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CHEMICAL CHANGE AND ENERGY OUTPUT DURING MUSCULAR CONTRACTION

By CLAUDE GILBERT, K. M. KRETZSCHMAR, D. R. WILKIE and R. C. WOLEDGE

From the Department of Physiology, University College London, W.C. 1

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SUMMARY

1. The production of heat and (internal) work and the changes in the amount of phosphocreatine (PCr), ATP, inorganic phosphate (Pi) and sometimes lactate have been measured from moment to moment during and after tetanic isometric contractions of isolated frog muscles at 0° C.

2. Heat production was measured by thermopiles and a novel apparatus was employed for freezing the muscles rapidly at a chosen instant so as to halt the chemical processes before analysis.

3. Using unpoisoned muscles in oxygen, it was shown that neither oxidative recovery processes nor glycolytic ones led to appreciable restitution of PCr or ATP during 15 sec of contraction. However, clear signs of recovery processes could be seen within a minute. In our preparations artificial 'ageing' by storage at low temperature did not interfere with the capacity for glycolysis.

4. Our clearest result was that the break-down of PCr was not nearly large enough to account for the rapid heat production during the first few sec of contraction. By the end of a 15 sec tetanus as much as 10 mcal/g remained unaccounted for.

5. The source of this heat is not clear. At no time is there any sign of net break-down of ATP; indeed there appears to be a slight increase of ATP in the stimulated muscle.

6. Break-down of PCr continues both during relaxation and during the minute following, while the muscle is at rest. Thus during contraction there is heat production without PCr break-down, while subsequently there is PCr break-down without heat production.

INTRODUCTION

It is a truism that the physical energy resulting from muscular contraction must come from chemical processes, and the nature of these chemical processes has been under intensive study for the last 100 years. In the past

decade interest has centred on the chemical processes that occur during contraction itself. By employing quick-freezing techniques to halt the chemical processes, and inhibitors to simplify them, R. E. Davis and W. F. H. M. Mommaerts and their various collaborators have done much to elucidate what happens during the early stages of contraction. However, a number of puzzling features remain which we have tried to resolve by employing the method of energy balance. Over any specified period of time the output of physical energy, heat + work ($h+w$) should be accounted for by the concurrent chemical changes. Following the first law of thermodynamics

$$h+w = n_1(-\Delta H_1)+n_2(-\Delta H_2) \text{ etc.},$$

where the n are the numbers of moles of the various chemical processes that have occurred during this period, and the ΔH are their molar enthalpy changes. If such a balance can be shown to hold, then it is quite probable that the description of the chemical events is complete, though this is not necessarily so. On the other hand, if the balance does not hold, it most certainly shows that the account of the chemical and other processes is incomplete or incorrect. The physical and chemical techniques to some degree compensate for one another's disadvantages, the complete non-specificity of the heat measurements serving as a check on the adequacy of the various specific chemical tests that have been employed. It is chastening to reflect that at the present time there is not a single experimental demonstration of a satisfactory energy balance during contraction and relaxation (Woledge, 1971).

In the work mentioned above, no attempt was made to relate chemical determinations to experimental heat measurements, though in an important paper Kushmerick & Davies (1969) have related chemical change to work performance. Even under the most favourable circumstances less than half the physical energy appears as work, so for the purposes of energy balance, heat measurements are essential. For various reasons that will be described later it is evident that little can be gained from the comparison of chemical investigations in one laboratory with heat measurements in another: it is essential that both types of measurement be carried out simultaneously. The following paper describes our attempts to do so.

METHODS

Because a thermopile could not be incorporated in a quick-freezing apparatus, the thermal and chemical changes could not usually be measured with a single muscle pair. This problem was surmounted by measuring chemical and mechanical changes with some frogs while others taken at random from the same batch were used to measure the thermal and mechanical changes.

Heat and work measurements. For most experiments the heat was measured with a constantan-chromel thermopile (L 40) similar in general construction to those described by Hill (1965, chapter 8). However, the thermopile is longer than those described by Hill so that it is possible to record the heat production from almost the whole of the length of the muscles. Stimulation is by gold electrodes evaporated onto the thermopile surface. For the heat production measurements, both sartorius muscles were used, mounted in the usual way on either side of the thermopile. The recorded heat productions were corrected for heat loss and for thermopile lag in the usual way (Hill, 1965) and the stimulus heat was subtracted. When the heat + work was required at a time when the muscles had developed tension but not yet relaxed, it was necessary to make two further corrections, for the thermoelastic heat absorption (Woledge, 1961) and for the work done in stretching the series elasticity. Since the thermoelastic heat-tension ratio (R) is rather variable (Woledge, 1963) and could not be determined for each muscle, a low value of R (0·01) was assumed to avoid any over-estimate of the heat production. The work done against the series elasticity was calculated from the observed compliance of the apparatus and from the average muscle compliance given by Jewell & Wilkie (1958): it amounted to about 0·9 mcal/g. In the case of isotonic contractions, the work done in lifting the load was also added.

In some experiments, in which rapid heat measurements were not required, an integrating thermopile (Wilkie, 1968) was used. In all thermal and mechanical experiments the tension and length changes were recorded with suitable transducers (Jewell, Kretzschmar & Woledge, 1967).

Experimental procedure. Both sartorius muscles were dissected from each frog. For the chemical experiments only one (E) was stimulated while the other (C) served as a control. To allow full recovery from any activity occurring during dissection the muscles were bathed in oxygenated Ringer solution for 1 hr at room temperature. In most experiments the muscles were unpoisoned and in that case they were soaked in oxygenated Ringer for 40 min at 0° C before stimulation. In some cases, however, this soaking was in Ringer bubbled with oxygen-free nitrogen and containing 0·5 mM iodoacetate (IAA).

Arrest of metabolism. The investigation of the chemical changes during short contractions required the rapid arrest of metabolism, which was achieved by rapid cooling of muscle. Since contractions of only 0·5 sec duration were to be investigated, it was necessary that the muscles should be completely frozen within 100 msec or less. Quick-freezing apparatus for this purpose, in which a container of liquid coolant is automatically raised at an appropriate moment to immerse and freeze the muscles, has already been described by Cain & Davies (1964) and Mommaerts & Schilling (1964). We built an apparatus based on the same principle in which continuously stirred and cooled isopentane at about − 150° C was propelled upwards by a powerful steel spring. The fall of temperature in the muscle was recorded by carefully threading a fine thermojunction into the muscle in such a way that the junction regions lay near the middle of the muscle's thickness. With this apparatus the delay before freezing (indicated by a sudden increase in the rate of cooling) was 200–300 msec, in agreement with the measurement given by Cain *et al.* and Mommaerts *et al.* However, the rate of immersion cannot be made very high without running into difficulties over the deceleration of the coolant fluid. Even when a new apparatus was constructed in which the muscles, attached to their transducers, were propelled at very high speed vertically downwards into well-stirred coolant, the freezing delay was scarcely diminished. Theoretical calculations showed that given the shape, size, and thermal constants of the muscles they could hardly be expected to freeze in less than 150 msec. Further details are given by Kretzschmar (1970).

A new approach was consequently devised (Kretzschmar & Wilkie, 1969) which

overcomes some of the problems associated with rapid freezing. This hammer apparatus first flattens, then freezes a muscle pair between the faces of two moving aluminium blocks precooled to $-196°$ C in liquid nitrogen. The rate of freezing was measured by placing a muscle on each side of a polyester sheet (8 μ thick) on which a thermojunction lay. This method seemed to provide the most accurate measure of freezing time, and certainly yielded the largest (and therefore least suspect) estimate of this quantity: muscles were found to be completely frozen in $\sim$ 80 msec.

Great care is required in order to avoid certain errors which tend to cause an underestimate of freezing times. For example, inaccurate positioning or movement of the thermojunction during freezing and the greater thermal conductivity of the thermojunction leads compared to muscle tissue would both tend to underestimate freezing times.

The indirect method for estimating the time course of freezing from changes of electrical impedance is even more prone to error, since the rapid rise of impedance corresponds to the first moment at which the electrical path is interrupted by ice at some point or other. This bears little relationship to the time course of freezing of the muscle as a whole. Moreover the electrodes are commonly superficially placed and are far more exposed to the action of the coolant than is the centre of the muscle. Presumably it is for these reasons that unbelievably rapid freezing rates have been mentioned in the literature (see Kushmerick, Larsen & Davis, 1969 and Mommaerts & Wallner, 1967).

The biological consequences of using the hammer apparatus are presented under Results.

Extraction and analysis. The preparation of muscle extracts and the analyses for phosphocreatine, total creatine, ATP and lactic acid were performed as described previously by Dydyńska & Wilkie (1966). It was not possible to weigh the squashed and frozen muscles or to be completely certain that no fragments had been lost. For this reason all our quantitative results are expressed 'per unit of total creatine'. The use of the total creatine content as an estimator of muscle size can be justified on experimental grounds (Wilkie, 1968, p. 176).

Estimation of inorganic phosphate (Pi). Although a standard method, originally due to Wahler & Wollenberger (1958) was employed, and procedures were closely similar to those used by Kushmerick *et al.* (1969), it was found that the method gave rise to errors. We were greatly helped in detecting these errors and in improving the standard technique by Dr Kushmerick, who was working in our laboratory (see Kushmerick 1971).

