_{O_2} was 0.106 μmol/(min · g blotted weight), ~71% of the maximum rate observed, whereas lactate production was negligible under these conditions. Q_I, $Q_T(= Q_I + Q_R)$, and Q_T/Q_I were 88.2, 181.5, 2.06 mJ/g blotted weight, respectively. The high-energy phosphate breakdown (Δ~P) breakdown during the first 3-s tetanus was not different from that during a contraction in the steady state and averaged 1.1 μmol/g blotted weight. Less than half of the initial heat could be accounted for in terms of the extent of the known chemical reactions occurring during contraction. From the stoichiometry of the theoretical biochemical pathways, the amount of ATP synthesized in the steady state exceeds Δ~P during contraction by more than twofold, corresponding to an apparent ADP:O ratio of 1.5. If it is assumed that carbohydrate oxidation is the only net chemical reaction in the steady state, the total heat production can be explained on the basis of the measured J_{O_2}. Under this assumption, heat production during recovery was less than that expected on the basis of the oxygen consumption and Δ~P during contraction. These observations support the hypothesis that the unexplained enthalpy production and low apparent ADP:O ratio are causally related, i.e., that the reaction(s) producing the unexplained heat during contraction is reversed during the recovery period.

INTRODUCTION

Within the past decade, two lines of experimental investigation have raised serious questions concerning our understanding of muscle energetics and metabolism. (a) It is now widely accepted (for reviews see Curtin and Woledge, 1978; Homsher and Kean, 1978) that the enthalpy (heat and work) change

produced by an isometrically contracting muscle exceeds that which can be expected on the basis of the known initial chemical reactions occurring during contraction and the molar enthalpy for these reactions. These observations suggest that reactions other than the known chemical breakdown (ATP and PCr) occur during contraction. (b) The extent of recovery metabolism after contraction was found to exceed that calculated from the initial $\Delta\sim P$ using standard biochemical stoichiometry (for review cf. Kushmerick, 1977). This biochemical imbalance was found both under aerobic (Kushmerick and Paul, 1976b) and anaerobic (Defuria and Kushmerick, 1977) conditions. Both observations may be related to the same underlying phenomena. The unknown reaction(s) liberating enthalpy during contraction could be reversed during the recovery period at the expense of additional $\Delta\sim P$, thus accounting for the biochemical imbalance. On the other hand, DeFuria and Kushmerick (1977, 1978) have suggested that these observations may not be causally related. They propose that the greater extent of recovery metabolism may not be related to reversal of some unknown reaction occurring during contraction but rather to the inability of muscle mitochondria in situ to achieve the theoretical stoichiometry during recovery. In particular, they have demonstrated the possibility of substrate cycling, as for example the cycle involving phosphorylation and dephosporylation of fructose 6-phosphate, which could considerably reduce the net phosphorylation efficiency. In this work, an energy balance study during recovery was used to differentiate between these mechanisms.

An energy balance study during recovery requires measurement of recovery metabolism, recovery heat production, and the net change in the high-energy phosphate level. At 0°C the first two types of measurement following single isometric tetani are technically difficult because of the small extent of reactions and the long time for recovery. Particularly long-term baseline stability of thermopiles required for recovery heat measurement can be a formidable problem (Wilkie, 1968). An alternative method for measurement of recovery heat production involves a protocol of repeated stimulation. This approach was first used by Bugnard (1934), who showed that a periodically stimulated muscle achieves a cyclic steady state in which the heat produced during each cycle is constant. In this steady state, both the initial heat and that produced between tetani for each cycle are constant. In effect, the recovery period is shortened to the duration between stimuli, which considerably reduces the effects of baseline change on the heat measured. Using this technique, Bugnard (1934) measured recovery heat production and his results on initial-to-recovery heat ratios agree well with more modern measurements made on recovery after single isometric tetani (Godfraind-DeBecker, 1972).

The choice of a steady state protocol also allowed the testing of the possibility that the abrupt transitions from rest to contraction and back might be a factor underlying the observed biochemical imbalance. In all previous studies, comparisons of recovery metabolism to $\Delta\sim P$ breakdown were made after single isometric tetani. Furthermore, one would anticipate (cf. Newsholme and Start, 1973) that the effects of substrate cycling would be minimized

under conditions of high metabolic flux concomitant with steady state stimulation.

In this study, biochemical and physical energy balances were measured during contraction and recovery in a muscle periodically stimulated to achieve a cyclic steady state. The following two alternative hypotheses were tested: (a) the discrepancy between recovery metabolism and initial high-energy phosphate breakdown observed after single contractions is minimized in the steady state; (b) the unexplained enthalpy production observed during contraction is reversed during recovery.

METHODS

Muscle Preparation

All experiments for physical and biochemical energy balance comparisons were performed on sartorius muscles of frogs (*Rana temporaria*), which were from a single batch obtained in the spring of 1977. Preliminary measurements of isometric force and oxygen consumption to determine steady state parameters were made in muscles from frogs of different batches. Muscles were dissected on the day before the measurements and were left to recover in Ringer solution at 4°C. The Ringer solution contained (millimoles per liter) 115.5 NaCl; 2 KCl; 1.8 CaCl$_2$; 2 Na phosphate (pH 7.0 at 0°C). Rest lengths were measured from tendon to tendon in the standard position, i.e., with the legs perpendicular to the midline of the body; all experiments were performed under isometric conditions at this length. The muscles were equilibrated for at least 40 min at 0°C before stimulation, which was achieved with condensor shocks (18 V, 0.5 μF) of alternating polarity at 10 Hz.

Measurements of Metabolism

OXYGEN CONSUMPTION   O$_2$ was measured polarographically; the details of the apparatus and measurement procedure have been described previously (Kushmerick and Paul, 1976a; Paul, 1976).

LACTATE ELIMINATION   Samples of the bathing medium were taken at intervals and were assayed for lactate as described below. The muscles were then subjected to extraction and analysis as described below.

Heat Production Measurements

Heat production was measured using a thermoelectric device (Th 1803) constructed of sintered bismuth telluride alloys (Thermagotron; MCP Electronics Ltd., Wembley, England) in a configuration designed by Dr. D. R. Wilkie. 54 elements were set in a field $\sim$5 × 29 mm. These devices and their calibration by the Peltier method have been described previously (Kretzschmar and Wilkie, 1975). In addition to the Peltier method of calibration, resistive heating methods and a novel, null detection method based on balancing Joule heating against Peltier cooling were used and found to agree within 3%. The Thermagotron used produced 7 mV K^{-1} (130 μV K^{-1} element^{-1}) and was characterized by a heat loss of 0.042 J s^{-1} K^{-1} (0.785 mJ s^{-1} K^{-1} element^{-1}) and a heat capacity of 0.65 J K^{-1}.

