J. Physiol. (1973), **229**, *pp.* 583–599
With 6 text-figures
Printed in Great Britain

DNP-INDUCED DISSIPATION OF ATP IN ANOXIC VENTRICULAR MUSCLE

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(*Received 24 August 1972*)

SUMMARY

1. During aerobic incubation in 5 mM glucose medium, 10^{-5} M-DNP reduced the action potential duration and amplitude and the developed tension of guinea-pig ventricular muscle more rapidly and to a greater extent than anoxia.

2. The DNP effect on electrical and mechanical activity was even more pronounced following prolonged anoxic incubation. Since the action potential duration and developed tension of anoxic ventricular muscle have previously been shown to be dependent on glycolytic ATP, and since the effects of DNP could not be duplicated with NaCN, it was concluded that DNP was exerting an effect in addition to its uncoupling of oxidative phosphorylation.

3. Anoxic muscle was incubated with 10^{-4} M-IAA or with 10^{-4} M-IAA + 10^{-4} M-DNP. The ATP content of IAA-treated muscle was significantly lower than control but in the presence of both IAA and DNP there was a further reduction in ATP and an increased lactate production.

4. Sodium azide (10^{-2} M), a potent inhibitor of mitochondrial ATPase, did not prevent the reduction of ATP in DNP-treated anoxic muscle.

5. Ouabain (10^{-7} M) partially prevented the rapid decline of action potential duration and developed tension of DNP-treated anoxic muscle. In addition, the glycoside partially blocked the DNP-induced break-down of ATP and stimulation of lactate production.

6. Oligomycin (10 μg/ml.) partially prevented the reduction in action potential duration and developed tension of DNP-treated anoxic muscle.

7. It was concluded that DNP induces an 'energy leak' by actively promoting the hydrolysis of an high energy glycolytic intermediate at least one step beyond the sites of ATPase inhibition by ouabain and oligomycin.

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INTRODUCTION

2,4-Dinitrophenol (DNP) is a classic uncoupler of oxidative phosphorylation and is thought to act by hydrolysing a high energy intermediate which is interposed between the electron transport chain and ATP (Ernster & Lee, 1964). However, it is apparent that DNP can also affect other energy pathways. In the isolated anaerobic turtle bladder, DNP markedly stimulated glycolysis and reduced ATP concentrations by about 25 % (Bricker & Klahr, 1966).

DNP has also been shown to depress the electrical and mechanical activity of anoxic guinea-pig ventricular muscle (McDonald, Hunter & MacLeod, 1971; McDonald & MacLeod, 1972). During anoxia, the action potential duration of ventricular muscle is extremely sensitive to the concentration of glucose in the medium as well as to agents which inhibit or stimulate glycolysis (MacLeod & Prasad, 1969), and changes in the action potential duration have been correlated with the availability of ATP from glycolysis (McDonald *et al.* 1971; McDonald & MacLeod, 1971). When ventricular muscle was incubated in medium containing 5 mM glucose and the equilibrating gas was nitrogen instead of oxygen the action potential duration declined from about 200 to 70 msec in 1 hr (McDonald *et al.* 1971). When the same medium also contained 10^{-6} M-DNP a similar decline was observed. However, in the presence of oxygen and either 10^{-5} M or 10^{-4} M-DNP, the action potential duration declined at a significantly greater rate. During aerobic incubation in the presence of 10^{-4} M-DNP the action potential declined to 70 msec duration within 10 min. During aerobic incubation the ATP content of DNP-treated muscles declined from 4·2 to 1·0 μmole/g wet wt. within 10 min. A similar decline required 60 min when untreated muscles were incubated under anoxic conditions.

To account for the finding that the shortening of the action potential and depletion of ATP were more severe during aerobic incubation with DNP than during anoxic incubation several explanations were considered: (1) The replacement of oxygen by nitrogen in the medium was not completely effective in blocking ATP production from oxidative phosphorylation. This was ruled out by the results of experiments in which 10^{-3} M-NaCN was added to the medium during a 1-hr anoxic incubation and its effect compared to that of 10^{-4} M-DNP. The presence of NaCN did not increase the rate of action potential shortening nor did it induce a greater depletion of ATP than that seen during anoxia alone (McDonald *et al.* 1971). The difference between NaCN and anoxia was not significant while the difference between DNP and NaCN or anoxia was highly significant ($P < 0{\cdot}001$). (2) DNP might reduce the level of ATP by inhibiting glyco-

lysis. The lactate production of anoxic muscles incubated with DNP was greater than during anoxia alone (see Results) and this suggested that the reduced action potential duration and ATP content of DNP-treated muscle was due to a greater utilization or break-down of ATP. This could be due to a stimulation of ATPase, particularly mitochondrial ATPase (Lardy & Wellman, 1953), and/or to an 'energy leak' resulting from the active hydrolysis of a glycolytic high energy intermediate. The latter mechanism has been proposed to explain the effects of DNP on anaerobic turtle bladder (Klahr, Bourgoigne & Bricker, 1968), and the results of the present investigation suggest that DNP induces a similar glycolytic energy leak in cardiac muscle.