The presence of phosphocreatine (PCr) in the assay mixture was found to interfere with the formation (or extraction) of the required inorganic phosphate/molybdate complex, possibly because PCr binds to the molybdate in competition with the Pi. The effect of this was to produce a 20 % underestimation of Pi present. Modification of the method avoids this error: the amount of molybdate used was increased fivefold so that it was in excess of not only the Pi but also the total PCr and Pi in the mixture.

The method of analysis inevitably requires a muscle extract to be in the presence of perchloric acid for a minute or so, and thus there is a danger of PCr-hydrolysis yielding Pi. Indeed, even after minimizing this period, a 3·5 % hydrolysis was found to occur. Since the concentration of PCr in resting muscle is about $8\times$ that of Pi, there was thus an overestimation of the Pi present of about 30 %. However, a Pi change (measured as the difference between the Pi contents of two muscles) will only be in error by $-3{\cdot}5$ % (if PCr and Pi changes are equal). There seems to be no modification to the method that would avoid this error; we have not made any correction for it.

During the course of the experimental work for this paper, the method for Pi

analysis was modified and improved. Thus many of the measurements were made using earlier methods which seemed to involve a 20% underestimation of both Pi contents and Pi differences. The probable errors associated with each set of results are given in detail in the Results section of this paper.

Statistical testing. Except where otherwise stated, statistical testing has been by a rigorous form of *t* test to determine the significance of the difference between sample means (see *Documenta Geigy Scientific Tables* 1962, p. 172). In this form of the *t* test quite a large allowance is made, by reduction of the total degrees of freedom, for any difference in the size, or in the standard deviation, of the two samples.

RESULTS

The first experimental results to be presented here will be concerned with validating certain aspects of our experimental technique and design, both of which differ from the procedures employed previously.

Quick-freezing: comparison of hammer apparatus and rapid immersion

The purely physical measurements to justify our advocacy of the hammer apparatus have been presented in the previous section. However, our chief interest is in the effectiveness of a given technique of freezing with regard to halting biochemical processes within the muscle, and permitting the various reactants to be taken unchanged into solution where they can be analysed.

Slow cooling might be expected to have at least three bad effects. It certainly leads to the formation of large ice crystals which disrupt the structure of the cell. In an active muscle chemical reactions will continue in the inner part for some time after the instant of immersion, which blurs the time-resolution of the procedure. Finally, it is probable that sudden chilling of a resting muscle itself stimulates muscle fibres; the less time available for the consequent chemical changes the better.

Effects on structure

Sections of muscles frozen by the hammer apparatus (*A* and *B*) and by cutting the pelvic tendon and immersing in isopentane chilled with liquid N_2 (*C* and *D*) are shown in Pls. 1 and 2. It is clear that the structure is well preserved in the former and not in the latter. One remarkable feature of the transverse section (*B*) is that the individual fibres are not conspicuously flattened in the squashed muscle. Evidently the lateral connective tissue is sufficiently weak to permit the fibres to become displaced sideways. The good preservation of the material, and the absence of voids caused by large ice crystals, suggests that the hammer technique might possibly be useful in histological investigation of contracting muscle.

In contrast, the sections of muscle frozen by immersion given an impression of disruption, and the large voids that are present indicate that slow

freezing is a major contributor to this disruption. Where the sarcomere length can be measured it has become reduced to about 1 μ, showing that at least some parts of the fibres have been activated even though the muscle as a whole has not shortened.

Biochemical effects

Since an unstimulated control muscle provides the base line from which the changes in the active muscles are estimated, it is important to try to assess what effect the freezing and extracting procedures have on a resting muscle. It is implicitly assumed this procedure will have the same action

TABLE 1. The levels of PCr and Pi in unstimulated muscle: the effects of freezing method and of poisoning. N.B. In this and subsequent Tables the dimensionless mole ratio (Pi/Cr) etc., can be converted approximately to μmole/(gram frozen weight) on the basis that resting muscles contain about 25 μmole/g of total (free creatine + phosphocreatine); thus μmole/g $\simeq$ mole ratio $\times$ 25

	PCr/Cr			Pi/Cr		
Experimental conditions	Mean	S.E.	N	Mean	S.E.	N
Muscle unpoisoned in O_2 Frozen by immersion, **Th**	0·823	0·0105	6	0·116	0·0044	6
Muscles poisoned with IAA and N_2 Frozen by immersion, **Th**	0·828	0·0087	6	0·141	0·0139	6
Muscle unpoisoned in O_2 Frozen by the hammer apparatus	0·876	0·0063	6	0·101	0·0030	6
Muscles poisoned with IAA and N_2 Frozen in the hammer apparatus	0·876	0·0094	5	0·094	0·0084	4

All experiments carried out 23. ix. 69–7. x. 69. Note that in this and subsequent Tables the chemical experiments were usually carried out on the hammer apparatus with heat measurements made independently on other frogs from the same batch. In some cases, indicated **Th**, experiments were carried out on a thermopile, the muscles being frozen later by immersion. In these cases heat and chemical change were estimated on the same muscles, though only the control observations are presented in this Table.

on the stimulated and on the control muscles, and the smaller the effect on the latter is found to be, the greater is the likelihood that our assumption is not leading to a large error.

This question was investigated by separating a single batch of twelve frogs into two groups, and freezing their unstimulated muscles by the two alternative techniques. Within each group half the muscles had been poisioned with IAA/N_2 while the other half were normal and in oxygen. The result, shown in Table 1, is clear-cut – the immersed muscles contain significantly less PCr and more Pi than the squashed ones. The null probability, P, is $< 0{\cdot}02$ in each case, and the results strongly suggest that about

1 μmole of extra PCr per gram is split when the muscle is frozen by immersion; the same effect is obtained no matter whether the muscles had been poisoned or not.

Of course, this does not prove that accidental splitting of PCr is completely absent when the muscles are frozen by squashing. Since the content of Pi is approximately equal to that of unphosphorylated creatine, the results in Table 1 are quite compatible with the view that in the resting muscle almost all the creatine present is phosphorylated but that the 2·5 μmole Pi/g observed in our experiments comes from the accidental break-down of an equivalent quantity of PCr during freezing and extraction. We have not been able to think of a way of testing this possibility directly.

The observed value of PCr/Cr in the extracts from resting muscle does thus not necessarily portray the true state of affairs in a resting muscle. Nevertheless, the higher this ratio is, the more confidence one can place in the experimental procedures. The value in Table 1, 0·876, is not quite the highest reported so far, for Carlson, Hardy & Wilkie (1967) (see also Wilkie, 1968, Table 3) obtained $0{\cdot}889 \pm 0{\cdot}0053$ (S.E., $N = 55$). In the majority of our experiments we, too, obtained higher values; for example, in the thirty-four resting muscles shown in Table 2, PCr/Cr was 0·902. This is but one example of the inconvenient but undeniable fact that one batch of frogs is liable to differ significantly from another.

Variation between batches of frogs

Examples of batch differences in otherwise completely comparable sets of experiments are shown in Table 2. Resting PCr level, tension development, production of $h+w$ and break-down of PCr are all seen to vary significantly. Part of the variation may arise from seasonal effects but it is a daunting task to disentangle these when it is known that the frogs available, being trapped in the wild, differ considerably in origin and nutritional history. Because of these variations, it is perilous to make any critical comparison, for example, between heat and chemical change, unless all the experiments of a given type can be carried out at random on the same group of frogs.

To poison or not to poison

In order to employ the method of energy balance it is desirable to keep the number of simultaneous chemical reactions to a minimum. For example, the study of the initial processes that accompany contraction is made much simpler if the recovery processes do not complicate the situation. This can be accomplished by poisoning the muscle with fluorodinitrobenzene (FDNB), iodoacetate (IAA) or anoxia (N_2). FDNB produces the greatest simplification because it inhibits creatine phosphokinase

TABLE 2. Variation between batches of frogs

Parameter	Units	'Batch A' Mean	S.E.	N	Date	'Batch B' Mean	S.E	N	Date	(A − B) difference Mean	S.E.	P
(PCr/Cr) Control	μmole/μmole Cr	0·902	0·005	34	26. xi. 68–9. i. 69	0·876	0·0063	6	30. ix. 69–2. x. 69	0·026	0·0080	< 0·01
[PL/Cr]*; IAA, N_2	g mm/μmole	873	8·0	4	25. ix. 69–30. ix. 69 **Th**	714	16·5	4	17 .iv. 69	159	18·3	< 0·001
[(h + w)/Cr] (15 + 2),† IAA, N_2; **Th**	mcal/μmole	2·41	0·119	6	24. ix. 69–7. x. 69	1·87	0·184	3	16. vii. 69	0·54	0·22	< 0·05
Δ(PCr/Cr) (15 + 60),† IAA, N_2	μmole/μmole Cr	−0·160	0·0076	5	25. ix. 69–30. ix. 69	−0·136	0·0073	5	8. vii. 69–17. vii. 69	0·024	0·0105	= 0·05

Notes. 'Batch *A*' and 'Batch *B*' are not the same in different rows.

