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ENERGY BALANCE IN DNFB-TREATED AND UNTREATED FROG MUSCLE

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SUMMARY

1. Heat production and chemical changes were measured in untreated and dinitrofluorobenzene (DNFB)-treated muscles during isometric tetani. Levels of total creatine (C_t), free creatine, ATP, ADP, AMP, inorganic phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, pyruvate, phosphoenolpyruvate, and lactate were measured. Changes in inosinic acid (IMP) were also measured.

2. DNFB effectively inhibited the creatine kinase reaction (Lohmann reaction).

3. Our major finding is that even after effective treatment with DNFB the observed heat + work after 2 sec and 5 sec of stimulation is significantly greater than the enthalpy change produced by the measured chemical changes. This confirms that an unidentified exothermic process occurs during muscle contraction; this conclusion was reached previously from studies of untreated muscle.

4. The unexplained heat + work is unlikely to be derived from glycolytic reactions since under anaerobic conditions no formation of lactate, pyruvate, phosphoenolpyruvate or fructose-1,6-diphosphate could be detected in either untreated or DNFB-treated muscles even 34 sec after a series of three 5 sec isometric tetani.

5. In the first 2 sec of stimulation the unexplained heat + work is less in DNFB-treated muscles than in untreated muscles. However from 2 to 5 sec of stimulation the unexplained heat + work is the same in treated and untreated muscles.

INTRODUCTION

Energy balance studies of untreated muscles have shown that more heat + work is produced than can be explained by the reactions that are believed to be involved. This discrepancy has been observed from moment to moment during isometric contractions (Gilbert, Kretzschmar, Wilkie &

Woledge, 1971; Homsher, Rall, Wallner & Ricchiuti, 1975) and it is still evident 3 sec after stimulation has ended and tension has fallen back to its resting level (Curtin & Woledge, 1974). Some unexplained heat + work is also produced during isovelocity shortening (Gilbert, Kretzschmar & Wilkie, 1972; Curtin, Gilbert, Kretzschmar & Wilkie, 1974). An unidentified exothermic reaction which occurs under all these conditions must contribute the unexplained part of the heat + work.

The unidentified reaction is not the creative kinase reaction,

$$\text{phosphocreatine} + \text{ADP} \rightarrow \text{creatine} + \text{ATP}$$

because it has been included in the energy balance and because it is endothermic, that is, it absorbs heat when it proceeds in this direction. However, there is some reason to suspect that the unidentified reaction may be involved with this reaction. If the endothermic creatine kinase reaction is blocked by DNFB treatment (Cain & Davies, 1962; Infante & Davies, 1965), and if no other reaction is influenced, the amount of heat + work would be expected to be greater than that produced by untreated muscles. However, Aubert (1964) found that the steady rate of heat production was, in fact, decreased by 30% after DNFB treatment. This result could be explained if DNFB had blocked not only the endothermic creatine kinase reaction, but also the unidentified exothermic reaction. The evidence for this is not conclusive since chemical measurements were not made; the decrease in heat output may have been due simply to a decrease in the extent of an exothermic reaction which is known to occur under normal conditions, such as ATP splitting. To clarify this point we have made an energy balance study of untreated and DNFB-treated muscles. A preliminary experiment was done to determine whether glycolytic reactions would be an important factor in the energy balance. In addition to the usual measurements of phosphocreatine, ATP and inorganic phosphate, the levels of ADP, AMP, and IMP were measured since it has been shown that these change in DNFB-treated muscles (Dydyńska & Wilkie, 1966; Kushmerick & Davies, 1969).

METHODS

The experiments were done on sartorius muscles of frogs (*Rana temporaria*) at 0° C. The Ringer solution contained (m-mole/l.): NaCl, 106; KCl, 2·0; $CaCl_2$, 1·8 and $NaHCO_3$, 10 and was gassed with either 5% CO_2 in O_2 or 5% CO_2 in N_2. DNFB treatment involved soaking the muscle in DNFB (0·38 m-mole/l. Ringer solution) at 0° C for 40 min immediately before use of the muscle. In all the experiments the stimuli were condenser shocks of alternating polarity, 27 V, 0·04 μF for each muscle, frequency 10 pulses/sec.

Chemical experiments

Anaerobic conditions were used in the experiment on glycolytic recovery and each pair of muscles was either untreated or DNFB-treated. One muscle of each pair remained unstimulated (control, *C*). The other muscle (experimental, *E*) performed three 5 sec isometric tetani at l_0. The interval between successive tetani was 25 sec and both muscles were frozen 99 sec after the start of the first tetanus.