Because of the relatively large heat capacity of these devices, thermopile lag could be appreciable. A delay with a time constant of $\sim$200 ms was required before the measured rate agreed with a steady rate of heat applied to an agar gel-simulated muscle. No correction for this lag in the Thermagotron response was made because

the exact time course of heat production at this level of resolution was not required. Before stimulating each muscle, its heat capacity was determined by the Peltier method; when the muscle was longer than 29 mm, the total capacity was calculated from the observations on the assumption that its heat capacity was proportional to its length. The heat records were corrected for heat loss algebraically as described by Wilkie (1968). The correction for stimulus heat was calculated from the number of stimuli and the parameters of the capacitor discharge stimulator used. This correction was ~3% of the total heat measured. Because the work done against the series elastic elements is dissipated as heat when the muscle relaxes and is included in the total heat measured, this experimental design did not necessitate a separate measurement of this work.

Measurements of the Chemical Reactions Occurring during Contraction

MUSCLE EXTRACTS   Chemical measurements were made on extracts of muscles rapidly frozen on a hammer apparatus (Kretzschmar and Wilkie, 1969). Right and left muscles were varied systematically in a protocol similar to that reported by Curtin and Woledge (1977) to avoid biasing the results. The frozen, flattened muscles were extracted for 4 d in 3 ml of a solution of EDTA (1.25 mM, pH 7.6) in methanol (50% vol/vol) at −30°C. Details of this extraction procedure have been reported (Kushmerick and Paul, 1976b; Curtin and Woledge, 1977). The muscle residue was then removed, rinsed with cold methanol (rinses were added to the extract), and freeze-dried. Muscles used for measurements of heat production and metabolism after determination of the blot weight were also frozen and similarly extracted for later analysis of total creatine. After further drying over P_2O_5, the muscle residue was weighed. The ratio of the blotted weight of a muscle to this extracted dry weight, used for comparisons on a blotted-weight basis, was 5.14 ± 0.13 (SE of mean of 16 observations).

ANALYTICAL TECHNIQUES   Total creatine (Ct) and free creatine (Cr) were measured by the technique of Ennor (1957). Phosphorylcreatine (PCr) was calculated using a 10.8% correction for creatinine formation. Inorganic phosphate (P_i) was determined by an enzymatic method based on that of Fawaz et al. (1966). Lactate was determined by an enzymatic method based on that of Hohorst (1962). All these techniques were adapted for use with an auto-analyzer (Technicon Instruments Corp., Tarrytown, NY). ATP, ADP, AMP, and P_i were measured by isotachophoresis, using a Tachophor (LKB Instruments, Inc., Rockville, MD). Application of this technique to the analysis of anions in muscle extracts is described by Gower and Woledge (1976, 1977).

EXTENT OF REACTIONS   The extent (ξ) of each relevant reaction was determined from the difference (Δ) in the level of metabolite between experimental and control members of a pair of sartorius muscles. This extent is based on the assumption that the content in the experimental muscle before treatment was identical to that of the control. Both the dry weight of the muscle residue and the total creatine content were used as estimates of muscle mass (see Results) in normalization of the data.

HIGH-ENERGY PHOSPHATE BREAKDOWN   This term refers to the extent of the reactions expected to occur during the tetanus:

ATP hydrolysis $ATP \rightarrow ADP + P_i$;   (1)

Creatine kinase reaction $ADP + PCr \rightarrow ATP + Cr$.   (2)

Because of the rapidity of the creatine kinase reaction and the absence of adenylate kinase or deaminase reactions (no significant change in AMP was detected;

see Results), the extents of reactions 1 and 2 are the same and can be estimated by three parameters, ΔPCr, ΔCr, and ΔP_i. The effects of normalization on variance and the independence of these estimates of phosphagen breakdown are discussed in Results.

TENSION TIME INTEGRALS $\int P dt$ was calculated from the myograms algebraically using Simpson's rule. In the experiments measuring O_2 consumption, only peak isometric force was measured. This value was converted to $\int P dt$ using the ratio $\dfrac{\int P dt}{P_{max}}$ = 3.47 ± 0.06, $N = 12$, obtained from the experiments in the series measuring chemical changes (3-s tetani).

REJECTION OF DATA   Chemical experiments on 32 muscle pairs (2 sets of 16 pairs) were technically successful. Values >2 SD away from the mean (including aberrant values) were rejected. Not more than one data point per group was deleted. Data are presented in terms of the mean and standard error of the mean.

RESULTS

Steady State Stimulation Pattern

The choice of a stimulation pattern to be used for steady state energy balance studies is limited by the following considerations: (*a*) stimulus duration should be of sufficient length to achieve a chemical breakdown of magnitude compatible with the resolution of the chemical measurement techniques, and (*b*) the intensity of stimulation should be sufficient to allow for considerable metabolic flux without exceeding the maximum oxidative capacity of the tissue. Preliminary experiments characterizing the effects of periodic stimulation on peak isometric force and O_2 consumption of frog sartorius muscle at $0°C$ were undertaken. As seen in Fig. 1, J_{O_2} attained a steady state, and peak force records were qualitatively similar to earlier reports (Aljure and Borrero, 1968), provided the stimulation intensity was such that the maximum oxidative capacity of the muscle was not exceeded. The rate of oxygen consumption (J_{O_2}) approaches a constant value with a time course similar to that of the isometric force. Using these experiments as a guide, a stimulation protocol of 3-s tetani at 256-s intervals was chosen to achieve a steady state compatible with requirements of an energy balance study.