* Maximum tetanic force × muscle length/total creatine.

† $(x+y)$ means x sec of stimulation followed by y sec of relaxation and recovery.

$(h+w)$/Cr can be approximately converted to mcal/g by multiplying by 25.

For explanation of **Th** see Table 1.

All chemical changes are given as (E–C); thus a negative number indicates break-down.

(CPK) so that during the early stages of activity the only net reaction appears to be the hydrolysis of ATP. However, this simplicity only persists for a second or two and the muscle soon becomes the site of a complex series of reactions (Dydyńska & Wilkie, 1966; Kushmerick & Davies, 1969).

Thus FDNB is not suitable for the present investigation of tetani of moderate duration. The classical method of isolating the initial processes by means of IAA and N_2 (Lundsgaard, 1930) is complicated by the fact that hexose phosphates may accumulate in the muscle, though this may not be a serious problem in frog muscle at 0° C (Carlson & Siger, 1960; Seraydarian, Mommaerts & Wallner, 1960: on the other hand, see Spronck, 1965).

However, both FDNB and IAA are known to react with the $-NH_2$ or —SH groups respectively of a wide variety of proteins. For example, FDNB is known to react with myosin (Bárány, Bárány & Bailin, 1968) and with other muscle enzyme systems (Infante & Davies, 1965). IAA is not so reactive, though at high enough concentration it will inhibit CPK and also prevent contraction (Carlson & Siger, 1960). This non-specificity suggests the possibility that there may be other unknown complications in addition to the known ones. Accordingly we decided to work with normal unpoisoned muscles in oxygen, making use of the fact that in frog muscle at 0° C the recovery processes start up relatively slowly so that for some time the muscle behaves as a closed system hydrolysing PCr but not replenishing it. This policy seemed quite reasonable when we began these experiments for we were concerned only with contractions lasting a few seconds; however, the initial findings were so puzzling that we were driven to investigate longer and longer periods of activity (up to 15 + 60 sec) so that it became necessary to undertake special experiments to find out what contribution recovery processes might be making to the energy balance.

Onset of oxidative and of glycolytic recovery

Heat studies

It has been known since the work of Hill (1913) and of Weizsäcker (1914) that the initial heat production resulting from muscular contraction is almost unaffected when oxygen is excluded, whereas the later recovery heat production is dramatically reduced. The extra heat that appears only in oxygen must arise from the oxidative recovery process, so its time of onset should indicate when oxidation begins (see Hill, 1965, Chapter 5). We could find no published results on the 15 sec tetani used in our longest experiments, so we were obliged to do some ourselves. We found that about 5 % of the oxidative recovery heat is produced during the minute following the end of stimulation. However, only about 1 % appeared during the first half of this period, so that we felt justified in neglecting the oxidative

recovery processes during contraction and relaxation under the conditions of our experiments. However, in experiments in which a minute of recovery is allowed, we would expect a contribution from oxidation: this is what we have in fact observed (see Table 3).

Unfortunately it is not feasible to attempt a similar experiment (comparison of heat production of an anoxic muscle before and after poisoning with IAA) in order to examine the onset of glycolytic recovery because the anaerobic recovery heat production is so small and variable that its onset could not be detected with any precision.

Chemical studies

If recovery does occur during a specified period of activity in oxygen it should show itself both as a smaller final break-down of PCr (because some will have been rebuilt; see Dydyńska & Wilkie, 1966, Fig. 1) and as a somewhat larger heat production, when compared with a similar period of activity in which recovery had been prevented by poisoning with IAA and N_2. However, experiments 1 and 2 (Table 3) show no sign of these differences during contraction itself. The conclusion is not absolutely clear-cut, because IAA and N_2 appear to have slightly reduced $(h+w)$ as well as ΔPCr/Cr.

It is only if the experimental period is extended that clear signs of oxidative recovery are seen, thus supporting the conclusion of the previous section. Comparison of Expts. 4 and 5 (unpoisoned and poisoned (15 + 60)) shows that more PCr remained broken down in the poisoned muscles ($P < 0{\cdot}001$) even though during the contractile period itself they had, if anything, broken down less (see Expts. 1 and 2). The conclusion seems inescapable that the small final PCr break-down in Expt. 4 is a sign that part of the PCr that had been broken down has already been rebuilt by the end of the experimental period. The amount of PCr rebuilt is approximately 1 μmole/g, about a quarter of that broken down.

The same conclusion is supported by a comparison of Expts. 3 and 4. The net PCr break-down at the end of (15 + 60) sec is actually less than that at the end of (15 + 2) sec, also suggesting that some PCr has been rebuilt. However, this result is not very significant statistically ($P < 0{\cdot}2$).

A quite different conclusion, which will be found to be of considerable importance later emerges from a comparison of Expts. 3 and 5. These experiments on a single batch of frogs suggest strongly that if recovery is prevented there is appreciably further break-down of PCr during the minute after contraction and relaxation have ended. Post-contractile break-down of PCr was first described by Lundsgaard (1934), and it is of such importance that we have undertaken a special series of experiments to investigate it, as will appear later.

TABLE 3. Various experiments on recovery

		ΔPCr/Cr			Δ lact./Cr			$(h+w)$/11 Cr			ΔATP/Cr			ΔPi/Cr		
		Mean	S.E.	*N*	Mean	S.E.	*N*	Mean	S.E.	*N*	Mean	S.E.	*N*	Mean	S.E.	*N*
1 Chem. 17. iv. 69; heat 24. iv. 69–6. v. 69	(15+2), O_2	−0·156	0·014	4	—	—	—	0·170	0·027	3	−0·0035	0·0028	4	—	—	—
2 Chem. 22. iv. 69; heat 24. iv. 69–6. v. 69	(15+2), IAA, N_2	−0·121	0·005	4	—	—	—	0·145	0·021	2	−0·002	0·0015	4	—	—	—
3 Chem. 30. ix. 69–2. x. 69; heat 1. x. 69—7. x. 69	(15+2), O_2	−0·146	0·013	6	+0·017	0·020	6	0·202	0·0238	5	+0·006	0·0042	6	+0·103	0·0155	6
4 1. x. 69–7. x. 69; **Th**	(15+60), O_2	−0·127	0·0047	6	+0·022	0·0025	6	0·208	0·023	6	−0·0025	0·0015	6	+0·095	0·011	6
5 Chem. 23. ix. 69–7. x. 69; heat 23. ix. 69–7. x. 69, **Th** (6)	(15+60), IAA, N_2	−0·173	0·009	11	—	—	—	0·222	0·012	6	+0·0035	0·0020	11	+0·105	0·0074	11
6 Chem. 19. v. 69–22. v. 69; heat 21. v. 69–23. v. 69	(2+8), O_2	−0·028	0·0056	8	−0·0006	0·0075	7	0·0614	0·0094	2	+0·002	0·0048	8	+0·024	0·015	8

Expts. 1 and 2 were performed on a single group of frogs.
Expts. 3, 4 and 5 were also performed on a single group.
Output of physical energy has been expressed as $(h+w)$/11 Cr to put it on same scale as PCr/Cr, see text. It can be converted approximately to mcal/g by multiplying by 275.
For sign convention of chemical change, see Table 2.

Glycolytic recovery

As mentioned in the previous section, there is little hope of assessing the participation of glycolytic recovery from the study of heat records. We have therefore tried to estimate the importance of this process by direct estimation of lactic acid production as shown by Expts. 3, 4, and 6 in Table 3. Only in Expt. 4 is the production of lactic acid statistically significant ($P < 0{\cdot}001$); in Expt. 3 there may have been some lactic acid formation that cannot be detected because of the large scatter in the individual determinations. However, we have another reason for thinking that not much lactic acid is formed during a 15 sec tetanus. If glycolysis did occur to an appreciable extent, poisoning the muscle with IAA should increase the break-down of PCr for a given amount of activity. Expts. 1 and 2 give no support whatever for such a conclusion.

Effect of ageing. One possible method of obtaining the advantages of IAA poisoning without its drawbacks is suggested by the work of Jöbsis (1963, see especially p. 958) on the tropical toad *Bufo marinus*. These muscles almost lose their capacity for formation of lactic acid if they are 'aged' for 36–48 hr at 0–3° C in Ringer solution. It seemed worth while to find out whether or not the same effect is shown by the temperate *Rana temporaria*. Accordingly a special set of experiments was undertaken on a single batch of frogs to investigate this point. The experimental conditions (100 isometric twitches at 3 sec intervals in N_2) were known to produce a relatively large amount of lactic formation, so that an effect of ageing, if it existed, would be most easily detected.

The results, shown in Table 4, are conclusive. Ageing (storage in Ringer at 5° C for approximately 24 hr) is totally without effect on any of the chemical changes measured, or on the production of heat and work. Table 4 shows a number of other points of interest. The increase in Pi is significantly less than the break-down of PCr. Originally we thought that this might be due to the incorporation of Pi as hexosephosphate. However, direct analyses for the hexose mono- and diphosphates by an enzymic method, kindly carried out for us by Dr R. K. Scopes (Meat Research Institute, Langford, Bristol) showed that the accumulation of these compounds was negligible. We now think that the discrepancy probably arises from a consistent underestimation in our technique for phosphate analysis, as explained under Methods.

