The Effect of 2,4-Dinitrofluorobenzene on the Activity of Striated Muscle*

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In 1959, Kuby and Mahowald (1) reported that 2,4-dinitrofluorobenzene can completely inhibit crystalline adenosine triphosphatase: creatine phosphotransferase. Treatment of muscles with DFB† has produced conditions in which a net breakdown of ATP in a single contraction has been found (2). The information reported here defines more clearly the effects of DFB on the contractile mechanism. Preliminary accounts of part of these results have been presented (3).

EXPERIMENTAL PROCEDURE

Rectus abdominis and sartorius muscles of the female frog (Rana pipiens) were used. The frog was decapitated and pithed, then the paired rectus abdominis or sartorius muscles were removed and allowed to rest for 2 to 4 hours at room temperature (25°) in an oxygenated physiological bicarbonate-saline solution (Ringer’s solution for toads): 87.7 mM NaCl, 25 mM NaHCO₃, 3.70 mM KCl, 2.0 mM CaCl₂ saturated with 5% CO₂:95% O₂. In most experiments, the muscles were then incubated at 0° for 40 min in 1500 volumes of a freshly prepared Ringer’s solution (frog) containing 0.38 mM DFB (Eastman Organic Chemicals). The DFB was dissolved after vigorous stirring for about 24 hours. This solution was maintained at pH 7.4 and kept anaerobic by continuous equilibration with 5% CO₂:95% N₂ during the incubation.

The usual rapid freezing, extraction, and assay techniques of this laboratory (4, 5) were used to study inorganic phosphate activity produced by electrical stimulation at 0°. All assays were done in duplicate or triplicate. Muscles incubated in DFB-free Ringer’s solution (frog) are described herein as untreated or normal.

Oxygen Uptake—The oxygen uptake of muscle was measured with an oxygen electrode chamber, and the rate of oxygen utilization was recorded. The electrode vessel contained 2.8 ml of the Ringer’s solution (frog) and was kept in a constant temperature bath for 30 to 60 min to allow temperature stabilization at 5° ± 0.5°. Two DFB-pretreated or untreated sartorius muscles, weighing about 100 mg each, were then added to the electrode chamber, and the rate of oxygen utilization was recorded. Since temperature greatly influenced the sensitivity of the electrode, only those experiments in which the final and initial temperatures agreed within 0.5° were used.

Lactate Measurements—Two or four sartorius muscles were incubated in DFB-Ringer’s solution as described and then tied at their rest lengths to a stainless steel rod. They were washed twice in 400 ml of Ringer’s solution (frog) for 10 min each time, and then placed in a small tube containing 4 ml of this solution gassed with 5% CO₂:95% N₂ at 0°. Their paired muscles were treated the same way, but with DFB-free Ringer’s (frog) throughout. After several intervals, the muscles were washed in 400 ml of Ringer’s solution (frog) at 0° for 10 min, and then placed in 4.0 ml of fresh Ringer’s solution (frog). The 4.0-ml solutions were assayed for lactate by the method of Barker and Summerson (6) or enzymatically by the method of Scholz et al. (7) as given in the Boehringer und Soehne (Mannheim, Germany) "Milchsäure Test" kits.

ATP:Creatine Phosphotransferase—Prior to extraction, DFB-treated muscles were washed twice for 5 min each time in a total of 4000 volumes of Ringer’s solution (frog) at 0°. This procedure avoided the possible destruction of the enzyme by any unreacted DFB during the extraction. The muscles were frozen in liquid nitrogen, pulverized, and extracted three times with a total of 25 volumes of 0.001 M KCl at 0° (8). The combined extracts were dialyzed overnight at 25° against 0.1 M Tris buffer, pH 7.2.

The transferase activity in these extracts was measured by the formation of creatine (9) in the presence of ADP and phosphocreatine. Final concentrations in a total of 2.0 ml were 0.80 mM ADP and 1.60 mM phosphocreatine in 0.1 M Tris buffer at pH 7.2. Incubation was for 5 min at 37°. The reaction was conveniently stopped by addition of the alkaline creatine assay reagent, 1% α-naphthol in 1.5 N NaOH + 1.5 M NaCO₃, containing 0.004 M EDTA. Protein was determined by the method of Lowry et al. (11).

