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THE BREAK-DOWN OF ADENOSINE TRIPHOSPHATE IN THE CONTRACTION CYCLE OF THE FROG SARTORIUS MUSCLE

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SUMMARY

1. It is confirmed that a fluorodinitrobenzene (FDNB)-treated frog sartorius muscle does not split phosphorylcreatine in the course of its contraction cycle, but does use adenosine triphosphate (ATP).

2. Good stoicheiometric relations between the diminution of ATP and the formation of adenosine diphosphate (ADP), adenosine monophosphate (AMP) and phosphate are obtained, and in a 0·2 sec tetanus at 0° C the net break-down of ATP amounts to 0·27, the total equivalent break-down to 0·34 μmoles/g.

3. There is no difference in this quantity between muscles interrupted at the height of contraction and those that have also relaxed, and, in experiments specifically designed to determine relaxation metabolism separately, no such metabolism is found. Thus, all the ATP-break-down occurs in the contraction phase.

INTRODUCTION

For a decade after the first attempts to establish the chemical reactions associated with the mechanical events in muscle (Fleckenstein, Janke, Davies & Krebs, 1954; Mommaerts, 1954), their identities remained uncertain. A turning point was reached after 1960 with the demonstration of phosphorylcreatine splitting as an early reaction in the frog sartorius (Mommaerts & Seraydarian, 1961; Mommaerts, Seraydarian & Wallner, 1962) as well as in the turtle rectus femoris muscle (Mommaerts, Olmsted, Seraydarian & Wallner, 1962), and of the corresponding ATP-splitting in muscles poisoned with fluorodinitrobenzene (Cain & Davies, 1962*a*; Infante & Davies, 1962).

In the demonstrations from our laboratory, the utilization of phosphorylcreatine (PC) appeared to take place during the ascending phase of the brief tetanic contractions: although no separate experiments were done to test for any additional metabolism during relaxation—apart from a subsequent

use of PC in the phosphohexokinase reaction—the sum of knowledge about the problem did not suggest such an event (see especially Maréchal & Mommaerts, 1963). Cain & Davies (1962*b*) studying the liberation of phosphate from an unidentified source, and Cain, Infante & Davies (1962) confirming PC breakdown in the frog rectus abdominis muscle in experiments similar to ours, state that there is no additional liberation of phosphate during relaxation. On the other hand, in reporting their findings on the FDNB-treated muscles, Infante & Davies (1962) specifically mention that ATP is broken down largely during the relaxation phase. This paradox is not resolved, but the latter result appears to be given preference in all later treatments by these authors and has played a crucial role in their proposed theory (Davies, 1963).

In view of the great significance of this point, we have devoted special attention to the temporal resolution of this chemical change in the course of the contraction cycle. The present publication will describe a set of experiments specifically designed to determine whether the break-down of ATP occurs during the contraction or the relaxation phase of a brief contraction.

METHODS

The rapid freezing method. The procedures for rapidly fixing contracting muscles at any desired state of activity (Mommaerts & Schilling, 1955) have been improved over the years, and have been described in general outline (Mommaerts & Schilling, 1964). In the present instance, a pair of frog sartorius muscles, treated as described below, is placed upon a double multi-electrode assembly designed to stimulate one muscle at a time, synchronously over most of its length. After a predetermined interval, both muscles are then suddenly immersed into isopentane cooled with liquid nitrogen. The uncertainty as to the actual moment of interrupting all activity is estimated to be of the order of 50 msec for the bulk of the muscle; this estimate refers to reaching a temperature of about $-10°$ C, from which point onward cooling proceeds more rapidly and the impedance function $d\Omega/dt$ shows a sharp rise (Mommaerts & Schilling, 1964). This time span must be compared to times of the order of 350 msec between the first stimulus and the plateau of contraction, and of 750 msec for the complete contraction cycle (Fig. 1).

Experimental design. In an actual experiment, the following sequence is programmed by the control devices. (*a*) Stimulation of one muscle by 5 square pulses of 1 msec duration, 50 msec repeat interval, just above threshold. (*b*) Stimulation of the contralateral muscle in similar fashion, starting 600 msec after the beginning of stimulation of the first muscle. (*c*) Immersion into the cooling bath about 300 msec later. This procedure results in the freezing of the muscle (*a*) after a complete 200 msec tetanus including relaxation (R), and of muscle (*b*) at the height of the contraction plateau (C). This will be referred to as the differential stimulation mode CR, an example of which is given in Fig. 1. In other experiments, one muscle is left unstimulated, and both are frozen when the second muscle is at the peak of contraction (difference OC), or has completed an entire cycle (difference OR). While it is easy to assure the freezing after a complete cycle (indeed at an arbitrary point after relaxation), the fixation at the plateau of contraction is less certain as to its accurate timing, and it might be questioned if such a muscle had received five stimuli or fewer. If, for example, in the mode CR, differences in composition had been encountered, this would have been a matter of concern, since it would then have to be debated whether the difference is due to

relaxation or to additional excitation. Since, however, no differences CR were found, this question does not arise, and either the muscles C did effectively receive five stimuli, or the number of stimuli, whether four or five, does not affect the chemical composition found afterwards. Furthermore (Fig. 1), since tension continues to rise after the last stimulus, and freezing is timed to occur at the maximum, it is indeed certain that the C muscle received five stimuli.