A further point of interest is that the $(h+w)$ observed is greater than can be accounted for by the break-down of PCr alone, allowing 11 kCal per mol. If the difference is all to be attributed to the formation of lactic acid this process apparently yields 16 kcal/mol lactate, rather less than might be expected (22 kcal/mole; Woledge, 1971, Table 2).

TABLE 4. The rate of glycolysis in fresh and 'aged' muscle. 100 twitches at 3 sec intervals in N_2

	$\Delta\frac{PCr}{Cr}$ μmole/μmole			$\Delta\frac{Pi}{Cr}$ μmole/μmole Cr			$\Delta\frac{Lact.}{Cr}$ μmole/μmole Cr			$\Delta\frac{Hex.\ Ph.*}{Cr}$ μmole/μmole C			$\frac{h+w}{Cr}$ mcal/μmole Cr		
	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N
Fresh	−0·419	0·017	6	+0·304	0·011	6	+0·137	0·011	6	+0·0125	0·0034	6	6·60	0·25	6
Aged	−0·438	0·022	5	+0·311	0·032	5	+0·130	0·018	4	+0·0166	0·0056	6	7·08	0·37	6

All experiments performed on a single batch of frogs, from 7. i. 70 until 13. i. 70.
All **Th** experiments, see Table 1.
* Hexose mono- and diphosphate.
For sign convention of chemical change, see Table 2.

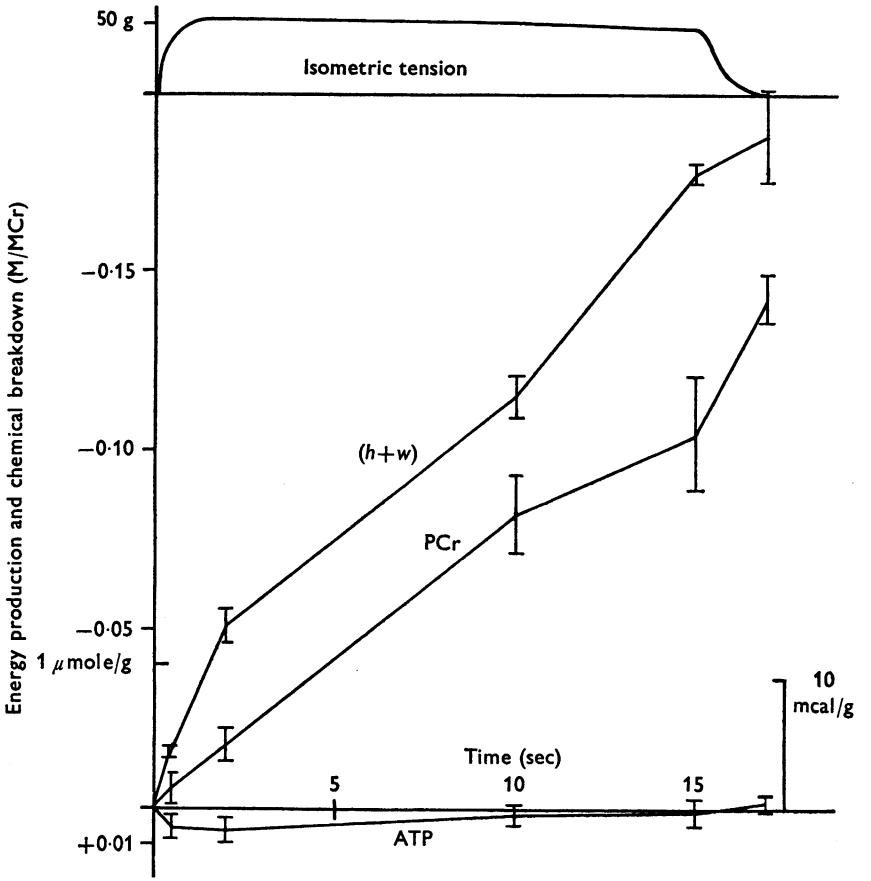
To summarize, our experience is that for short contractions, say, less than a 10 sec tetanus at 0° C, it is not necessary to poison the muscle. The initial processes are sufficiently faster than the recovery processes so that no complication arises from the latter. However, for contractions lasting longer than 10 sec the situation becomes a little uncertain and for such experiments it is probably better to poison with IAA and N_2, and to accept whatever risks arise from that procedure.

Chemical changes during an isometric tetanus

15 *sec tetani*

Our earliest experiments were on brief isometric and isotonic tetani, but their results did not seem to make sense until after we had investigated longer contractions. For this reason the results of the later experiments will be presented first. In Fig. 1 are shown the mechanical tension development, the production of heat and work, and a number of the chemical changes during and immediately following a 15 sec isometric tetanus. The changes in PCr and ATP are plotted on the ordinate in the usual way as mole/mole Cr; break-down is plotted upwards in both cases. In order to relate the physical energy output $(h+w)$ to the chemical changes, the assumption has been made that the physical energy comes from a chemical process that yields 11 kcal/mol. This seems to be the most likely value for the heat accompanying hydrolysis of PCr (see Wilkie, 1968) and this reaction might well have been the source of the physical energy. However, since the publication of that paper the interpretation of this value has become somewhat uncertain. On the one hand we have performed more recently sets of experiments similar to those of the 1968 paper (unpublished observations and this paper, Tables 3 and 6) some of which yield values up to 14 kcal/mole. On the other hand Woledge (1971) has pointed out that *in vitro* measurements permit no more than 7 kcal per mole of PCr split. Comparison between chemical and physical measurements is made much clearer if one particular value is assumed. Under the present somewhat confused circumstances 11 kcal/mole seemed the most reasonable value to assume, though its limitations should always be borne in mind. Assuming this value and also that splitting of PCr was indeed the only reaction occurring, the curve for $(h+w)/11$ Cr should lie on top of the curve for ΔPCr/Cr. It is clear from Text-fig. 1 that this is not the case and that no other value of the numerical constant would make them superimpose. The break-down of PCr seems to proceed at a constant rate of about 0·2 μmole/g–sec from the beginning of stimulation, whereas the heat production, as is well known, starts at a high rate and only later settles down to a lower steady one. Our heat results are closely similar to those already published (Aubert, 1956; Hill & Woledge, 1962; Carlson *et al.* 1967; summarized Woledge,

1971). After the first 3 sec of stimulation the rate of heat production is steady and it runs parallel with the rate of PCr splitting; thus for the remainder of contraction and relaxation it appears that about 11 kcal are indeed obtained per mol of PCr split. During the initial period additional



Text-fig. 1. The physical and chemical changes during and after a 15 sec isometric tetanus. 0° C, O_2.

The uppermost graph represents the tension during a typical 15 sec isometric tetanus. The first stimulus occurs at time zero.

The lower graphs show the physical and chemical changes, measured in mole/mole creatine (M/M Cr, Cr = total creatine). A calibration in mcal/g and μmole/g is also shown, but they are only approximate because the experimental method precluded measurement of muscle weight.

The heat + work ($h+w$), measured experimentally in mcal/m-mole Cr, is plotted in terms of equivalent chemical units (M/M Cr) using a conversion factor of 11 kcal/mole (for details see text).

PCr break-down, ATP break-down and ($h+w$) production are plotted upwards. The bars indicate ± S.E. Experiments performed Dec. 68–May 69, nos. 7, 8, 15, 16, 17 in Table 5.

heat amounting to almost 10 mcal per gram has been given out, apparently from some other source than the splitting of PCr. During relaxation, that is between 15 and (15+2) sec, PCr continues to be broken down (mean $\Delta PCr/Cr = -0{\cdot}038$, S.E. = 0·018, $N = 18$, $P < 0{\cdot}1$) while the heat production, as is already known, remains small. However, even at the end of relaxation the extra heat that had been emitted during the first 3 sec of contraction remains unaccounted for by the observed splitting of PCr.

2 sec tetanus

The details of the left-hand end of Text-fig. 1 are shown in Text-fig. 2, which is plotted in an exactly similar way. The experiments shown there actually concern 2 sec tetani with various subsequent periods for relaxation and rest. However, during the first 2 sec the muscle cannot have known for how long stimulation would continue, so it is legitimate to use the points at 0·5 and 2·0 sec to delineate the early part of Text-fig. 1. Full details of all the experiments are given in Table 5. The brief contractions demand the highest possible time resolution, and they are also most seriously affected by statistical fluctuations because the chemical changes to be detected are so small. For these reasons, and also because these were the first experiments to be performed (December 1968 to May 1969) we felt somewhat cautious about the results. As shown in Table 5, some of the experiments have been repeated in April 1970 and January–February 1971 after various modifications of technique had been introduced. The results show the batch-to-batch variation to which we are now resigned. In addition Expt. 11 does not show the slight increase in ATP observed in all the other experiments.