ATP:AMP Phosphotransferase—Muscles treated with DFB were freed of unreacted DFB by washing twice in 1200 volumes of vigorously stirred Ringer’s solution (frog) for 10 min each time. Three sartorius muscles were frozen in liquid nitrogen, ground to a smooth powder, and extracted twice with 2 volumes of cold 0.03 M KOH + 0.002 M EDTA. Myokinase free of ATPase and nucleotides was prepared from the combined extracts (approximately 2.0 ml) as described by Colowick (12). The activity of the preparation was determined as the rate of ATP production from ADP. ATP was measured fluorimetrically by the method described by Estabrook and Maitra (13).

Actomyosin Adenosine Triphosphatase—The actomyosin ATPase was prepared according to the method described by Mom-
maerts (14). Five pairs of rectus abdominis or sartorius muscles were finely minced and homogenized with an all glass homogenizer in 5 volumes of solution containing 0.6 m KCl, 0.04 m NaHCO3, and 0.01 m NaCO3. The total weight of tissue was about 1 g. DFB-pretreated muscles were washed free of unreacted DFB as described in the ATP:creatinine phosphotransferase preparation. The extract was stirred for 24 to 28 hours and then centrifuged at 14,000 rpm (International centrifuge head No. 296) for 45 min; all operations were at 0-4°. Actomyosin was precipitated from the supernatant fluid by dilution with 6 volumes of water. The suspension was allowed to stand for about 30 min, and then the voluminous precipitate was collected by centrifugation and dissolved by the addition of 2.0 m KCl to a final concentration of 0.5 m. Precipitation was repeated three more times. The activity of the preparation was the amount of inorganic phosphate produced from ATP in an assay mixture which contained 0.1 m histidine, pH 7.0, 1.0 mm CaCl2, 0.6 m KCl, and 1.5 mm ATP in a final volume of 3.0 ml. The reaction was stopped after 5 min at 37° by the addition of 1.0 ml of 20% trichloroacetic acid, and the inorganic phosphate measured by the method of Berenblum and Chain (15). The protein concentration was measured by the biuret reaction.

Glycerol Extracted Fibers—Normal and DFB-pretreated muscles were extracted in 50% glycerol at -20°, and the fibers were prepared as described by Szent-Györgyi (16).

RESULTS

After 40 min of incubation in DFB-Ringer's solution at 0°, frog sartorius muscles contained the normal amount of ATP, phosphorylcreatine, creatine, and inorganic phosphate. Tables I and II give the changes in these compounds during isotonic contractions of isolated muscles at 0°. Single working contractions of normal rectus abdominis and sartorius muscles resulted in net equivalent increases of free creatine and inorganic phosphate. In DFB-treated muscles during single contractions, the same amount of inorganic phosphate was formed as in normal muscles, but the change of free creatine was not significantly different from zero. There was no measurable net change in the ATP level during a single contraction of normal muscle (Table II), but there was an equivalence between ATP breakdown and inorganic phosphate formation during a single contraction of DFB-treated sartorius muscle.

**Table I**

Production of inorganic phosphate without net change in free creatine during single contraction at 0° of frog rectus abdominis and sartorius muscles pretreated with 2,4-dinitrofluorobenzene

<table>
<thead>
<tr>
<th>Type of muscle</th>
<th>No. of muscle pairs</th>
<th>External work</th>
<th>Δ Inorganic phosphate</th>
<th>Δ Creatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sartorius</td>
<td></td>
<td>g cm/g</td>
<td>μmole/g muscle</td>
<td>μmole/g muscle</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>100 ± 5</td>
<td>+0.62 ±0.09</td>
<td>+0.68 ±0.07</td>
</tr>
<tr>
<td>DFB</td>
<td>12</td>
<td>100 ± 5</td>
<td>+0.67 ±0.11</td>
<td>+0.04 ±0.32</td>
</tr>
<tr>
<td>Rectus abdominis</td>
<td>12</td>
<td>125 ± 10</td>
<td>+0.95 ±0.23</td>
<td>+0.93 ±0.19</td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>125 ± 10</td>
<td>+0.97 ±0.17</td>
<td>+0.18 ±0.16</td>
</tr>
</tbody>
</table>

**Table II**

Breakdown of adenosine triphosphate and production of inorganic phosphate during single working isotonic contraction at 0° of frog sartorius muscles pretreated with 2,4-dinitrofluorobenzene

The muscles were isolated and treated as described in Table I. ATP and inorganic phosphate were measured in 0.25 M perchloric acid extracts (2). Results are ± standard error of the mean per g of muscle. The numbers in parentheses are the number of muscle pairs used.