Steady State Metabolic Rate

OXYGEN CONSUMPTION   Measurements of metabolism for this steady state stimulation procedure on muscles from the batch of frogs used for the energy balance comparisons are given in Table I. The basal and maximum J_{O_2} for all muscles studied were 15.6 ± 1.6 (SEM, $N = 15$) and 148 ± 11.3 (SEM, $N = 9$) nmol/(min·g blotted weight). These values were consistent with the values obtained for the primary batch of muscles (Table I), which suggests these parameters were not as sensitive to batch differences as measurements of initial heat and phosphocreatine breakdown in single tetani reported by Gilbert et al. (1971). Although basal and maximal values of O_2 consumption showed typical variations, presumably because of differences between animals, suprabasal oxygen consumption (ΔJ_{O_2}) was considerably more reproducible
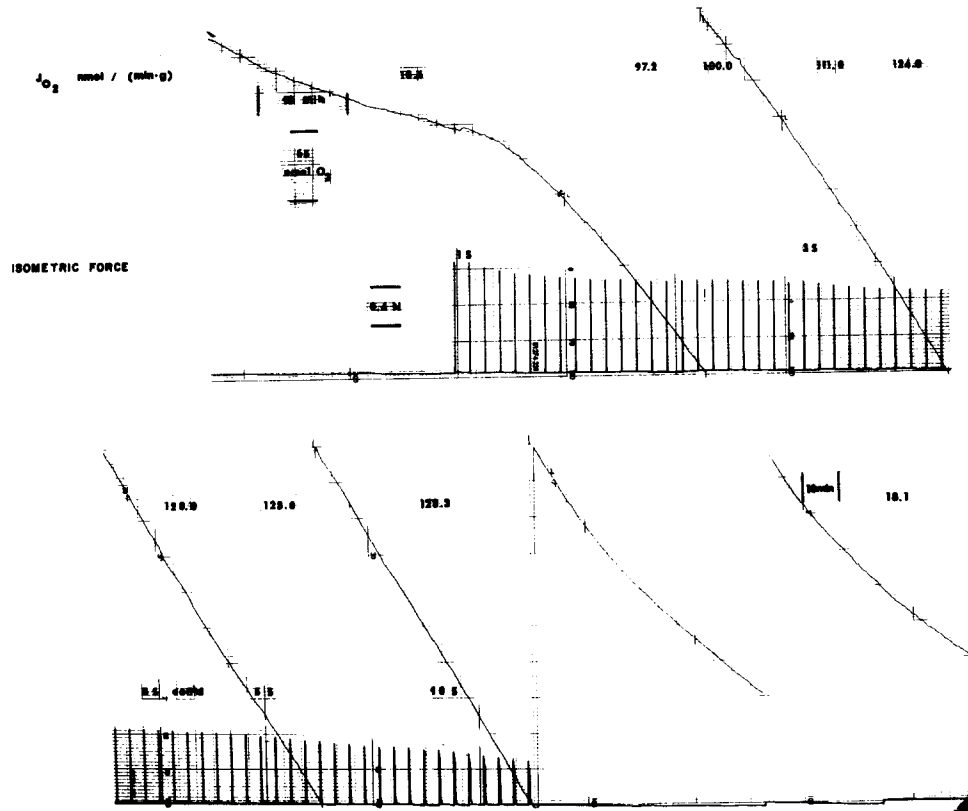
FIGURE 1. Experimental record of simultaneous measurement of oxygen consumption and isometric force vs. time. The upper thin trace is the output of the polarographic electrode. As the chamber is a closed system, the slope of the trace is a measurement of the rate of oxygen consumption. Measurements were made between partial fractions of oxygen of 0.42 and 0.27 atm, well above the diffusion limit for these preparations. The output of the electrode is biased such that a full scale deflection represents a 10% change in the initial oxygen content; a rapid vertical rise indicates a change of this bias. The bold numbers above the polarograph traces indicate the rate of oxygen consumption in nanomoles per minute per gram calculated from the slope of the line. The lower heavier lines are the force transducer output during the periodic isometric tetani. J_{O_2} was linearly related to P_oD/I (P_o, isometric force; D, stimulus duration; I, interval between stimuli), a measure of the average tension time integral, a parameter previously used (Kushmerick and Paul, 1976b). For five experiments the average slope was 0.021 ± 0.008 μmol/(min·g blotted weight) per mN/mm^2; the intercept was 0.057 ± 0.01 μmol/(min·g blotted weight) with an average correlation coefficient of 0.92 ± 0.02.

as judged by standard deviations (Table I). Furthermore, for a particular muscle, ΔJ_{O_2}, after an increase when stimulation duration was changed to 5 s, returned to within 2.5% of the previous value at 3 s when the duration was

decreased to this value. The suprabasal recovery O_2 consumption associated with each cycle is $^{256}/_{60} \times \Delta J_{O_2} = 0.392 \pm 0.006$ μmol/g blotted weight.

LACTATE PRODUCTION   The values of lactate elimination are also given in Table I. As there was negligible change in muscle lactate content (see below), these values can be interpreted as lactate production. These levels of lactate production are considerably lower than the small but statistically significant lactate production during aerobic recovery from a single tetanus that was reported by Kushmerick and Paul (1976a).

Measurements of Muscle Heat Production

The Thermagotron recording of temperature for each of 10 cycles of a muscle stimulated for 3 s every 256 s are shown superimposed in Fig. 2. After four to five cycles, the records were superimposible and indicated that a steady state had been obtained. The time required to achieve this steady state was 21.2 $\pm$ 2.9 min and agreed closely with the time of 20.8 $\pm$ 1.6 estimated from O_2 consumption measurements.

TABLE I

OXYGEN CONSUMPTION AND LACTATE ELIMINATION OF
FROG SARTORIUS MUSCLE AT 0°C

Condition	J_{O_2}	J_{lac}
		nmol/(min·g blotted weight)
Basal	15.3±2.3; $n = 7$	0.9±0.3; $n = 5$
3 s every 256 s	105.6±5.8; $n = 7$	1.9±0.8; $n = 6$
10 s every 256 s	148.0±9.6; $n = 4$	3.3±0.6; $n = 2$
	ΔJ_{O_2}	
3 s every 256 s	92±1.6; $n = 7$	

When corrected for heat loss, the temperature records indicate a rapid evolution of heat complete in ~4–6 s. For the first contraction, a slower evolution of heat begins ~60 s later. For a contraction in the steady state, a second linear phase is observed which immediately follows the rapid phase and continues throughout the remainder of the cycle. This behavior is shown in Fig. 3. Though delayed in time because of the thermal characteristics of the Thermagotron, these results are consistent with two phases (initial and recovery heat) reported for previous measurements (Hill, 1965) using themopiles of greater temporal resolution. The magnitude of the initial heat production was taken as the total deflection complete by 6 s, and for a contraction in the steady state, the total deflection at 6 s was extrapolated from the linear second phase (Fig. 3). The initial heat production in the first 3-s contraction was 108.9 $\pm$ 2.0 mJ/g ($\pm$ SEM; $N = 10$). For a 3-s contraction in the steady state the initial heat was 88.2 $\pm$ 2.5 mJ/g ($\pm$ SEM; $N = 10$) and the total heat evolved over a 256-s steady state cycle was 181.5 $\pm$ 4.4 mJ/g ($\pm$ SEM; $N = 10$). This separation of an initial heat, Q_I, from the total heat, Q_T, results in a measured Q_T/Q_I ratio of 2.06 $\pm$ 0.03 ($\pm$ SEM; $N = 10$), which agrees with many reports of this ratio (cf. Woledge, 1971).

