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RELATIONSHIP BETWEEN INITIAL CHEMICAL REACTIONS AND OXIDATIVE RECOVERY METABOLISM FOR SINGLE ISOMETRIC CONTRACTIONS OF FROG SARTORIUS AT 0° C

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SUMMARY

1. Measurements of initial chemical changes and recovery oxygen consumption (ΔO_2) were made in unpoisoned aerobic frog sartorius muscles at 0° C to provide independent measures of the chemical energy used for isometric tetani of various durations.

2. ΔO_2 was measured polarographically and increased in a curvilinear fashion with stimulus duration. For stimulations longer than 4 sec ΔO_2 was a linear function of the tension–time integral.

3. Measurements of the changes in the content of phosphorylcreatine, 'free' creatine and inorganic phosphate were made in muscles rapidly frozen during a tetanus. The average of these quantities, $\Delta \sim P$, was used to measure the initial 'high energy' phosphate utilization. No break-down of 'high-energy' phosphate compounds was detected up to 200 sec after relaxation of tension. Changes in the content of ATP were not observed except for a small decrease ($-0{\cdot}25 \pm 0{\cdot}1$ μmole/g) in muscles tetanized for 1 sec.

4. $\Delta \sim P$ also increase curvilinearly with tetanus duration and, for tetanic durations greater than 4 sec, was a linear function of the tension–time integral.

5. Both ΔO_2 and $\Delta \sim P$ were quantitatively related by a constant scaling factor of about 4·3 ($\Delta \sim P/\Delta O_2$) throughout the range of tetanic durations studied. The constancy of this ratio provides evidence against the hypothesis that a significant 'missing reaction' provides energy during any one portion of the tetanus. Several hypothesis may account for the numerical value of the ratio $\Delta \sim P/\Delta O_2$.

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INTRODUCTION

Recovery O_2 consumption, ΔO_2, was discussed in the previous paper (Kushmerick & Paul, 1976) as a useful measure of the total energy utilization for contraction. We now describe experiments using this approach to study chemical energy balance. The amount of O_2 consumption during recovery was compared with the extent of chemical reactions involving 'high-energy' phosphate compounds adenosine 5'-triphosphate, ATP, and phosphorylcreatine, PCr, splitting, which are known to occur during contraction. The method differs from, and may be a useful alternate to, myothermal methods.

Myothermal experiments have raised two important questions regarding energy balance. The first concerns a substantial discrepancy between the enthalpy of PCr splitting measured by calorimetry, -8 kcal/mole (Woledge, 1972, see also Maréchal, 1964) and the value obtained from measurements in muscle of the output of heat plus work divided by the PCr hydrolysed, -11 kcal/mole (Wilkie, 1968; Chaplain & Frommelt, 1972; Homsher, Rall, Wallner & Ricchiuti, 1975). The discrepancy continues and even increases throughout the course of a prolonged tetanus at 20° C (Canfield, Lebacq & Maréchal, 1973). Together, these experiments raise the possibility that an unknown exothermic reaction occurs simultaneously with and (for brief stimulations) proportional to PCr hydrolysis. A second type of discrepancy was reported by Gilbert, Kretzschmar, Wilkie & Woledge (1971): more heat plus work was released during the first few seconds of an isometric tetanus at 0° than could be explained by any single value for the enthalpy of PCr splitting. Their results lead to the possibility that another missing reaction provides energy at the start of a contraction. Thereafter, as the postulated energy source is depleted, energy is obtained from known reactions. The existence of yet unknown exothermic processes in contraction could explain both discrepancies concerning energy balance. Alternatively, as pointed out by Caplan (1971), there may be theoretical difficulties hindering the interpretation of muscle heat production in terms of simultaneous chemical events.

Changes in the content of known 'high-energy' phosphate compounds during contraction and O_2 consumption during recovery were compared in similar isometric tetanic contractions at 0° C. Our experiments tested whether there is a proportionality between the extents of PCr break-down during a tetanus and ΔO_2 for a range of tetanic durations. If this were the case, there would be a constant proportionality between the known initial chemical reaction and the total chemical energy utilization and the hypothesis that a missing reaction provides energy for contraction *during any one portion* of an isometric tetanus would be rejected. These experiments

also provide information on the chemical coupling of oxidation recovery metabolism to 'high-energy' phosphate resynthesis in frog muscle. Previous estimates of this coupling were made from measurements of isolated mitochondria or, in intact muscle, from studies of muscles in anaesthetized dogs (Piiper & Spiller, 1970).

METHODS

Muscle preparation. Rana pipiens were obtained from North American biological supply houses and kept 2–10 weeks at 10° C without feeding. Dissected muscles were soaked in a phosphate-buffered physiological saline (pH 7·1, 0° C) equilibrated with air unless specified otherwise. Experimental protocols and analytical methods are given in detail in the preceding paper (Kushmerick & Paul, 1976).

Stimulations were made by applying alternating condenser discharges of 9 V with a pair of platinum electrodes. For muscles stimulated in a gas phase (rapid freezing experiments) a 0·3 μF capacitor was used; for measurements of ΔO_2, muscle stimulation in Ringer was made with a 1·3 μF capacitor. A frequency of 10 Hz just produced a fused tetanus for both. Muscle length (L_0) was measured with the frog supine and the hip abducted and knee flexed to 90°. The muscle length on the apparatus was set to that which maintained a passive load of 0·5 g wt. The combined compliance of our force transducer (Harvard Apparatus Co., Millis, Mass.) and muscle connexions allowed a shortening of about 1 mm at full tetanic tensions; most of the compliance resided in the knots and thread used. Tension was recorded as a function of time with a Gould-Brush (model 444) recorder with dynamic response accurate to 50 Hz. The tension–time integral was obtained from the area under the myogram by a Hewlett-Packard calculator.