In the energy balance experiment, some of the muscles were used for heat measurements, which will be described later, and the others were used for chemical measurements. Each pair of muscles was either untreated and oxygenated or was DNFB-treated and anaerobic. In the chemical experiment each muscle was either stimulated under isometric conditions at l_0 for 5 sec (5) or for 2 sec (2) or remained unstimulated (0). Paired muscles (*E vs. C*) were used in one of three ways: 2 *vs.* 0, 5 *vs.* 0, or 5 *vs.* 2. The moment of freezing was set so that the muscles were frozen at the last stimulation. In the case of the 5 *vs.* 2 experiment the onset of stimulation was set so that both muscles were frozen simultaneously. In all the chemical experiments tension was recorded and the muscles were frozen on the hammer apparatus (Kretzschmar & Wilkie, 1969; Kretzschmar, 1970). Extracts were prepared as described by Dydyńska & Wilkie (1966). The amounts of free creatine (Cr) and total creatine (C_t) were measured by the method of Ennor (1957), and ATP, ADP, AMP, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (F-1,6-P), pyruvate, phosphoenolpyruvate (PEP), lactate and inorganic phosphate (P_i) were measured by the enzymic methods described by Scopes (1972*a*, *b*).

The change in the level of IMP was determined from the optical density (O.D.) at 249 and 260 nm. If we could assume that the adenine nucleotides and IMP are the only compounds which absorb at these wave-lengths, the actual amounts of these compounds could be found from the observed O.D. However, this assumption was not justified because the DNFB in the extracts of the treated muscles also absorbs at these wave-lengths. Comparison of extracts of DNFB-treated and untreated muscle suggests that 10–20% of the O.D. of the extracts of treated muscles at both test wave-lengths was due to DNFB. We tried to determine the amount of DNFB in each extract using the O.D. at a third wave-length, 240 nm, but this was not successful because there appeared to be some other substance, besides adenine nucleotide, IMP and DNFB, which absorbs at 240 nm. This prevented us from determining the absolute levels of these compounds. We were nevertheless able to determine the difference between the IMP contents of the experimental and control muscles because it could be shown that both muscle extracts contained the same amounts (per unit C_t) of the interfering materials. They thus provide a constant base line against which any conversion of AMP to IMP can be detected. To establish that the amounts of the interfering materials were indeed constant we made use of the fact that the *true* amount of (adenine nucleotide + IMP)/C_t should be the same in the two muscle extracts, experimental and control. Thus any difference between the *estimated* amount of (adenine nucleotide + IMP)/C_t for the two muscles, would point to a difference in the amount of the interfering materials. In fact we found no evidence of such a difference, Δ (adenine nucleotide + IMP)/C_t = 0·003 ± 0·005, mean ± S.E., n = 41.

To test the accuracy of our determination of ΔIMP/C_t we made a series of measurements on 'internal standards', that is, known amounts of IMP added to aliquots of muscle extract. When ΔIMP/C_t was determined from the O.D. measurements on the (extract + IMP) and the extract alone, the ΔIMP/C_t was 91·6% ± 1·3 (mean ± S.E., n = 12) of the expected value. This seems acceptable since the inaccuracy is small and makes a very minor contribution to the energy balance calculation.

Extent of reaction. According to the 'textbook' description of metabolism during brief contractions, the reactions (in schematic form) which are important in the energy balance are:

ATP splitting:

$$\text{ATP} \rightarrow \text{ADP} + \text{P}_\text{i}, \tag{1}$$

creatine kinase reaction:

$$\text{PCr} + \text{ADP} \rightarrow \text{Cr} + \text{ATP}, \tag{2}$$

adenylate kinase reaction:

$$2\text{ADP} \rightarrow \text{ATP} + \text{AMP}, \tag{3}$$

adenylate deaminase reaction:

$$\text{AMP} \rightarrow \text{IMP} + \text{NH}_3, \tag{4}$$

glucose-1-phosphate formation:

$$(\text{C}_5\text{H}_{10}\text{O}_5)_n + \text{P}_\text{i} \rightarrow \text{G-1-P} + (\text{C}_5\text{H}_{10}\text{O}_5)_{n-1}. \tag{5}$$

In most previous energy balance studies only reactions (1) and (2) were considered and in some studies reaction (2) has been assumed to proceed to equilibrium. In this simple case the extents of reaction (1) and (2), the values needed for the energy balance calculation, are equal to the measured change in the level of PCr. However, when all five reactions occur they form a network; the products of one reaction are the reactants of one or more other reactions. In this more complex case the extent of a reaction cannot be determined from the measured change in a single compound. However, the extent of each reaction (ξ, units mole) can be determined by combining the measured chemical changes as shown in the following equations.

The extent of ATP splitting was estimated in three ways. The first was based on the change in ATP level.

$$\xi_{1a} = -\Delta\text{ATP} + \Delta\text{AMP} + \Delta\text{IMP} - \Delta\text{PCr}. \tag{6}$$

The second was based on the change in ADP level

$$\xi_{1b} = +\Delta\text{ADP} + 2\Delta\text{AMP} + 2\Delta\text{IMP} - \Delta\text{PCr}. \tag{7}$$

The third was based on the change in P_i level

$$\xi_{1c} = +\Delta\text{P}_\text{i} + \Delta\text{G-1-P} + \Delta\text{G-6-P} + \Delta\text{F-6-P}. \tag{8}$$

No significant differences were found between the extent of ATP splitting based on the changes in ATP level and that based on ADP level, ξ_{1a} and ξ_{1b}. They were therefore combined

$$\xi_{1ab} = -\Delta\text{PCr} - (\tfrac{1}{2})\Delta\text{ATP} + (\tfrac{1}{2})\Delta\text{ADP} + (\tfrac{3}{2})\Delta\text{AMP} + (\tfrac{3}{2})\Delta\text{IMP}. \tag{9}$$