Measurement of Muscle Metabolites

The two types of experiments performed are shown schematically in Fig. 4. In the first type, the experimental muscle, E, received a 3-s tetanus, while the control muscle, C, remained unstimulated. Both muscles were frozen simultaneously on the hammer apparatus at the last shock of the tetani, facilitating comparison with previous studies (Kushmerick and Paul, 1976*b*). Chemical change refers to the difference in contents of the appropriate chemical species and rests on the assumption that the content of the experimental muscle
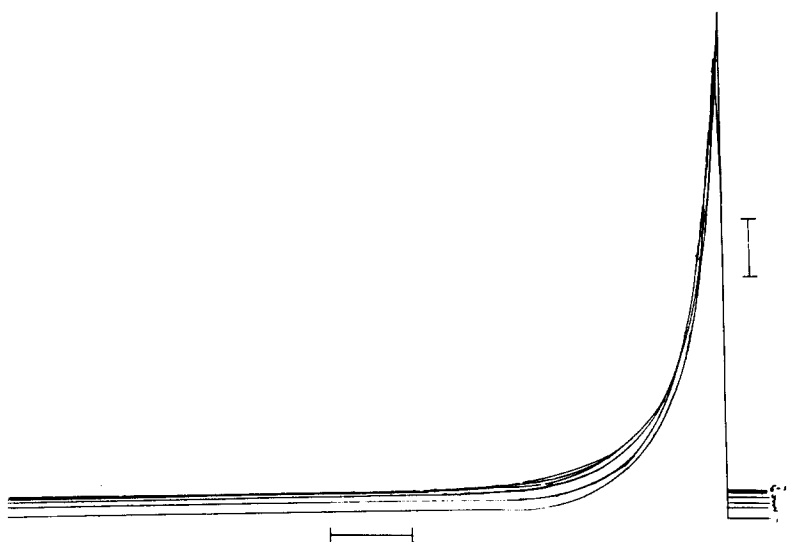

FIGURE 2.   Thermagotron records of temperature of a muscle stimulated isometrically for 3 s every 256 s. 10 cycles are shown superimposed; the number given to the right of each curve indicates the temporal order. The vertical bar indicates a Thermagotron output of 7 μV, corresponding to a temperature change of 1 m°C. Time runs from right to left; the horizontal bar corresponds to 0.5 min. The muscle had a blot weight of 50.5 mg and l_o of 3.2 cm. Traces essentially superimpose after the fifth cycle; for contractions 5–10, Thermagotron outputs before stimulus were identical and peak values had a coefficient of variation of 1%. Peak isometric forces (not shown) in contractions 3–10 were identical.

before stimulation was the same as the control muscle. In the second type of experiment, both muscles were given 3-s tetani at 256-s intervals. Mechanical reproducibility could be achieved after as few as four stimulations (three cycles) and thermal and oxygen consumption measurements indicated a steady state was achieved after ~21 min. To assure a steady state, both muscles were stimulated at least eight times (29.9 min). On the subsequent cycle, the control muscle was not stimulated and both muscles were frozen simultaneously at the time of the last tetanic shock to the experimental muscle. Chemical change under this steady state regimen is also measured as the
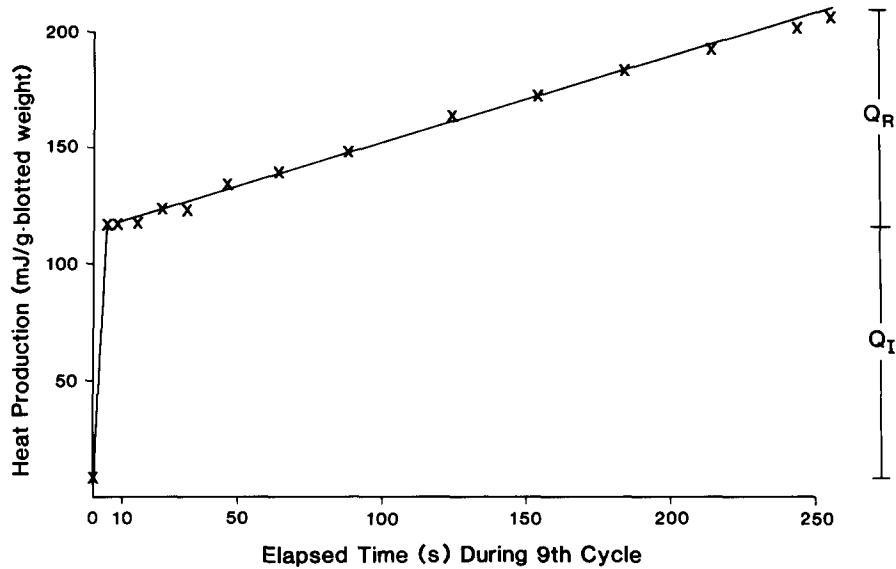
FIGURE 3.   Heat production during the ninth cycle of stimulation consisting of a 3-s isometric tetanus every 256 s. Values calculated from thermal records were corrected for heat loss. Because of the thermal characteristics of the Thermagotron, the time course shown here would slightly lag the heat production of the muscle. The initial heat (Q_I) was extrapolated from the linear portion of the heat record in the recovery period as shown.
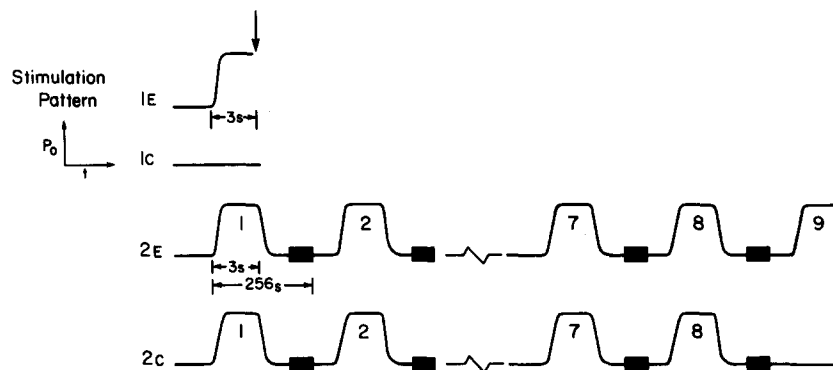

