Biochemische Zeitschrift 345, 286–299 (1966)

Adenosine Triphosphate and Phosphorylcreatine Breakdown in Resting and Stimulated Muscles after Treatment with 1-Fluoro-2,4-Dinitrobenzene*

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Received February 3, 1966

* This paper is dedicated to Prof. H. H. Weber.

Sartorius muscles of male *Rana temporaria* were poisoned with 1-fluoro-2,4-dinitrobenzene. The ATP content of the resting muscles decreases rapidly within 15 min, at a rate nearly equal to that expected if the normal resting usage of ATP goes on, and the ATP production is inhibited. After 35 min incubation in 0.41 mM 1-fluoro-2,4-dinitrobenzene at 0°C, a 10 sec isometric tetanus provokes the breakdown of 1.14 micromoles ATP and 1.54 micromoles phosphorylcreatine per gram muscle; a 20 sec tetanus doubles these values. After 60 min incubation in 0.82 mM 1-fluoro-2,4-dinitrobenzene, a 10 sec tetanus provokes the breakdown of 0.83 micromoles ATP per gram, but no breakdown of phosphorylcreatine. After such tetanus, the muscles relax completely and there is no resynthesis of ATP within 200 sec. The ATP used is not found as ADP or AMP, but is dephosphorylated and deaminated to IMP.

Introduction

In a thorough and monumental work, WEBER and his collaborators established beyond any doubt the central role of adenosine triphosphate (ATP) in the contraction and the relaxation of glycerinated muscle models. As the mechanical properties and the birefringence changes during the contraction are nearly alike in the muscle models and in the intact muscles themselves, they provided a firm foundation to the theory that the dephosphorylation of ATP is the primary source of energy for the muscular contraction [29].

However, many efforts made to investigate a possible breakdown or turnover of ATP associated with muscle contraction failed to be accepted as proving or disproving a direct role for ATP in intact muscle contraction [24], until CAIN, INFANTE, and DAVIES [3] observed a breakdown of ATP in single tetanic contractions of frog rectus abdominis muscles poisoned with 1-fluoro-2,4-dinitrobenzene. As the phosphorylcreatine of these muscles does not change, the facts are explained by a complete inhibition of the creatine-ATP phosphoryltransferase, which is very sensitive to the drug [15].

These observations, besides establishing the reality of ATP breakdown in intact muscle, provide us with a tool to probe deep in the early

events of a muscular contraction. But, first of all, we should know to what extend the contraction and its chemical processes are still normal after fluorodinitrobenzene poisoning. It is known that while iodoacetate (IAA) poisoning does not interfere with the maintenance heat production, fluorodinitrobenzene does [2]. It is shown in this paper that fluorodinitrobenzene (FDNB) reduces very much the rate of high-energy phosphates expenditure during the isometric tetanus.

Methods

General methods. The frogs (male specimen of *Rana temporaria* supplied by "*Reptilia*", Aarschot, Belgium) were kept by groups of seven in a cold room (5° C) for at least 4 days prior to the experiment. After pithing the seven frogs, their sartorius muscles were dissected out, their standard length l_0 was measured [10] and the paired muscles still attached to the pelvic bone were left overnight in a Ringer saline (NaCl, 113.5 mM; KCl, 2.0 mM; $CaCl_2$, 1.8 mM; Phosphate buffer: 1.9 mM, pH 7.3), gassed with air at a temperature of 5° C.

The next morning, the muscles were poisoned at 0° C with 1-fluoro-2,4-dinitrobenzene (FDNB). Half a ml of the drug (BDH laboratories, London) was dissolved in 9.5 ml absolute ethanol. This mixture was kept at 5° C, and appears to be stable. Just prior to the intoxication, 0.1 or 0.2 ml of this solution was added to 100 ml of the Ringer cooled at 0° C (final concentration 0.408 or 0.816 mM-FDNB). The mixture must be well stirred because the poison has a tendency to lie in a surface layer. After 3 hours, the solution begins to get brownish and must be discarded. The stated duration of intoxication is the time elapsed between the first contact of the muscle with the drug and the freezing.

Each muscle pair is mounted at length l_0 on the electrode assembly of a rapid freezing apparatus [19], similar to that of Mommaerts and Schilling [23], and is kept at 0° C in nitrogen. Each muscle connected by a steel wire to a separate strain gauge can be stimulated independently; their forces are displayed on a double channel recorder (Sanborn model 320).

The maximal force exerted by the muscle (P_0 in gram-force) and the force at the end of the tetanus (P_f) are given multiplied by the standard length (l_0 in cm), and divided by the weight M of the muscle (in g). The ratio Pl_0/M is approximately equal to the tension of the muscle in gcm^{-2}.

