Experimentally induced defects of mitochondrial metabolism in rat skeletal muscle

Biological effects of the NADH: coenzyme Q reductase inhibitor diphenyleneiodonium

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(Received 7 December 1984/28 February 1985; accepted 11 March 1985)

An animal model for the human condition of mitochondrial myopathy has been established and characterized physiologically and biochemically. The NADH: coenzyme Q reductase inhibitor diphenyleneiodonium [Bloxxham (1979) *Biochem. Soc. Trans.* 7, 103–106] was either infused acutely *in vivo* into rat hind limb or injected chronically into rats. Both modes of delivery resulted in a reduced muscle oxidative capacity and increased fatigue. Analysis of muscle metabolites by h.p.l.c. and ³¹P-n.m.r. indicated that ATP concentrations were similar to control values during periods of stimulation and these were maintained by the phosphocreatine pool. During the recovery period after muscle stimulation in the experimental animals the muscle pH remained depressed and the rate of phosphocreatine synthesis was markedly delayed as compared with controls. Factors thought to be involved in the fatigue response are discussed in relation to this model.

The provision of energy (ATP) for muscular contraction may be met by either anaerobic glycolysis or aerobic metabolism. The predominant pathway under resting conditions and during moderate work regimes is the oxidative (mitochondrial) process. In the preceding paper (Byrne *et al.*, 1985) it has been demonstrated that the classical mitochondrial uncoupler, dinitrophenol, depleted high energy phosphate compounds in rat hind limb *in vivo* and that contracture ensued. It was further noted that administration of this agent served as a poor model for the human condition termed mitochondrial myopathy (Byrne *et al.*, 1985).

There are a range of defects which have been reported within the mitochondrial myopathy syndrome (Clark *et al.*, 1983, 1984; Morgan-Hughes *et al.*, 1984). However, the most commonly recorded defect lies within the NADH: coenzyme Q

reductase (Complex I) portion of the respiratory chain (Clark *et al.*, 1983, 1984; Morgan-Hughes *et al.*, 1984). In the present paper the effects of the administration *in vivo* of a potent irreversible inhibitor of the NADH: coenzyme Q reductase, diphenyleneiodonium (see Bloxxham, 1979), have been investigated. This compound was either infused intra-arterially in a rat hindlimb preparation or injected subcutaneously for 2–3 days. The gastrocnemius muscle from the animals was stimulated via the sciatic nerve and the muscle was freeze-clamped for metabolite assay. Administration by either route resulted in muscle fatigue but usually without contracture development. If contracture was present it was as a late finding (see Fig. 1).

The development of this animal system appears to provide a more appropriate model for the mitochondrial myopathic condition observed in human patients and should also allow a more detailed investigation of the relationship between aerobic and anaerobic energy production and muscle fatigue.

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Materials and methods

Reagents

Diphenyleneiodonium was prepared from 2-aminobiphenyl by the methods of Collette *et al.* (1956) and H. S. A. Sherratt, (personal communication). 2-Aminobiphenyl was obtained from the Aldrich Chemical Co. All other reagents were purchased from suppliers as previously detailed (Morgan-Hughes *et al.*, 1977, 1979, 1982).

Animals

Adult male Wistar rats (180–300 g body wt.) were used. They were maintained on a standard laboratory diet (SDS Ltd., Witham, Essex, U.K.) and drinking water was always freely available. Animals were anaesthetized with urethane [25% (w/v) in saline; 5 ml/kg body wt.]. Additional boluses of 1 ml/kg body wt. were given as required.

Administration of diphenyleneiodonium

Intra-arterial infusion. Following anaesthesia, the left femoral artery was cannulated with the tip of the cannula located at the iliac bifurcation (see Byrne *et al.*, 1985). The right gastrocnemius muscle was dissected free and attached to a strain gauge held in a rigid animal frame (see Byrne *et al.*, 1985). Isometric twitch tensions were recorded with either 1 or 5 Hz stimulation, using equipment previously described (Byrne *et al.*, 1985). The diphenyleneiodonium was dissolved in warm 5% (w/v) glucose (pH 7.0) at a final concentration of 4 mg/ml. Prior to infusion the cannula was flushed with the glucose solution, then the diphenyleneiodonium was infused at 4–8 mg/kg body wt.