FIGURE 4.   Schematic outline of the isometric myograms in the protocol used to measure the chemical changes during the reactions in each member of a sartorius pair. The upper drawing (1E and 1C) represents the conventional pattern for measuring change in a single contraction using an unstimulated muscle as the control. The lower drawing (2E and 2C) represents the pattern for measuring change in a contraction in the steady state; the control muscle in this case receives one stimulation less than the other muscle of the pair. Muscles are rapidly frozen on hammers cooled to liquid nitrogen temperature at the time indicated by the vertical arrow.

difference in muscle contents, and this again assumes that the content of the experimental muscle before the last stimulus was the same as the control muscle. It is assumed that the rate of synthesis during the contraction is the same in the experimental and control muscles, though this is less critical because it represents only a small fraction (3/256) of the total recovery metabolism. The metabolite content and tension-time integrals of muscles for the

TABLE II
METABOLITE CONTENT AND TENSION-TIME INTEGRALS FOR INITIAL AND STEADY STATE ISOMETRIC TETANI

Pattern of stimulation: substance	Chemical change experiments											
	3 s			O			N 3-s tetani every 256 s			$(N-1)$ 3-s tetani every 256 s		
	$\bar{X}$	$\sigma_{\bar{x}}$	n	$\bar{X}$	$\sigma_{\bar{x}}$	n	$\bar{X}$	$\sigma_{\bar{x}}$	n	$\bar{X}$	$\sigma_{\bar{x}}$	n
$\mu mol/g$ dry												
Ct	180.86	7.28	15	181.92	7.69	15	184.48	3.02	15	184.46	2.51	15
PCr	148.74	5.99	15	153.25	6.76	15	137.31	3.27	16	140.22	3.08	15
Cr	34.24	1.18	15	27.26	1.52	15	48.12	1.60	15	44.25	2.28	15
P_i	24.39	0.89	15	19.77	0.92	15	29.94	1.12	15	27.47	1.30	15
ATP	10.62	0.90	15	10.60	0.85	15	11.37	0.45	16	11.81	0.63	16
ADP	3.99	0.33	15	3.94	0.30	15	4.52	0.27	15	4.59	0.32	15
AMP	2.01	0.18	15	2.03	0.15	15	2.27	0.21	16	2.23	0.16	16
Lactate	0.34	0.03	15	0.40	0.05	15	0.60	0.06	16	0.61	0.06	16
$\frac{l_o}{g}\int P dt$ (Ns cm^{-2})	79.3	3.5	16				80.8	2.8	16			
Metabolism experiments												
Ct	178.59	4.31	6									
$\frac{l_o}{g}\int P dt$	73.7	2.0	7				72.6	1.6	7			
Heat production experiments												
Ct	186.00	6.8	10									
$\frac{l_o}{g}\int P dt$	85.3	3.9	10				75.5	4.2	10			

Metabolite content and tension-time integrals for initial and steady state isometric tetani. Parameters for sartorius muscles given the following treatments before freezing: 3-s isometric tetanus (3-s), paired muscle unstimulated (O); N of 3-s isometric tetani, where N indicates the number of stimulations, paired muscle given $(N-1)$ 3-s tetani. $\bar{X}$, $\sigma_{\bar{x}}$, and n indicate the mean, standard error of the mean, and number of muscles, respectively. For direct comparison with the heat production and metabolism experiments, $\int P dt$ for the chemical experiments were corrected to account for 21 ± 1% of the total $\int P dt$ that occurs during relaxation.

various types of experiments are given in Table II. The muscles appear to be quite uniform based on the total creatine content and mechanical responses. Differences in the tension-time integrals or total creatine content for the muscle groups used in the comparisons discussed below were not statistically significant.

Comparison of the Chemical Content of Muscles in the Resting and Steady States

As anticipated, the phosphocreatine content was lower in the steady state than at rest, the values of PCr/Ct being 0.76 ± 0.01 and 0.85 ± 0.01 (± SEM, n = 15), respectively. The coefficients of variation for the values of the various chemical contents of a muscle in the steady state are generally less than or near the values found for the unstimulated muscles. The reproducibility of the chemical parameters in the steady state is at least as good as that of the resting state. This supports the assumption underlying the use of a muscle stimulated $N - 1$ times as a control, against which the chemical changes during a steady state contraction are measured.

No statistically significant differences in ATP, ADP, or AMP content under any conditions were found (Table II). A small but significant increase in the lactate content of muscles in the steady state compared with that of muscles at rest was found. If this difference represents a lactate production that is assumed to continue in the steady state, the measured changes in bath content would underestimate the true aerobic glycolysis by a factor of 2. As such, the rate of lactate production would still be some 50-fold less than J_{O_2} and negligible (<1%) in terms of the total rate of ATP synthesis.

Chemical Changes during Contraction

Estimates of chemical change are given in Table III. Changes in ATP, ADP, AMP, and lactate were small and not statistically significant for either measurement protocol, and thus were not used in the determination of $\Delta\sim P$. In both series, no statistically significant difference between the total creative contents of the control and experimental members of the paired sartorius muscles was found, so that total creatine could be used as an estimator of muscle mass. Consistent with previous reports (Wilkie, 1968), the use of total creatine to normalize chemical change was found to significantly reduce the variance in the mean value of ΔPCr compared with that found using the extracted dry weight. Using this normalization, however, reduces the number of independent estimators of initial chemical change as ΔPCr/Ct = ΔCr/Ct. For this reason ΔCr/g dry weight is included in Table III. For comparison, all values are expressed in terms of grams of dry weight, as indicated in Table III. It should be noted that the best estimates of chemical change based on paired differences using the normalization procedures described are presented for comparative purposes in terms of micromoles per gram dry weight. These values are not necessarily equal to differences in the group mean values of metabolite content given in Table II.