Chemical analysis. ΔO_2 was measured using a polarographic technique and a glass and stainless steel chamber and expressed in units of μmole/g blotted weight. It is the total increment in O_2 consumption from base line to base line after a single tetanus (see Fig. 2 in Kushmerick & Paul, 1976). Chemical measurements were made of extracts of muscles rapidly frozen on a hammer apparatus similar to that described by Kretzschmar & Wilkie (1969). The initial chemical change refers to the difference in chemical content of relevant compounds between an unstimulated control and experimental member of a pair of sartorii frozen at a given time during a tetanus; the sign convention used is experimental minus control. The following measures of the chemical content of each muscle were made from the primary data: PCr/Ct, P_i/Ct, ATP/Ct, Cr/g and Ct/g, where Ct is the total creatine content (Ct = PCr + Cr), Cr is creatine, P_i, inorganic phosphate and g, the blotted wet weight. Estimates of chemical content based on the dry weight of the extracted muscle and Ct had approximately equal coefficients of variation ($\sigma/\bar{X}$). Blotted weights for the frozen muscles were obtained by multiplying the dry weight by 5·02, the mean value of the ratio of blotted to dry weight of extracted muscles. All values of chemical content and chemical change are expressed per gram blotted weight since Ct/g is always measured.

There are three measures of net PCr hydrolysis: disappearance of PCr and appearance of the products, Cr and P_i. Each is the result of an independent chemical analysis or assumption with respect to the content of the experimental and control muscle. Changes in the content of Cr are estimated on the assumption that Cr/g is identical in experimental and control muscles before the stimulation. Changes in PCr and P_i contents are based on the assumptions that PCr/Ct and P_i/Ct, respectively, are initially the same in each member of the pair. We define $\Delta \sim$P/g as the mean, for any pair of muscles, of values of $-\Delta$PCr/g, ΔCr/g and ΔP_i/g; a positive

value indicates utilization of chemical energy. For any experimental series mean values of $\Delta \sim P/g$ were often found to have lower variance than mean values for $\Delta PCr/g$, $\Delta Cr/g$ or $\Delta P_i/g$. $\Delta \sim P/g$ is used as the most precise estimate of the extent of initial 'high energy' phosphate utilization.

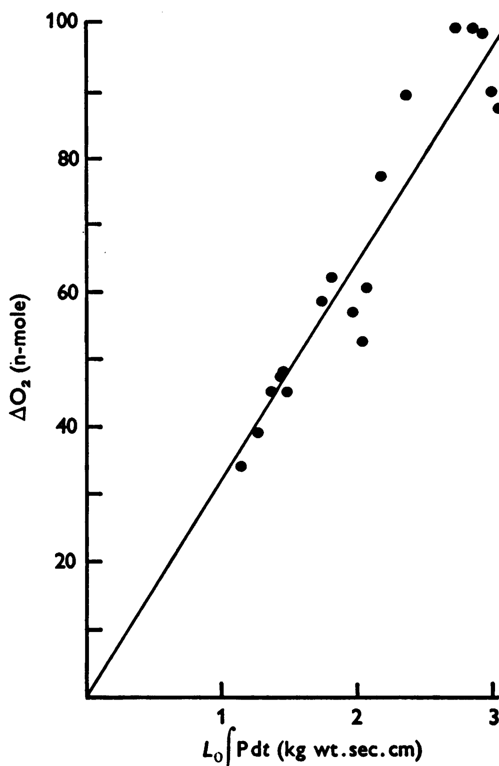

Fig. 1. Relation between the extent of recovery oxygen consumption (ΔO_2) following a 10 sec tetanus and the tension–time integral ($L_0 \int P dt$)s plotted to test the procedure for normalizing ΔO_2 to $L_0 \int P dt$. Each point represents one muscle; twenty muscles were used from ten animals. The regression equation (with the S.D. of ratio) is: $\Delta O_2 = 32{\cdot}2 \pm 2{\cdot}3$ kg wt.cm.sec $+ 0{\cdot}3 \pm 5{\cdot}2$ n-mole ($r = 0{\cdot}957$).

The measured extent of any chemical reaction would depend on the muscle mass (g) and on the time-averaged force (tension–time integral, $\int P dt$) per cross-section area (A) as well. The postulated proportionality for a population of muscles is thus: $\Delta O_2 \propto (g/A) \int P dt$. With the approximation $A = g/\rho L_0$ one can write $\Delta O_2 \propto L_0 \int P dt$; we use a value 1 for the constant, ρ, a number sufficiently close for our purposes to a measured value of 1·06 at 0° (Hill, 1931). A test of this proportionality is presented in Fig. 1; ΔO_2 is linear function of $L_0 \int P dt$ over a twofold range of muscle size and the line passes through the origin. Similar graphs were obtained for other stimulus durations and for the relation between $\Delta \sim P$ and $L_0 \int P dt$. Hence, it is valid to normalize a measurement of chemical change by force per cross-sectional area obtained for that tetanus; calculations of mean values of $\Delta O_2/(L_0 \int P dt)$

and $\Delta \sim P/(L_0 \int P dt)$ for several stimulus durations will be used to compare $\Delta \sim P$ and ΔO_2. There is also a statistical reason for including a factor for the force per cross-section area in a normalization procedure. The coefficients of variation ($\sigma/\bar{X}$) for mean values of $\Delta O_2/g$ and $\Delta O_2/L_0 \int P dt$ obtained from the data given in Fig. 1 were respectively 17·8 and 8·7%. Thus for the same level of statistical significance in studying a population of frog muscles the number of experiments required may be reduced (about fourfold in this example) by the use of this normalization factor.

Experimental design. Each measurement of $\Delta \sim P$ requires one animal; thus only mean values from a population of frogs can be studied. A previously unstimulated pair of sartorii was mounted on the apparatus, kept for 40 min in air-equilibrated Ringer at 0° C and both members of the pair were frozen during a maintained isometric tetanus of the experimental muscle. We used this common protocol in order that our results could be compared directly with values obtained in other laboratories. $\Delta \sim P$ was studied as a function of stimulus duration (1–60 sec) and of tension–time integral. In contrast, ΔO_2 can be studied as functions of stimulus duration and of tension–time integral by making repeated measurements in a single muscle as well as by calculating mean values of a population of frogs. A result obtained from a single muscle can be tested in a population of animals.