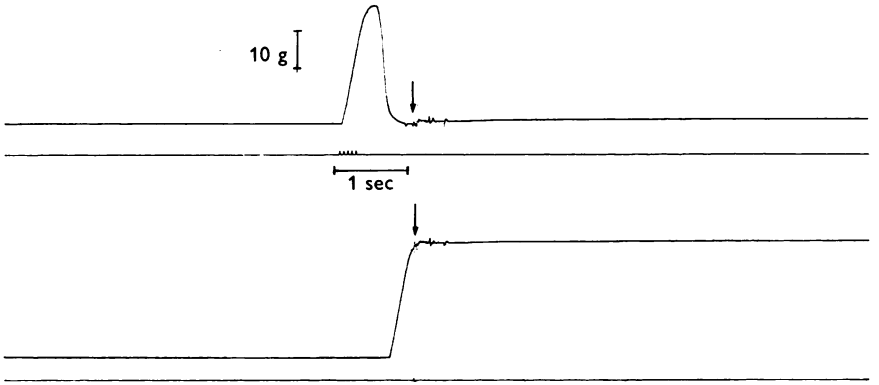

Fig. 1. Example of an experiment in the mode CR (see text). Isometric recording of a pair of muscles, one of which is frozen at the peak of contraction, the other after a complete cycle (arrows). The stimulus signal applies to the upper record, none was available for the lower, but this can easily be inferred.

In one block of experiments executed on a single day, 6–8 pairs of muscles were used. A stratified design was preferred, in which all different modes were represented in each block: two each according to OC and OR, and two to four in the mode CR; of each of these combinations, half is done isometrically at standard length L_0 with a minimal external compliance (a cotton loop of about 1 mm diameter attached to a heavy steel wire leading to the transducer), half is done isotonically with a light load, 4 g, starting from L_0. Within each block, all experiments were randomized with respect to the position (left or right) in the apparatus, the selection (left or right) of the stimulus or the first stimulus, and the sequence of modes within one block.

The muscles were attached to the isotonic and isometric variable-capacitance gauges of this laboratory (Schilling, 1960).

Treatment of the muscles. Frogs (*Rana pipiens*) were purchased from Northern suppliers in the fall or winter, and kept dormant at 6° C in moist tanks with a daily rinse of cold water. The day before the experiment, the animals were each given 0·6 mg of (+)-tubocurarine in the dorsal lymph sac, and the sartorius muscles, attached to the pelvic bone, dissected free. They were left aerated overnight at 4° C in a Ringer solution of 95·3 mM-NaCl, 20·0 mM-$NaHCO_3$, 2·5 mM-KCl, 1·0 mM-$MgSO_4$, 1·0 mM-$CaCl_2$, the gas phase being O_2 with 2 % CO_2. Before each experiment, each pair was soaked for 40 min at 0° C in Ringer solution containing 0·38 mM FDNB in an atmosphere of N_2 with 2 % CO_2 (as practised by Infante & Davies, 1962), then 5 min again in the same medium without the drug. They were then mounted in the apparatus and kept for 10 min at 0° C in N_2 with 2 % CO_2 moistened at the vapour pressure of the Ringer solution at that temperature. The experiment is then performed in the indicated fashion. In experiments before 1965, not described, the poisoning was conducted aerobically, but when the results obtained were found to differ from those of Infante & Davies (1962), the procedure was then made to conform with theirs.

Analytical procedures. After freezing, the muscles are detached from the instrument, blotted and weighed (wrapped in tared pieces of Parafilm), and stored in liquid nitrogen until analysis. The muscles are individually powdered at low temperature and extracted with 0·25 N perchloric acid with our standard procedures (Seraydarian, Mommaerts, Wallner & Guillory, 1961); in other experiments individually discussed, extraction was done with citrate-ethanol at −20° C (Seraydarian, Mommaerts & Wallner, 1962).

The following analytical procedures were used (Mommaerts, 1957): inorganic phosphate (P_i) with the alkaline $CaCl_2$ precipitation procedure of Seraydarian *et al.* (1961); free creatine, and total creatine after hydrolysis, according to Ennor & Rosenberg (1952); a 10·8 % correction is often applied to compensate for creatinine formation in the determination of phosphocreatine, but there has been some difference of opinion about the size of this correction; since no changes in phosphocreatine were encountered, this point is of less relevance to our work, and the analyses are given as obtained, without correction.

Adenine nucleotides were determined by enzymic-fluorimetric methods. Enzyme preparations from Biochimica-Boehringer (Mainz, Germany) were used throughout. For ATP, the determination is based upon the formation of 1,3-diphosphoglycerate from 3-phosphoglycerate and ATP, followed by the formation of pyridine nucleotide from reduced pyridine nucleotide in the phosphoglyceraldehyde dehydrogenase reaction. For ADP, the procedure is that of Seraydarian *et al.* (1962), while the additional presence of adenylate kinase gives the amount of AMP. The relative abundance of adenine and hypoxanthine nucleotides has not been studied systematically by us, but spectrophotometric determinations relevant to that question are mentioned in the text. The enzymic determination for ATP and ADP would also cover any inosinetriphosphate (ITP) and inosinediphosphate (IDP), but inosinemonophosphate (IMP), the only deaminated nucleotide likely to be present, is not included in the analyses.

All analyses are given in μmoles/g wet muscle.

RESULTS

Presentation of results. The results of all experiments, with the omission of two whose outcomes were evidently divergent (both in the mode OA, isometric), are listed in Table 1. We considered using the total creatine contents as a reference for the true active muscle mass, and to correct the analyses on that basis. In the present work, none of the mean values in any of the subgroups showed any difference in total creatine contents; the correction was therefore omitted, since it would not have altered any of the comparisons, although if applied to individual pairs it might have reduced the standard errors.

Fewer determinations, in which citrate-ethanol was used as the extractant, are listed in Table 3. This solvent (Seraydarian *et al.* 1962) serves to extract free ADP selectively without actin-bound ADP, and lessens the formation of inorganic phosphate from excessively acid-labile precursors.