The extents of the other reactions were determined from the following equations:

$$\xi_2 = -\Delta\text{PCr}, \tag{10}$$

$$\xi_3 = +\Delta\text{AMP} + \Delta\text{IMP}, \tag{11}$$

$$\xi_4 = +\Delta\text{IMP}, \tag{12}$$

$$\xi_5 = +\Delta\text{G-1-P} + \Delta\text{G-6-P} + \Delta\text{F-6-P}. \tag{13}$$

The sign convention was that the extent of reaction has a positive sign if the reaction proceeds in the direction given above. The S.E. of each extent of reaction was calculated assuming that the measured chemical changes (ΔATP etc.) for an individual pair of muscles were not correlated.

For the purpose of performing statistical tests we needed to know the numbers of degrees of freedom associated with estimates obtained by combining various means. These were calculated using the formulae given in Documenta Geigy Scientific Tables (p. 172, 1962).

Rejection of data. A chemical measurement was rejected if it was more than 2 standard deviations away from the mean of the group. The S.D. and mean used in this test were calculated including the value thought to be aberrant. In the chemical experiments, of 683 otherwise satisfactory values, forty-five were rejected in this way.

Best estimates. In the energy balance experiment we have, for each experimental pattern, both a direct (ξ_d) and an indirect (ξ_i) estimate of the extent of each reaction. For example, the indirect estimate of ξ for 5 *vs.* 0 is the sum of the direct estimates of ξ for 2 *vs.* 0 and for 5 *vs.* 2. We have combined these direct and indirect estimates into a best estimate, ξ_b. Since we found no significant differences between the variances of the direct estimates, $V_{2\ vs\ 0}$, $V_{5\ vs\ 0}$, and $V_{5\ vs\ 2}$, all of them were taken as estimates of the same population variance, V_p. The best estimate of the extent of reaction is thus given by

$$\xi_b = (2/3)\xi_d + (1/3)\xi_i.$$

The variance of ξ_b is $(2/3)\ V_p$.

The derivation of these formulae, for the more general case in which the variances of the direct estimates are significantly different, is given by Curtin *et al.* (1974).

Heat experiments

These measurements paralleled the chemical experiments for the energy balance. Each pair of muscles was either untreated and oxygenated or DNFB-treated and anaerobic. Both muscles of a pair were stimulated simultaneously for 5 sec. Each pair was stimulated only once and tension was recorded. Heat was measured with a thermopile as described by Dickinson & Woledge (1973). After the experiment the muscles were removed from the thermopile and frozen, and their weights and total creatine content were determined. The heat production records were corrected in the usual way for heat loss and thermopile lag and for the heat produced by the stimuli. For these experiments it was necessary to calculate the amount of thermoelastic heat production and the work done by the muscle. The thermoelastic heat calculation was made using a value of 0·01 for the ratio of thermoelastic heat to tension × muscle length. This is the average value found by Woledge (1961, 1963). The work done against the series elasticity of the muscle itself was calculated from the data of Jewell & Wilkie (1958), and that done against the compliance in the connexions and the transducer was calculated from the observed stress–strain curve of the apparatus.

RESULTS

Onset of glycolytic recovery

The possibility exists that glycolytic recovery after stimulation could start sooner and proceed faster in DNFB-treated than in untreated muscle. If so glycolytic reactions might contribute significantly to the heat + work production even in brief contractions and therefore have to be considered in an energy balance study. To investigate this possibility we performed an experiment in which muscles, under anaerobic conditions and either untreated or DNFB-treated, were tetanized three times, for 5 sec, with

25 sec intervals between tetani and then frozen after a further 34 sec. Control muscles remained unstimulated. The chemical changes observed in these experiments are shown in Table 1. As expected a considerable decrease in PCr was observed in the untreated muscles, but there was no significant change in the case of DNFB-treated muscles. This indicates the creatine kinase was effectively inhibited by DNFB treatment. Little evidence for glycolytic recovery was found in either DNFB-treated or untreated muscles. No significant formation of lactate, pyruvate, PEP, or F-1,6-P was detected. In untreated muscles small but significant amounts

TABLE 1. Chemical changes in untreated and DNFB-treated muscles

The chemical changes (Δ in n-mole/μmole C_t) were determined by comparison of paired muscles (*E–C*) one of which, *C*, was not stimulated, the other, *E*, performed three 5 sec isometric tetani with an interval of 25 sec between tetani. The muscles were frozen 99 sec after the start of the first tetanus. A positive sign indicates an increase in the amount of the substance concerned. PCr is phosphocreatine, C_t is total creatine (PCr + free creatine), G-1-P is glucose-1-phosphate, G-6-P is glucose-6-phosphate, F-6-P is fructose-6-phosphate, F-1,6-P is fructose-1,6-diphosphate, PEP is phosphoenolpyruvate, Pyr pyruvate, and Lac is lactate