Measurements of ΔO_2 for the first tetanus of previously unstimulated muscles were not used (see the preceding paper, Kushmerick & Paul, 1976). A control experiment was made where measurements of ΔO_2 were made twice in each of a pair of muscles and compared with a measurement of $\Delta \sim P$ made in the otherwise usual way for the third tetanus.

Another difference between measurements of $\Delta \sim P$ and ΔO_2 is that for ΔO_2 the range of stimulus durations which could be studied was limited by the sensitivity of our apparatus usually to tetani longer than 3 sec and by the possibility that axial regions of the muscle become hypoxic in tetani longer than 30 sec. We usually studied ΔO_2 for stimulus durations 5, 10 and 20 sec over which range data could be obtained readily; some observations were made for other stimulus durations.

Statistical calculations. S.D. of the slope and intercepts of least-squares linear regressions were calculated according to formulae found in Snedecor & Cochran (1967). The S.D. of the slope of readings, X, is equal to $S_{yx}/\Sigma(X-\bar{X})^2$, where S_{yx} is the sample S.D. from regression. The S.D. of the intercept is equal to

$$S_{yx}\sqrt{(1/n+(\bar{X})^2/\Sigma(X-\bar{X})^2)},$$

where n is the number of data points. S.D. of the ratios $\Delta \sim P/\Delta O_2$ can only be approximately calculated. Variance (V_y) of a ratio $y = \bar{a}/\bar{b}$, here $\Delta \sim P/\Delta O_2$, can be estimated by the formula (Armitage, 1971):

$$V_y = \frac{V_a}{(\bar{b})^2} + \frac{V_b(\bar{a})^2}{(\bar{b})^4}.$$

RESULTS

Initial chemical changes

The main results are given in Fig. 2. $\Delta \sim P/g$ increases with the duration of stimulation in a curvilinear fashion (Fig. 2*A*). Graphs of $\Delta \sim P/g$ as a function of tension–time integral ($L_0 \int P dt/g$) become linear after 4 sec stimulation (Fig. 2*B*). This result reflects the fact that isometric tension declines during a maintained tetanus; the rate of decrease was

approximately 0·007 L_0 per sec of stimulation. For 1, 2 and 3 sec tetani values of $\Delta \sim P/g$ fall somewhat below the regression line. The curvilinear relation between $\Delta \sim P/g$ and stimulus duration for tetani longer than 4 sec can be explained by the decline in tension.

Isometric tension became maximal within 2 sec of stimulation but the apparent rate of hydrolysis of 'high-energy' phosphate compounds only becomes steady for stimulus durations longer than 4 sec. The apparent rate is greater for shorter stimulus durations. These results stand in contrast to the results of Maréchal & Mommaerts (1963) and of Gilbert *et al.* (1971) who reported that the amount of PCr split during an isometric tetanus increased linearly with tetanus duration. A regression line for $\Delta \sim P/g$ and tension–time integral has a statistically significant intercept. The relation between $\Delta \sim P/g$ and $L_0 \int P\,dt/g$ remains linear up to 60 sec of a maintained tetanus of aerobic muscle.

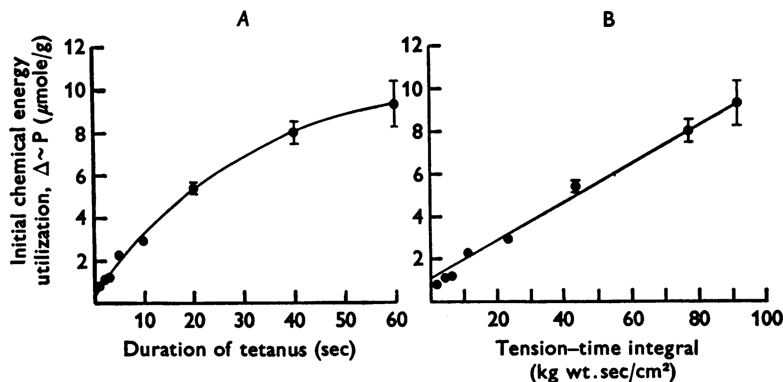

Fig. 2. Relationships between initial changes in 'high energy' phosphate compounds, $\Delta \sim P/g$ (see text), and stimulation duration (*A*) and tension–time integral (*B*). Bars indicate one S.E. of mean; no bars are visible in some points because the diameter of the circle is larger. The number of muscle pairs represented by each point, beginning with the datum for a 1 sec tetanus are: 19, 15, 8, 30, 22, 20, 6 and 6. A smooth curve is drawn through the data in *A*. In *B*, the line drawn is the least-squares regression for the data obtained for stimulations 5–60 sec: $\Delta \sim P/g = 0{\cdot}0896\ L_0 \int P\,dt/g + 1{\cdot}21\ \mu mole/g$ ($r = 0{\cdot}99$).

Stoichiometry of the initial reactions

An implicit assumption in the calculation of $\Delta \sim P/g$ is that the Lohmann reaction is the only quantitatively significant reaction involving release and uptake of P_i. Mean values of PCr splitting agree well with the observed production of Cr and P_i (Table 1). The discrepancy between PCr break-down and production of P_i was statistically significant at 20 sec ($P < 0{\cdot}01$). The correct interpretation of this observation is not known since increases in the content of hexose monophosphates were negligible

at the moment the muscles were frozen; these findings are consistent with those of Gilbert *et al.* (1971). A few measurements were made of muscles during the course of aerobic recovery. The content of hexose monophosphates increased transiently during the recovery period in the range 0·5–0·8 μmole/g at 15 and 30 min after a 20 sec tetanus. Net changes in the content of ATP were observed only for 1 sec tetani; ΔATP/g was $-0{\cdot}25 \pm 0{\cdot}10$ μmole/g (S.E. of mean, $n = 19$, $P < 0{\cdot}025$). This amount of net ATP hydrolysis is included in the datum for 1 sec stimulations given in Fig. 2. This observation conflicts with the synthesis of ATP described by Gilbert *et al.* (1971).