METHODS

Electrophysiology

Papillary muscles were obtained from the right ventricle of guinea-pig heart. Animals were killed by cervical dislocation and the heart removed as quickly as possible. Dissection of the muscles was carried out in cool modified Krebs solution (see below). Papillary muscles were usually selected from the same position within the right ventricle and were about 3–5 mm in length and 0·5 mm in diameter. The muscles were mounted horizontally in a jacketed 25 ml. bath at 35° C. Resting tension was 100–200 mg. The muscles were held at one end in a plastic clamp and stimulated at 60/min through platinum electrodes attached to the clamp. The other end of the muscle was tied by a short length of silk thread to an insulated stainless steel rod connected to the head of a Statham force displacement transducer. The length of the muscle was adjusted through movement of the transducer by a micrometer screw. Gas was supplied to the bathing medium through a fritted disk 15 mm in diameter. This disk was situated about 10 mm below the muscle.

Single cell electrical activity was recorded using conventional micro-electrodes. Electrodes were mounted rigidly or floated using the technique of Woodbury & Brady (1956). Potential measurements were made through a Medistor negative capacitance electrometer, monitored on a Tektronix 504 oscilloscope, and recorded either on film or on a Grass polygraph. Action potential duration was measured at 75% repolarization.

Solutions

Modified Krebs medium had the following composition in milliequivalents per litre; Na 138·5, K 4·6, Ca 4·9, Mg 2·3, HCO_3 21·91, PO_4 3·48, Cl 124·91, and glucose 50 mM. Media contained 5 or 50 mM glucose and were equilibrated with either 95% O_2:5% CO_2 (oxygen) or 95% N_2:5% CO_2 (nitrogen).

ATP determinations

ATP content was determined in right ventricular strips weighing between 15 and 25 mg. The method used was a modification of the firefly luminescence technique of Strehler & McElroy (1957). Complete details are provided elsewhere (McDonald & MacLeod, 1971).

Lactate determinations

Lactate production was measured in right ventricular strips weighing between 15 and 25 mg. Lactate in the medium was determined using the enzymic method of Hohorst (1965). Complete details are provided elsewhere (McDonald & MacLeod, 1972).

Experimental procedure

All muscles were equilibrated in 50 mM glucose medium gassed with 95% O_2:5% CO_2 for 1 hr before beginning an experiment. Anoxic conditions were achieved by replacement with fresh medium and gassing with 95% N_2:5% CO_2. The fresh medium had previously been equilibrated with 95% N_2: 5% CO_2. In long-term experiments, solutions were renewed every hour.

Drugs

Ouabain (Schwartz/Mann), iodoacetic acid (Sigma) and 2,4-dinitrophenol (Sigma) were dissolved in distilled water and the stock solutions adjusted to pH 7 as required.

Oligomycin (Sigma) was dissolved in ethanol. The volume of drug solutions added never exceeded 1% of the medium volume.

RESULTS

The action potential duration of guinea-pig ventricular muscle declined during anoxic incubation in medium containing 5 mM glucose or during aerobic incubation in the presence of DNP. These effects are illustrated in Fig. 1. When the medium containing 5 mM glucose was bubbled with 95% N_2:5% CO_2 (N_2, Fig. 1*A*) instead of 95% O_2:5% CO_2, the action potential duration declined to about 40% control in 60 min. When oxidative phosphorylation was inhibited by the addition of 10^{-5} M-DNP during aerobic incubation in 5 mM glucose medium, the action potential duration declined more rapidly and to a greater extent than during anoxia (Fig. 1*B*), and the action potential amplitude was reduced by some 35 mV. Similarly, DNP depressed developed tension to a greater degree than anoxia.

The action potential duration and developed tension of ventricular muscle becomes more sensitive to changes in the external glucose concentration with increasing anoxic preincubation (McDonald *et al.* 1971), maximum sensitivity usually being achieved after 3 hr. At this time the effect of DNP on the electrical and mechanical activity was, perhaps, even more pronounced than in anaerobic muscle. This point is illustrated in Fig. 2. Papillary muscles were incubated under anoxic conditions for 3 hr in 50 mM glucose medium before changing the medium to 5 mM glucose without (filled symbols) or with 2×10^{-5} M-DNP (open symbols). In comparison to control, DNP rapidly reduced both the action potential duration and developed tension.

The rate of decline of the action potential duration has been related to muscle ATP content and it has been shown that DNP-treated muscle has a lower ATP content than NaCN-treated muscle or anoxic muscle (McDonald *et al.* 1971). The greater effect of DNP on the action potential duration and ATP content could be attributed to either a decreased production of ATP or to an increased dissipation. The latter explanation was considered the more likely since DNP stimulates the lactate production of anoxic muscle (McDonald *et al.* 1971).