Evaluation of data. In experiments performed in mode CR, the differences Δ_{CR} reflect the difference in composition correlated with the occurrence of relaxation in addition to contraction; $\bar{\Delta}_{CR}$ is the mean of these differences, and S_{CR} the standard error of this mean. In modes OC or OR, the differences similarly refer to the metabolic processes occurring between

TABLE 1. Compilation of data in groupwise arrangement. Means and standard errors in μmoles/g muscle

		Free creatine	Total creatine	P_i
Control *vs.* contracted				
(1) Isometric	$n = 16$	O = 7·88 C = 7·83 $\Delta = -0{\cdot}05$ $S_\Delta = 0{\cdot}32$	O = 30·78 C = 30·73 $\Delta = -0{\cdot}05$ $S_\Delta = 0{\cdot}83$	O = 3·81 C = 4·11 $\Delta = +0{\cdot}30$ $S_\Delta = 0{\cdot}19$
(2) Isotonic	$n = 17$	O = 7·30 C = 7·17 $\Delta = -0{\cdot}13$ $S_\Delta = 0{\cdot}18$	O = 30·50 C = 30·40 $\Delta = -0{\cdot}10$ $S_\Delta = 0{\cdot}50$	O = 4·07 C = 4·56 $\Delta = +0{\cdot}49$ $S_\Delta = 0{\cdot}15$
(3) All	$n = 33$	O = 7·59 C = 7·50 $\Delta = -0{\cdot}09$ $S_\Delta = 0{\cdot}18$	O = 30·64 C = 30·56 $\Delta = -0{\cdot}08$ $S_\Delta = 0{\cdot}47$	O = 3·94 C = 4·33 $\Delta = +0{\cdot}39$ $S_\Delta = 0{\cdot}12$
Control *vs.* relaxed				
(4) Isometric	$n = 17$	O = 7·08 R = 7·13 $\Delta = +0{\cdot}05$ $S_\Delta = 0{\cdot}13$	O = 30·75 R = 30·71 $\Delta = -0{\cdot}04$ $S_\Delta = 0{\cdot}48$	O = 3·81 R = 4·04 $\Delta = +0{\cdot}23$ $S_\Delta = 0{\cdot}12$
(5) Isotonic	$n = 17$	O = 7·98 R = 7·96 $\Delta = -0{\cdot}02$ $S_\Delta = 0{\cdot}12$	O = 31·90 R = 31·87 $\Delta = -0{\cdot}03$ $S_\Delta = 0{\cdot}50$	O = 4·00 R = 4·45 $\Delta = +0{\cdot}45$ $S_\Delta = 0{\cdot}10$
(6) All	$n = 34$	O = 7·53 R = 7·54 $\Delta = +0{\cdot}01$ $S_\Delta = 0{\cdot}08$	O = 31·32 R = 31·29 $\Delta = -0{\cdot}03$ $S_\Delta = 0{\cdot}34$	O = 3·90 R = 4·24 $\Delta = +0{\cdot}34$ $S_\Delta = 0{\cdot}08$
Control *vs.* contracted or relaxed				
(7) Isometric	$n = 33$	O = 7·48 C+R = 7·48 $\Delta = 0{\cdot}00$ $S_\Delta = 0{\cdot}17$	O = 30·76 C+R = 30·72 $\Delta = -0{\cdot}04$ $S_\Delta = 0{\cdot}47$	O = 3·81 C+R = 4·07 $\Delta = +0{\cdot}26$ $S_\Delta = 0{\cdot}11$
(8) Isotonic	$n = 34$	O = 7·64 C+R = 7·56 $\Delta = -0{\cdot}08$ $S_\Delta = 0{\cdot}24$	O = 31·20 C+R = 31·13 $\Delta = -0{\cdot}07$ $S_\Delta = 0{\cdot}35$	O = 4·03 C+R = 4·50 $\Delta = +0{\cdot}47$ $S_\Delta = 0{\cdot}09$
(9) All	$n = 67$	O = 7·56 C+R = 7·52 $\Delta = -0{\cdot}04$ $S_\Delta = 0{\cdot}10$	O = 30·98 C+R = 30·92 $\Delta = -0{\cdot}06$ $S_\Delta = 0{\cdot}28$	O = 3·92 C+R = 4·28 $\Delta = +0{\cdot}36$ $S_\Delta = 0{\cdot}07$
Contracted *vs.* relaxed				
(10) Isometric	$n = 24$	C = 7·20 R = 7·21 $\Delta = +0{\cdot}01$ $S_\Delta = 0{\cdot}13$	C = 29·16 R = 29·21 $\Delta = +0{\cdot}05$ $S_\Delta = 0{\cdot}45$	C = 4·02 R = 4·06 $\Delta = +0{\cdot}04$ $S_\Delta = 0{\cdot}10$
(11) Isotonic	$n = 23$	C = 8·15 R = 8·17 $\Delta = +0{\cdot}01$ $S_\Delta = 0{\cdot}11$	C = 30·62 R = 30·58 $\Delta = -0{\cdot}04$ $S_\Delta = 0{\cdot}46$	C = 4·27 R = 4·34 $\Delta = +0{\cdot}07$ $S_\Delta = 0{\cdot}11$
(12) All	$n = 47$	C = 7·68 R = 7·69 $\Delta = +0{\cdot}01$ $S_\Delta = 0{\cdot}08$	C = 29·89 R = 29·89 $\Delta = 0{\cdot}00$ $S_\Delta = 0{\cdot}32$	C = 4·14 R = 4·20 $\Delta = +0{\cdot}06$ $S_\Delta = 0{\cdot}07$
		ATP	**ADP**	**AMP**
(1) Isometric	$n = 16$	O = 2·19 C = 1·89 $\Delta = -0{\cdot}30$ $S_\Delta = 0{\cdot}03$	O = 0·86 C = 1·03 $\Delta = +0{\cdot}17$ $S_\Delta = 0{\cdot}03$	O = 0·34 C = 0·40 $\Delta = +0{\cdot}06$ $S_\Delta = 0{\cdot}03$
(2) Isotonic	$n = 17$	O = 2·17 C = 1·87 $\Delta = -0{\cdot}30$ $S_\Delta = 0{\cdot}04$	O = 0·96 C = 1·10 $\Delta = +0{\cdot}15$ $S_\Delta = 0{\cdot}03$	O = 0·36 C = 0·45 $\Delta = +0{\cdot}09$ $S_\Delta = 0{\cdot}02$
(3) All	$n = 33$	O = 2·18 C = 1·88 $\Delta = -0{\cdot}30$ $S_\Delta = 0{\cdot}02$	O = 0·91 C = 1·07 $\Delta = +0{\cdot}16$ $S_\Delta = 0{\cdot}02$	O = 0·35 C = 0·42 $\Delta = +0{\cdot}07$ $S_\Delta = 0{\cdot}12$