Though the values for ΔP_i tended to be somewhat lower, there was general agreement among ΔP_i, ΔPCr, and ΔCr as estimators of $\Delta\sim P$. In one comparison, ΔPCr and ΔP_i for the initial contraction, the difference in group means showed a marginal ($P = 0.02$) statistical significance when tested using an unpaired means t test. Using a nonparametric sign test (Colquhoun, 1971) for differences in ΔP_i and ΔPCr on the individual muscle pairs, however, did not indicate a significant difference. On the whole, these measurements indicate

that the creatine kinase reaction was close to equilibrium. Thus measurements of ΔPCr, ΔP_i, and ΔCr can each provide a measure of $\Delta{\sim}P$. The variance weighted mean of these values was used (Kushmerick and Paul, 1976b) to provide an unbiased estimator of $\Delta{\sim}P$. It can be argued that since $\Delta PCr/Ct = \Delta Cr/Ct$, $\Delta Cr/g$ dry weight may not be a true independent estimate. Estimates based on all three parameters and only $\Delta PCr/Ct$ and $\Delta P_i/Ct$ are given in Table III. Both estimates are similar; however, only the latter will be used in subsequent calculations.

$\Delta{\sim}P$ during a contraction in the steady state was nearly identical to that

TABLE III
CHEMICAL CHANGE DURING INITIAL AND STEADY STATE ISOMETRIC TETANI

	Substance	Initial 3-s tetanus 3-s(E) − 0s(C)			Steady state tetanus N of 3-s(E) − $(N − 1)$· 3-s(C)		
		$\bar{X}$	$\sigma_{\bar{x}}$	n	$\bar{X}$	$\sigma_{\bar{x}}$	n
	μmol/g dry weight						
ΔCt	$(C_t/g)_E − (C_t/g)_C$	−1.04	3.32	15	+0.19	2.97	15
1. ΔPCr	$\{(PCr/C_t)_E − (PCr/C_t)_C\} \cdot (C_t/g)_E$	−6.89	0.76	15	−6.07	1.37	16
2. ΔCr	$(Cr/g)E − (Cr/g)C$	6.22	1.02	15	5.44	1.13	15
3. ΔP_i	$\{(P_i/C_t)E − (P_i/C_t)C\} \cdot (C_t/g)E$	4.31	0.65	15	5.10	0.77	15
ΔATP	$(ATP/g)E − (ATP/g)C$	0.02	0.56	15	−0.44	0.39	16
ΔADP	$(ADP/g)E − (ADP/g)C$	0.04	0.18	15	−0.03	0.20	15
ΔAMP	$(AMP/g)E − (AMP/g)C$	−0.02	0.11	15	0.04	0.12	16
$\Delta Lactate$	$(Lactate/g)E − (Lactate/g)C$	−0.04	0.03	15	−0.02	0.06	16
	Phosphagen breakdown variance weighted mean of:	$\bar{X}$	$\sigma_{\bar{x}}$		$\bar{X}$	$\sigma_{\bar{x}}$	
	1 and 3	5.81	0.72		5.86	1.23	
	1, 2, and 3	6.21	0.77		5.34	0.98	

Chemical change during initial and steady state isometric tetani. Chemical change is based on the difference in content of a pair of sartorius muscles from the same animal. Initial 3-s tetanus refers to the difference between one muscle (E) stimulated for 3-s and its pair (C) unstimulated. 3-s tetanus in the steady state refers to the difference between one muscle (E) stimulated N times for 3 s at 256-s intervals and its pair (C) stimulated $N − 1$ times for 3 s at 256-s intervals (see text and Fig. 4 for details). Various normalization procedures as described in the text were used to obtain the best estimates of these chemical changes, which for comparative purposes are all expressed in terms of micromols per gram weight. $\bar{X}$, $\sigma_{\bar{x}}$, and n indicate the mean, standard error of the mean, and number of muscle pairs, respectively.

during the first tetani. This was true for the tension-time integrals as well. Thus, in terms of the chemical and mechanical changes during an isometric tetanus, the first contraction and contractions in the steady state are indistinguishable.

Comparisons of High-Energy Phosphate Breakdown, Heat Production, and Oxygen Consumption

To facilitate comparison with previous studies, each type of measurement has been presented to this point in the units commonly used in each type of study.

Comparisons of the energy balance parameters standardized to muscle size based on blotted weight, weight of the muscle residue after extraction and drying, or total creatine yielded similar results. As no significant reduction of variance was associated with any particular estimate of muscle mass, comparisons will be presented in terms of the more familiar blotted weight of the muscle.

Comparison of High-Energy Phosphate Breakdown during Contraction with Steady State ATP Synthesis

Using the theoretical value of the stoichiometry of the standard biochemical pathways, the predicted ATP synthesis based on the extent of the steady state metabolism occurring between tetani is $6.5 \times \Delta O_2 = 2.58 \pm 0.038$ μmol/g blotted weight. This is more than twofold greater than can be accounted for by $\Delta{\sim}P$ during contraction of 1.14 ± 0.24 μmol/g blotted weight. The apparent ADP:O ratio based on $\Delta{\sim}P$ during contraction and steady state oxygen consumption is 1.5, a value much lower than the theoretical value of

TABLE IV

PHYSICAL ENERGY BALANCE

	Observed enthalpy	Explained enthalpy	Unexplained enthalpy
		mJ/g blotted weight	
During contraction			
1st 3-s tetanus	97.2±2.0	38.4±4.8	58.8±6.1
Steady state 3-s tetanus	76.5±2.5	38.8±8.1	37.7±10.2
During recovery	93.3±2.4	141.8±12.2*	−48.5±17.5
(steady state)		148.4±12.2‡	−55.1±17.5
Total (initial + recovery)	181.5±4.4	180.6±3.2*	1.1±5.9
(steady state)		187.2±3.2‡	−5.7±5.9

* Substrate oxidation enthalpy of 460.2 kJ/mol O_2 (mixed, see text).
‡ Substrate oxidation enthalpy of 477.0 kJ/mol O_2 (carbohydrate).

3.25 and also lower than the value of 2.8 reported for isolated frog muscle mitochondria (Kushmerick, 1977; Skoog et al., 1978).

Energy Balance during Contraction

The calculated energy balance during contraction is presented in Table IV. The initial heat measured in these experiments includes heat produced during mechanical relaxation. For an exact comparison with the chemical breakdown measured during contraction, the measured initial heat must be corrected for the heat production associated with chemical reactions occurring during relaxation. The temporal resolution of the Thermagotron does not allow for accurate determination of this correction, which is on the order of 10% of the initial heat measured. A precise estimate of this heat production, however, has been determined by Curtin and Woledge (1974) as 11.7 mJ/g. This figure was used to correct the initial heat measurements in determination of observed enthalpy used for energy balance comparison during contraction. The ex-

THE JOURNAL OF GENERAL PHYSIOLOGY · VOLUME 81 · 1983

plained enthalpy is determined by multiplying the measured value of $\Delta{\sim}P$ by 34 kJ/mol, the molar enthalpy, $h_{\sim p}$, of the known reactions (cf. Curtin and Woledge, 1978). The difference between the observed enthalpy and the explained enthalpy, i.e., the unexplained enthalpy, was considerable for both the initial 3-s isometric tetanus and a tetanus of similar duration in the steady state. The absolute values of the unexplained enthalpy were 58.8 ± 6.1 and 37.7 ± 10.2 mJ/g blotted weight, respectively (values that are not significantly different from each other, $P > 0.05$). The process or processes underlying the unexplained enthalpy observed in single isometric tetani appear to be nearly fully apparent in the steady state.