Subcutaneous injection. Animals were injected daily with diphenyleneiodonium (1.5–3 mg/kg body wt.). The hypoglycaemic action of this agent (Holland & Sherratt, 1972; Holland *et al.*, 1973; Bloxham, 1979) was minimized by fortifying the drinking water with 5% (w/v) glucose. When animals developed marked weakness the physiological and biochemical parameters were studied as outlined above, except that a cannula was not inserted. Hypoglycaemia if present was corrected by intraperitoneal injection of 10% (w/v) glucose.

Metabolite assay

At appropriate times the gastrocnemius muscle was rapidly frozen between two brass plates (77 K) and quickly quenched in liquid N₂. Frozen muscle samples were stored at –70°C prior to assay. Nucleotides were extracted by the method of Lush *et al.* (1979) and quantified by a reverse-phase ion-pairing h.p.l.c. technique (Knox & Jurrand, 1981; see Byrne *et al.*, 1985, for details). Phosphocreatine and lactate were measured by standard spectro-

photometric assays (Bergmeyer, 1974), glucose was estimated by the glucose oxidase/peroxidase method (Sigma Diagnostic Kit 510). ³¹P-n.m.r. studies were carried out by methods previously described (Byrne *et al.*, 1985). Calculation of metabolic concentrations was carried out as indicated in the previous paper (Byrne *et al.*, 1985).

Mitochondrial studies

Liver mitochondria were isolated essentially by the method of Hogeboom *et al.* (1948) except that isolation medium consisted of 225 mM-mannitol/75 mM-sucrose/10 mM-Tris/HCl/0.1 mM-EDTA, pH 7.2. The isolated mitochondrial pellets were resuspended in isolation medium at a protein concentration of approx. 40 mg/ml. Skeletal muscle mitochondria were isolated essentially as described by Morgan-Hughes *et al.* (1982) except that Sigma Protease Type VII (10 mg/g of muscle with a 5 min digestion period) was used. The isolated mitochondrial pellet was resuspended in heparin-free isolation medium (see above) at a protein concentration of approx. 10 mg/ml.

The mitochondrial respiratory activities were studied polarographically at 25°C using methods described elsewhere (Morgan-Hughes *et al.*, 1977). Protein was measured by the method of Lowry *et al.* (1951) using fat-free bovine serum albumin to construct standard curves.

Results

Observations in vivo

In preliminary experiments involving intra-arterial infusion of diphenyleneiodonium (see the Materials and methods section) hypoglycaemia developed after 60–90 min. In the experiments reported in this study hypoglycaemia was prevented by injection of 100 µl boluses of 10% (w/v) glucose at approx. 30 min intervals.

Animals injected subcutaneously with 3 mg/kg body wt. of diphenyleneiodonium daily developed unsteadiness of gait with splaying of limbs after 2–4 days. Similar signs developed in rats given a lower dose (1.5 mg/kg body wt., 4 or 5 days/week) between 10 and 35 days after commencement of the injections. Estimation of blood glucose levels were made from the tail vein and if hypoglycaemia was indicated (glucose levels < 1.8 mM), this was corrected by intraperitoneal injection of 10% glucose. The glucose levels remained stable at > 5.6 mM, but the weakness persisted. In some animals the weakness resolved within 24 h following cessation of injections of diphenyleneiodonium (1.5 mg/kg body wt.) but reinstitution of diphenyleneiodonium injections provoked a second acute episode of weakness.