	ΔPCr	ΔIMP	ΔG–1–P	ΔG–6–P	ΔF–6–P	ΔF–1,6–P	ΔPEP	ΔPyr	ΔLac
Untreated									
mean	−168·5	2·0	0·9	3·7	1·6	0·3	0·2	0·2	0·7
S.E.	25·9	3·1	0·2	0·5	0·3	0·5	0·2	0·2	0·7
n	8	8	7	6	6	8	7	7	7
DNFB-treated									
mean	0·1	40·4	0·2	0·6	0·2	2·7	−0·2	0·4	0·1
S.E.	6·2	4·5	0·9	6·1	1·7	1·6	0·3	0·4	1·5
n	7	6	7	7	7	7	7	7	6

of hexose monophosphates were formed. In DFNB-treated muscles no significant changes in these compounds were seen. DNFB probably increases glycolytic activity in resting anaerobic muscle since resting DNFB-treated muscle contained significantly more G-6-P, F-1,6-P and lactate than untreated muscles did. However, there is no evidence that following stimulation the glycolytic reactions proceed faster in DNFB-treated than in untreated muscle. As the amount of glycolysis is so small under these conditions, three tetani and a recovery period, it seems reasonable to assume that any contribution to the heat + work is negligible in our energy balance experiment, which involves only the first 5 sec of stimulation.

In this experiment we also measured the formation of IMP (Table 1). As expected a significant amount was formed in DNFB-treated muscle, but not in untreated muscle. Clearly, deamination is a process which must be considered in an energy balance study on DNFB-treated muscle.

Energy balance experiments

In the chemical experiments three types of comparison between paired muscles were made: 2 sec of stimulation with unstimulated (2 *vs.* 0), 5 sec of stimulation with unstimulated (5 *vs.* 0), and 5 sec of stimulation with 2 sec of stimulation (5 *vs.* 2). In the heat production experiments the

TABLE 2. Chemical changes in untreated and DNFB-treated muscles during stimulation under isometric conditions at l_o

The chemical changes (Δ in n-mole/μmol C_t) were determined by comparison of paired muscles (E–C); 2 *vs.* 0 indicates that the experimental muscle (*E*) was stimulated for 2 sec and the control muscle (*C*) was not stimulated; 5 *vs.* 0 indicates that *E* was stimulated for 5 sec and *C* was not stimulated; 5 *vs.* 2 indicates that *E* was stimulated for 5 sec and *C* for 2 sec. All stimulated muscles were frozen at the last stimulus, before relaxation began. A positive sign indicates an increase in the amount of the substance concerned. ΔIMP was not measured in the untreated muscles. *Since hexose monophosphatases were not measured for untreated muscles 2 *vs.* 0, the indirect estimates are given. Indirect Δ for 2 *vs.* 0 = (measured Δ for 5 *vs.* 0) − (measured Δ for 5 *vs.* 2)

	ΔPCr	ΔATP	ΔADP	ΔAMP	ΔIMP	ΔP_i	ΔG–6–P	ΔG–1–P	ΔF–6–P
Untreated									
2 *vs.* 0 mean	−31·4	−0·9	5·2	−3·3	—	−1·3	0·9*	0·3*	−0·2*
S.E.	6·1	8·7	4·8	2·9	—	12·0	0·7	0·3	0·4
n	8	8	7	6	—	9			
5 *vs.* 0 mean	−36·1	4·0	1·0	−1·2	—	37·5	0·4	0·4	0·3
S.E.	12·6	4·3	1·2	0·5	—	12·5	0·6	0·2	0·2
n	8	8	9	8	—	8	8	8	8
5 *vs.* 2 mean	−36·4	−0·6	−3·5	−2·3	—	27·7	−0·5	0·1	0·5
S.E.	10	4·2	2·1	1·1	—	19·3	0·4	0·2	0·4
n	8	8	8	8	—	8	6	6	6
DNFB-treated									
2 *vs.* 0 mean	−5·7	−21·0	4·9	5·2	3·5	2·7	−0·9	−0·8	−1·2
S.E.	3·6	7·8	3·2	1·1	4·6	10·3	1·2	0·4	0·7
n	13	14	11	10	14	13	12	13	12
5 *vs.* 0 mean	0·4	−15·5	7·9	8·8	6·0	37·5	1·3	0·4	0·5
S.E.	3·0	5·3	3·1	1·7	2·4	6·6	1·4	0·2	0·5
n	15	15	14	14	16	15	14	14	14
5 *vs.* 2 mean	2·0	−3·0	−1·7	4·5	−2·3	−1·7	−0·1	0·6	0·8
S.E.	5·1	3·9	2·6	1·6	3·9	6·0	1·3	0·4	0·5
n	13	14	13	10	13	14	11	11	10

heat + work produced was determined at 2 sec and at 5 sec. The experiments were performed with both untreated and DNFB-treated muscles. The mean value of the tension $[(Pl_0)/C_t]$ at 1, 2 and 5 sec was the same in untreated and DNFB-treated muscle. The chemical changes observed are shown in Table 2. The extents of the various reactions were determined from these observed changes as described in the Methods and are shown in Table 3*A*. The results clearly show that creatine kinase was inhibited by