<table>
<thead>
<tr>
<th>Type of muscle</th>
<th>External work</th>
<th>Inorganic phosphate</th>
<th>Δ Inorganic phosphate</th>
<th>ATP</th>
<th>ΔATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sartorius (normal)</td>
<td>0</td>
<td>1.70</td>
<td>+0.80 ±0.10 (6)</td>
<td>3.40</td>
<td>-0.01 ± 0.12 (6)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.81</td>
<td>+0.64 ±0.10 (8)</td>
<td>3.20</td>
<td>-0.70 ± 0.15 (8)</td>
</tr>
<tr>
<td>Contracted</td>
<td>100 ± 5</td>
<td>2.50</td>
<td></td>
<td>3.39</td>
<td></td>
</tr>
<tr>
<td>Sartorius (DFB)</td>
<td>0</td>
<td>2.45</td>
<td></td>
<td>2.50</td>
<td></td>
</tr>
</tbody>
</table>
which time the tension fell to a low value at the rate of about 10 g per sec.

Fig. 2 is a kymograph tracing of isotonic contractions by a tetanically stimulated sartorius muscle preincubated in DFB-Ringer's solution and then transferred to normal Ringer's solution (frog). The first two contractions of this DFB-treated muscle were apparently identical to those of its untreated control in all respects, and in particular in the speeds of contraction and relaxation, the amount of shortening, and hence the work done. In this experiment, the muscle performed about 80 g-cm of external work per g of muscle during each tetanic contraction. The number of contractions at 0° depended on the work per contraction, and in sartorius muscles it was reduced from many hundred to about four under these conditions. With single 0.4-msec supramaximal electrical pulses, the muscles could do 9 or 10 twitches, performing about 25 g-cm of work per g in each. Although the rates of shortening and relaxation were normal in the first two contractions (Fig. 2), they were markedly reduced in the following contractions. A short rest, in this case of 10 min, enabled the muscle to perform another contraction, and an additional delay sometimes enabled it to do a very small extra contraction. The muscle then became incapable of performing any significant amount of external work on further electrical stimulation, but it was able to shorten against no external load once or twice. The muscle gradually became stiff and entered rigor at 0° while still containing about 0.4 µmole of ATP per g.

During the entirely normal first contraction of the DFB-treated sartorius muscles shown in Tables I and II, the external work done per g of muscle was 150 g-cm per µmole of ATP used. This is the same as the 150 g-cm per µmole of phosphorylcreatine found for normal muscles in Table I. It compares favorably with the value of 120 g-cm of work per µmole of phosphorylcreatine calculated from the data of Mommaerts, Seraydarian, and Maréchal (17) in extensive studies of large numbers of contractions of iodoacetate-treated sartorius muscles. On the basis of about 10 Kcal per mole of ATP for the conditions in the muscle, the results represent an efficiency of energy transduction of about 35%.

The ATP concentrations of DFB-treated muscles after various amounts of activity are shown in Fig. 2 below the recordings; each value is an average of 4 to 6 muscles. Throughout this activity and even at rigor, the phosphorylcreatine levels remained constant at 22 to 25 µmole per g which is normal for these muscles.

It is interesting that there was no apparent net synthesis of ATP during the 10-min rests, and indeed a net increase of ATP was never observed at any time in DFB-treated sartorius muscles. This suggested that DFB treatment stopped oxidative and glycolytic metabolism in this tissue. We tested the oxygen consumption at 5-6°, and as shown in Fig. 3, there was no detectable oxygen uptake by the DFB muscle, whereas the QO2 for normal sartorii was -0.7 µl per mg, dry weight, per hour. Glycolytic activity was examined by measuring the rate of lactate production under anaerobic conditions at 1°. Table III shows that lactate was produced by both normal and DFB-pretreated muscles at a similar rate for the first 3 hours, i.e. until the DFB-muscle had been in rigor for some time. Indeed, after 10 hours, the rate of lactate production of the DFB-muscle became significantly higher than that of normal muscle. After 24 hours at 1°, the lactate concentration in DFB-sartorii was approxi-
mately 4 times higher than in their untreated pairs (15.6 and 3.5 pmoles per g, respectively). At the end of this time, the normal muscles could still contract readily.