Nevertheless, the main conclusion seems to be quite clear. During the early stages of an isometric tetanus there is substantial heat production without nearly enough break-down of PCr or ATP to account for it. Even at (0·5+0) sec where the changes are small and the scatter relatively large, the difference between $(h+w)/11$ Cr and $\Delta PCr/Cr$ is statistically significant in one of the two experiments ($P < 0{\cdot}1$ for Expt. 7, $P < 0{\cdot}05$ for Expt. 11). With these short tetani there was substantial production of physical energy at a time when none of the chemical changes examined could be distinguished statistically from zero. At (2+0) sec the significance of the discrepancy is greater ($P < 0{\cdot}001$ for Expt. 8, $P < 0{\cdot}01$ for Expt. 13). These calculations have disregarded the apparent changes in ATP: if these are accepted as genuine, then the discrepancy between physical energy output and its supposed chemical origin becomes even more striking.

As shown in Table 5, the changes in Pi are almost always what would be expected on the assumption that they arose from the changes in ATP and PCr. The only significant exceptions are that in Expts. 7 and 8 the libera-

TABLE 5. Tetanic contractions: unpoisoned muscles in oxygen

Experiment	type		ΔPCr/Cr			ΔATP/Cr			ΔPi/Cr			$(h+w)$/11Cr		
			Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N
(7)	(0·5+0)	A	−0·057	0·0060	16	+0·0054	0·0035	16	+0·021	0·0067	16	0·0177	0·0019	3
(8)	(2+0)	A	−0·018	0·0050	21	+0·006	0·0040	21	+0·029	0·0050	20	0·0511	0·0034	8
(9)	(2+2)	A	−0·030	0·0056	16	+0·002	0·0019	16	+0·033	0·0067	16	0·0457	0·0023	7
(10)	(2+8)	A	−0·028	0·0056	8	+0·002	0·0048	8	+0·024	0·015	8	0·0614	0·0094	2
(11)	(0·5+0)	B	−0·0023	0·0080	15	−0·0011	0·0021	15	−0·0047	0·0085	15	0·0229	0·0019	4
(12)	(0·5+0)	A	−0·0003	0·0061	15	+0·0046	0·0028	15	+0·036	0·0144	15	0·0255	0·0047	3
(13)	(2+0)	C	−0·038	0·0041	7	+0·0011	0·0034	7	+0·043	0·0071	7	0·078	0·0064	3
(14)	(1·1+0)	C	−0·044	0·0057	6	+0·0057	0·0063	7	+0·030	0·0118	7	0·066	0·0069	4
(15)	(10+0)	A	−0·082	0·011	4	+0·002	0·003	4	+0·066	0·019	4	0·115	0·0063	3
(16)	(15+0)	A	−0·105	0·016	4	+0·001	0·0038	4	+0·135	0·024	4	0·178	0·003	2
(17)	(15+2)	A	−0·143	0·0075	14	−0·0017	0·0027	13	+0·113	0·0132	10	0·189	0·0128	10

All experiments were isometric save for (12) which were isotonic against a small load, and (14) which were isometric for 0·43 sec followed by 1 cm of free shortening.

Dates of experiments: A December 1968–May 1969; B April 1970; C January–February 1971.

For sign convention of chemical change, see Table 2.

tion of Pi appears to be greater than could be accounted for by this source and in Expt. 17 it appears to be less. However these were our earliest experiments (dogged by technical difficulties, as explained under Methods) and we do not feel prepared to draw far-reaching conclusions from them.

Relaxation and early recovery. The interval from 2 to 4 sec in Text-fig. 2 covers the period of relaxation. The fall in $(h+w)$ during this period, which is not statistically significant, gives a somewhat startling impression for it is well known that during relaxation from an isometric tetanus heat is produced rather than absorbed. The individual heat records obtained in the (2 + 2) sec experiments confirmed that this was indeed the case. However, it will be remembered that each particular type of experiment is made on an individual batch of frogs so that comparison within the batch should be valid even though comparison between one batch and the next may be less so. The fall in the $(h+w)$ curve is simply a result of the fact that the frogs used for the (2 + 2) point happened to have an unusually low production of $(h+w)$ for their content of Cr.

The only other feature of interest is that some PCr appears to be broken down during relaxation. This result considered alone is not very highly significant ($P \simeq 0{\cdot}1$) but the conclusion is supported by the similar finding in Text-fig. 1.

During the 6 sec following the relaxation period, very little happens. The discrepancy between $(h+w)$ output and ΔPCr that arose during the early stages of contraction persists.

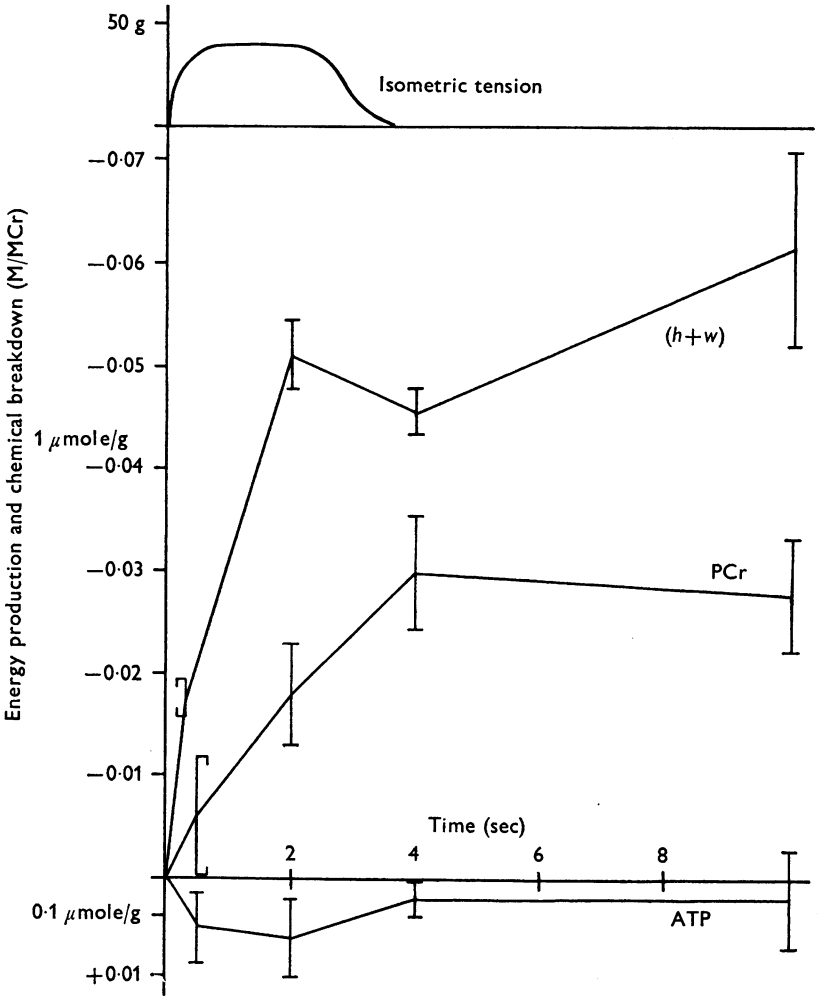
Are the apparent changes in ATP genuine?

One of the most surprising features of Text-fig. 2 is the apparent increase in ATP/Cr in the early stages of contraction. After this result first began to emerge we took special pains to eliminate obvious artifacts such as might arise from unequal treatment of the experimental and control muscles. For example, in the earliest experiments preliminary stimuli followed by a 40 min recovery period in oxygen were given to both E and C muscles to test the electrical and mechanical apparatus. The effects may not have been identical in both muscles, so such stimuli were elimiated. Another possible explanation, that Cr decreases as a result of stimulation, can also be discounted (see Wilkie, 1966, p. 27).

Further to avoid bias we systematically alternated the following factors: E and C muscles from left or right leg; position on electrode assembly of E and C muscles; which frozen muscle was removed first; which was crushed and extracted first. During the chemical analyses a different Latin square arrangement was used for the same purpose.

Nevertheless, the change tended to persist, though tantalizingly close to the noise level set by statistical fluctuations. Neither the 0·5 sec nor

the 2·0 sec point in Text-fig. 2 is itself significantly different from zero at $P < 0{\cdot}05$ nor are the changes shown on the last line (12) of Table 5, which were found in 0·5 sec isotonic low-load tetani ($0{\cdot}2 > P > 0{\cdot}1$).



Text-fig. 2. The physical and chemical changes during and after a 2 sec isometric tetanus. 0° C, O_2. Plotted exactly as Text-fig. 1. Experiments performed Dec. 1968–May 1969, nos. 7, 8, 9, 10 in Table 5.

The accumulation of further results has shown that although this result is not always seen (Expt. 11) all the other experiments do show it to varying degrees. Taking all the eighty-one short tetani of Table 5 together, the mean change in ATP/Cr is + 0·0039, S.E. = 0·00153 leading to $P < 0{\cdot}02$. All the experiments save Expt. 11 were performed on winter frogs: if the

summer frogs are excluded the significance of the result is naturally much higher. However, we are in no position at present to assert that this is a genuine seasonal effect. Incidentally it seems perfectly legitimate to lump our results together in this way: it merely corresponds to the null hypothesis that under no circumstances does the ATP content change as a result of stimulation.

Shortening heat

A comparison between experiments 7 and 12 in Table 5 shows that, if anything, the isotonic contraction caused less break-down of PCr, or (PCr + ATP), than the isometric one even though it produced more heat (shortening heat). A significant difference of this type was first reported by Kushmerick *et al.* (1969; see especially their Table 8 which relates to a 0·18 sec tetanus).