The muscles are stimulated by 230 or 460 supra-maximal condenser discharges (time constant 3 msec, frequency 23/sec) of alternating polarities, applied through 6 platinum electrodes. The muscles are frozen in isopentane cooled to — 160° C with liquid nitrogen, usually 50 msec after the last stimulus, at a time when the relaxation has not yet begun. As for the resting muscles, they are frozen on the electrode assembly at any convenient time. 456 muscles were used in the experiments reported in this paper, of which 104 were used for mechanical experiments and 352 were frozen and analysed for their content in adenosine triphosphate, free creatine and phosphorylcreatine. All analyses are expressed in micromoles per gram wet muscle.

Extraction and assay method. After freezing, each muscle was rapidly weighed, put into a stainless-steel cartridge cooled with liquid nitrogen, ground, and extracted with 2 ml 0.25 M-perchloric acid.

After neutralization with 0.4 N-KOH, the extract is brought up to 10 ml. The free creatine (C_F) is measured by its reaction with diacetyl-alpha-naphtol [7]. After acid hydrolysis of phosphorylcreatine (10 min at 65° C in 0.8 N HCl and neutralization with NaOH), the total creatine (phosphorylcreatine + free creatine) is measured by the same reaction. During this hydrolysis, some phosphorylcreatine (PC)

is turned into creatinine, and a correction was applied to take care of this effect. In a preceding work [19] this correction was overlooked, with the result that all the PC determinations were too low and should be multiplied by 1.22. The increase in the total creatine content with stimulation that was observed in this earlier work is entirely due to that omission. The ATP is measured by the firefly method [28].

Statistical designs. Much care was paid to the statistical design of the experiments. In general, the variance between muscles of the same frog is lower than the variance between muscles from different frogs. Although this rule suffers some exceptions, the comparison between treatments should take this fact into account. If two treatments only are compared, then each muscle pair may include both. If three treatments (A, B, and C) are compared, they are combined two by two: AB, BC, and AC. The complete set requires three muscle pairs, and two such sets can be done in one run. If four treatments are compared, the same principle holds, but six pairs are now required to get one balanced set. This type of design is known as "incomplete balanced blocks" or "symmetrical pairs" design [16].

The elementary block of each experimental run included six frogs. A seventh frog was added and prepared as the other ones (see general methods): in this way we were able to keep the balance of the experiment, even if some technical error occurred.

The lay-out of each experiment was carefully randomized with a table of random numbers. The randomization was made in three steps. In the first, the position of the muscle pair on the electrodes is determined. This step takes care of any bias in the stimulation of the muscle due to the slightly different geometry of the two sets of electrodes and of any systematic difference between right and left muscles of the frogs. In the second step six pairs of treatments were alloted to the six muscle pairs. In the third step, each muscle was assigned its treatment.

In the study of the action of FDNB on resting muscle it was necessary to compare ten treatments. The number of muscle pairs required becomes then too large to be performed in one run. The symmetrical pair design could not be applied, and was replaced by a plain randomization. The two muscles of the same pair received the same treatment, and six treatments were included in one run. Five runs in one case (3° C) and six in the other (20° C) were completed: the treatments were alloted randomly.

The muscles used in one run were numbered 1—12, according to the order of their freezing, and the chemical analyses were performed systematically in the same order. There is no need to randomize the chemical analysis, because the randomization is already very thorough, and the systematization decreases the risk of confusion.

Chromatography of the nucleotides. Adenosine triphosphate (ATP) adenosine, diphosphate (ADP), adenosine monophosphate (AMP), and inosine monophosphate (IMP) are separated by thin layer chromatography (method of Randerath, 1962 [25] slightly modified).

The muscle powder (at — 15° C) was treated with 0.5 ml of 10% trichloroacetic acid. Fifty microliters of this extract are removed for the determination of total creatine, and 100 microliters for the estimation of free creatine. The remaining portion is shaken twice with 5 ml diethylether and the trichloroacetic acid is thus removed. The ATP is measured in 20 microliters by the firefly method; another 20 microliters were used for the thin layer chromatography.

The plates were covered with DEAE-cellulose (250 μ thick Macherey, Nagel & Co., Düren, Germany). The developping solution was 1 vol propanol-1 vol 0.06 N-HCl. After 1—2 hours nucleotides are separated. The measured R_F values, referred to AMP, were: ATP: 0.01; ADP: 0.26, IMP: 0.60; AMP 1.00. The

nucleotides were detected by illumination at 260 mμ. The spots were removed and eluted in 0.1 N-HCl. After centrifugation, the absorption was read at 257 mμ for the adenine nucleotides, and at 248 mμ for IMP with a Zeiss spectrophotometer, and compared with the absorption at 290 mμ. A further correction was applied to take care of the rather high blank due to the cellulose powder, and the result was compared to the specific absorbancy of nucleotides. It is known that iodoacetate poisoned muscles hydrolyse their ATP after a long tetanus, and that the amounts of ADP, AMP and IMP increase [9]. This system was used to test the method. It was found that the amount of ATP in muscle as estimated by the chromatographic method was consistently higher by 20—50% to that estimated by the firefly method. However the decrease in ATP induced by the stimulation is exactly equal to the increase in ADP, AMP and IMP. Although the method is not yet considered satisfactory as to the absolute changes in nucleotides, it is considered that the percentage changes are correctly evaluated.