TABLE 1. Observed chemical changes during single isometric tetani of unpoisoned muscles at 0° C

Duration of tetanus (sec)	n	ΔPCr/g (μmole/g) Mean	ΔPCr/g S.E. of mean	ΔCr/g (μmole/g) Mean	ΔCr/g S.E. of mean	ΔP_i/g (μmole/g) Mean	ΔP_i/g S.E. of mean
1	19	−0·64	0·09	0·54	0·10	0·72	0·14
2	15	−1·16	0·12	1·07	0·14	1·21	0·11
3	8	−1·28	0·13	1·13	0·17	1·13	0·12
5	30	−2·44	0·15	2·44	0·13	2·07	0·13
10	22	−2·99	0·21	3·01	0·19	3·01	0·14
20	20	−5·83	0·17	5·65	0·17	4·70	0·17
40	6	−8·23	0·77	8·16	0·77	7·62	0·39
60	6	−9·61	1·19	9·16	1·19	9·25	0·46

Recovery O_2 consumption

The main results are given in Fig. 3. The amount of O_2 consumption above the base line (ΔO_2) increases with tetanus duration in a curvilinear fashion (Fig. 3*A*). These results were obtained by making repeated measurements of one muscle. Similar curvilinear relations were observed in other muscles although we usually studied a narrower range of stimulus durations, 5–20 sec. ΔO_2 is a linear function of the tension–time integral (Fig. 3*B*), and the intercept of the least squares regression line is statistically significant. The curvilinearity of ΔO_2 as a function of stimulus duration can also be explained adequately by the decline in isometric tension during a maintained tetanus. ΔO_2/g as a function of tension–time integral ($L_0 \int P\,dt/g$) was studied in eleven muscles; regression analyses of the data obtained are given in Table 2. The large correlation coefficients indicates that the regression lines fitted the data well in all cases. Thus the linearity between ΔO_2 and $L_0 \int P\,dt$ (Fig. 3) observed in a single muscle is the same as observed between ΔO_2/g and $L_0 \int P\,dt/g$ in population of animals and the observed linearity is not an artifact of normalization by muscle weight.

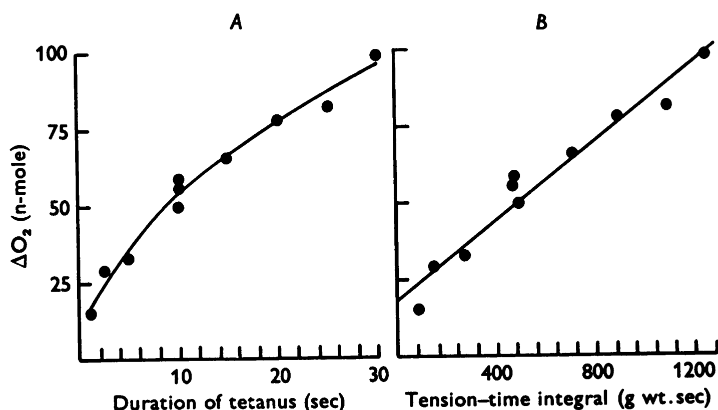

Fig. 3. Relationships between recovery O_2 consumption (ΔO_2) and tetanus duration (A) and tension–time integral (B). All data are obtained from one muscle and are not normalized to muscle weight. A tetanus of 10 sec duration was repeated three times during the experiment. A smooth curve was drawn through the data in A. In B, the line drawn is the least squares linear regression of all the observations: $\Delta O_2 = 0{\cdot}0648$ (g wt.sec) + 18·6 n-mole ($r = 0{\cdot}975$). Muscle weight was 60·18 mg; L_0 was 32 mm.

TABLE 2. Regression of $\Delta O_2/g$ on $L_0 \int P\,dt/g$ for isometric tetani in each of eleven muscles

Slope (μmole/g ÷ kg wt.sec.cm/g)	Intercept (μmole/g)	n	r
0·0178	0·338	6	0·93
0·0146	0·488	6	0·88
0·0154	0·395	6	0·99
0·0142	0·470	6	0·87
0·0274	0·128	4	0·99*
0·0261	0·275	5	0·99†
0·0281	0·155	7	0·95
0·0301	0·125	6	0·97
0·0184	0·191	5	0·93‡
0·0202	0·309	10	0·98§, ‖, ‡
0·0209	0·308	9	0·90‖

Slope Mean	Slope S.E. of mean	Intercept Mean	Intercept S.E. of mean
0·0212	0·0017	0·289	0·039

The experimental points were from tetanic durations predominantly 5, 10 and 20 sec, and each duration was usually repeated for each muscle. In some cases muscle size was appropriate for making measurements in the ranges < 5 and > 20 sec. The muscles containing such data are indicated: §, 1 and 2·5; †, 1·7; *, 3·3; ‖, 25; ‡, 30 sec tetanus. Also, n is the number of data collected and r is the product moment coefficient of correlation.

In four muscles it was possible to obtain a measure of ΔO_2 for stimulus durations < 5 sec; these experiments are identified in Table 2 and an example of one of these is given in Fig. 3. Those points always fell below the regression line but too few observations were made to establish that result clearly.