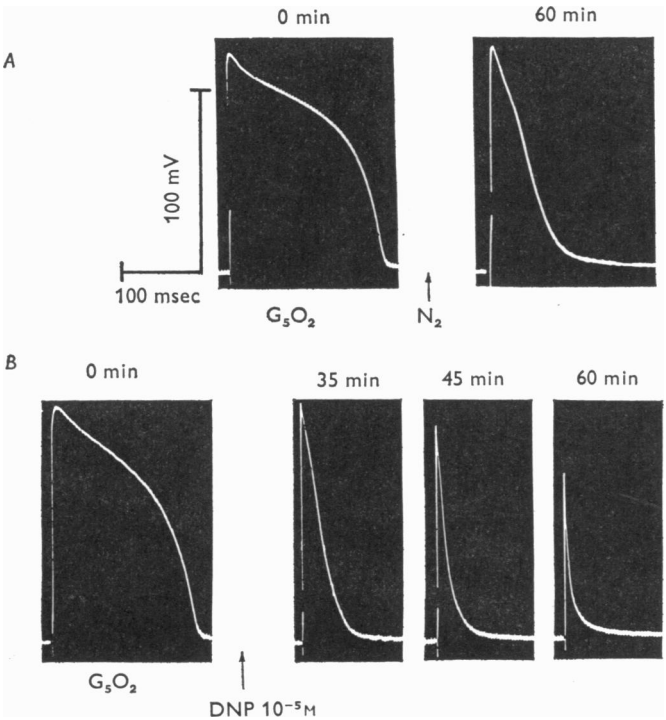

Fig. 1. Comparative effects of (*A*) anoxia (N_2) and (*B*) 2,4-dinitrophenol (DNP) on the action potential of guinea-pig papillary muscle. Control action potentials at 0 min were obtained during incubation in medium containing 5 mM glucose and oxygen (G_5O_2). The incubation medium was then changed to either (*A*) 5 mM glucose and nitrogen or (*B*) 5 mM glucose and 10^{-5} M-DNP. Time continuous from 0 min.

To test this hypothesis, glycolysis in anoxic muscle was inhibited by the addition of 10^{-4} M iodoacetic acid (IAA), and lactate production and ATP content were compared to that of muscles treated with IAA alone or in combination with 10^{-4} M-DNP (Table 1). The control anoxic incubation (Table 1*A*) was carried out for 30 min in medium containing 5 mM

glucose (G_5N_2). During this period the ATP content declined from 4·24 to 1·29 μmole/g wet wt. and lactate production during the 15–30 min period was 48·6 μmole/g.hr. All anoxic muscles treated with IAA alone or in combination with DNP were first incubated in 5 mM glucose medium for 5 min, treated with 10^{-4} M-IAA for 10 min, and then with either IAA or IAA + 10^{-4} M-DNP for the next 15 min (Table 1*B*). Ten minutes after the addition of IAA, the ATP content had declined to 0·94 μmole/g, significantly ($P < 0·05$) lower than the control anoxic value of 1·44 μmole/g.

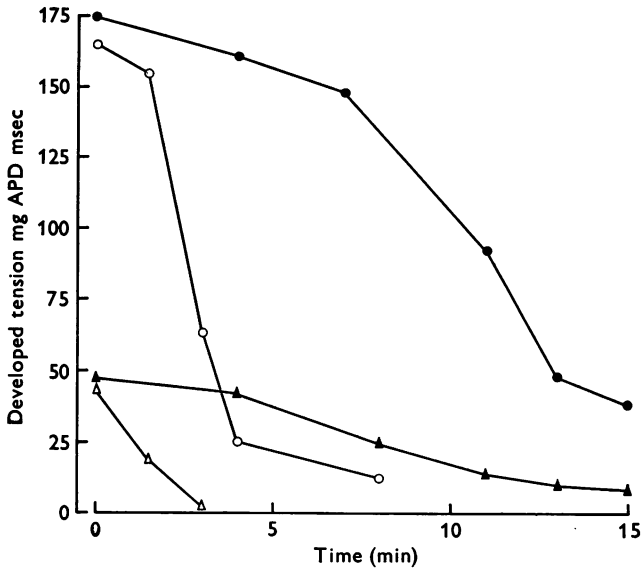

Fig. 2. The effect of DNP on the action potential duration (APD) and developed tension of papillary muscle preincubated for 3 hr under anoxic conditions in 50 mM glucose medium. The action potential duration during anoxia in 50 mM glucose medium is maintained at about 90% aerobic value while developed tension usually stabilizes at about 25% aerobic value (McDonald *et al.* 1971). Medium containing 5 mM glucose was added at 0 min. Control (filled symbols) and with 2×10^{-5} M-DNP (open symbols); action potential duration (circles) and developed tension (triangles).