Results

1. The resting muscle

According to INFANTE, KLAUPIKS and DAVIES [14], the frog sartorius muscle in 0.38 mM-FDNB becomes stiff and inexcitable in 90 min at 0° C, and in 15 min at 20° C. We have also observed that the resting muscle in FDNB goes into rigor, but with somewhat longer delay. This is reminiscent of the spontaneous rigor that occurs in iodoacetate (IAA) treated muscle in about a dozen hours [26]. The IAA-rigor is most certainly due to the disappearance of ATP, and it is probable that the FDNB induced rigor is due to the same origin.

Fifty-four muscles were left 15, 30, or 60 min at 3° C in a solution of FDNB either of 0.41 or 0.82 or 1.22 mM; six other muscles were not poisoned.

Table 1 shows that the ATP content of the muscles decreases by 1.40 ± 0.25 micromoles $\cdot g^{-1}$ after 15 min in 0.41 mM-FDNB, and by 2.00 ± 0.25 micromoles $\cdot g^{-1}$ after 15 min in 1.22 mM-FDNB. As the duration of intoxication is raised to 30 min or 60 min, there is a further fall in the ATP content, at a somewhat slower rate. The phosphorylcreatine (PC) does not change. The amount of free creatine drops from 5.01 micromoles $\cdot g^{-1}$ in the control muscles, to 4.35 micromoles $\cdot g^{-1}$ in the muscles poisoned 15 min by 0.4 mM-FDNB. The difference 0.66 ± 0.46 is not statistically significant. As the concentration of FDNB is raised to 0.82 or 1.22 mM, the difference in free creatine content between control muscles and muscles poisoned 15 min rises to 1.11 ± 0.46 and 1.29 ± 0.46 ($P < 5\%$).

In a second experimental series another fifty-four muscles were treated according to the same scheme with only one modification: the temperature was 20° C. Eighteen unpoisoned muscles were used as controls. It is seen in Table 1 that the results are on the main the same, taking into account the accelerating effect of a rise in temperature. The ATP content decreases sharply even after 15 min of intoxication, and continues to fall during the next 15 and 45 min. All the muscles which

Table 1. *Breakdown of adenosine triphosphate and decrease in free creatine without change in phosphorylcreatine of resting frog sartorius muscles incubated in Ringer containing 1-fluoro-2,4-dinitrobenzene at various concentrations*

incubation period in FDNB in min		ATP micromoles · g^{-1}			C_F micromoles · g^{-1}			PC micromoles · g^{-1}		
		15	30	60	15	30	60	15	30	60
FDNB concentration										
3° C	0.41 mM	2.48	2.18	2.11	4.35	3.40	4.18	26.5	26.3	28.0
	0.82 mM	2.33	1.47	1.20	3.90	3.52	3.95	28.0	24.7	24.6
	1.22 mM	1.88	1.43	1.00	3.72	3.52	3.44	28.5	26.5	26.3
	SEM ($n = 6$)		±0.22			±0.39			±0.5	
	untreated muscles		3.88			5.01			28.8	
	SEM ($n = 6$)		±0.12			±0.25			±1.3	
FDNB concentration										
20° C	0.41 mM	2.81	1.21	0.45	3.45	2.62	3.55	25.4	22.1	22.1
	0.82 mM	2.20	0.58	0.27	4.13	3.25	3.25	27.2	24.3	22.4
	1.22 mM	1.10	0.36	0.19	3.72	3.45	3.60	23.2	24.2	22.5
	SEM ($n = 6$)		±0.17			±0.21			±1.1	
	untreated muscles		5.98			7.42			23.4	
	SEM ($n = 18$)		±0.18			±0.60			±0.5	

SEM: standard error of the means; n: number of replicates.

stayed 60 min in FDNB were contracted, and so were most of those poisoned 30 min in 1.22 mM-FDNB. The PC content seems to rise after a mild poisoning; but this increase does not occur in muscles poisoned 30 or 60 min. The poisoned muscles have about half the free creatine content of the control muscles; the differences are highly significant ($P < 0.001$).

These experiments demonstrate that the level of ATP in a muscle poisoned with FDNB decreases rapidly. It is therefore very important to control rigorously the concentration of the poison, the temperature of incubation, and duration of contact with the drug, if it is required to estimate the effect of a tetanization on the ATP content by comparing a stimulated muscle to an unstimulated one. These three factors were carefully controlled in the next experiments; the success of this control is shown by the fact that the ATP content of the unstimulated muscles in the next experiments (Tables 2—5) agrees quite well with the results reported in Table 1 (taking into account that the next experiments were performed at 0° C, while those of Table 1 were performed at 3° C and 20° C).