Total Energy Balance

The hypothesis that the process(es) underlying the unexplained enthalpy production during contraction is reversed during recovery can be tested by evaluating the total energy balance. If reversal occurred during recovery, then the total heat production would be equal to that expected from the oxidation of substrate. The explained total enthalpy production rate would be $J_Q = h_{O_2} \cdot J_{O_2}$, where h_{O_2} is the "calorific value" of one mole of O_2, which is dependent on the substrate used. These experiments were performed in the absence of exogenous substrate and glycogen would presumably be the substrate oxidized, for which h_{O_2} would be 477 kJ/mol O_2 (Curtin and Woledge, 1978). The literature values for the respiratory quotient for isolated muscle, 0.9–0.95 (Hill, 1940b; Fenn, 1927), suggest, however, that up to 25% of the substrate oxidized may be fatty acid; the h_{O_2} for this case would be 460.2 kJ/mol O_2 (Woledge, 1971).

The explained enthalpy production based on the steady state rate of O_2 consumption would range from 0.70 ± 0.01 to 0.73 ± 0.01 mW/g blotted weight, depending on the substrate. This value is in close agreement with the observed enthalpy of 0.71 ± 0.02 mW/g blotted weight. Thus, an energy balance for the overall reaction of substrate oxidation is achieved.

Energy Balance during Recovery

If resynthesis of the high-energy phosphate broken down during contraction at the expense of substrate oxidation is the only net reaction occurring during recovery, the explained enthalpy change would be:

$$\Delta H = \text{explained enthalpy} = h_{O_2} \cdot \Delta O_2 - h_{\sim p} \cdot \Delta{\sim}P.$$

Using the values for ΔO_2 and $\Delta{\sim}P$ in Table IV, the explained enthalpy production during the steady state recovery would range from 141.5 to 147.7 mJ/g blotted weight, depending on the substrates. The observed enthalpy during recovery was substantially less (93.3 mJ/g blotted weight), which suggests that the imbalance established during contraction is reversed during recovery (see Curtin and Woledge, 1978).

DISCUSSION

The measurement of high-energy phosphate breakdown during a 3-s tetanus is in good agreement with those recently reported by Curtin and Woledge

(1979) for 2- and 5-s tetani in *Rana temporaria*, as well as with the results of many previous experiments summarized in recent reviews (Curtin and Woledge, 1978; Homsher and Kean, 1978). The literature values for the initial enthalpy production in a 3-s tetanus for *R. temporaria* extrapolated from the values tabulated in recent reviews (Homsher and Kean, 1978; Curtin and Woledge, 1978) range from 2.7 to 3.5 expressed in units of millijoules per micromole total creatine. The value observed in this study is 3.12 ± 0.065. The maximum rate of oxygen consumption measured in these experiments, 0.15 mmol/min·g blotted weight, agrees with the value of 0.18 μmol/min·g blotted weight reported by Hill (1940*a*). Furthermore, the rates of oxygen consumption were in good agreement with myothermal measurements based on the known overall reactions. This agreement is of significance in validating the use of these very different techniques, particularly in view of the recent differences in ADP:O ratios reported for single isometric tetani at 20°C (Mahler, 1979). Thus, the discrepancy between the high-energy phosphate breakdown during contraction, the expected ATP synthesis, and enthalpy production is unlikely to be an artifact of the measurement techniques.

Under these steady state conditions, in which the effects of metabolic transitions and substrate cycling should be minimized, high-energy phosphate breakdown during contraction accounted for less than half of that expected from measurements of O_2 consumption results similar to those reported earlier for single isometric tetani (Kushmerick and Paul, 1976*b*). Therefore, the discrepancy between the predicted synthesis during recovery and high-energy phosphate breakdown during contraction does not appear to be associated with the transitions in metabolism concomitant with a single tetanus. This conclusion is further supported by the energy balance comparisons. The observed energy balance is most simply interpreted as reflecting the oxidation of substrate as the only net reaction occurring in the steady state. Myothermal data, however, cannot rule out the presence of thermally neutral reactions or a series of reactions in addition to substrate oxidation whose enthalpy production fortuitously equals zero, although this is unlikely. The design of these experiments provides support for the assumption that substrate oxidation is the only net reaction because the attainment of a steady state implies that one has a complete recovery at the end of each cycle, i.e., the concentration of all metabolic intermediates is the same at the beginning and end of each steady state cycle.

The assumption that the recovery was complete at the end of each cycle was tested by two techniques. The first was measurement of the reproducibility of the heat production during each cycle in the steady state, a test first used by Bugnard (1934). The superimposition of the thermal records in the steady state (Fig. 2) strongly implies that the underlying metabolic reactions are reproduced over each cycle. For the metabolic reactions to show this periodicity, the initial concentrations of metabolites must be the same for each cycle, i.e., recovery is complete. Myothermal evidence for cyclical recovery in the steady state is strengthened by the constancy of the rate of O_2 consumption achieved. The steady state of O_2 consumption reflects periodic constancy of the underlying reactions and implies no net change of metabolite concentra-

tion from cycle to cycle in the steady state. Thus, although it is indirect, the evidence for the constancy of metabolic intermediates at the beginning and end of each steady state cycle supports the assumption that substrate oxidation is the only net reaction. If no other net reactions occur, the process underlying the unexplained enthalpy production during contraction must be reversed during recovery. This idea is supported by comparison of the amount of unexplained enthalpy during contraction and during recovery (Table IV), the amounts are both equal and of opposite sign.

The evidence presented here supports the hypothesis that the process(es) underlying the unexplained enthalpy production during contraction is reversed at the expense of substrate oxidation during recovery. Thus, the initial unexplained enthalpy production and the low apparent ADP:O ratios are likely to be causally linked. If the theoretical ADP:O ratio of 3.25 is actually achieved, reversal of the unknown processes occurring during a 3-s steady state isometric tetanus requires at maximum 1.4 μmol/g blotted weight ATP breakdown during recovery. Although there is no direct evidence that reversal is coupled to ATP hydrolysis, such a process would require 1 μmol ATP per 26.9 mJ/g blotted weight unexplained initial enthalpy production.