TABLE 3. Ratios of $\Delta \sim P/\Delta O_2$ for 5, 10 and 20 sec tetani stimulations

A, ratio obtained from observed chemical changes

Duration of stimulation (sec)	$\Delta \sim P$/g (μmole/g)			ΔO_2/g (μmole/g)				
	Mean	S.D. of observation	*n*	Mean	S.D. of observation	*n*	$\frac{\Delta \sim P}{\Delta O_2}$	S.D. of observation
5	2·26	0·75	30	0·555	0·062	8	4·1	1·4
10	2·94	0·52	22	0·849	0·148	14	3·5	0·9
20	5·39	0·85	20	1·23	0·26	11	4·4	1·2

B, ratio obtained from chemical changes normalized to the tension–time integral

Duration of stimulation (sec)	$\Delta \sim P/L_0\int P\,dt$ (μmole/kg wt.sec.cm)			$\Delta O_2/L_0\int P\,dt$ (μmole/kg wt.sec.cm)				
	Mean	S.D. of observation	*n*	Mean	S.D. of observation	*n*	$\frac{\Delta \sim P}{\Delta O_2}$	S.D. of observation
5	0·209	0·060	30	0·043	0·006	8	4·9	1·6
10	0·130	0·029	22	0·034	0·007	14	3·8	1·2
20	0·124	0·016	20	0·028	0·003	11	4·5	0·7

Comparison of initial chemical energy utilization with recovery metabolism

If the initial chemical energy utilization and ΔO_2 both measure the total chemical energy cost of contraction, the relationship of one measure to the other should be related by a constant scaling factor. The simplest quantitative comparison between $\Delta \sim P$/g and ΔO_2/g is obtained by calculating the ratio of mean values obtained for 5, 10 and 20 sec tetanic stimulations (Table 3). There is no obvious correlation between stimulus duration and the ratio $\Delta \sim P/\Delta O_2$.

Muscles used for measurement of $\Delta \sim P$/g were frozen during the tetanus whereas those muscles used for measurement of ΔO_2/g relaxed completely. Correspondingly, the mean value of the tension–time integral ($L_0\int P\,dt$/g) for each tetanus duration was slightly larger for muscles used in recovery O_2 measurements. The difference increases as tetanus duration decreases since relaxation time was relatively constant. Small systematic differences between the two types of experiments were taken into account by calculating the mean values of $\Delta \sim P/L_0\int P\,dt$ and $\Delta O_2/L_0\int P\,dt$ and taking the

ratios of those quantities (Table 3). The justification for this normalization was described in the Methods section. These estimates of the ratio $\Delta \sim P/\Delta O_2$ differ little from the previous ones. The simplest interpretation of these calculations is that the ratio is constant, the average value being 4·2, for muscles stimulated for 5, 10 and 20 sec.

TABLE 4. Ratios of $\Delta \sim P/\Delta O_2$ obtained from linear regression analyses of chemical change as a function of the tension–time integral (see text)

	$\Delta \sim P$	ΔO_2	$\Delta \sim P/\Delta O_2$
A. Slopes (μmole/g ÷ kg wt.sec. cm/g)	0·104 ± 0·008	0·0263 ± 0·0019	4·0 ± 0·4
Intercepts (μmole/g)	0·69 ± 0·23	0·138 ± 0·058	5·0 ± 2·7
n	36	15	
B. Slopes (μmole/g ÷ kg wt.sec. cm/g)	0·094 ± 0·006	0·0212 ± 0·0058	4·4 ± 1·2
Intercepts (μmole/g)	1·10 ± 0·17	0·289 ± 0·129*	3·8 ± 1·8
n	72		

The values are given with the S.D. of the observation; *n* is the number of data used to calculate the regressions.

* Mean value of regression parameters for eleven muscles.

The form of the curves relating $\Delta \sim P$ and ΔO_2 with stimulus duration and tension–time integral is similar (Figs. 2 and 3). Another comparison between $\Delta \sim P/g$ and $\Delta O_2/g$ can be obtained easily from the population regressions against the tension–time integral because the data are accurately fitted by a straight line for stimulus durations > 4 sec. We can test whether the regression slopes and intercepts can be scaled by the same factor, that is, whether the relation between the measures of initial and recovery chemical energy utilization as functions of tension–time integral are superimposable. The obvious alternate possibility is that the regression lines are only parallel. The same information could be obtained by curvilinear regression analyses of $\Delta \sim P/g$ and $\Delta O_2/g$ against stimulus duration, but we chose the simpler linear regression analysis against tension–time integral. The analysis of the regression parameters is given in Table 4. In *A* the regression of $\Delta O_2/g$ and tension–time integral were made from equal numbers of experiments (five) for 5, 10 and 20 sec tetanic durations chosen from experiments made during July and December 1972 such that each animal was represented only once. The regression of $\Delta \sim P/g$ and tension–time integral in the same row represents all data obtained in the same period and equal numbers of experiments (twelve) for 5, 10 and 20 sec tetanic durations; thus each measurement also represents one

animal. These data have been presented (Paul & Kushmerick, 1974). ΔO_2/g as a function of tension–time integral is most accurately obtained from repeated measurements on a single muscle; $\Delta \sim P$/g can be measured only once for one stimulus duration in one pair of muscles. The data for ΔO_2/g from Table 2 are summarized in *B* and are the mean values of the regression parameters for multiple measurements on each of eleven muscles. All measurements of $\Delta \sim P$/g made for 5, 10 and 20 sec stimulations are included in the other regression given in the same row. The ratios, $\Delta \sim P/\Delta O_2$, are calculated from the slopes and intercepts. The ratios of the slopes, 4·0 and 4·4, are not very different from ratios derived in Table 3. There is larger error in the values for the regression intercepts and in the ratios obtained. Nevertheless, those ratios are not significantly different from the ratios derived from the slopes or from the ratios derived in Table 3. The result seems to be that the same ratio as obtained from the data in Table 3 will superimpose the regression of $\Delta \sim P$/g and ΔO_2 against the tension–time integral.

Superposition means, first of all, that the ratio $\Delta \sim P/\Delta O_2$ is constant for tetanic durations 5–20 sec. Data obtained on tetani of longer durations (up to 60 sec in experiments measuring $\Delta \sim P$/g and up to 30 sec in experiments measuring ΔO_2/g) also fall on the same regression lines. Therefore the same relation between $\Delta \sim P$/g and ΔO_2/g is obtained in more prolonged tetani. Superposition also implies that the same ratio of $\Delta \sim P$/g to ΔO_2/g is obtained for stimulations less than 5 sec (values of tension–time integral less than 11 kg wt.sec/cm^2). Otherwise the linear dependence of $\Delta \sim P$/g and ΔO_2/g on tension–time integral would be only parallel.