2. The isometric mechanogram

The shape of the mechanogram is hardly affected after 60 min incubation at 0° C in 0.1 mM-FDNB. Muscles poisoned at 0° C with FDNB at a concentration between 0.1 and 0.8 mM can develop a maximal tension similar to that of iodoacetate-poisoned muscles (see Table 2). If the concentration of FDNB is raised to 1.6 mM, the maximal tension is much

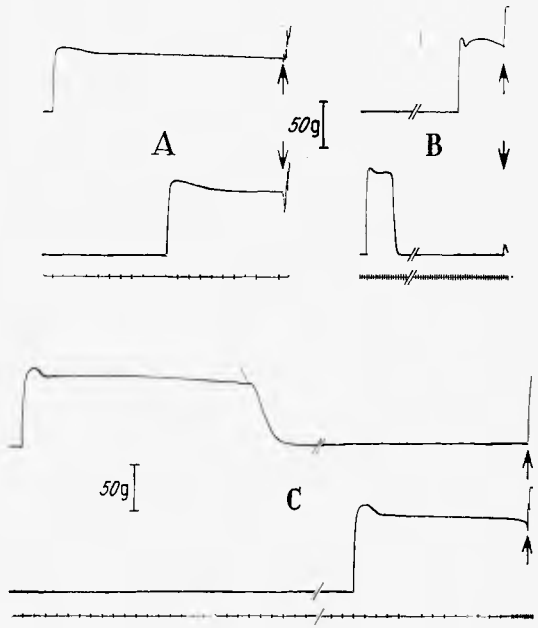

Fig. 1 A—C. *Isometric tetanus of frog sartorius muscles treated with 1-fluoro-2,4-dinitrobenzene.* A Muscle pair poisoned 35 min in 0.41 mM-FDNB. This is one of the experiments reported in Table 3a. Upper trace: muscle stimulated 20 sec; lower trace: muscle stimulated 10 sec (temp. 0° C); time marker: 1 sec. The muscles are frozen at the arrows, 50 msec after the last stimulus; the increase in tension during the freezing is an artefact resulting from the different magnitude of shortening of the frozen muscle and the electrode assembly in the isopentane at —160° C. Weights: 70 and 70 mg; length: 2.7 cm. The muscles contained respectively 9.8 and 7.8 micromoles · free creatin per g; 18.1 and 23.4 micromoles · PC per g; 0.92 and 2.11 micromoles · ATP per g. B Muscle pair poisoned 60 min in 0.82 mM-FDNB. This is one of the experiments reported in Table 4. Upper trace: muscle stimulated 20 sec and frozen 50 msec after the last stimulus. Lower trace: muscle stimulated 10 sec and frozen 200 sec after the last stimulus. Weights: 103 and 108 mg; length: 2.9 cm; free creatine: 3.2 and 3.4 micromoles · g^{-1}; PC: 31.3 and 27.3 micromoles · g^{-1}; ATP: 1.05 and 1.32 micromoles · g^{-1}. C Muscle pair poisoned 60 min in 0.82 mM-FDNB. This is one of the experiments reported in Table 4, Both muscles are stimulated 20 sec, but frozen 200 sec and 50 msec after the last stimulus. Weights: 108 and 111 mg (Upper and lower trace); length 3.0 cm; free creatine: 2.3 and 2.6 micromoles · g^{-1}; PC: 25.4 and 26.9 micromoles · g^{-1}, ATP: 1.19 and 1.35 micromoles · g^{-1}

lowered, even if the muscle is poisoned for 20 min only. At a concentration of 3.2 mM for 20 min at 0°C, the muscle is inexcitable.

After poisoning with 0.4 mM-FDNB the maximal tension decreases slowly during a 20 sec tetanus, but not quicker than in iodoacetate poisoned muscle (Table 2; Fig. 1); in 0.8 mM-FDNB, there is often a sharp drop in tension at the beginning of the tetanus, often followed by a slow secondary increase (Fig. 1B).

The rate of rise in tension is somewhat slower in FDNB-treated muscle, than in control fresh muscle: it does not change much in a few successive short tetanus if the interval between them is 2 min or more; if this interval is reduced to 15 sec, the decrease between successive tetanus becomes quite definite (Fig. 2).

Relaxation is usually complete, even after a 20 sec tetanus at 0° C (Fig. 1 B and C) but its rate is quite slow and depends on the concentration of FDNB and on the previous amount of tetanization (Fig. 2).

If FDNB-poisoned muscles are stimulated longer than about 30 sec at 0° C or 1 sec at 20° C they enter into rigor (Fig. 3).