Delayed break-down of PCr

The results in Table 3 partially confirm Lundsgaard's finding (1934) that appreciable break-down of PCr occurs during the minute or more following relaxation. In order to check this point and also to extend our knowledge of the changes during relaxation itself a special series of experiments was undertaken whose results are shown in Table 6. It was necessary to poison with IAA and N_2 in order to avoid complications arising from recovery.

Relaxation. As the first two lines of Table 6 show, PCr appears to be broken down during relaxation, though as found before (Expts. 8 and 9 and 16 and 17 in Table 5) the result is not very significant statistically. What is needed is a method of pooling all these results together. Unfortunately the outcome of our experiments was unknown to us when we began them and their consequent unbalanced arrangement did not lend itself to treatment by the analysis of variance. A simpler approach is to consider the six results as samples from six independent normal populations, all with different means and standard deviations. If there were truly no PCr break-down during relaxation, then the changes in PCr concentration during relaxation would be zero on the average after each of the three contraction patterns studied. The sum of these three changes and thus the statistic: $[2+2]-[2+0]+[15+2]-[15+0]+[15+2;\ \text{IAA}]-[15+0;\ \text{IAA}]$ would also be zero. (The quantities in square brackets are the *population* mean PCr break-downs in each type of contraction denoted in our usual fashion.)

It is easy to show from sampling theory (see e.g. Snedecor & Cochrane 1967) that if *samples* are drawn from the six populations and *their* means are inserted in the above expression then it would be distributed with variance $\Sigma(\sigma^2/N)$ (summed over the six samples) thus enabling the null hypothesis to be tested. Within-sample standard deviations, which in our

TABLE 6. Changes during relaxation and 'recovery': IAA, N_2 poisoned muscle

Experiment type		ΔPCr/Cr			ΔATP/Cr			ΔPi/Cr			$(h+w)$/11 Cr			Date chem.	Date heat
		Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N		
(8)	(15+0)	−0·103	0·012	5	+0·0012	0·0036	5	+0·096	0·010	5	0·160	0·0162	3	8. vii. 69–17. vii. 69	16. vii. 69
(9)	(15+2)	−0·112	0·009	5	−0·0026	0·0028	5	+0·094	0·011	5	0·170	0·0167	3		
(20)	(15+60)	−0·136	0·0073	5	+0·0002	0·0043	5	+0·106	0·0094	5	0·174	0·0205	3		

All experiments performed on a single batch of frogs.
For sign convention of chemical change, see Table 2.

case were all of similar size, were used as best estimates of the population standard deviations. The above statistic amounts to 0·059; $\Sigma(\sigma^2/N)$ was 0·000594; P was therefore approximately 0·02. It thus appears that there is indeed significant break-down of phosphocreatine during relaxation.

In order to obtain the best estimate of the magnitude of this break-down it is probably best to weight the individual means according to their reduced degrees of freedom (see Methods). This estimate of Δ(PCr/Cr) is then $-0{\cdot}014$, about 0·35 μmole/gram. In so far as this conclusion might be expected to apply also to muscles poisoned with FDNB, it supports the results of Kushmerick *et al.* (1969, Table 4), and contradicts those of Mommaerts & Wallner (1967).

'*Recovery.*' Table 7 fully confirms ($P < 0{\cdot}05$) that PCr continues to be split after the stimulus has ended and the muscle has returned to its original mechanical state, during a period when the heat production is small. Indeed, for the first 30 sec or so there is probably a slight heat absorption though this cannot be seen in Text-fig. 4. This confirms the result of Lundsgaard (1934) but disagrees with that of Marechal & Mommaerts (1963) who concluded that there was no break-down of PCr from 1 to 1000 sec following a tetanus.

Summary

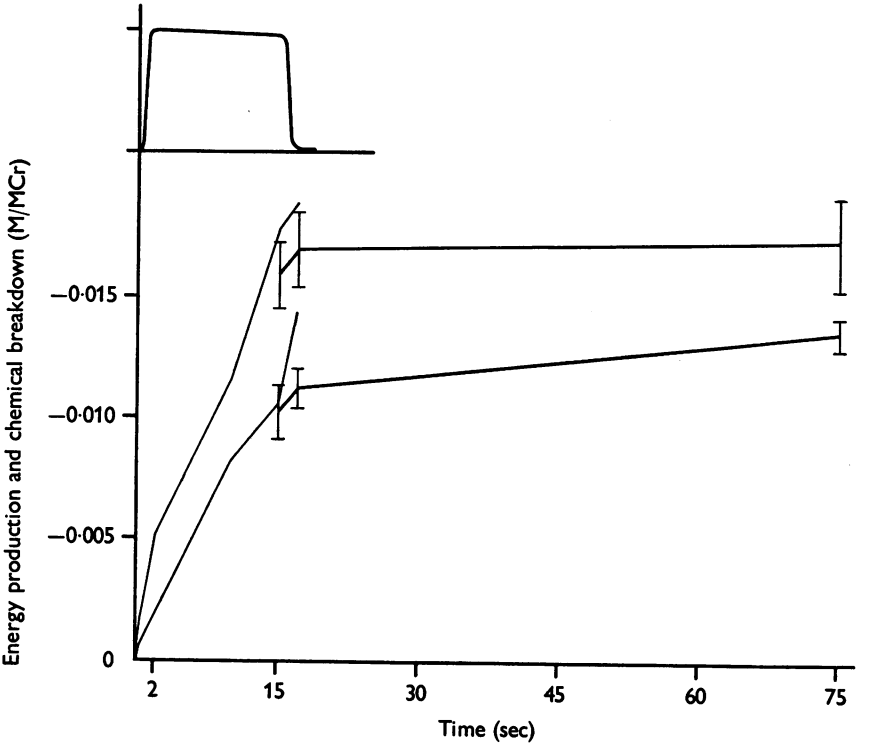
The results that we regard as reasonably established are shown in Text-fig. 3. Allowing as usual for 11 kcal of physical energy per mole of PCr split, it seems that during the first few sec of isometric contraction there is heat production amounting to perhaps 10 mcal/g without corresponding break-down of PCr or any other identified reaction. For the remainder of the contraction, hydrolysis of PCr does correspond approximately with the now steady rate of heat production. A small quantity of PCr hydrolyses during relaxation but not enough to account for the total physical energy that has been observed. This gap slowly diminishes later as PCr is split without corresponding heat production.

DISCUSSION

The most dramatic, as well as the clearest, of our results is the finding that the heat production in short tetanic contractions cannot be accounted for by splitting of PCr or ATP. In a 2 sec tetanus, most of the (heat + work) comes from some other process. This is the first direct evidence for a substantial source of (heat + work) during the early stages of contraction other than the hydrolysis of ATP or PCr. The effect is so large that we can be quite certain of it in spite of any uncertainty concerning the exact amount of (heat + work) to be expected from splitting one mole of PCr in the

muscle. On the most probable assumption about this quantity the unexplained (heat + work) is about 10 mcal/g (Text-fig. 1), a substantial amount, at least equal to the total amount of (heat + work) produced in two twitches.

Before discussing the possible origins of this heat we should enquire why this large effect has not been found earlier. One reason is that we have used



Text-fig. 3. Production of heat and work, and break-down of PCr during and following a 15 sec tetanus, plotted as in Text-figs. 1 and 2. Left-hand points and light lines: normal muscles in O_2 from Text-fig. 1. Right-hand points, heavy lines and ±1 S.E. bars: IAA, N_2 poisoned muscles, from Table 6.

a freezing method giving substantially quicker arrest of metabolism than was previously possible. Another point is that our experiments were expressly designed to compare (heat + work) and chemical change without the uncertainties involved in measuring the two quantities in different laboratories, on different species, or even at different temperatures. Where direct comparisons can be made there are some striking contrasts between our results and those of earlier workers. For example, we find, in a muscle frozen at 2 sec in an isometric tetanus, that (ATP + PCr) 0·2–0·4 μmole/g

were used; whereas Kushmerick *et al.* (1969, their Table 8) report a similar ATP usage of 0·2 μmole/g in a contraction of FDNB-poisoned muscle of less than one tenth the duration (0·18 sec instead of 2 sec). It does not seem likely that the difference is due to the use of FDNB since, in other experiments, Kushmerick *et al.* (1969) find the chemical changes to be similar in poisoned and in unpoisoned muscles. Possibly the rise in the temperature of the mucle prior to stimulation (p. 320 of their paper) was responsible for at least part of this discrepancy. Certainly something strange is going on, for a quite different result is given in Table 4*a* of Kushmerick *et al.* (1969). This indicates a rate of break-down of 0·2 μmol/g.sec, similar to our own estimate and also to that of Maréchal & Mommaerts (1963). Although these disagreements cannot at present be explained in detail, there is no reason to suspect the validity of our results because of them.