After a further 15-min exposure to IAA the ATP content had declined to 0·64 μmole/g compared to 1·29 in the control anoxic muscles. Muscles treated with both IAA and DNP during this 15-min period had an ATP content of 0·36 μmole/g, significantly lower ($P < 0·001$) than muscles treated with IAA alone. Lactate production by muscles treated with IAA + DNP was greater than that of muscles treated with IAA alone but less than one third that of control anoxic muscles.

DNP stimulates a latent mitochondrial ATPase (Lardy & Wellman, 1953) and it was possible that by this mechanism DNP induced an increased hydrolysis of ATP. Since sodium azide is a potent inhibitor of mitochondrial ATPase (Vigers & Ziegler, 1968), it should block this DNP effect. Table 2 presents the data from experiments designed to this hypothesis. In control experiments there was no significant difference in the ATP content of muscles incubated under anoxic conditions for 30 min (G_5N_2) or under the same conditions in the presence of 10^{-2} M sodium

TABLE 1. The lactate production and ATP content of anoxic ventricular muscle treated with IAA alone or in combination with DNP. All incubations were in 5 mM glucose medium in the absence of oxygen (G_5N_2). The doses of IAA and DNP were 10^{-4} M. The experimental protocol used in *B* was as follows: muscles were incubated in G_5N_2 medium for 5 min, in G_5N_2 + 1AA for the next 10 min, and in either G_5N_2 + 1AA or G_5N_2 + 1AA + DNP for the last 15 min. All values are mean ± S.E. and the number of determinations is shown in parentheses

Time	Medium	ATP content (μmole/g wet wt.)	Lactate production (μmole/g wet wt. hr) 15–30 min period
	A Control anoxic incubation		
0 min	G_5N_2	4·24 ± 0·23 (16)	—
5 min	G_5N_2	2·11 ± 0·09 (12)	—
15 min	G_5N_2	1·44 ± 0·10 (12)	—
30 min	G_5N_2	1·29 ± 0·08 (12)	48·6 ± 2·2 (6)
	B IAA; IAA + DNP		
0 to 5 min	G_5N_2	2·11 ± 0·09 (12)	—
5 to 15 min	G_5N_2 + IAA	0·94 ± 0·04 (7)	—
15 to 30 min	G_5N_2 + IAA	0·64 ± 0·01 (9)	5·1 ± 0·8 (6)
15 to 30 min	G_5N_2 + IAA + DNP	0·36 ± 0·01 (9)	15·0 ± 3·1 (6)

azide. When anoxic muscles were incubated for the first 15 min with 10^{-2} M sodium azide and the next 15 min with both sodium azide and 10^{-4} M-DNP, the ATP content was significantly ($P < 0·01$) lower than control, but not significantly different from the control 15-min exposure to DNP alone. It has also been reported that a short period of anoxia prevents the stimulation of mitochondrial ATPase (Chefurka, 1960), yet DNP induced an extra break-down of ATP following a 15-min anoxic preincubation (Table 2).

Klahr *et al.* (1968) have postulated that DNP lowers the ATP content of anaerobic turtle bladder by hydrolysing a high energy intermediate evolving from glycolytically produced ATP. The concept of a high energy intermediate involved in plasma membrane ATPase activity has been documented earlier (Post, Sen & Rosenthal, 1965; Kahlenberg, Gals-

TABLE 2. The ATP content of anoxic ventricular muscle treated with sodium azide and DNP

Experimental conditions	n	ATP content ± S.E. of mean μmole/g wet wt.
30 min 5 mM glucose, N_2	(5)	1·31 ± 0·11
30 min $G_5N_2 + 10^{-2}$ M azide	(6)	1·28 ± 0·09
15 min $G_5N_2 + 10^{-2}$ M azide, then 15 min $G_5N_2 + 10^{-2}$ M azide + 10^{-4} M-DNP	(6)	0·89 ± 0·06
15 min G_5N_2, then 15 min $G_5N_2 + 10^{-4}$ M-DNP	(4)	0·85 ± 0·07