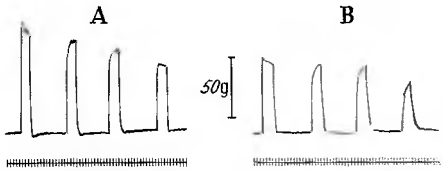

Fig. 2 A and B. *Series of four isometric tetanus of sartorius muscles treated with FDNB.* The muscles were poisoned 20 min at 0° C in 0.41 mM-FDNB (A) and 0.82 mM-FDNB (B). They are stimulated at 15 sec intervals for 3 sec at 0° C (23/sec). Weights: A: 70 mg; B: 81 mg. Length: 2.9 cm (A); 3.2 cm (B)

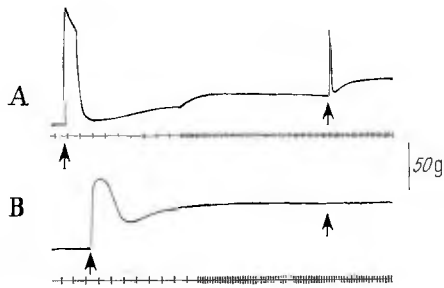

Fig. 3 A and B. *Rigor induced by the tetanization of a sartorius muscle treated with FDNB.* Muscles poisoned at 0° C for 60 min in 0.82 mM-FDNB (B), or for 30 min in 0.4 mM-FDNB (A). They are then warmed to 20° C, and stimulated twice at the arrows, 2 sec (B) or 1 sec (A). Time markers: 1 sec; note that the speed of recorder is changed after a few seconds

This behaviour is very similar to that of a iodoacetate-poisoned muscle tetanized for 12—15 sec at 20° C [17, 26]. When rigid, the muscles respond to stretches in a way very similar to the muscles in iodoacetate-rigor [18]: it seems very probable that the state of rigor is the same in both cases and that it is caused by the same mechanism.

The above observations are indicative of interesting changes in the mechanical characteristics of FDNB-treated muscles, which should deserve further investigation. They suggested that the experiments on the metabolism of ATP and PC should be performed on muscles poisoned at 0° C for 30—60 min with 0.41—0.82 mM-FDNB.

3. The ATP and PC changes during an isometric tetanus

The aim of these experiments was primarily to obtain quantitative estimations on the energy requirement of an FDNB-treated muscle during the isometric tetanus, as estimated by the ATP and PC changes.

These are well known in the case of an iodoacetate-poisoned muscle [19]. The first step to be taken is to compare FDNB treated muscles with iodoacetate poisoned ones in the same set of experiments.

a) Comparison between FDNB and IAA poisoned muscles. Pairs of sartorius were poisoned 1 hour with 0.4 mM iodoacetate or FDNB (three pairs each). One muscle of each pair was stimulated 20 sec, the other remained unstimulated. The two muscles were frozen after the end of relaxation. The six muscle pairs were handled and analyzed the same day. This experiment was repeated five times, but two muscle pairs were lost; the results of the two series of fourteen muscle pairs are given in Table 2.

Table 2. *Breakdown of adenosine triphosphate and phosphorylcreatine during a 20 sec isometric tetanus at 0° C of frog sartorius muscles pretreated with iodoacetate or 1-fluoro-2,4-dinitrobenzene*

Treatment of muscles	Weight mg		$\frac{P_0 l_0}{M}$		$\frac{P_f l_0}{M}$		ATP micro-moles · g^{-1}		C_F micro-moles · g^{-1}		PC micro-moles · g^{-1}	
	IAA	FDNB	IAA	FDNB	IAA	FDNB	IAA	FDNB	IAA	FDNB	IAA	FDNB
Unstimulated	72.0	74.1	0	0	0	0	4.51	4.03	7.7	7.3	26.4	27.1
Stimulated 20 sec	75.8	73.4	2,350	2,360	1,670	1,510	4.91	1.44	18.8	9.9	15.4	24.4
SED ($n = 14$)	2.0	2.1	60	60	20	20	0.17	0.20	0.8	1.0	1.0	1.4

SED: Standard error of the difference between two means of the same column. n: number of replicates. — The muscles were poisoned 1 hour at 0° C in 0.4 mM iodoacetate or 0.41 mM FDNB.

The IAA poisoned muscles used 11 micromoles · g^{-1} PC. The FDNB poisoned muscles used 2.7 micromoles PC per gram, and 2.59 micromoles ATP per gram. At first sight the sum of high energy phosphates hydrolysed would be 5.31 micromoles · g^{-1}, i.e. only half the amount of energy needed for the isometric tetanus of an IAA-poisoned muscle; but if the myokinase enzyme is still active, as it probably is (see further, § 4), then the amount of high-energy phosphates is 7.90 micromoles · g^{-1}, 72% only of that needed by the IAA-poisoned muscles.

b) The influence of stimulus duration. According to the experiments just described the total cost of an isometric tetanus, in term of high-energy phosphates, is depressed by the FDNB poisoning. In order to obtain some information about the time course of the chemical changes during contraction, we have compared muscles stimulated for either 10 sec or 20 sec with unstimulated control muscles. The three treatments were balanced in pairs (see methods), so that the standard error of the difference between any two means remains the same. All the muscles were poisoned with FDNB. In the previous experiments (IAA-FDNB),

the muscles cleaved some PC, suggesting that the creatine phosphoryltransferase was not completely inhibited. Accordingly, we have completed two experimental series: in the first one, the muscles were poisoned 35 min in 0.41 mM-FDNB, and in the second one they stayed 60 min in 0.82 mM-FDNB. The muscles were frozen 50 msec after the last stimulus, before any relaxation had time to occur.