TABLE 3

A, best estimates of the extents of reactions during contraction of untreated and DNFB-treated muscles The reactions are:

ATP splitting, $ATP \rightarrow ADP + Pi$, (1)

creatine kinase, $PCr + ADP \rightarrow Cr + ATP$, (2)

adenylate kinase, $2\ ADP \rightarrow ATP + AMP$, (3)

adenylate deaminase, $AMP \rightarrow IMP + NH_3$, (4)

formation of glucose-1-phosphate, $(C_6H_{10}O_5)_n + Pi \rightarrow (C_6H_{10}O_5)_{n-1} + G\text{-}1\text{-}P$. (5)

A positive sign indicates that the reaction proceeded in the direction shown.
The extent of reaction (1) determined from the change in the ATP level, ξ_{1a}, and the change in ADP level, ξ_{1b}, is

$$\xi_{1ab} = (\xi_{1a} + \xi_{1b})/2.$$

The extent of reaction (1) determined from the change in P_i level is ξ_{1c}. See the methods for the appropriate equations for calculating the extents of the reactions and their S.E. The three best estimates for a particular reaction have the same S.E. because the best estimates were calculated assuming that the direct estimates had equal S.E. (see methods).

B, the molar enthalpy changes (ΔH_m) determined from *in vitro* calorimetric measurements. A negative sign indicates that heat is produced when the reaction proceeds as shown above. Sources of ΔH_m values: for reactions (1) and (2), Woledge (1972), and for reactions (3), (4), and (5) Woledge (1971).

C, the enthalpy change produced by each reaction is the product of the extent of reaction, ξ, and the molar enthalpy change, ΔH_m. A negative sign indicates heat + work is produced by the reaction which occurred in the muscle.

A Extent of reaction (n-mole/μmole C_t)

	ξ_{1ab}		ξ_{1c}		ξ_1		ξ_2		ξ_3		ξ_4		ξ_5	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Untreated														
2 *vs.* 0	20·1		3·4		11·8		20·8		−1·8		—		1·0	
5 *vs.* 0	42·2	8·9	34·9	12·2	38·6	7·6	46·7	8·1	−2·7	1·5	—		1·1	0·6
5 *vs.* 2	22·1		31·5		26·8		25·8		−0·8		—		0·1	
DNFB-treated														
2 *vs.* 0	31·6		13·2		22·4		4·3		10·0		5·1		−1·6	
5 *vs.* 0	33·6	6·5	26·3	6·5	29·9	4·6	1·0	3·3	13·5	3·3	4·4	3·1	0·9	1·2
5 *vs.* 2	1·9		13·0		7·5		−3·4		3·5		−0·7		2·6	

B Molar enthalpy change ΔH_m for reaction (kJ/mole reaction)

Reaction	(1)	(2)	(3)	(4)	(5)
ΔH_m	−48	+14	−6	−12	−12

C Enthalpy changed produced by reaction (μJ/μmole C_t)

Reaction	(1)		(2)		(3)		(4)		(5)	
$\Delta H_m \times \xi$	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Untreated										
2 *vs.* 0	−566		+291		+11		—		−12	
5 *vs.* 0	−1853	365	+654	113	+16	9	—		−13	7
5 *vs.* 2	−1286		+361		+5		—		−1·2	
DNFB-treated										
2 *vs.* 0	−1075		+60		−60		−61		+19	
5 *vs.* 0	−1435	221	+14	46	−81	20	−53	37	−11	14
5 *vs.* 2	−360		−48		−21		+8		−31	

DNFB treatment, since in treated muscles, ξ_2, the extent of the creatine kinase reaction is not significantly different from zero, although a substantial and significant amount of ATP splitting, ξ_1, was detected. Another difference between the untreated and treated muscles was found in the extent of the adenylate kinase reaction, ξ_3, which is significant in the treated muscles, but not in the untreated muscles.

TABLE 4. The energy balance: a comparison of the heat + work which is produced and the enthalpy change due to chemical reactions which occur during contraction. The observed heat + work was measured as described in the methods. A negative sign indicates that heat + work is produced by muscle. The explained enthalpy change is the sum of the enthalpy changes given in Table 3*C* for individual reactions. The difference between the observed heat + work and the explained enthalpy change is given in the last section of the Table. A negative sign indicates that more heat + work is produced by the muscle than can be explained by the enthalpy changes of the observed reaction. We have tested the significance of the difference between zero and the values and give the *P* values.