Four measurements were made of ΔO_2/g for stimulus durations less than 5 sec: 0·248, 1 sec; 0·364, 2 sec; 0·479, 2·5 sec and 0·311 μmole/g, 3 sec. Comparison of $\Delta \sim P$/g with ΔO_2/g, especially for these brief stimulus durations, requires that the chemical changes be normalized to the tension–time integral (see above). Each normalized value ($\Delta O_2/L_0\int P\,dt$) was compared with the mean value of $\Delta \sim P/L_0\int P\,dt$ for the corresponding duration of tetanus (a linearly interpolated value was used for 2·5 sec). The ratios $\Delta \sim P/\Delta O_2$ obtained were: 6·1, 1 sec; 4·5, 2 sec; 4·7, 2·5 sec and 3·8, 3 sec. These values are similar to the ratios obtained for longer tetani (Table 3) and from the regression analysis (Table 4) except for the first value (6·1, 1 sec) which is subject to greatest experimental error. The mean values of the ratios for stimulations less than 5 sec is 4·8 ± 1·0 (S.D. of ratio) and is consistent with the average ratio obtained for longer tetani. Therefore we conclude that $\Delta \sim P$/g and ΔO_2/g are proportional over the range of tetanic stimulus durations studied.

There may be a systematic error introduced into the calculation of the ratio $\Delta \sim P/\Delta O_2$ which arises from the experimental design. The first

tetanus of previously unstimulated muscles was used for measurements of $\Delta \sim P/g$ whereas the first tetanus was not used for measurements of $\Delta O_2/g$ (see the preceding paper, Kushmerick & Paul, 1976). A control experiment has been made (Paul & Kushmerick, 1974). The muscles were stimulated, frozen and the extracts were analysed as usual *after* the muscles had been stimulated twice for measurements of ΔO_2. The data for the first tetanus were not discarded and $\Delta O_2/g$ for both tetani were similar. Values for $\Delta O_2/g$ and $\Delta \sim P/g$ for 10 sec tetani were similar to those given in Table 3. The mean of these ratios, $\Delta \sim P/\Delta O_2$, was $3{\cdot}4 \pm 0{\cdot}14$ (S.D. of the mean; $n = 5$). This estimate of $\Delta \sim P/\Delta O_2$ is in the range of the other estimates but it is also approximately 20% lower than the mean (4·3) of the values given in Tables 3 and 4 and thus could reflect a genuine systematic error of that magnitude.

Splitting of PCr after relaxation

It is clear that continued splitting of PCr continues after the last shock of a tetanus more or less in proportion to declining muscle activity (Curtin & Woledge, 1974; see also Kushmerick & Davies, 1969; Gilbert *et al.* 1971). We made further experiments to test the possibility that extra PCr splitting occurred after complete relaxation of isometric tension. Muscles were anaerobic and poisoned with 0·5 mM iodoacetate to prevent synthesis of 'high-energy' phosphate compounds. Both muscles of a pair were given identical tetanic stimulations (2 or 10 sec duration). The control muscle was frozen after relaxation of tension (approximately 2·7 sec after the last shock) and the experimental muscles were frozen at longer intervals after the last shock. Our experimental design is incomplete in the sense that the total duration of aerobic recovery (approximately 45 min) was not studied. It was practicable to study intervals up to 200 sec after tetanus. The quantity, $\Delta \sim P/g$, for these experiments only, is the mean of $\Delta P_i/g$ and $\Delta Cr/g$. The results are given in Table 5.

No significant chemical change was observed in any group. Thus PCr splitting, in so far as it was measured by the appearance of Cr and P_i, was not observed up to 60 sec after a 2 sec tetanus and up to 200 sec after a 10 sec tetanus. These results also provide evidence against a hypothesis that PCr splitting after relaxation continues in proportion to the PCr splitting during the tetanus. According to that hypothesis the predicted chemical break-down after a 10 sec tetanus would be approximately threefold greater than the break-down after a 2 sec tetanus (see Table 1). The mean $\Delta \sim P/g$ after 2 sec tetani (grouped data) was $0{\cdot}14 \pm 0{\cdot}12$ (S.E. of mean) μmole/g and after 10 sec tetani (grouped data) $\Delta \sim P/g$ was $0{\cdot}05 \pm 0{\cdot}11$ (S.E. of mean) μmole/g; a break-down of 0·3–0·4 μmole/g could have been detected.

TABLE 5. Tests of PCr splitting after relaxation of tension

Duration of stimulation (sec)	Interval after last shock (sec)	$\Delta \sim P/g$ (μmole/g) Mean	S.E. of mean	n
2	15	0·18	0·14	22
2	60	0·02	0·25	7
All 2 sec tetani		**0·14**	**0·12**	**29**
10	15	0·01	0·17	13
10	60	0·32	0·30	8
10	200	0·06	0·24	8
10*	200	−0·14	0·25	10
All 10 sec tetani		**0·05**	**0·11**	**39**

Both muscles of a pair were tetanically stimulated. The control muscle was frozen at the end of relaxation and the experimental muscle was frozen at intervals thereafter (see text). Muscles were poisoned for 45 min at 0° C with 0·5 mM iodoacetate in Ringer gassed with N_2 except the group marked (*) which were aerobic and unpoisoned. Quantities in bold type were grouped.