The evidence presented supports the hypothesis that an unknown reaction occurs during contraction and is reversed at the expense of ATP hydrolysis after the contraction. The energy cost of this unknown process can be substantial. About 50% of the total metabolic cost of a 3-s isometric contraction is thus not related to the phosphagen breakdown, presumably because of the actomyosin ATPase that occurs during contraction, but is expended during recovery to reverse some unknown exothermic reaction associated with contraction.

REFERENCES

Aljure, E. F., and L. M. Borrero. 1968. The evolution of fatigue associated with isometric contraction in toad sartorius. *J. Physiol. (Lond.).* 194:289–303.

Bugnard, L. 1934. The relation between total and initial heat in single muscle twitches. *J. Physiol. (Lond.).* 82:509–519.

Colquhoun, D. 1971. Lectures on Biostatistics. Oxford University Press, Oxford. 153.

Curtin, N. A., and R. C. Woledge. 1974. Energetics of relaxation in frog muscle. *J. Physiol. (Lond.).* 238:437–446.

Curtin, N. A., and R. C. Woledge. 1977. A comparison of the energy balance in two successive isometric tetani of frog muscle. *J. Physiol. (Lond.).* 270:455–471.

Curtin, N. A., and R. C. Woledge. 1978. Energy changes and muscular contraction. *Physiol. Rev.* 58:690–761.

Curtin, N. A., and R. C. Woledge. 1979. Chemical change and energy production during

contraction of frog muscle: how are their time courses related? *J. Physiol. (Lond.)*. 288:353–366.

DeFuria, R. R., and M. J. Kushmerick. 1977. ATP utilization associated with recovery metabolism in anaerobic frog muscle. *Am. J. Physiol.* 232:C30–C36.

DeFuria, R. R., and M. J. Kushmerick. 1978. Energetic cost of metabolism: the simultaneous *in vitro* activity of fructose 1,6-diphosphate and phosphofructokinase. *Fed. Proc.* 37:376. (Abstr.)

Ennor, A. H. 1957. Determination and preparation of *N*-phosphates of biological origin. *In* Methods of Enzymology. S. P. Colwick, and N. O. Kaplan, editors. Academic Press, Inc., New York. 3:850–856.

Fawaz, E. N., L. Roth, and G. Fawaz. 1966. The enzymatic estimation of inorganic phosphate. *Biochem. Z.* 344:212–214.

Fenn, W. O. 1927. The gas exchange of isolated muscles during stimulation and recovery. *Amer. J. Physiol.* 83:309–322.

Gilbert, C., K. M. Kretzschmar, D. R. Wilkie, and R. C. Woledge. 1971. Chemical change and energy output during muscular contraction. *J. Physiol. (Lond.)*. 218:163–193.

Godfraind-DeBecker, A. 1972. Heat production and fluorescence change of toad sartorius muscle during aerobic recovery after a short tetanus. *J. Physiol. (Lond.)*. 223:719–734.

Gower, D. C., and R. C. Woledge. 1976. Isotachophoretic analysis of muscle extracts. *J. Physiol. (Lond.)*. 258:46–47P.

Gower, D. C., and R. C. Woledge. 1977. The use of isotachophoresis for analysis of muscle extracts. *Sci. Tools.* 24(2):17–21.

Hill, A. V. 1965. *Trails and Trials in Physiology*. Arnold, London.

Hill, D. K. 1940*a*. The time course of oxygen consumption of stimulated frog's muscle. *J. Physiol. (Lond.)*. 98:202–227.

Hill, D. K. 1940*b*. Hydrogen ion concentration changes in frog's muscle. *J. Physiol. (Lond.)*. 98:467–479.

Hohorst, H. J. 1962. L(+) Lactat Bestimmung mit Lactat-Dehydrogenase und DPN. *In* Methoden der Enzymatischen Analyse. H. V. Bergmeyer, editor. 266–270.

Homsher, E., and C. J. Kean. 1978. Skeletal muscle energetics and metabolism. *Annu. Rev. Physiol.* 40:90–131.

Kretzschmar, K. M., and D. R. Wilkie. 1969. A new approach to freezing tissues rapidly. *J. Physiol. (Lond.)*. 202:66–67P.

Kretzschmar, K. M., and D. R. Wilkie. 1975. The use of the Peltier effect for simple and accurate calibration of thermoelectric devices. *Proc. R. Soc. Lond. B Biol. Sci.* 190:315–321.

Kushmerick, M. J. 1977. Energy balance in muscle contracation. A biochemical approach. *Curr. Top. Bioenerg.* 6:1–37.

Kushmerick, M. J., and R. J. Paul. 1976*a*. Aerobic recovery metabolism following a single isometric tetanus in frog sartorius at 0°C. *J. Physiol. (Lond.)*. 254:693–709.

Kushmerick, M. J., and R. J. Paul. 1976*b*. Relationship between initial chemical reactions and oxidative recovery metabolism for single isometric contractions of frog sartorius at 0°C. *J. Physiol. (Lond.)*. 254:711–727.

Kushmerick, M. J., and R. J. Paul. 1977. Chemical energetics in repeated contractions of frog sartorius muscles at 0°C. *J. Physiol. (Lond.)*. 267:249–260.

Mahler, M. 1979. The relationship between initial creatine phosphate breakdown and recovery oxygen consumption for a single isometric tetanus of the frog sartorius muscle at 20°C. *J. Gen. Physiol.* 73:159–174.

Newsholme, E. A., and C. Start. 1973 Regulation in Metabolism. John Wiley & Sons, New York.

Paul, R. J. 1976. Polarographic measurement of O_2 consumption in frog sartorius muscle at 0°C. *J. Physiol. (Lond.).* 258:47–48P.

Skoog, L., U. Kromer, R. W. Mitchell, J. Hoogstraten, and N. L. Stephens. 1978. Characterization of frog muscle mitochondria. *Am. J. Physiol.* 234(1):C1–C6.

Wilkie, D. R. 1968. Heat, work and phosphorylcreatine in muscle. *J. Physiol. (Lond.).* 195:157–183.

Woledge, R. C. 1971. Heat production and chemical change in muscle. *Prog. Biophys. Mol. Biol.* 22:37–74.