	Observed (h + w)			Explained enthalpy change		Observed (h + w) − Explained enthalpy change		
	mean	S.E.	*n*	mean	S.E.	mean	S.E.	*P*
	μJ/mole C_i			μJ/μmole C_i		μJ/μmole C_i		
Untreated								
2 *vs.* 0	−1880	110		−276		−1604	398	< 0·001
5 *vs.* 0	−3340	220	6	−1196	382	−2144	441	< 0·001
5 *vs.* 2	−1458	115		−921		−537	399	0·20
DNFB-treated								
2 *vs.* 0	−1710	100		−1117		−593	251	0·02
5 *vs.* 0	−2930	200	10	−1566	230	−1364	305	< 0·001
5 *vs.* 2	−1220	100		− 452		−768	251	< 0·005

As described in Methods the amount of ATP splitting can be estimated in three ways; it can be based on the change in ATP level, ξ_{1a}, or based on the change in ADP level, ξ_{1b}, or based on the change in P_i level, ξ_{1c}. The estimates ξ_{1a} and ξ_{1b} are in good agreement, and in the Table only their mean, ξ_{1ab}, is given. The mean ξ_{1ab} is larger than the mean ξ_{1c} for the 2 *vs.* 0 and 5 *vs.* 0 experiments on both untreated and DNFB-treated muscles; although these differences are not significant. These discrepancies could be due to an extra reaction involving ADP and ATP, or P_i; this would, of course, be very important. If such a reaction were occurring, a more sensitive measure of it would be the difference, $\xi_{1ab} - \xi_{1c}$, determined for each muscle pair. This quantity was determined for each pair for which we had measured all the relevant chemical changes. Taking all the results for pairs with resting controls, the difference, $\xi_{1ab} - \xi_{1c}$, was not significant,

$-4{\cdot}5 \pm 5{\cdot}8$ ($n = 32$, $P = 0{\cdot}45$). Since this more sensitive test did not give any evidence for the existence of an extra reaction involving ADP and ATP, or P_i, we have averaged the mean ξ_{1ab} and mean ξ_{1c} to determine the extent of ATP splitting, ξ_1.

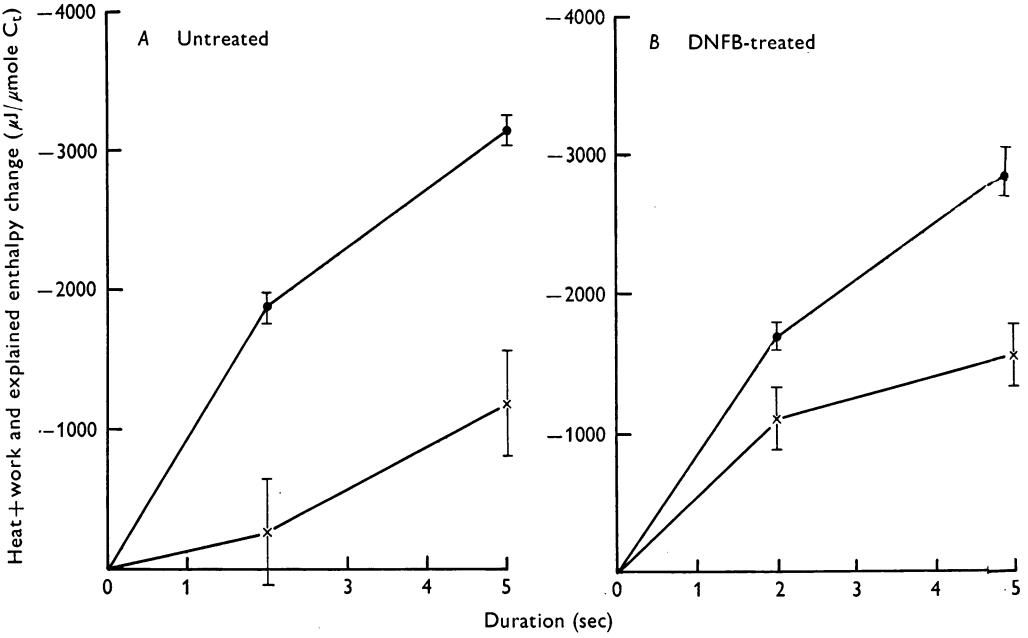

Fig. 1. Heat + work produced during an isometric tetanus of untreated muscle (*A*) and DNFB-treated muscle (*B*). The observed quantities of heat + work (Table 4) are shown as (●). The explained enthalpy change, [$= \Sigma(\Delta H_m \times \xi)$, Table 4] are shown as (×). A negative sign indicates that heat + work was produced by the muscle. The bars show ± 1 S.E.

The first step in the energy balance calculation is shown in Table 3*C*. The values given there show the enthalpy change produced by the amount of reaction which occurred in the muscle. Each value is the product of an extent of reaction, given in part *A* of the Table, and the appropriate ΔH_m shown in part *B*. The total explained enthalpy change, the sum of these terms for the five reactions considered, is shown in Table 4. In most cases the largest term in the sum is that due to ATP splitting; however in untreated muscles the contribution from the endothermic creatine kinase reaction is also important. The enthalpy changes from the other reactions make only a minor contribution to the total. Table 4 shows also the observed heat + work, which in every case is greater than the explained enthalpy change (see Fig. 1). The differences, the unexplained heat + work,

their standard errors and P values are shown in Table 4 and Fig. 2. In both untreated and treated muscles there is a significant amount of unexplained heat + work after 2 sec and after 5 sec of stimulation. Our result for untreated muscle is thus in agreement with earlier work