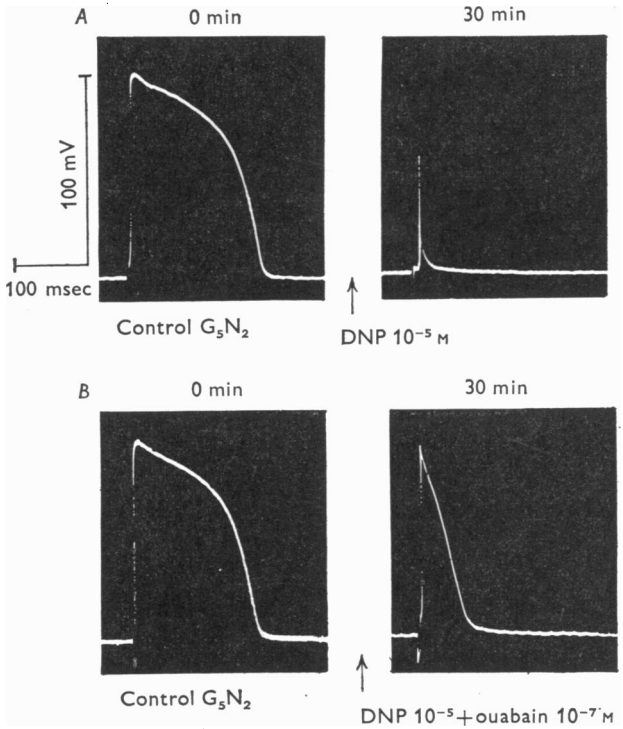

Fig. 3. The effect of ouabain on the action potential duration of DNP-treated muscle incubated under anoxic conditions. Papillary muscles were incubated in 5 mM glucose medium in the absence of oxygen (G_5N_2) for 5 min following which the bathing medium contained either (*A*) 10^{-5} M-DNP or (*B*) 10^{-5} M-DNP and 10^{-7} M ouabain for 30 min.

worthy & Hokin, 1967). Klahr reasoned that if DNP hydrolysed the intermediate, and if the site of action of DNP was beyond the formation of glycolytic ATP and its hydrolysis by plasma membrane ATPase, then

inhibitors of ATPase might block the effects of DNP on anaerobically produced ATP.

Therefore, the effect of ouabain on DNP-treated muscle was examined. Fig. 3*A* shows the effect of 10^{-5} M-DNP on the action potential duration of an anoxic papillary muscle. The muscle was incubated in 5 mM glucose medium for 5 min before the addition of DNP and after 30 min both the action potential duration and amplitude were severely depressed. The experiment illustrated in Fig. 3*B* followed the same protocol except that 10^{-7} M ouabain was added along with the DNP. The addition of ouabain,

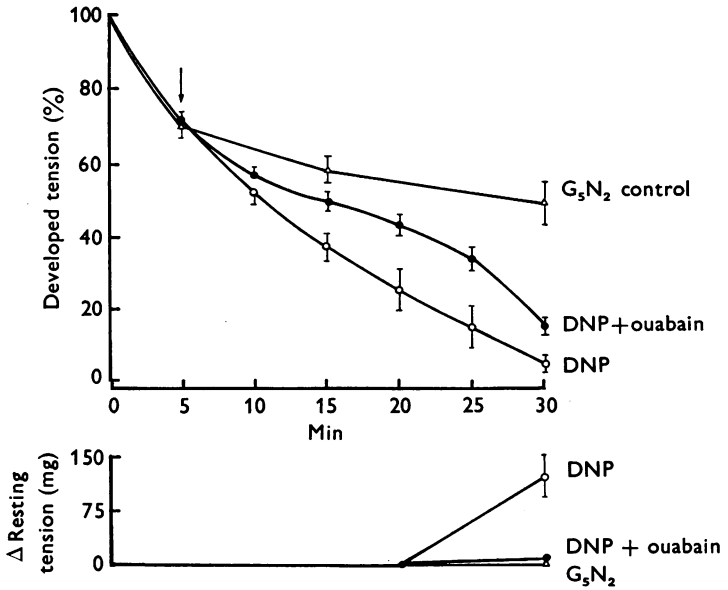

Fig. 4. The effect of ouabain on the developed tension (upper) and resting tension (lower) of DNP treated muscle incubated under anoxic conditions. Values are mean ± S.E., $n = 4$.

which by itself slightly increased the rate of decline in action potential duration of anoxic muscle (Prasad & MacLeod, 1969), reduced the effect of DNP on both the action potential duration and amplitude. Similar results were obtained in three additional experiments.

Fig. 4 summarizes the effect of ouabain on the developed tension and resting tension of DNP-treated anoxic muscle. Muscles were incubated under anoxic conditions for 5 min and then for a further 25 min either under control conditions (G_5N_2), in the presence of 10^{-5} M-DNP, or in the presence of 10^{-5} M-DNP and 10^{-7} M ouabain. Developed tension declined at a greater rate in DNP + ouabain than in control, but not as rapidly as

in DNP alone. Further, the resting tension of muscle incubated with DNP + ouabain did not increase by more than 10 mg during the observation period while the resting tension with DNP alone increased by an average of 125 mg.

Since the action potential duration and developed tension of anoxic ventricular muscle have been related to muscle ATP levels (McDonald *et al.* 1971; McDonald & MacLeod, 1971) the effect of ouabain on the electrical and mechanical activity of DNP-treated muscle suggested an

TABLE 3. The lactate production and ATP content of anoxic ventricular muscle treated with ouabain and DNP. All incubations were in 5 mM glucose medium in the absence of oxygen (G_5N_2). The doses of ouabain and DNP were 10^{-7} M and 10^{-5} M respectively. All values are mean ± S.E. and the number of determinations is shown in parentheses.