The effects of DFB on some of the physical and enzymatic properties of the contractile elements were examined. Fiber bundles prepared from muscles treated for 40 min at 0° in DFB-Ringer's solution and extracted 2 to 3 weeks in 50% glycerol at −20°, were quite normal in the velocity and extent of shortening induced by addition of ATP.

The actomyosin ATPase activities of normal and DFB-treated muscles are given in Table IV. It is of interest that this activity had not been reduced in muscles incubated in DFB, but it had been increased by 20 to 50%. In contrast to this, the activity of the myokinase isolated from muscles pretreated in DFB for 40 min was reduced by about 20 to 50% of the values found in normal muscle.

Fig. 4 shows that the very active ATP:creatine phosphotransferase present in extracts of normal muscle was completely absent in extracts of DFB-muscles. Indeed under optimal conditions, in which the total extracts of normal muscles converted 6 pmoles of phosphorylcreatine to creatine in approximately 4 min, there was no detectable (+0.02 µmole) creatine formed in 30 min with the DFB-muscle extract of same protein content. The possible objection that small amounts of physically bound unreacted DFB were inhibiting the enzyme in vitro after the extraction was examined by extracting together an untreated muscle and an unwashed DFB-treated muscle. The total trans

**TABLE III**

*Lactate production in normal and DFB-treated muscle at 1°*  
The values are averages of three experiments.

<table>
<thead>
<tr>
<th>Time of incubation (hrs)</th>
<th>Lactate production (µmoles/g)</th>
<th>Rate (µmoles/µl/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal muscle</td>
<td>DFB-muscle</td>
</tr>
<tr>
<td>1</td>
<td>0.78</td>
<td>0.83</td>
</tr>
<tr>
<td>3</td>
<td>2.48</td>
<td>2.46</td>
</tr>
<tr>
<td>6</td>
<td>4.30</td>
<td>8.00</td>
</tr>
<tr>
<td>10</td>
<td>7.10</td>
<td>13.2</td>
</tr>
<tr>
<td>24</td>
<td>18.2</td>
<td>79.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The experiments of Lundsgaard (18), Carlson and Siger (19), and of Mommaerts, Soraydarian, and Maréchal (17), with iodoacetate-treated muscles showed clearly that phosphorylcreatine is used by muscles during prolonged activity. The utilization of phosphorylcreatine in single working isotonic contractions or short isometric contractions has recently been demonstrated in untreated (20–22), 2,4-dinitrophenol-treated (4, 21, 23), or iodoacetate-treated (24) frog muscles. This chemical process was directly associated with the energy output of the muscle during the contractions (4, 20, 21, 23). In all of these studies, there was no net breakdown of ATP during brief muscular activity (cf. Reference 17). However, as found by Lohmann in 1934 (25), and others (26, 27), dialyzed muscle extracts are incapable of hydrolyzing phosphorylcreatine without the addition of adenine nucleotides. The work of Engelhardt, Szent-Györgyi, Weber, and many others (see Reference 28) with myosin, actomyosin threads, and glycerinated-muscle fibers indicated that ATP is intimately associated with contraction. This has led to the following theory for the chemical mechanism of muscle.
lar contraction which is generally accepted even though changes in ATP had not been observed in living muscle except in rigor.

\[ \text{ATP} \rightarrow \text{ADP} + P_i \]
\[ \text{ADP + phosphorylcreatine } \rightarrow \text{ATP + creatine} \]
\[ \text{phosphorylcreatine } \rightarrow P_i + \text{creatine} \]

The assumption is that the cleaving of ATP by the action of the actomyosin ATPase provides energy for contraction and the ADP that is formed is rapidly phosphorylated by phosphorylcreatinthrough the action of ATP:creatine phosphotransferase. The net reaction is thus cleavage of phosphorylcreatine with formation of Pi and free creatine.

It is clear from Tables I and II that phosphorylcreatine was broken down during a single contraction of frog sartorius muscle at 0°, and that this source of energy was blocked in the DFB-treated muscle. Thus, the breakdown of ATP was not reversed. Indeed, the ATP:creatine phosphotransferase appeared to be completely inhibited in vivo since ATP was not restored even after a very long time (Fig. 2). This agrees with the observation that there was no transferase activity in extracts of DFB-treated muscle, and that the phosphorylcreatine content of the muscle remained constant even after the ATP had been greatly reduced.