Table 3. *Breakdown of ATP and PC during an isometric tetanus in frog sartorius muscles pretreated with 1-fluoro-2,4-dinitrobenzene*

Duration of the tetanus (sec)	Weight mg	$\frac{P_o l_o}{M}$	$\frac{P_f l_o}{M}$	ATP micromoles · g^{-1}	C_F micromoles · g^{-1}	PC micromoles · g^{-1}
a) Muscles incubated 35 min in 0.41 mM FDNB at 0°C						
0	80.5	0	0	3.97	5.55	25.1
10	79.7	2,340	2,120	2.83	7.08	23.9
20	80.5	2,210	1,850	1.81	8.20	22.3
SED (n=16)	±2.8	±130	±130	±0.16	±0.80	±0.9
b) Muscles incubated 60 min in 0.82 mM FDNB at 0°C						
0	106.9	0	0	2.33	2.73	26.7
10	105.7	2,420	2,260	1.50	2.75	27.6
20	104.2	2,390	1,880	0.88	2.73	27.9
SED (n=16)	±1.6	±90	±130	±0.14	±0.14	±0.6

SED: Standard error of the difference between any two means in the same column. n: number of replicates.

The results are reported in Table 3. While the decay of the tension during the tetanus is about the same, irrespective of the FDNB dose, it is clear that the high dose inhibits completely the creatine phosphoryltransferase. It is also clear that the amount of high-energy phosphate used during the 20 sec tetanus is nearly twice that used during the 10 sec tetanus and is lower in the muscles more heavily intoxicated. However, an accurate estimation of the energy expenditure of these muscles must take into account the fact that the myokinase reaction is active, and that the AMP produced is deaminated to inosine monophosphate (IMP) (see § 4).

c) ATP and PC changes after the termination of activity. The muscles were poisoned 60 min with 0.81 mM FDNB, and stimulated isometrically during 10 or 20 sec. They were frozen either 50 msec, or 200 sec after the last stimulus. The four treatments were given according to a 'symmetrical pairs' design; the results are reported in Table 4.

There is no change in free creatine or in phosphorylcreatine. The ATP-content just at the end of the tetanus is the same as that found in the previous experimental series (Table 3b). During the 200 sec after tetanization there hardly occurs any further change in the ATP content.

Table 4. *Absence of ATP or PC resynthesis after an isometric tetanus in frog sartorius muscles pretreated 60 min with 0.81 mM 1-fluoro-2,4-dinitrobenzene at 0° C*

Treatment of muscles	Weight mg	$\frac{P_o l_o}{M}$	$\frac{P_f l_o}{M}$	ATP micro-moles · g^{-1}	C_F micro-moles · g^{-1}	PC micro-moles · g^{-1}
10 sec tetanus	112.9	2,290	2,030	1.45	2.92	24.5
20 sec tetanus	109.6	2,380	1,760	0.81	2.96	25.3
10 sec tetanus + 200 sec rest	109.0	2,370	2,010	1.41	2.89	24.2
20 sec tetanus + 200 sec rest	110.9	2,310	1,720	0.69	3.07	24.1
SED ($n = 12$)	±2.9	±110	±110	±0.10	±0.21	± 0.5

SED: Standard error of the difference between any two means in the same column. n: number of replicates.

4. Changes in adenosine and inosine nucleotides during an isometric tetanus

Myokinase is very active in muscle, and is only partly inhibited by FDNB [13]. In order to get a correct evaluation of the amount of high-energy phosphates used up during an isometric contraction, it is necessary to know whether this reaction occurs in our experiments as well, and to what extent.

Three pairs of sartorius muscles were poisoned for 35 min with 0.41 mM FDNB and received three treatments in "symmetrical pairs": no stimulation, one isometric tetanus of 10 sec and a tetanus with forced lengthening. Two such sets were made the same day, and three runs were completed. The muscles were frozen 50 msec after the last stimulus, and analysed for free creatine, PC, ATP by the firefly method; ATP, ADP, AMP, and IMP were separated by thin layer chromatography. The results are reported in Table 5, except for the stretched muscles the study of which lies outside the scope of this paper.