Experimental conditions	ATP content (μmole/g wet wt.)	Lactate production (μmole/g wet wt. hr)
30 min G_5N_2 (control)	1·26 ± 0·06 (12)*	52·7 ± 2·4 (6)*
30 min G_5N_2 + ouabain	1·20 ± 0·08 (14)	55·2 ± 2·2 (6)
5 min G_5N_2 then 25 min G_5N_2 + DNP	0·95 ± 0·05 (14)***	69·4 ± 3·8 (6)**
5 min G_5N_2 + ouabain, then 25 min G_5N_2 + ouabain + DNP	1·11 ± 0·05 (14)	60·6 ± 2·8 (6)

* Not significantly different than the value immediately below.
** Significantly ($P < 0·05$) different than control and significantly ($P < 0·10$) different than the value immediately below.
*** Significantly ($P < 0·01$) different than control and significantly ($P < 0·01$) different than the value immediately below.

ATP 'sparing' action. Direct verification of this was sought in measurements of the ATP content of anoxic ventricular muscle exposed to DNP alone or in combination with ouabain. As a corollary, we might also expect ouabain to partially block the DNP-induced increase in lactate production. Anoxic ventricular muscles exposed to either ouabain or DNP alone, or to a combination of these drugs, were monitored for lactate production and analysed for ATP; the data are presented in Table 3. The lactate production and ATP content of muscles incubated with 10^{-7} M ouabain were not significantly different than control. In 10^{-5} M-DNP medium, muscles produced more lactate and had a lower ATP content ($P < 0·01$) than control muscles. However, in DNP + ouabain medium muscles had a higher ATP content ($P < 0·01$) and produced less lactate ($P < 0·10$) than muscles treated with DNP alone. The interaction of ouabain and DNP was also studied at doses of 2×10^{-7} M and 2×10^{-5} M respectively. Measurements of action potential duration, tension, ATP content and lactate production were in good agreement with those obtained at the lower concentrations.

It was desirable to demonstrate that the effect of DNP was blocked by ATPase inhibitors other than ouabain. Oligomycin has been shown to inhibit Na-K-ATPase activity (Whittam, Wheeler & Blake, 1964; Hokin *et al.* 1965) and in control experiments oligomycin (10 μg/ml.) did not affect the action potential duration of anoxic muscle. The experimental procedure was as follows: Papillary muscle was incubated under anoxic conditions for 3 hr in 50 mM glucose medium. The effect of 10^{-4} M-DNP on the action potential duration and developed tension was monitored and, following washout and recovery in 50 mM glucose medium, the muscle was pre-treated with oligomycin (10 μg/ml.) before the addition of

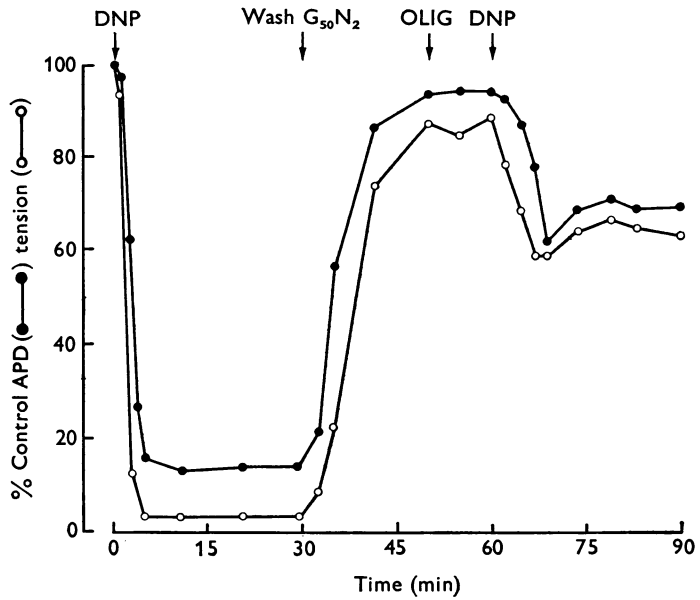

Fig. 5. The effect of DNP on the action potential duration (APD) and developed tension of anoxic papillary muscle before and after treatment with oligomycin (OLIG). The experiment was begun after 1 hr of aerobic incubation and 3 hr of anoxic incubation in 50 mM glucose medium. Control action potential duration at 0 min was 87% of aerobic value and control developed tension was 23% of aerobic value. 10^{-4} M-DNP rapidly reduced both parameters in the absence of oligomycin. Following recovery after washing with 50 mM glucose medium at 30 min, oligomycin 10 μg/ml. partially blocked the effect of a second dose of 10^{-4} M-DNP.