Since oxidative metabolism was also completely inhibited, a possible resynthesis of ATP could only occur in the following ways: (a) through the glycolytic pathway or (b) from ADP in the myokinase reaction. The normal rate of lactate production in the DFB-treated sartorius muscle (i.e. approximately 0.01 μmole per g per min at 0°) is clearly insufficient by a factor of 500 times to interfere with measurements of chemical changes that occur in contractions lasting only 0.2 to 1.0 sec, which result in a breakdown of 0.2 to 1.0 μmole of ATP per g. Despite a normal rate of lactate production (Table III), there was no evidence of a net resynthesis of ATP after its usage in working contractions of DFB-muscles. It should be noted that the small amounts of ATP expected from glycolysis in 10 min (Fig. 2) may have been missed, but this is unlikely. Also the ATP levels began to fall in muscles after 60 min in 0.38 mM DFB at 0° (Fig. 1) when lactate formation was not less than normal (Table III). The explanation appears to be that in these muscles the production of hexose phosphates proceeds at a greater rate than they are used by the glycolytic enzymes (29).

The equivalence between ΔP, and ΔATP in Table II shows that myokinase was not active in these single isotonic contractions of DFB-treated sartorius muscles. The same equivalence was obtained in isometric contractions lasting as much as 1.5 sec (30). This is different from the situation in rectus abdominis where myokinase activity was observed even in the first contraction of the DFB-muscle (2, 21). Since the myokinase activity was largely unaffected by DFB, this difference is probably accounted for by the fact that the ATP content of the sartorius is much higher than that of the rectus abdominis (2, 21, 31). Hence, myokinase activity would become apparent only after several contractions, i.e. after much work has been done and the ATP:ADP ratio much reduced. This has recently been demonstrated (29).

It is interesting that the Ca++-activated actomyosin ATPase activity of DFB-treated muscles is higher than in normal muscles. Kiely and Bradley have shown that titration of about one-half of the sulfhydryl groups of myosin resulted in a 3- to 4-fold increase in its Ca++-activated ATPase (32). It is possible that under these conditions DFB operates similarly in vivo. This increased activity did not seem to have any marked effect on the contraction of the glycerol-extracted muscle model or on the first few contractions of the intact muscle.

Since the ADP levels increased during contractions of DFB-muscles (2, 21), the inhibitory effect of ADP on myosin ATPase (33) may result in the reduced rate of shortening after several normal contractions of the DFB-muscle. The fact that an exhausted DFB-sartorius muscle could do more work after a short rest, without a significant change in ATP level, suggests that the ATP moved from distant sites to the region occupied by the contractile proteins. ADP has recently been shown to inhibit the vesicular calcium pump which has been implicated in the relaxation process (34–36). Therefore, an increase in ADP may account for the reduced rate of relaxation in the last contractions of the DFB-treated sartorius muscle. Another effect may be a reduction of the amount of ATP available to this calcium pump (36).

Thus, the results show that the DFB-treated muscle can be used to study muscular contraction during single isotonic or short isometric contractions under conditions when the large stores of phosphorylcreatine cannot be used. This preparation has provided the means to show that ATP is broken down even in a single isotonic twitch lasting about 0.2 sec (31), to relate isometric contractions with ATP usage (30), and to clarify the chemical changes associated with the stretching of an activated muscle (37).

SUMMARY

Incubation of isolated frog muscles in 2, 4-dinitrofluorobenzene appears to inhibit completely their adenosine triphosphate: creatine phosphotransferase activity. Studies in vivo with rapid freezing methods showed that, although single anaerobic isotonic contractions of normal muscles at 0° produced equivalent increases in inorganic phosphate and free creatine without a change of ATP content, in DFB-treated muscles there was an increase in inorganic phosphate and an equivalent decrease in ATP without a net formation of creatine. DFB-treated muscles contained the normal concentrations of ATP, phosphorylcreatine, inorganic phosphate, and creatine, and physically appeared perfectly normal in brief contractile activity at 0°. However, on prolonged incubation at 0° in DFB the ATP concentration fell and the muscles began to enter rigor mortis.

There was no demonstrable oxygen uptake in DFB treated muscles, but they produced lactate at a normal rate for at least 3 hours after preincubation. The calcium activated actomyosin ATPase activity in extracts of the DFB-treated muscles was increased by 20 to 50%, and the ATP:AMP phosphotransferase was about 30% inhibited. During the first contraction, the external work done was 150 g-cm per g of muscle per μmole of ATP. At 10,000 cal of free energy per mole of ATP, this is an efficiency of about 35%.

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