Table 5. *Balance of adenosine and inosine nucleotides after an isometric tetanus in sartorius pretreated with 1-fluoro-2,4-dinitrobenzene 0.41 mM for 35 min*

Treatment of muscles	Weight mg	$\frac{P_o l_o}{M}$	$\frac{P_f l_o}{M}$	C_F	PC	ATP_{FF}	ATP	ADP	AMP	IMP
Unstimulated	76.4	0	0	4.71	23.8	2.93	4.47	0.77	0	0.18
Stimulated 10 sec	75.5	2,540	2,410	5.86	24.4	1.85	2.93	0.20	0	2.85
SED (n=18)	±1.7	±114	±76	±0.35	±1.2	±0.18	±0.22	±0.18		±0.40

SED: Standard error of the difference between two means of the same column. n: number of replicates. ATP_{FF}: ATP as measured by the firefly-method. ATP, ADP, AMP and IMP measured by the chromatographic procedure. All concentrations expressed in micromoles per gram muscle.

The increase in free creatine, $+ 1.15 \pm 0.35$ micromoles $\cdot g^{-1}$, and the decrease in ATP (measured by the firefly method), $- 1.08 \pm 0.18$ micromoles $\cdot g^{-1}$, are in good agreement with the results of the experiments reported in Table 3. As mentioned earlier (see methods), the ATP measured by the chromatographic procedure is about 50% higher than that measured by the firefly method. The ATP which disappears, $- 1.54 \pm 0.22$ micromoles $\cdot g^{-1}$, plus the decrease in ADP, $- 0.57 \pm 0.18$ micromoles $\cdot g^{-1}$, nearly equals the IMP that appears, $+ 2.66 \pm 0.40$ micromoles $\cdot g^{-1}$. There is no AMP. Thus, the ATP used during the isometric tetanus is dephosphorylated twice, and deaminated to IMP.

Discussion

1. Resting muscles

The incubation of muscle in 1-fluoro-2,4-dinitrobenzene produces in 15 min a large fall in the ATP and the free creatine content. The phosphorylcreatine level does not change, except perhaps for small transients.

The fall in ATP is 1.4—2.0 micromoles $\cdot g^{-1}$ at 3° C (according to the concentration in FDNB) and 3.2—4.9 micromoles $\cdot g^{-1}$ at 20° C. These values are a minimum estimation of the decrease in high energy phosphates, as they might be doubled by the action of myokinase. They however fit rather well the normal resting metabolism. At 20° C, the resting sartorius muscle produces 1.7—2.4 mcal $\cdot g^{-1} \cdot min^{-1}$ [11]; assuming an enthalpy of hydrolysis of ATP equals to $- 8$ kcal $\cdot$ mole^{-1}, this is equivalent to a rate of 0.21—0.28 micromoles ATP per gram, or in 15 min, 3.1—4.2 micromoles $\cdot g^{-1}$. The Q_{10} of the resting heat production is about 2.5 [11]; in the case of the ATP usage of the resting FDNB-treated muscle, Q_{10} is nearer to 1.6. The oxygen consumption at 20° C of resting muscles has been measured by Gillis [8] on frogs coming from the same batch as those used in our own experiments. It is about 25—50 nanomoles $O_2\ g^{-1} \cdot min^{-1}$, which is equivalent to 2.3—4.6 micromoles ATP per gram in 15 min, if we accept a P/O = 3.

Thus, it appears that the FDNB interferes rapidly with the production of ATP rather than with its utilisation. The respiration of the muscle is stopped; the glycolysis however proceeds at its usual pace for at least 3 hours [13], but does not lead to any ATP production (Davies [4, 5], and this work 3, c of the results). After the rapid initial fall, the decrease in ATP content of the FDNB-treated muscle occurs slower and slower: either FDNB blocks some of the ATP-using processes, or there is an automatic regulatory process which adjusts the ATP usage to its level.

The action of FDNB on the creatine content is more puzzling: while PC does not change, free creatine decreases after 15 min in 0.41—1.22 mM FDNB by 0.7—1.3 micromoles $\cdot g^{-1}$ at 0° C, and by 3.3—4.0 micromoles $\cdot g^{-1}$ at 20° C. There is no explanation for these variations in free

creatine. The FDNB itself does not react with creatine. It might be that FDNB lowers the permeability of the membrane to the creatine, which can then diffuse out more easily into the surrounding Ringer-solution.

The PC content of resting muscle does not change even when the ATP level is very low. This can easily be understood if FDNB inhibits completely the ATP-creatine phosphoryltransferase. But, it seems probable that a complete inhibition does not occur, at least at the lowest dosage of FDNB used (0.41 mM). At this concentration, there still occurs a slight breakdown of PC during an isometric tetanus. It is probable that FDNB slows down the rate of the Lohmann reaction to such an extent that the ADP produced by the resting metabolism is used up by other more rapid reactions, such as those catalysed by myokinase and adenosine deaminase; only during the isometric tetanus the breakdown of ATP would occur at a rate high enough to increase the ADP content up to a level which accelerates significantly its phosphorylation via the PC breakdown.