DISCUSSION

Correlation of initial PCr break-down and recovery O_2 consumption

The major finding is that ΔO_2 is proportional to initial PCr splitting in the range of tetanic durations from 5 to 20 sec and the available evidence argues for the same proportionality for tetanic durations less than 5 and greater than 20 sec. The ratio, $\Delta \sim P/\Delta O_2 = 4{\cdot}2$, which was obtained from mean values of each quantity for 5, 10 and 20 sec tetani (Table 3), was similar to the ratios obtained for tetanic stimulations less than 5 sec and was close to the scaling factor (4·4) which superimposes the regressions of $\Delta \sim P/g$ and $\Delta O_2/g$ against the tension–time integral (Table 4). We conclude the regressions of $\Delta \sim P/g$ and $\Delta O_2/g$ as a function of tension–time integral are superimposable, not merely parallel. The error associated with the ratio $\Delta \sim P/\Delta O_2$ can be estimated by calculating the mean and S.D. of the ratios given in Tables 3 and 4 and average of those given in the text for tetanic stimulations less than 5 sec (mean = 4·4 and S.D. of ratios = 0·5). One specific hypothesis, which proposes that an unknown reaction providing a substantial amount of chemical energy during the first few sec of a tetanus (Gilbert *et al.* 1971) can be considered. If such a reaction were coupled to recovery processes (and it must, for otherwise it could not be resynthesized) the quantity $\Delta \sim P/\Delta O_2$ should decrease for short tetanic stimulations by at least 50 %. Our present results contradict such a hypothesis since the ratio $\Delta \sim P/\Delta O_2$ bears no consistent relation to the stimulus duration. While variations of the ratio $\Delta \sim P/\Delta O_2$ within that

range of error stated above ($4 \cdot 4 \pm 0 \cdot 5$ S.D. of ratios) cannot be excluded, the decrease in the ratio required by the data of Gilbert *et al.* (1971) could have been detected.

Interpretation of the ratio $\Delta \sim P/\Delta O_2$

To assess whether ΔO_2 can account for the initial chemical energy utilization ($\Delta \sim P$), a value for the number of moles of ADP re-phosphorylated per mole O_2 consumed must be used. A standard biochemical value for $\Delta \sim P/\Delta O_2$ is obtained from the stoichiometry of known pathways and from measurements on suspensions of mitochondria *in vitro* and is 6·5 for the complete oxidation of glucosyl units from glycogen. The predominant substrate for amphibian muscle is likely to be carbohydrate since the respiratory quotient is close to unity (Fenn, 1927; Hill, 1940) and because exogenous substrate was not added to our muscles. We observed a ratio of $\Delta \sim P/\Delta O_2$ closer to 4·3. Several hypotheses might explain this observation.

1. *Phosphorylation ratio in isolated mitochondria.* The phosphorylation ratio, P/O, at 0° for mitochondria isolated from frog skeletal muscle may be closer to 2 than the value 3 more commonly observed in mammalian preparations at higher temperatures. Several studies were made (M. J. Kushmerick & Yeung, C. 1973, unpublished) using mitochondria isolated from frog leg muscle with glutamate and malate as substrates. Mitochondrial O_2 consumption following additions of ADP was measured with the same apparatus used to study the whole muscle. Respiratory control ratios ranged from 2 to 4. The average observed ratio of ADP added to O_2 consumed in three preparations, using serial additions of three different amounts of ADP, was $3 \cdot 1 \pm 0 \cdot 3$ (S.D. of ratio). These results at 0° C agree with values obtained by Skoog & Stephens (1973) for frog muscle mitochondria at 17–37° C. Hence we have no evidence that in frog mitochondria at 0° C the P/O ratio or the control of oxygen consumption by ADP levels at saturating substrate concentrations differs substantially from that observed in other mitochondrial preparations. It remains an interesting possibility that mitochondria within intact muscle cells may be partly uncoupled at least under the conditions of our experiments.

2. *Systematic error in measurements of* $\Delta \sim P$ *and* ΔO_2. The possibility is excluded that there was simultaneous PCr hydrolysis and resynthesis during the tetanus, such that net PCr hydrolysis was underestimated. Calculations based on the results in our previous paper (Kushmerick & Paul, 1975) show that negligible oxidative phosphorylation during the tetanus could have occurred because the time constant for oxygen consumption is of the order of 15 min. Further, those results showed only about 6–9 % of high-energy phosphate resynthesis occurred by glycolysis

during the whole of recovery and even in a 20 sec tetanus, lactate production at the end of the stimulation was not detected. Lastly, initial chemical energy utilization in a single tetanus was not different in normal and anaerobic and iodoacetate-poisoned muscles (Paul & Kushmerick, 1974).

Measurements of initial PCr splitting were made in muscles frozen during the first tetanus after dissection whereas measurements of ΔO_2 were made repeatedly in each muscle and the datum for the first tetanus was usually not collected (Kushmerick & Paul, 1976). Thus the possibility that the first tetanus is energetically different from subsequent ones must be raised. The best evidence that this factor does not lead to a substantial error comes from previously reported experiments wherein initial and recovery chemical measurements were made in the same muscle pair. $\Delta O_2/g$ was measured in two tetani; then in the third tetanus of the experiment PCr splitting was measured (Paul & Kushmerick, 1974). In those studies of 10 sec tetani PCr splitting and $\Delta O_2/g$ was not significantly different from those measured in the usual way; however $\Delta \sim P/\Delta O_2$ was approx. 20% lower than values obtained in the present study. The difference, which may be genuine and the large amount of ΔO_2 occasionally found for the first tetanus of a series (see the preceding paper, Kushmerick & Paul, 1976) indicates differences in the serial order of contractions ought to be studied in detail.

3. *Delayed PCr splitting*. Oxidative resynthesis measured over the course of many minutes cannot be used to distinguish a burst of PCr splitting during contraction from a slow net hydrolysis during recovery which would be included in our measurements of $\Delta O_2/g$. Since a constant scaling factor superimposes the regressions of $\Delta \sim P/g$ and $\Delta O_2/g$ on the tension–time integral, any post-contractile break-down is constrained to be proportional to $\Delta \sim P/g$, the extent of the known net chemical driving reactions during the contraction. Our experiments demonstrate (Table 5) not only that this is not observed, but that there is no evidence for any significant net PCr hydrolysis up to more than 3 min after relaxation of tension. There is no evidence to suggest such additional PCr splitting thereafter; nor can such a possibility be excluded by the available evidence. Curtin & Woledge (1974; see also Kushmerick & Davies, 1969) carefully studied the relaxation phase of a tetanus and observed a small splitting of PCr consistent with declining contractile activity; certainly no large burst of PCr splitting was found.