TABLE 1 (*cont.*)

			ATP		ADP		AMP	
Control *vs.* relaxed								
(4) Isometric	$n = 17$	O = 2·21	R = 2·04	$\Delta = -0{\cdot}17$, $S_\Delta = 0{\cdot}07$	O = 0·94	R = 1·05	$\Delta = +0{\cdot}10$, $S_\Delta = 0{\cdot}04$	O = 0·37	R = 0·45	$\Delta = +0{\cdot}08$, $S_\Delta = 0{\cdot}03$
(5) Isotonic	$n = 17$	O = 2·17	R = 1·87	$\Delta = -0{\cdot}30$, $S_\Delta = 0{\cdot}06$	O = 0·90	R = 1·05	$\Delta = +0{\cdot}15$, $S_\Delta = 0{\cdot}03$	O = 0·40	R = 0·48	$\Delta = +0{\cdot}08$, $S_\Delta = 0{\cdot}02$
(6) All	$n = 34$	O = 2·19	R = 1·95	$\Delta = -0{\cdot}24$, $S_\Delta = 0{\cdot}05$	O = 0·92	R = 1·05	$\Delta = +0{\cdot}13$, $S_\Delta = 0{\cdot}02$	O = 0·38	R = 0·46	$\Delta = +0{\cdot}08$, $S_\Delta = 0{\cdot}02$
Control *vs.* contracted or relaxed										
(7) Isometric	$n = 33$	O = 2·20	C+R = 1·96	$\Delta = -0{\cdot}24$, $S_\Delta = 0{\cdot}04$	O = 0·90	C+R = 1·04	$\Delta = +0{\cdot}13$, $S_\Delta = 0{\cdot}03$	O = 0·36	C+R = 0·42	$\Delta = +0{\cdot}06$, $S_\Delta = 0{\cdot}02$
(8) Isotonic	$n = 34$	O = 2·17	C+R = 1·87	$\Delta = -0{\cdot}30$, $S_\Delta = 0{\cdot}03$	O = 0·93	C+R = 1·07	$\Delta = +0{\cdot}15$, $S_\Delta = 0{\cdot}02$	O = 0·38	C+R = 0·46	$\Delta = +0{\cdot}08$, $S_\Delta = 0{\cdot}02$
(9) All	$n = 67$	O = 2·18	C+R = 1·91	$\Delta = -0{\cdot}27$, $S_\Delta = 0{\cdot}02$	O = 0·91	C+R = 1·06	$\Delta = +0{\cdot}14$, $S_\Delta = 0{\cdot}02$	O = 0·37	C+R = 0·44	$\Delta = +0{\cdot}07$, $S_\Delta = 0{\cdot}01$
Contracted *vs.* relaxed										
(10) Isometric	$n = 24$	C = 1·78	R = 1·74	$\Delta = -0{\cdot}04$, $S_\Delta = 0{\cdot}03$	C = 0·87	R = 0·91	$\Delta = +0{\cdot}04$, $S_\Delta = 0{\cdot}02$	C = 0·40	R = 0·41	$\Delta = +0{\cdot}01$, $S_\Delta = 0{\cdot}01$
(11) Isotonic	$n = 23$	C = 1·69	R = 1·71	$\Delta = +0{\cdot}02$, $S_\Delta = 0{\cdot}03$	C = 1·07	R = 1·05	$\Delta = -0{\cdot}02$, $S_\Delta = 0{\cdot}03$	C = 0·40	R = 0·42	$\Delta = +0{\cdot}02$, $S_\Delta = 0{\cdot}01$
(12) All	$n = 47$	C = 1·73	R = 1·72	$\Delta = -0{\cdot}01$, $S_\Delta = 0{\cdot}02$	C = 0·97	R = 0·98	$\Delta = +0{\cdot}01$, $S_\Delta = 0{\cdot}02$	C = 0·40	R = 0·42	$\Delta = +0{\cdot}02$, $S_\Delta = 0{\cdot}01$

TABLE 2. Reference square of all mean differences.
Amounts are in μmoles/g; differences OC, OR, CR as in the text.