For the most part, our determinations of ΔPi, ΔPCr and ΔATP agree well, as they should if the only source for Pi production were ATP or PCr break-down. In two early experiments, the Pi production was significantly greater than the ATP and PCr break-downs, but as we have noted above, we do not have complete confidence in this result. Even if it is genuine it cannot be the result of a process essential to muscular contraction since, in other experiments, there was no excess Pi. Certainly this isolated result is not enough to raise the spectre of an unidentified phosphagen! Thus we have found nothing in our chemical analyses to suggest a source for the early (heat + work) which, therefore, must be associated with at least one process other than ATP break-down or the Lohmann reaction.

We have now to suggest some process which could produce about 10 mcal/g during the first two seconds of a tetanus and which is not reversed during even a fairly long tetanus. Perhaps reactions involving proteins as reactants might provide the source. Two types of process seem worth considering: binding of ions, particularly calcium ions, to proteins, and conformational changes of the proteins themselves. Certainly there are movements of calcium ions early in a tetanus (Winegrad, 1970); and presumably myosin, acting and troponin are all greatly changed when the muscle becomes active. 10 mcal/g of muscle is equivalent to 140 kcal/mole of troponin, 15 kcal/mole of actin or 70 kcal/mole of myosin. Heats of this order have been reported for conformational changes of some proteins (Lumry & Biltonen, 1969) but we know of no direct experimental evidence concerning the proteins of muscle. Certainly the discrepancy is far greater than can be attributed to the splitting of one ATP molecule at each active site in the muscle.

Muscle contains 0·6 μmole of exchangeable calcium per gram of muscle, of which about 0·4 μmole/g probably moves from one site within the

sarcoplasm to another during a tetanus (Winegrad, 1970), though only about 0·07 μmole/g is necessary fully to activate troponin in the muscle (Ebashi, Endo & Ohtsuki, 1969). The unexplained heat is thus equivalent to 25 kcal/mole of moved calcium or to 140 kcal/mole of calcium bound to troponin. Clearly the latter figure is too large to be accounted for by calcium binding alone, although if calcium binding produced structural changes in troponin then such a figure is well within the bounds of possibility. The figure of 25 kcal/mole also seems to be rather large: when calcium binds to a protein it may do so by exchanging with two hydrogen ions. These would react with intracellular buffers to produce about 7 kcal/mole of hydrogen ion (i.e. 14 kcal/mole of calcium ion). Thus the remaining 11 kcal/mole represents the difference in the binding heats of the initial and final binding sites of calcium. This quantity seems too large for a calcium binding heat (Coleman, 1952), let alone the difference between two (presumably similar) binding heats, but unfortunately there seems to be no direct calorimetric data for the biochemical system under investigation here. Nonetheless it seems unlikely that the excess (heat + work) can be explained in terms of calcium movement *per se*, although it is possible that such movements give rise to structural changes of proteins, and large quantities of heat can be produced in this way (Lumry & Biltonen, 1969; Fasman, 1967).

We do not know in detail the time course of the unexplained (heat + work) but our results are compatible with the assumption that PCr splitting occurs at a steady rate of about 0·2 μmole/g.sec from the start of the tetanus, whereas the heat rate, as is well known, is greatest at the start of the tetanus and declines to a steady rate. Aubert (1956) has called this latter the 'stable' heat rate and part of the early excess over this the 'labile' heat. Later work from his laboratory has suggested that these components might be the result of different processes within the muscle (for a summary, see Woledge, 1971, p. 60). Our steady rate of PCr splitting would account for the stable heat production if, as seems to be the case, 11 kcal are produced when each mole of PCr is split in the muscle. Our results are thus compatible with the idea that the unexplained (heat + work) corresponds to Aubert's labile heat, plus the heat and work produced rapidly during the first second of isometric contraction (see Woledge 1963) that Aubert deliberately omitted from his analysis. Woledge (1971) has argued, on other grounds, that the labile heat might be the net result of calcium movements to and from the sarcoplasmic reticulum. In this argument ATP (or PCr) splitting by the S.R. was suggested as the process producing the heat. Our present evidence excludes this but there remains, as a possible source of heat, the calcium movements suggested by the work of Winegrad and others. It is possible that Aubert's labile heat, Winegrad's

calcium movements and our unexplained (heat + work) are all connected. While this idea at least suggests some further experiments, we obviously have no evidence to exclude alternative hypotheses, for example, that the heat is the result of some conformational change of the contractile proteins themselves as they become active. A slight difficulty in this case would be the relative slowness with which the unexplained (heat + work) appears compared with the time course of tension development or active-state development. There are several ways in which a hypothesis could avoid this difficulty. At present, however, it does not seem worth while to carry these speculations any further.

The (heat + work) per mole of PCr splitting

Wilkie (1968) showed that for a variety of different types of contraction, of IAA and N_2-poisoned muscle, the (heat + work) seemed to be proportional to the PCr split if the changes were examined some 30 sec after the end of the contraction. The constant of proportionality was 11 kcal/mole. This we have confirmed above though in other experiments, as yet unpublished, we find the constant to vary between batches of frogs from 10 to 14 kcal/mol. Expts. 5 and 20 (Tables 3 and 6) were of this type: both yielded 14·1 kcal/mole. Walsh & Woledge (1970) argued that 9 kcal/mole was the most that could come from PCr splitting itself and the associated phosphate deionization and buffer ionization. More recently a revision of the heat of ionization of phosphate (R. C. Woledge, unpublished) has led to the conclusion that only 7 kcal/mole can be accounted for in this way. It seems, therefore, that some other process occurs side by side with PCr splitting. Our present experiments seem to show that, whatever this process may be, it keeps pace with PCr splitting during contraction and relaxation. Thus the rate of PCr splitting from 2 to 15 sec in a tetanus is related to the rate of heat production by the familiar 11 kcal/mole. There is no reason to suppose there is any connexion between this unknown process, producing heat in proportion to the PCr split, and the process discussed above which is restricted to the early seconds of a tetanus.

Working contractions

In all the contractions reported in this paper the work is but a small fraction of the (heat + work). We are therefore unable to answer the very interesting question whether the unknown process is responsible for any of the work output of the muscle. Gilbert & Kushmerick (1970) have used the hammer freezing apparatus to study contractions in which a considerable amount of work was done. They found that the PCr split was always enough to provide free energy equivalent to the work done. There is thus no need to suppose that the muscular machinery is capable of

obtaining any work from the unknown process that we have been discussing, although this possibility is, of course, not excluded. It is also quite possible that the unknown process is one which has quite a small free energy change – the heat coming predominantly from an entropy change. This would be the case for conformational changes of proteins and for the ionic binding processes discussed above.

Delayed PCr splitting

Lundsgaard (1934) was the first to report that PCr splitting continued after contraction had ended. In his experiments (25 sec tetanus at 2° C) about 25 % of the total PCr splitting occurred either during relaxation or during the following 3 min. Spronck (1965) has also reported considerable break-down of PCr during the minute following 5 twitches at 2° C. On the other hand, Maréchal & Mommaerts (1963) found no evidence for post-contractile PCr splitting between 1 and 1000 sec after a six-second tetanus. This is a worrying discrepancy for it is not easily explained by any difference in the methods used. However, our experiments seem clearly to show post-contractile PCr break-down in the minute after a 15 sec tetanus. There is a somewhat similar discrepancy over the changes during relaxation of FDNB-poisoned muscle, for Kushmerick *et al.* (1969) found that about half the total ATP splitting occurs after peak tension in a twitch, whereas Mommaerts & Wallner (1967) found no ATP splitting after peak tension in a 0·3 sec tetanus, which is little more than a twitch. Here again there is no obvious technical explanation for the discrepancy. Our own results bearing on this problem show a significant PCr splitting during relaxation.

Taking our own results into account, it seems likely that delayed splitting of PCr really does occur. IAA-poisoned muscle produces little or no (heat + work) during the minute after relaxation: there may even be a heat absorption (D. K. Hill, 1940). PCr splitting is certainly exothermic, therefore an endothermic process must be occurring in the muscle at the same time as the PCr splitting. It seems reasonable to suppose that this may be the reversal of the unidentified exothermic process occurring early in the contraction. The reason that this occurs at the same time as PCr splitting might well be that it is coupled to PCr splitting and that the latter provides the free energy required to cause the unknown process to go in reverse. It could, of course, be just chance that the heats of the PCr splitting and of the reversed unknown process are about equal and opposite. However, we should note that this would also come about if the entropy change for both processes was small and if only just enough PCr was split to bring about the coupled process.

The time course of reversal of the calcium movements occurring during a tetanus has been studied by Winegrad (1970). He finds a half-time of

28 sec at 4° C and a Q_{10} of 1·7. The estimated half-time at 0° C is thus 35 sec. This is somewhat similar to the time course of the post-contractile PCr splitting, which we do not yet know in detail. Aubert (1967) has studied the time course of the reappearance of the capacity to produce labile heat, which is lost after a long tetanus. The half-time of this process is 3 min, apparently significantly greater than the time course of post-contractile calcium movements.

Further experimentation will be needed to delineate the time course of the delayed PCr splitting. If it is not complete by about 40 sec after the end of relaxation, this would have led to systematically too high values for the heat obtained per mol of PCr split in experiments such as those of Wilkie (1968) and Carlson *et al.* (1967). Moreover, if in some batches of frogs its rate is appreciably lower than in the majority, which might well happen as a result of seasonal or other effects, this might account for the fact that occasional batches of frogs give values of the (heat + work)/ ΔPCr ratio that are unusually high.