10^{-4} M-DNP again. The results are shown in Fig. 5. Within 5 min, DNP reduced both the action potential duration and developed tension to less than 20% control value (100% control refers to the action potential duration and developed tension immediately before the addition of DNP, and was 87% and 23% respectively of aerobic control values). Upon

washing out the DNP, both parameters recovered and were not affected by the addition of oligomycin, 10 μg/ml. When 10^{-4} M-DNP was again added to the medium, the action potential duration and developed tension declined to about 65% control in 10 min and thereafter remained stable. In two additional experiments the dose of DNP following oligomycin was raised to 3×10^{-4} M but no further depression could be observed.

DISCUSSION

The action potential duration of anoxic guinea-pig ventricular muscle has been shown to be extremely sensitive to metabolic intervention. The duration could be varied at will by manipulating the concentration of glucose in the medium (MacLeod & Prasad, 1969); in 5 mM glucose medium the action potential duration was shortened to about 30% of its normal value while in 50 mM glucose medium it was maintained at, or restored to, about 90% of its aerobic value. Other sugars were ineffective in this regard. Agents which inhibited glucose transport or glycolysis reduced the action potential duration while agents which increased glucose transport or stimulated glycolysis lengthened the action potential duration (Prasad & MacLeod, 1969). These results, along with measurements of muscle ATP content and lactate production (McDonald *et al.* 1971; McDonald & MacLeod, 1971) provide a solid basis for our working hypothesis that the duration of the action potential is dependent on the level of glycolytic ATP.

DNP, a classic uncoupler of oxidative phosphorylation (Ernster & Lee, 1964) might be expected to affect the action potential duration and ATP content of aerobic muscle in a manner similar to that of anoxia. In fact, DNP reduced the action potential duration and ATP content of aerobic muscle at a greater rate and to a greater extent, than did anoxia when both experiments were done in 5 mM glucose medium (McDonald *et al.* 1971). NaCN, on the other hand, produced effects quite comparable to anoxia (McDonald *et al.* 1971) leading to the conclusion that DNP was exerting an action in addition to its inhibition of oxidative phosphorylation.

When muscle was exposed to DNP in the absence of oxygen, the ATP content was lower and the lactate production was greater than that of anoxic muscle. Also, when anoxic muscle was exposed to both IAA and DNP the ATP content was lower and the lactate production greater than muscle treated with IAA alone. Thus, the lower ATP content of DNP treated muscle must have been due to an increased break-down of ATP rather than a reduced rate of synthesis.

It was possible that the extra break-down of ATP induced by DNP

was related to an ATPase stimulation. ATPase systems which must be considered include myosin-ATPase, mitochondrial ATPase, Mg^{2+}-activated ATPase, and Na^{+}-K^{+}-Mg^{2+} activated ATPase. DNP has been shown to activate myosin-ATPase (Greville & Reich, 1956) but only at doses exceeding 10^{-3} M. In the present study 10^{-5} M-DNP significantly reduced the ATP content of anoxic muscle suggesting that myosin-ATPase stimulation can be ruled out. Mitochondrial ATPase stimulation does not appear to be responsible for the DNP effect on the following grounds. (1) Sodium azide, a potent inhibitor of mitochondrial ATPase, did not prevent the reduction in ATP. (2) Anoxic pretreatment blocks mitochondrial ATPase stimulation (Chefurka, 1960) but did not block the effect of DNP. Na^{+}-K^{+}-Mg^{2+} activated ATPase is not affected by DNP in red blood cells (Hoffman, 1962; Wins & Schoffeniels, 1966), electric organ (Glynn, 1963) or turtle bladder (Klahr *et al.* 1968) although a Mg^{2+}-activated ATPase in red blood cell ghosts has been reported to be stimulated by DNP in the range 10^{-5}–10^{-3} M (Laris & Letchworth, 1967). That the latter possibility could contribute to the reduced ATP cannot be ruled out but, in view of the results with ouabain and oligomycin, a further alternative (the 'energy leak' hypothesis of Klahr) appears to be a more suitable explanation.

Klahr *et al.* (1968) found that DNP stimulated glycolysis in the anaerobic turtle bladder but this stimulation was accompanied by lower ATP levels and an inhibition of ATP utilization. Ouabain or ethacrynic acid prevented both the glycolytic stimulation and the reduction in ATP, leading to the conclusion that DNP exerted an operational ATPase effect by hydrolysing a high energy intermediate interposed between the ATPase site inhibited by ouabain and the site mediating active transport. In the presence of DNP the hydrolysis of the high energy intermediate would force the reaction to the right and thus dissipate ATP and increase lactate production. DNP may act in a similar way on anaerobic yeast cells (Riemersma, 1968) and such an explanation may account for the interaction of DNP with ouabain or oligomycin in the present study.