During contraction ATP may well have been used to drive processes in addition to the activated actomyosin system. Examples include protein phosphorylations and active Na^+, K^+ and Ca^{2+} transport. However, all of these energy expenditures during contraction would be included in the measurement of $\Delta \sim P/g$ via the Lohmann reaction. If they occurred

throughout recovery it is not obvious why such energy costs should be proportional to $\Delta \sim P/g$ during contraction.

4. *Unknown sources of chemical energy.* Suppose a reaction of the type $X \sim Y \rightarrow X + Y +$ energy occurs during or following a contraction and provides some of the energy released as a consequence of contraction. If $X \sim Y$ is not in equilibrium with the ATP–PCr pool then its restoration during recovery or any other time is unlikely in accord with the weight of biochemical knowledge. It is also unlikely that there be an irreversible pool of chemical energy in the cell. Our experiments cannot exclude the existence of such an unidentified reaction, $\Delta X \sim Y$, but do set the following limitations: (*a*) $\Delta \sim P$ and the extent of the unknown reaction $\Delta X \sim Y$ are proportional, because $\Delta \sim P$ and ΔO_2 are proportional; (*b*) $\Delta X \sim Y$ probably occurs during contraction, not afterwards, because the time course of resynthesis of $\Delta \sim P$ and recovery O_2 consumption follow similar exponential time courses and have the same time constants (see the preceding paper, Kushmerick & Paul, 1976); (*c*) the chemical energy provided by $\Delta X \sim Y$ is approximately a half that provided by $\Delta \sim P$, since, if the true mitochondrial $\Delta \sim P/\Delta O_2$ ratio in cells were 6 (see hypothesis 1), the observed $\Delta \sim P/\Delta O_2$ ratio is 4·3. This hypothesis coincides with the first one described in the Introduction which is based on myothermal measurements and which has been discussed, in terms of the balance of heat and chemical changes, at length (Gilbert *et al.* 1971; Woledge, 1971, 1972; Canfield *et al.* 1973).

The evidence from our work as well as that of others cannot exclude any of these hypotheses, although at the moment the second hypothesis seems unlikely. Perhaps the first and fourth are 'most interesting' especially since the underlying biochemical mechanism is clearly distinct but the functional manifestations of each would be similar.

REFERENCES

Armitage, P. (1971). *Statistical Methods in Medical Research*, p. 97. Oxford, Edinburgh: Blackwell Scientific.

Canfield, P., Lebacq, J. & Maréchal, G. (1973). Energy balance in frog sartorius muscles during an isometric tetanus at 20° C. *J. Physiol.* **232**, 467–484.

Caplan, S. R. (1971). Non-equilibrium thermodynamics and its application to bioenergetics. *Curr. Top. Bioenerg.* **4**, 1–79.

Chaplain, R. A. & Frommelt, B. (1972). The energetics of muscular contraction. I. Total energy output and phosphorylcreatine splitting in isovelocity and iso tonic tetani of frog sartorius. *Pflügers Arch. ges. Physiol.* **334**, 167–180.

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

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

GILBERT, C., KRETZSCHMAR, K. M., WILKIE, D. R. & WOLEDGE, R. C. (1971). Chemical change and energy output during muscular contraction. *J. Physiol.* **218**, 163–193.

HILL, A. V. (1931). Myothermic experiments on a frog gastrocnemius. *Proc. R. Soc.* B **109**, 267–303.

HILL, D. K. (1940). Hydrogen-ion concentration changes in frog's muscle following activity. *J. Physiol.* **98**, 467–479.

HOMSHER, E., RALL, J. E., WALLNER, A. & RICCHIUTI, N. V. (1975). Energy liberation and chemical change in frog skeletal muscle during single isometric tetanic contractions. *J. gen. Physiol.* **65**, 1–21.

KRETZSCHMAR, K. M. & WILKIE, D. R. (1969). A new approach to freezing tissues rapidly. *J. Physiol.* **202**, 66–67*P*.

KUSHMERICK, M. J. & DAVIES, R. E. (1969). The chemical energetics of muscle contraction. II. *Proc. R. Soc.* B **174**, 315–353.

KUSHMERICK, M. J. & PAUL, R. J. (1976). Aerobic recovery metabolism following a single isometric tetanus in frog sartorius muscle at 0° C. *J. Physiol.* **254**, 693–709.

MARÉCHAL, G. (1964). *Le Métabolisme de la Phosphorylcréatine et de l'Adénosine Triphosphate durant la Contraction Musculaire*, pp. 88–90. Brussels: Editions Arscia.

MARÉCHAL, G. & MOMMAERTS, W. F. H. M. (1963). The metabolism of phosphocreatine during an isometric tetanus in the frog sartorius muscle. *Biochim. biophys. Acta* **70**, 53–67.

PAUL, R. J. & KUSHMERICK, M. J. (1974). Apparent P/O ratio and chemical energy balance in frog sartorius muscle *in vitro*. *Biochim. biophys. Acta* **347**, 483–490.

PIIPER, J. & SPILLER, P. (1970). Repayment of O_2 debt and resynthesis of high-energy phosphates in gastrocnemius muscle of the dog. *J. appl. Physiol.* **28**, 657–662.

SKOOG, C. M. & STEPHENS, N. L. (1973). Oxidative phosphorylation in frog skeletal muscle mitochondria. *Proc. Can. Fedn Biol. Socs* **16**, 34 (abstract 136).

SNEDECOR, G. W. & COCHRAN, W. G. (1967). *Statistical Methods*, 6th edn., pp. 135–171. Ames, Iowa, U.S.A.: Iowa State University Press.

WILKIE, D. R. (1968). Heat work and phosphorylcreatine break-down in muscle. *J. Physiol.* **195**, 157–183.

WOLEDGE, R. C. (1971). Heat production and chemical change in muscle. *Prog. Biophys. mol. Biol.* **22**, 37–74.

WOLEDGE, R. C. (1972). *In vitro* calorimetric studies relating to the interpretation of muscle heat experiments. *Cold Spring Harb. Symp. quant. Biol.* **37**, 629–634.