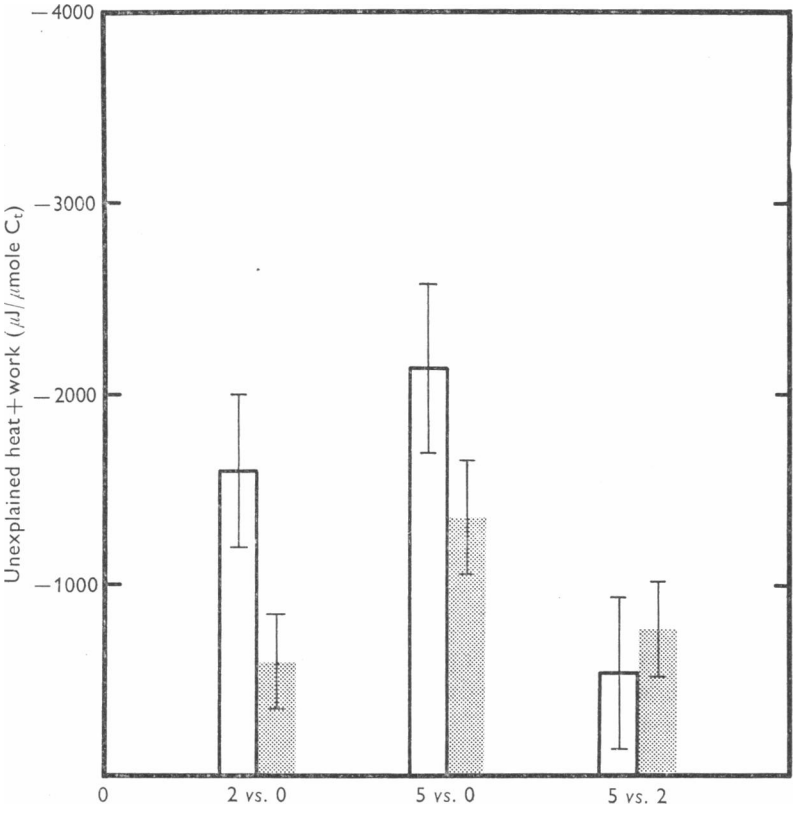

Fig. 2. A comparison of the unexplained heat + work in untreated and DNFB-treated muscle. Each block represents the unexplained heat + work from Table 4 for one type of experiment. The unshaded blocks are for untreated muscle and the shaded blocks are for DNFB-treated muscle. The bars show ± 1 S.E.

(Gilbert *et al.* 1971; Gilbert *et al.* 1972; Curtin & Woledge, 1974; Curtin *et al.* 1974) in indicating that a substantial amount of heat + work is derived from an unidentified source. We can now add that the same conclusion is also true for DNFB-treated muscle. The results also show that the mean amount of unexplained heat + work is less in treated than in untreated muscle (Fig. 2).

DISCUSSION

In untreated muscle we found that a large part of the heat + work produced during isometric contraction cannot be accounted for by the identified chemical reactions. This confirms the original finding of Gilbert *et al.* (1971). Our experiments are, however, more comprehensive in that we have analysed the muscle extracts for more compounds and included more reactions in the energy balance calculation. Thus we can definitely establish that the unidentified reaction is not the adenylate kinase, adenylate deaminase or glycolytic reactions. Another new aspect of our energy balance calculation is that we have used the calorimetrically-determined ΔH_m values which are now available. If we recalculate the energy balance using the results of Gilbert *et al.* for a 2 sec isometric tetanus and these ΔH_m values, the amount of unexplained heat + work is -2026 ± 351 μJ/μmole C_t, which is larger than, but not significantly different from the results reported here. Similar results have been obtained by Homsher *et al.* (1975) using *Rana temporaria*; although they found considerably less unexplained heat + work in similar experiment with *R. pipiens*. It thus seems well established from studies of untreated muscle that an unidentified exothermic process occurs during contraction. Our results show that even after DNFB-treatment this unidentified process continues to contribute a significant part of the heat + work produced by the muscle. Since the energy balance for DNFB-treated muscle is largely independent of the measured ΔPCr and the ΔH_m for the creatine kinase reaction, the apparent existence of an unidentified exothermic process could not be due solely to an error in the measurement of these quantities.

Possible errors in the energy balance calculation

The ΔH_m values used in our energy balance calculation include contributions from the interaction of a buffer with the H^+ ions absorbed or released by each reaction. We have taken the imidazole group in carnosine and in proteins to be the buffering group since it has the appropriate pK and is present in reasonable quantity (Woledge, 1971). It may seem that if some other process were acting as the buffering reaction that it could be the unidentified reaction responsible for the excess heat + work. Although quantitatively it is most unlikely for this to be the explanation, the results of previous experiments could not exclude it. The new results for DNFB-treated muscle can now rule out this possibility. Reaction (1), ATP splitting, *produces* 1/2 mole H^+ ions per mole of reaction, and reaction (2), the creatine kinase reaction, *absorbs* 1 mole H^+ ions per mole reaction. In

untreated muscle the net effect of reactions (1) and (2) is to *absorb* H^+ ions which are provided by the buffer. In DNFB-treated muscle, reaction (2) is prevented and reaction (1) *produces* H^+ ions which the buffer must absorb. The buffer reaction must proceed in opposite directions in untreated and in DNFB-treated muscle so its heat of ionization cannot explain the fact that there is a significant amount of excess heat + work in both untreated and DNFB-treated muscle.