2. Stimulated muscles

In FDNB-treated muscles there is a definite breakdown of ATP, and, if the concentration of the drug reaches 0.82 mM, there is no more PC usage; the ATP is quantitatively replaced by IMP, there is a slight decrease in ADP and no AMP appears in detectable amount.

These findings can be interpreted if the myokinase and the AMP deaminase are not totally inhibited by FDNB: the ADP produced by the actomyosin ATP-ase is turned into ATP and AMP by the myokinase reaction; the ATP can then be re-used by the contractile machinery, while the AMP is deaminated to IMP. Myokinase is inhibited by FDNB to a certain extend, but it is still active in FDNB treated rectus abdominis [3] and sartorius, at least after several contractions [13]. Deamination was not reported by these authors, but it frequently occurs in states of great exertion of the muscle, such as iodoacetate-rigor [9].

The amount of high-energy phosphate bonds split during isometric tetanus in our experiments is the sum of twice the amount of ATP split minus the amount of creatine set free: the chemical energy used for the first 10 sec and the last 10 sec of a 20 sec tetanus can be computed from the Tables 3, 4, and 5, and the results are reported in Table 6.

These results can now be compared with the energy spent by a muscle poisoned with iodoacetate during an isometric tetanus. Such a muscle splits PC at a constant rate b, the stable metabolism, plus a certain amount, A, connected with the processes of the early part of the tetanus and of the relaxation, the so-called extrametabolism [22]:

$$\Delta PC = A + bt.$$

The sartorius muscle of *R. temporaria* cleaves PC at a rate of 0.51 micromoles $\cdot g^{-1} \cdot sec^{-1}$, i.e. 5.1 micromoles $\cdot g^{-1}$ during a 10 sec tetanus:

Table 6. *The high-energy phosphates usage during the tetanus of muscle pretreated with 1-fluoro-2,4-dinitrobenzene*

Experiment	Concentration mM-FDNB	ATP micromoles · g^{-1}	C_F micromoles · g^{-1}	2 ATP—C_F micromoles · g^{-1}
Table 3a				
0—10 sec	0.41	— 1.14	+ 1.54	— 3.82
10—20 sec		— 1.02	+ 1.12	— 3.16
Table 3b				
0—10 sec	0.82	— 0.83	+ 0.02	— 1.64
10—20 sec		— 0.64	— 0.02	— 1.22
Table 4				
10—20 sec	0.82	— 0.64	+ 0.04	— 1.24
Table 5				
0—10 sec	0.41	— 1.08	+ 1.15	— 3.31

the extrametabolism of the same muscle is 0.78 micromoles · g^{-1} for one tetanus [19]. This figure however includes the energy set free during relaxation [12] and that used after the tetanus [6, 27], which should not be taken into account in our experiments, as the muscles are frozen before relaxation sets in. The energy needed to produce the elastic work at the beginning of the tetanus is about 0.7 mcal · g^{-1} [19], and the labile part of the maintenance heat [1] is about 3 mcal · g^{-1}. The sum, 3.7 mcal · g^{-1}, is the energy needed in addition to the stable maintenance metabolism. This is equivalent to 0.4 micromole high-energy phosphate per gram, assuming $\Delta H_{ATP} = -8$ kcal · $mole^{-1}$. Therefore, the amount of high-energy phosphates split during the first half of a 20 sec tetanus should be 5.5 micromoles · g^{-1}, and during the second half (relaxation excluded) 5.1 micromoles · g^{-1}.

It is clear that FDNB decreases the amount of high-energy phosphates needed for an isometric tetanus by about 30% at a concentration of 0.41 mM, and by about 70% at a concentration of 0.82 mM. Moreover, the muscles split 0.4—0.6 micromoles · g^{-1} more during the first 10 sec of the tetanus than during the 10 last sec. This suggests that the energy needed in addition to the stable metabolism is unaltered. An interesting confirmation of this view is that FDNB 0.41 mM decreases the stable maintenance heat production by 30%, while the labile maintenance heat remains normal [2].

If these conclusions are correct, then the amount of high-energy phosphates split during the first second or so of a contraction should be very near normal, because the decrease expected in the maintenance metabolism is overshadowed by the production of work and of the labile maintenance heat. This is in fact what Davies and his co-workers found for the FDNB treated rectus abdominis [13, 14] and the sartorius [13].

The reason why the maintenance metabolism slows down after a few seconds of stimulation is as yet not clear. FDNB does not inhibit acto-

myosin ATP-ase, on the contrary it stimulates it [13]. The muscular fatigue is also characterized by a much greater decrease in energy output than in maintained tension [20, 21].

Fluorodinitrobenzene is the only chemical known to us which can depress the rate of energy dissipation during the tetanus of the intact muscle, and the study of its effect could very well lead to a better knowledge of the mechanisms wich control the maintenance metabolism.

Acknowledgement. The authors are indebted to Prof. X. AUBERT for interesting discussions and valuable advices during the preparation of this paper.

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