		(1) ATP	(2) ADP	(3) AMP	(4) P_i	Sum of 2 and twice 3; should equal value 4	Number in Table 1
Isometric	O–C	$-0{\cdot}30 \pm 0{\cdot}03$	$+0{\cdot}17 \pm 0{\cdot}03$	$+0{\cdot}06 \pm 0{\cdot}03$	$+0{\cdot}30 \pm 0{\cdot}19$	+0·29	1
Isotonic	O–C	$-0{\cdot}30 \pm 0{\cdot}04$	$+0{\cdot}15 \pm 0{\cdot}03$	$+0{\cdot}09 \pm 0{\cdot}02$	$+0{\cdot}49 \pm 0{\cdot}15$	+0·33	2
Both	O–C	$-0{\cdot}30 \pm 0{\cdot}02$	$+0{\cdot}16 \pm 0{\cdot}02$	$+0{\cdot}07 \pm 0{\cdot}02$	$+0{\cdot}39 \pm 0{\cdot}12$	+0·30	3
Isometric	O–R	$-0{\cdot}17 \pm 0{\cdot}07$	$+0{\cdot}10 \pm 0{\cdot}04$	$+0{\cdot}08 \pm 0{\cdot}03$	$+0{\cdot}23 \pm 0{\cdot}12$	+0·26	4
Isotonic	O–R	$-0{\cdot}30 \pm 0{\cdot}06$	$+0{\cdot}15 \pm 0{\cdot}03$	$+0{\cdot}08 \pm 0{\cdot}02$	$+0{\cdot}45 \pm 0{\cdot}10$	+0·31	5
Both	O–R	$-0{\cdot}24 \pm 0{\cdot}05$	$+0{\cdot}13 \pm 0{\cdot}02$	$+0{\cdot}08 \pm 0{\cdot}02$	$+0{\cdot}34 \pm 0{\cdot}08$	+0·29	6
Isometric	O–(C or R)	$-0{\cdot}24 \pm 0{\cdot}04$	$+0{\cdot}13 \pm 0{\cdot}02$	$+0{\cdot}06 \pm 0{\cdot}01$	$+0{\cdot}26 \pm 0{\cdot}11$	+0·25	7
Isotonic	O–(C or R)	$-0{\cdot}30 \pm 0{\cdot}03$	$+0{\cdot}15 \pm 0{\cdot}02$	$+0{\cdot}08 \pm 0{\cdot}01$	$+0{\cdot}47 \pm 0{\cdot}09$	+0·31	8
Both	O–(C or R)	$-0{\cdot}27 \pm 0{\cdot}02$	$+0{\cdot}14 \pm 0{\cdot}01$	$+0{\cdot}07 \pm 0{\cdot}01$	$+0{\cdot}36 \pm 0{\cdot}07$	+0·28	9
Isometric	C–R	$-0{\cdot}04 \pm 0{\cdot}03$	$+0{\cdot}04 \pm 0{\cdot}02$	$+0{\cdot}01 \pm 0{\cdot}01$	$+0{\cdot}04 \pm 0{\cdot}10$	+0·06	10
Isotonic	C–R	$+0{\cdot}02 \pm 0{\cdot}03$	$-0{\cdot}02 \pm 0{\cdot}03$	$+0{\cdot}02 \pm 0{\cdot}02$	$+0{\cdot}07 \pm 0{\cdot}11$	+0·02	11
Both	C–R	$-0{\cdot}01 \pm 0{\cdot}02$	$+0{\cdot}01 \pm 0{\cdot}02$	$+0{\cdot}02 \pm 0{\cdot}01$	$+0{\cdot}06 \pm 0{\cdot}07$	+0·05	12

the onset of excitation and the peak or the end of the contraction cycle; OC and OR are also combined, for the total of all experiments of rest versus activity. Finally, the difference $\bar{\Delta}_{OC} - \bar{\Delta}_{OR}$ is an alternative, but less accurate, route to the detection of additional relaxation metabolism. These quantities are grouped in several ways in Table 1 and the crucial numbers summarized in Table 2.

The occurrence of ATP-break-down in contractile activity. These data are compiled in Table 1 (and 2), entries 1–9, in different combinations. The most inclusive statement derives from line 9, summarizing all experiments in which one muscle was kept as the unstimulated control, the other frozen either at the height of contraction or after a complete cycle, and combining both isotonic and isometric experiments. This series shows with considerable certainty the following points. (*a*) There is no change in total creatine (free plus phosphorylated), thus there is no transformation of PC into, for example, creatinine nor is there any formation of a substance reacting like creatinine. In these brief spans of activity, this finding has no particular significance. (*b*) There is no change in the free creatine, neither in the aggregate, where the total tabulated change is $-0{\cdot}04$ ($\pm 0{\cdot}10$), hence the insignificant diappearance rather than a formation, nor in any of the subgroups. Thus, the ATP: creatine phosphotransferase reaction is indeed satisfactorily suppressed, as was already concluded by Infante & Davies (1962) but criticized by Wilkie (1966), see also Maréchal & Beckers-Bleukx (1966). This point depends, of course, on just how completely the phosphotransferase is inhibited, and how great its excess is. (*c*) There is a breakdown of ATP. In the aggregate, this averages 0·27, but to obtain a measure of actual ATP breakdown one must add the amount of AMP formed, so obtaining an ATP usage of 0·34. This is of the same magnitude as previous results on the FDNB-poisoned frog sartorius (Infante & Davies, 1962) although those were obtained on twitches and a rather larger change was reported after a complete cycle; the result corresponds accurately to the findings on single contractions of untreated muscles (about 0·40/g/0·3 sec tetanus, Mommaerts, Seraydarian & Wallner, 1962) and in series of contractions of iodoacetate-poisoned muscles (0·29/g/twitch, Carlson & Siger, 1960; 0·45/0·4 sec tetanus, Mommaerts, Seraydarian & Maréchal, 1962). (*d*) There is a formation of both ADP and AMP, and a satisfactory stoicheiometric correspondence between these increases and the decrease of ATP (as in the first brief publication by Cain & Davies, 1962*a*, on the rectus abdominis muscle). No significant formation of IMP seems to occur within the allowed time span, although this becomes pronounced in more prolonged activity (Maréchal & Beckers-Bleukx, 1966). (*e*) There is a formation of P_i and a good correspondence between this and the breakdown of ATP if the formation of both ADP and AMP is considered. (*f*) The com-

parisons between isometric and isotonic experiments come out less explicitly (entries 7–8; also 1–2 and 4–5); they do not show that the isotonically contracting muscles display the same or less chemical change than those contracting isometrically (Carlson, Hardy & Wilkie, 1963; Mommaerts, Seraydarian & Maréchal, 1962; Davies, Kushmerick & Larson, 1967), but this is largely due to a low run of values in entry 4; this point is not a subject of the present investigation.