The role of ATP in contraction

One conclusion that does seem to be quite certain is that no net breakdown of ATP has appeared under any conditions of contraction. If this process does occur it must be completely nullified, presumably by the rapid operation of the Lohmann reaction. The usual result, as shown in Table 5, is for the ATP to increase slightly during the first couple of seconds of contraction and although this result is near the noise level set by statistical fluctuations it has persisted over a sufficiently long period of time to justify some discussion.

The mean increase in ATP is about 0·1 m-mole/kg and our first problem concerns the source of the ADP which has become phosphorylated. Certainly enough ADP is present (0·5–0·65 m-mole/kg, Seraydarian, Mommaerts & Wallner, 1962) but only about 0·04 m-mole/kg of this is thought to be in the pool accessible to the creatine kinase system (Carlson & Siger, 1959; Chance & Weber, 1963). Of the ADP that is segregated, a large fraction is known to be bound to F-actin: there is independent evidence that this ADP cannot be phosphorylated unless the structure of the F-actin has been weakened, for example, by bile salts or sonication (Moos, 1964; see also Bendall, 1969, p. 13). Possibly some comparable change in structure occurs during the early stages of contraction, rendering some of the segregated ADP available for phosphorylation.

In recent years attention has been drawn to the participation in the contractile cycle of nucleotide attached to myosin. In schemes such as that of Taylor, Lymn & Moll (1970) a cycle is envisaged in which (1) ATP becomes bound to myosin, (2) it is hydrolysed *in situ* and (3) the resulting

ADP then dissociates from the myosin. The question of interest in relation to our results is at what stage this process is halted when the muscle relaxes. If it were between (2) and (3) one would expect an increase in the total ATP of the muscle soon after activation, when the ADP liberated by stage (3) would be rapidly rephosphorylated via the creatine kinase system. Taylor *et al.* (1970) did indeed find that stage (3) was the limiting one in solutions of myosin. It is possible that it becomes even more limiting when actin is present and the troponin-tropomyosin relaxing system is operating, that is, in a situation like that of a muscle at rest.

Two preliminary reports of this work have already appeared (Gilbert, Kretzschmar, Wilkie & Woledge, 1969, 1970) but our subsequent experiments have led us to modify some of our earlier conclusions. Thus we no longer believe that the increase in Pi is greater than can be attributed to changes in PCr and ATP. On the other hand, we feel more inclined to believe that the slight increase in ATP is genuine, and we are very much more confident that there is a genuine discrepancy between the physical energy observed and the chemical processes that we have investigated.

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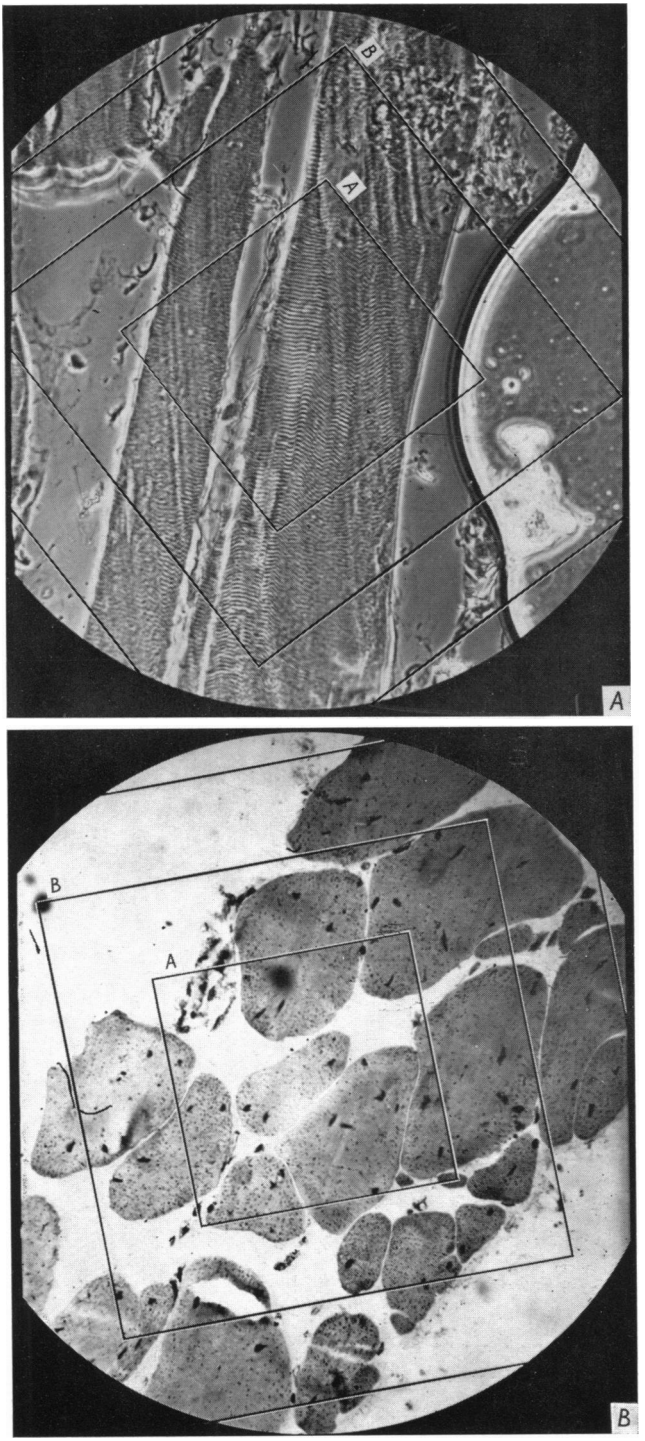
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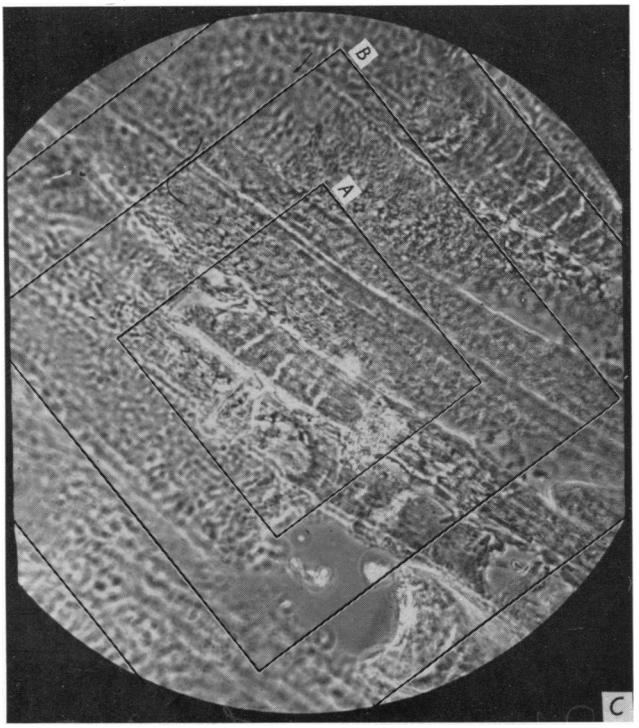
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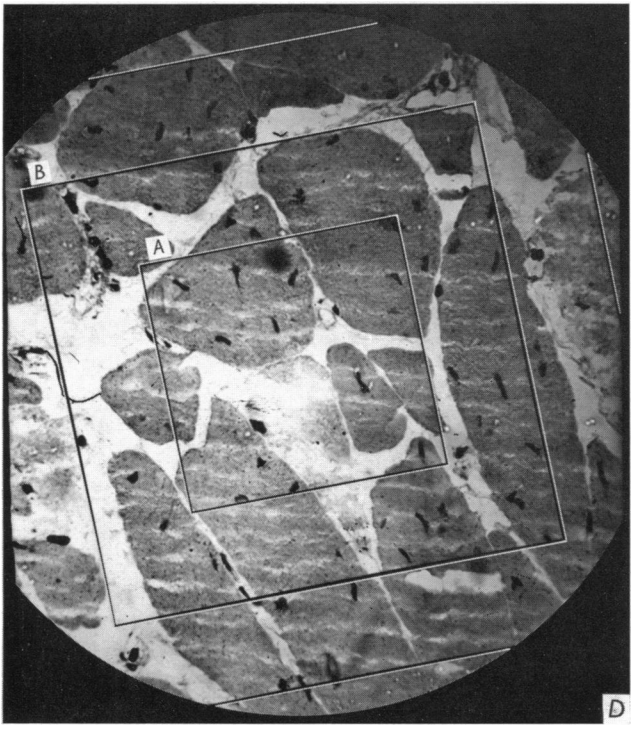
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EXPLANATION OF PLATES

PLATE 1

Side of square $A = 147\ \mu$.

A. Longitudinal section of muscle frozen in the hammer apparatus. Light microscope; phase contrast.

B. Transverse section, hammer apparatus. Light microscope: haematoxylin and eosin.

PLATE 2

C. Longitudinal section; frozen by immersion. Phase contrast.

D. Transverse section; immersion. Haematoxylin and eosin