Both ouabain and oligomycin are known to inhibit Na^{+}-K^{+}-Mg^{2+}-activated ATPase (Whittam *et al.* 1964) and, assuming that DNP acts on anoxic ventricular muscle in the same way as on turtle bladder, these agents would be expected to inhibit the effect of DNP on the action potential duration, developed tension, ATP content, and lactate production. All of these effects were partially inhibited by ouabain and the reduction in action potential duration and tension were partially blocked by oligomycin. It is worth considering why the effects of DNP were not completely inhibited. Oligomycin treatment may have only partially inhibited ATPase activity (Van Gronigen & Slater, 1963; Robinson, 1971) while the dose of

ouabain used ($1–2 \times 10^{-7}$ M) may also have been insufficient. Larger doses were impractical since anoxic muscle is quite sensitive to ouabain toxicity. Ouabain was found to be more effective in inhibiting the DNP-induced changes in ATP content and lactate production than in inhibiting the decline in action potential duration. Since DNP also appears to interact with the cell membrane and produce increases in potassium permeability and efflux (Godfraind, Krnjević & Pumain, 1970; Haas, Kern & Einwächter, 1970; McDonald & MacLeod, 1972) the ameliorating effect of both ouabain and oligomycin on the action potential duration might not be expected to be complete.

One difficulty which arises in comparing the present results with those of Klahr *et al.* is that the DNP dissipation of ATP in anaerobic turtle bladder was accompanied by an inhibition of active ion transport. In ventricular muscle the effect of DNP on ion transport was not as clear cut (McDonald & MacLeod, 1972). At 10^{-4} M there was no increase in sodium

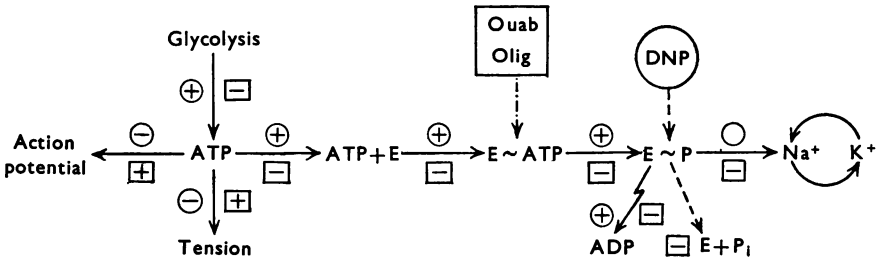

Fig. 6. A model describing the interaction of DNP with ouabain or oligomycin on the action potential, tension, and anaerobic metabolism of ventricular muscle. The basic ATPase-ion transport pathway is that of Klahr *et al.* (1968) where E is an enzyme or enzymes and E ~ P is the high energy intermediate. The effect of DNP is represented by the symbols in circles: ⊕ stimulation, ⊖ inhibition and ○ (no change). Ouabain or oligomycin either inhibit (⊟) the increased fluxes induced by DNP or stimulate (⊞) fluxes decreased by DNP.

during the first 30 min but a downhill movement thereafter. At 10^{-5} M there was no increase during the first hour but an increase thereafter. There was an immediate large dose-dependent loss of potassium due mainly to increased efflux and since the uptake of ^{42}K was not significantly lower than anoxia during the first 30 min, it was concluded that DNP had no 'extra' effect on active ion transport. The data can still be reconciled with Klahr's hypothesis, as shown schematically in Fig. 6. DNP hydrolyses an high energy intermediate (E ~ P) involved in ion transport, stimulating glycolysis and diverting ATP to this pathway from others including that ATP destined for electrical and mechanical activity. The

increased ATP flux can, depending on the glucose supply and dose-duration of DNP treatment, satisfactorily maintain the pumping rate at anoxic levels. The addition of ouabain or oligomycin partially prevents the formation of the high-energy intermediate and subsequent DNP hydrolysis and thus conserves ATP for other purposes.

The oft noted observation that DNP treatment of cardiac muscle incubated under aerobic conditions leads to a more rapid and complete depression of electrical activity than incubation of muscle under anoxic conditions (Webb & Hollander, 1956; Macfarlane, 1960; Prasad & MacLeod, 1969; McDonald *et al.* 1971) or with NaCN (Macfarlane, 1960) can be partially explained by invoking Klahr's hypothesis of an energy leak. In this regard, it is interesting to note that oligomycin prevented the DNP-induced inhibition of beating in cultured heart cells and ouabain also had a beneficial effect (Harary & Slater, 1965). The authors attributed the oligomycin protection to a blocking effect on mitochondrial ATPase stimulation by DNP. In view of the present work, these results could also be interpreted in terms of the glycolytic energy leak hypothesis.

In summary, the present study suggests that when DNP is used for the sole purpose of blocking oxidative phosphorylation, the results should be interpreted with caution.

We thank Mrs Jean Crozsman for excellent technical assistance. This work was supported by the Medical Research Council of Canada.

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