In summary, it is found that all comparisons, between resting controls and muscles that have contracted, demonstrate a breakdown of ATP to ADP, AMP and P_i, and an absence of breakdown of PC.

The time course of breakdown of ATP: contraction as compared to relaxation. The experiments comparing the difference CR directly provide the most explicit and accurate information on this point. In the aggregate, they show (entry 12) with considerable certainty that there is no additional breakdown of ATP coincident with relaxation ($-\Delta = 0{\cdot}01 \pm 0{\cdot}02$) nor is any such breakdown indicated in the isometric or isotonic subgroups (items 10 and 11). With respect to the products ADP and AMP, the results are identical, only differences in the second decimal figure being encountered. With respect to P_i formation, the differences are somewhat larger, 0·04 and 0·07 in the subgroups, but this is still within the error margin.

Equally, the conclusion could be based upon a comparison of the differences OC and OR (entries 3 and 6); this is not further commented upon, as this comparison is less precise.

Thus, it is concluded that no changes in ATP and its reaction products are demonstrably associated with the relaxation process, and that such changes, of a physiologically significant magnitude (see the Discussion) are excluded by the experiments.

Concentrations of metabolites. All the analyses listed so far were obtained on FDNB-treated muscles exclusively, but these will be compared with determinations on unpoisoned muscles illustrated by a small number of analyses (Table 3) which bear out the impression gained from the main body of the experiments. Thus, it is found that the ATP contents of FDNB-treated muscles are always lower than those of untreated ones, and the ADP (and AMP) contents higher. The determinations of free ADP with citrate-ethanol (Seraydarian *et al.* 1962) show that in unpoisoned muscles this is of the order of 0·15 μmoles/g as was found previously; but that in poisoned muscles it is this quantity which is markedly higher. Specific tests (the application of PCA after the extraction with citrate ethanol is illustrated in Table 3) indicate that the actin-bound ADP is not altered as could conceivably have occurred had the FDNB reacted with a crucial sulphydryl group on this protein.

TABLE 3. Nucleotide analyses in citrate-ethanol and in residual PCA-extracts, in normal and FDNB-treated muscles. Entries on each line refer to muscles of a pair. Analyses are in μmoles/g

	Unpoisoned				FDNB-treated				Difference due to FDNB			
	ATP	ADP	AMP	P_i	ATP	ADP	AMP	P_i	ATP	ADP	AMP	P_i
Ethanol	2·24	0·21	0·03	3·38	1·45	0·65	0·07	3·81	−0·79	+0·44	+0·04	+0·43
PCA	0·37	0·22	0	0·33	0·27	0·21	0	0·29	−0·10	—	0	—
Sum	2·61	0·43	0·03	3·71	1·72	0·86	0·07	4·10	−0·89	0·44	+0·04	0·43
	2·46	0·19	0·03	3·49	1·84	0·64	0·07	3·86	−0·62	+0·45	+0·04	+0·37
	0·34	0·24	0	0·31	0·27	0·22	0	0·29	−0·07	—	0	—
	2·80	0·43	0·03	3·80	2·11	0·86	0·07	4·15	−0·69	+0·45	+0·04	+0·37
	2·34	0·17	0·03	2·80	1·92	0·52	0·05	3·30	−0·42	+0·35	+0·02	+0·50
	0·37	0·22	0	0·31	0·29	0·22	0	0·31	−0·08	—	0	—
	2·71	0·39	0·03	3·11	2·21	0·74	0·05	3·61	−0·50	+0·35	+0·02	+0·50
	2·55	0·11	0·03	2·54	2·05	0·54	0·07	3·04	−0·50	+0·43	+0·04	+0·50
	0·23	0·20	0	0·14	0·18	0·16	0	0·14	−0·05	—	0	—
	2·78	0·31	0·03	2·68	2·23	0·70	0·07	3·18	−0·55	+0·43	+0·04	+0·50
	2·37	0·13	0·03	2·98	1·45	0·53	0·08	3·55	−0·92	+0·40	+0·05	+0·57
	0·37	0·20	0	0·27	0·21	0·21	0	0·31	−0·16	—	0	+0·04
	2·74	0·33	0·03	3·25	1·66	0·74	0·08	3·86	−1·08	+0·40	+0·05	+0·61
	1·98	0·21	0·03	2·88	1·49	0·51	0·05	3·66	−0·49	+0·30	+0·02	+0·78
	0·31	0·25	0	0·32	0·24	0·28	0	0·35	−0·07	+0·03	0	+0·03
	2·29	0·46	0·03	3·20	1·73	0·79	0·05	4·01	−0·56	+0·33	+0·02	+0·81
	2·39	0·17	0·03	2·31	1·95	0·53	0·05	2·69	−0·44	+0·36	+0·02	+0·38
	0·38	0·25	0	0·28	0·30	0·28	0	0·36	−0·08	+0·03	0	+0·08
	2·77	0·42	0·03	2·59	2·25	0·81	0·05	3·05	−0·52	+0·39	+0·02	+0·46
	2·20	0·22	0·03	2·09	1·67	0·60	0·07	2·50	−0·53	+0·38	+0·04	+0·41
	0·33	0·27	0	0·31	0·25	0·30	0	0·43	−0·08	+0·03	0	+0·12
	2·53	0·49	0·03	2·40	1·92	0·90	0·07	2·93	−0·61	+0·41	+0·04	+0·53
	1·85	0·22	0·03	2·33	1·43	0·51	0·06	2·60	−0·42	+0·29	+0·03	+0·27
	0·24	0·24	0	0·31	0·18	0·28	0	0·43	−0·06	+0·04	0	+0·12
	2·09	0·46	0·03	2·64	1·61	0·79	0·06	3·03	−0·48	+0·33	+0·03	+0·39
	1·88	0·24	0·03	2·24	1·37	0·54	0·05	2·76	−0·51	+0·30	+0·02	+0·52
	0·31	0·24	0	0·29	0·23	0·27	0	0·46	−0·08	+0·03	0	+0·17
	2·19	0·48	0·03	2·53	1·60	0·81	0·05	3·22	−0·59	+0·33	+0·02	+0·69
Means	2·55	0·42	0·03	2·99	1·90	0·80	0·06	3·51	−0·64	+0·38	+0·03	+0·52