A possible source of error in these energy balance studies is the occurrence in the muscle of reactions, other than reactions (1) to (5), involving ATP, ADP, or P_i. Such reactions might produce systematic errors in the estimate of the extent of ATP splitting, ξ_1. For example, if a protein kinase catalysed reaction occurred, in which ATP was a reactant and ADP a product, ξ_{1ab} would over-estimate the amount of ATP splitting, but ξ_{1c} would be unaffected. Another possibility is that a phosphorylase-catalysed reaction might occur in which P_i would be a reactant. In this case ξ_{1c} would underestimate the amount of ATP splitting, but ξ_{1ab} would be unaffected. We have not found any convincing evidence that the mean values of ξ_{1ab} and ξ_{1c} are significantly different, but the possibility of this type of error remains. To examine the consequences of this possibility we have calculated the energy balance using only ξ_{1ab} as a measure of ATP splitting, and again using only ξ_{1c}. In either case there remains a significant amount of unexplained heat + work in both untreated and DNFB-treated muscle. In either case we can also conclude, as described later, that there is significantly less unexplained heat + work in DNFB-treated muscles than in untreated muscles. However the actual amounts of unexplained heat + work and the time course of its appearance are somewhat affected by which estimate is used for the extent of ATP splitting. Thus our conclusions about the presence of unexplained heat + work and the action of DNFB on it are unaffected by the possible occurrence of such a kinase or phosphorylase reaction.

The effect of DNFB treatment on the unexplained heat + work

The simplest hypothesis about DNFB treatment is that it has only one action, that of preventing the rephosphorylation of ADP by the creatine kinase reaction. Since this process absorbs heat, the hypothesis requires that DNFB treatment should increase the total heat + work production, but should not change the amount of unexplained heat + work. The results, however, do not bear out either of these predictions. The amount of total heat + work is not changed significantly by DNFB treatment (Table 4, Fig. 1 *A* and *B*), and the amount of unexplained heat + work is reduced by DNFB treatment. During the first 2 sec of stimulation significantly less unexplained heat + work is produced by treated than untreated muscles

($P < 0{\cdot}05$, Fig. 2). A similar, although not significant, difference is seen in the 5 *vs.* 0 experiments. It seems that the difference between treated and untreated muscles does not change in the period between 2 sec and 5 sec of stimulation; this is supported by the fact that there is no difference between the 5 *vs.* 2 results for treated and untreated muscle (Fig. 2). Accepting this, the differences for 2 *vs.* 0 and 5 *vs.* 0 can be combined to give a better estimate of the amount by which DNFB treatment reduces the unexplained heat +work. The resulting value, -896 ± 357 μJ/μmole C_t, is significantly different from zero ($P < 0{\cdot}02$). Thus we conclude that, in addition to stopping the rephosphorylation of ADP by PCr, DNFB has a second energetically significant effect on muscle; it reduces the amount of unexplained heat +work produced by the muscle.

One possibility is that DNFB prevents a reaction which is stoichiometrically linked to the creatine kinase reaction. Any reaction involving creatine or phosphocreatine or participating H^+ ions would be an example of this. The creatine kinase reaction occurs at an approximately steady rate during contraction, and it is completely blocked by DNFB treatment. These characteristics should apply to any process stoichiometrically linked to the creatine kinase reaction. However, DNFB alters the amount of unexplained heat + work produced during the first 2 sec, but it does not influence that released during the next 3 sec of contraction; the effect of DNFB is not to block a steady source of heat + work. This makes it unlikely that a reaction, stoichiometrically linked to the creatine kinase reaction, is blocked.

Since DNFB reacts with proteins, it could reduce the unexplained heat + work by preventing an exothermic change in a protein. As DNFB certainly reacts with creatine kinase the question arises whether an exothermic change in creatine kinase could produce enough heat to explain our observations. DNFB reduced the unexplained heat + work by about -900 μJ/μmole C_t. Rabbit muscle contains 1·5 n-mole creatine kinase/μmole C_t (Kuby, Noda & Lardy, 1954). Assuming this figure to apply to frog muscle we can calculate that the ΔH_m of the change in creatine kinase would have to be -600 kJ/mole to explain our results; this value seems impossibly large. It is thus unlikely that DNFB reduces the unexplained heat + work by preventing an exothermic change in creatine kinase.

It is known that DNFB can react with myosin (Bárány & Bárány, 1970). A generous estimate of the myosin content of frog muscle (Perry, 1967) is 8 n-mole of myosin/μmole C_t. For a change in myosin to explain our results, the ΔH_m would have to be -112 kJ/mole. This value seems possible since Yamada, Shimizu & Suga (1973) have shown that myosin undergoes an exothermic change on binding of ATP or ADP which produces about -200 kJ/mole. Thus the reduction of the unexplained heat + work by

DFNB could be explained if DNFB stops an exothermic change in a protein, possibly myosin.

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