Note: Some of the analyses in this table are somewhat lower than usual, especially the actin-bound ADP (as indicated by the PCA-extracted ADP) is about 0·24 instead of 0·3–0·4. The muscles in the upper half of the table were from frogs kept several months in the cold without food; those in the lower half of the table were from spring frogs freshly obtained. Also, the treatment of the muscles differed from that in the paper in which the magnitude of the bound ADP was established (Seraydarian *et al.* 1962). Since the PCA-extracted ATP values tend to be higher, it is conceivable that some of the actin-bound nucleotide may be ATP.

Occasional recordings of the ultraviolet absorption spectra of muscle extracts have not shown that a significant degree of deamination of nucleotides occurs during the poisoning or during the activity as carried out here. Of some concern is the report by Dydynska & Wilkie (1966) that not only a good deal of deamination occurs during poisoning, but also the sum of the resulting hypoxanthine and adenine nucleotides is in sizable excess over that in normal muscle. During the poisoning period, as we could establish by weighing blotted muscles, some 7 % shrinkage occurs (as is also shown by Dydynska & Wilkie, 1966, in their Table 2), but this fluid loss is reversed when the reagent is washed out, and does not suffice to account for their apparent increase in nucleotide which is about 25 %. Since these authors determined the adenine- and hypoxanthine compounds by spectrophotometry at two wave-lengths, and do not appear to have removed excess FDNB, we believe that their findings must be ascribed to the remaining drug appearing in the extracts, where, having a major absorption band at 240 mμ like hypoxanthine nucleotides, its presence would lead to an erroneous result in just this sense. When the muscle is bathed in ordinary Ringer solution, the drug is removed sufficiently, and no significant deamination is found. As to the events in contraction, the stoicheiometric balance of ATP disappearance and the formation of ADP, AMP and P_i indicates that within the times allowed no significant deamination takes place; the enzymic assays would also respond to IDP or ITP but these are not found in muscle; IMP is not included in the enzymic assays. Maréchal & Beckers-Bleukx (1966) did, however, show a considerable deamination after a prolonged tetanus; this difference may be a matter of timing, or perhaps the adenylate kinase is subjected to allosteric control and is not activated until after a certain amount of activity. These authors, as well as Cain, Kushmerick & Davies (1964) studying the proportions of adenine and hypoxanthine nucleotides in a different context, employed chromatographic procedures instead of two-point spectrophotometry.

For all resting FDNB-muscles, perchloric acid extracted, these are the mean concentrations of the adenine nucleotides: 2·19 (ATP), 0·92 (ADP) and 0·37 (AMP). When entered into the equation for the ATP:AMP phosphotransferase equilibrium, this would give a K value of 0·96; and of 3·2 if the free ADP were corrected to 0·5; Davies (1965) has reported that the enzyme is only about 30 % inhibited in the FDNB-treated muscles. Even if admitting that the equilibrium constant which is dependent upon the Mg concentration and other factors (cf. Noda, 1962), may be higher than the 0·45 considered as standard (according to Eggleston & Hems, 1952, and Green, Brown & Mommaerts, 1953), the nucleotides do not seem to be in equilibrium unless additional assumptions are made. However, when drawing data from all paired comparisons of rest versus activity

($\Delta_{ATP} = -0{\cdot}27$, $\Delta_{ADP} = +0{\cdot}14$, $\Delta_{AMP} = +0{\cdot}07$; ATP reformed in the myokinase reaction $= +0{\cdot}07$), the quotient $(0{\cdot}07 \times 0{\cdot}07)/(0{\cdot}14)^2$ would be close to a possible value of the equilibrium constant. It may be concluded, therefore, that much of the ADP formed is subjected to transphosphorylation, while in experiments reported by Davies *et al.* (1967) this reaction was insignificant until at a somewhat later time; this may well be due to a different treatment of the muscles.

DISCUSSION

Since this appears to be the first extensive study, since the original brief announcements by Cain & Davies (1962*a*) and Infante & Davies (1962), of the chemical events in the contraction of FDNB-poisoned muscle, some general comments on this preparation, developed to eliminate the ATP: creatine phosphotransferase reaction, may be in order. It was unlikely, beforehand, that FDNB, which should react with all accessible amino, imidazole and sulphydryl groups of all proteins, would exert such a specific inhibition at such a desired point. Davies (1965) comments that the possibility was not tested for two years after the inhibition of the transphosphorylase was first described by Kuby & Mahowald (1959), and we (Mommaerts, 1965) had declined to attempt it altogether. Yet, the results, as confirmed here, show that the reagent can indeed be so used; such side effects as do occur do not matter in this specific context.

Among the side effects is the finding, here emphasized, that the ATP content is lower (previously reported by Murphy, 1966), and the ADP and AMP content higher than in unpoisoned muscle, doubtless because of an inhibition or uncoupling of all restitution reactions and a continued and possibly enhanced basal ATP breakdown between the poisoning and the experiment. While in normal muscle the high ratio ATP:ADP might be thought to increase the effective free-energy of the ATP-driven processes, this would be much less the case under the circumstances described here, especially since the free ADP is increased by a significant proportion. Yet, the same force seems to be developed, although possibly somewhat more slowly (Aubert, 1964); only when the composition becomes more drastically changed does the mechanical performance decrease markedly (e.g. Murphy, 1966).

In one major respect, then, we have confirmed the findings by Cain & Davies (1962*a*) and specifically by Infante & Davies (1962): the FDNB-poisoned sartorius muscle does not use PC, but splits ATP to ADP, AMP and phosphate, in sensible amounts. We differ from these authors in another major respect: the ATP breakdown occurs exclusively within the contraction phase, not in relaxation.

To be sure, our experiments were conducted differently in that brief tetani instead of twitches were used. We have come to prefer this form of experimentation because, the duration of activation being more explicitly set and complete activation becoming externally manifest, it lends itself in general better to bio-energetic and chemical studies, although in the present instance it has no specific advantage. The difference in method would not cloud the issue because, while the activation metabolism associated with five stimuli would be increased (see below) and its relaxation might partly fall within the active phase, it would be increased only fractionally, not 5-fold; and it would increase equally in the stimulation patterns C and R. The separate contribution of relaxation would not be diminished. To put it differently: a twitch is a physiological unit in a sense, in that it represents the response cycle elicited by one excitatory event. A brief tetanus as employed here maintains the active state somewhat longer, and this serves the study of energy transformation phenomena more optimally. The number of stimuli, whether one or five, matters little in a context where the post-excitatory mechanisms are emphasized. This follows from the fact that the total breakdown of high-energy phosphate donors in our contraction, 0·34 μmoles, is but little more than the 0·29 μmoles involved in a twitch.

The positive side of the findings is, then, that a quantity of ATP, of a magnitude in accurate agreement with earlier determinations, breaks down during the ascending phase of a contraction cycle. Thus, it is in this phase that a major fraction of the chemo-mechanical events are to be placed. According to present insights (Gibbs, Ricchiuti & Mommaerts, 1966; Gibbs, Mommaerts & Ricchiuti, 1967), the following quantities may enter into the energetics of a contraction cycle: activation and its maintenance, tension development or the tension-time integral, work, and shortening; the entity recognized by Hill (1964) as feed-back heat may or may not be identifiable as an increase of the tension-time factor. According to Davies (1963), the activation heat eventually results from the splitting of ATP needed to return calcium ions to the sarcotubular system so that activation metabolism occurs during relaxation. We can estimate its quantity on the basis of our results (Gibbs *et al.* 1966) that the activation heat in a twitch amounts to about 0·4 times the isometric initial heat of 3 mcal; or, out of a total of 0·3 μmoles of ATP used in a twitch, some 0·12 μmoles, comparable to the 0·15 μmoles of PC reported, by Distèche (1964), to be broken down under conditions eliminating the mechanical response. This would also be the quantity to count with in our experiments, since in the mode CR it is the equivalent of the last activation, rather than the combined activations, which reverses in the relaxation phase. We have shown, then, that this process is excluded, as is the

somewhat larger change postulated by Davies (1963) and reported by Infante & Davies (1962) to occur in the relaxation phase of a cycle. With respect to the latter investigations, the difficulty extends into the most recent publications. Davies *et al.* (1967), reiterating their view on the nature of the relaxation process and referring to the delayed ATP-breakdown of 0·2 μmoles/g/cycle according to Infante & Davies (1962), state (on p. 148) that 'a breakdown of ATP at this time and in the expected amount has been observed'. Yet, further on (p. 149 and Table 1), they limit the activation metabolism to 0·05 μmoles/g/cycle, and feel that this too 'is the amount expected from studies of the energy requirements for pumping calcium in isolated vesicles'. These views are contradictory; our current biochemical and myothermal experiments (W. F. H. M. Mommaerts, N. V. Ricchiuti & A. Wallner, unpublished) are more in harmony with the latter; but these, and our present results, eliminate the original experimental foundation of Davies's (1963) view of the relaxation process and the activation heat.

This finding does in no way contradict the present concepts concerning the role of the calcium ion in the activation process (e.g. Sandow, 1952; Podolsky & Constantin, 1964). However, the mechanism of its migrations is still an entirely open question, and the stoicheiometry of the ATP usage in this process (Hasselbach & Makinose, 1963) is likewise unsettled (Ebashi & Yamanouchi, 1964; Seraydarian & Mommaerts, 1965; W. F. H. M. Mommaerts & A. Wallner, unpublished).

While our findings clearly contradict that the activation metabolism is actually delayed to the relaxation phase and on that basis identified with the return of calcium ions, we must express our factual difference with the report of Infante & Davies (1962) with restraint. It is possible, of course, that their figures result from the run of luck that may occur in series of small size and not performed with paired controls. It is also possible, however, that dependent upon the frog population or the physiological state of the muscles, the ATP splitting is sometimes delayed and perhaps increased as compared with its normal occurrence; we too, in fact, performed a small series in 1963 suggesting such an outcome. This explanation might apply to a fraction equivalent to the metabolism of the calcium pump if this were to become less efficient and use more ATP, or were to fail to utilize certain non-ATP energy donors that have been considered as possible intermediates (Carsten & Mommaerts, 1964); (the abnormally large relaxation heat reported by Aubert (1964) could be indicative of an inefficient calcium pump under the conditions of his experiment, although we obtained no such indication in twitches). Or, it might apply to chemomechanically involved ATP if the primary transduction process were to consist of the formation or activation of a myosin-ATP complex (Nanninga

& Mommaerts, 1960*a*, *b*), and its normally rapid hydrolysis (e.g. Tonomura & Kanazawa, 1965) were to be delayed by a change of one of the rate constants (cf. Nanninga, 1962). Our present experiments, however, represent a situation in which all ATP breakdown whether related to activation, shortening, or force, occurs in the contraction phase, as does the active heat production. Thus, it is not found that a definite quantity of ATP is generally broken down in relaxation.

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