Physiological studies after intra-arterial infusion of diphenyleneiodonium

Stimulation at 1 Hz. The isometric twitch tension stabilized at a mean steady state tension of 76% (range 67–85%) of the initial value, which was 468 ± 69 g (mean $\pm$ S.D., $n = 15$) (see Edstrom & Kugelberg, 1968; Kugelberg & Edstrom, 1968). The diphenyleneiodonium was infused after 60 min of stimulation and additional boluses were given at 75 or 90 min (at a total dose of 2–4 mg) to produce a severe force failure. The rate of twitch tension failure with nerve stimulation and with interpolated single direct muscle stimuli was identical. This force failure was closely associated with the fall in muscle action potential amplitude (Fig. 1). As the twitch tension fell the twitch half-relaxation times became longer, a feature associated with fatigue (Edwards *et al.*, 1975a,b). In a group of five animals a 60 min period of recovery followed the 60 min stimulation run. The mean twitch tension at the end of stimulation was 74 g (range 10–140 g) and had risen to a mean value of 160 g (range 60–280 g) following the rest phase, but decreased rapidly upon restimulation.

Stimulation at 5 Hz. Diphenyleneiodonium was infused once the steady-state twitch tension had stabilized (after 5–7 min; see Edstrom & Kugelberg, 1968; Kugelberg & Edstrom, 1968), and caused a rapid decline in the twitch response. The

twitch tension fell from the mean steady state value of 61% to 17% of the initial response (see Table 1). If the muscle was allowed to recover for 15 min then the twitch response rose to a mean value of 47% of the initial response. This represented an improvement of 64% above the value at the end of stimulation (see Table 1). A similar improvement in twitch tension was not observed in control rats (Table 1).

In another series of experiments the stimulation at 5 Hz was interrupted by rest phases (see Fig. 2 for a typical experiment). A stimulation pattern caused rapid fatigue responses with a delayed recovery in twitch tension, which upon restimulation fatigued.

Physiological studies after subcutaneous injection of diphenyleneiodonium

Animals were studied after weakness had developed. The hypoglycaemia if present was corrected (see the Materials and methods section) and the animals were mounted in the animal frame. The gastrocnemius muscle was stimulated at 1 Hz and the isometric twitch tension fell to less than 20% of the initial value after 15 min of stimulation (Table 2). The tension evoked with direct muscle activation was identical with that when the nerve was stimulated. The failure in tension was accompanied by a marked prolongation of half-relaxation times and by a parallel fall in the action potential amplitude. The muscle was rested for 15 min prior to restimulation at 1 Hz for 15 min. Some improvement in the twitch response

Fig. 1. Diphenyleneiodonium infusion during stimulation at 1 Hz

Rat gastrocnemius muscle was stimulated at 1 Hz. Diphenyleneiodonium (DPI) was infused (2 mg) at the time indicated. The isometric twitch tension either by nerve (●) or direct muscle activation (○) was recorded. The action potential (□) and resting tension (■) are also shown. Contracture was a late finding. Solid bar represents period of stimulation.

Fig. 2. Diphenyleneiodonium infusion during repeated 5 Hz stimulation trains

Rat gastrocnemius muscle was repeatedly stimulated at 5 Hz (0–22, 85–95, 220–225 and 260–262 min) by either nerve (●) or direct muscle (○) stimulation. Muscle action potential (□) paralleled the twitch response. Diphenyleneiodonium (DPI) was infused at 6 and 15 min (1 mg each time).

Table 1. Metabolite levels and twitch tension of rat gastrocnemius muscle infused with diphenyleneiodonium and stimulated at 5 Hz

Gastrocnemius muscle was infused with diphenyleneiodonium (2–4 mg/kg body wt.) during which the muscle was stimulated at 5 Hz for 30 min and either freeze-clamped immediately (group A) or allowed a 15 min recovery period and then freeze-clamped (group B). The results are the means $\pm$ s.d. for four rats in each group. Student's *t*-test between control and experimental: ^a*P* $\leq$ 0.001, ^b*P* $\leq$ 0.002, ^c*P* $\leq$ 0.005, ^d*P* $\leq$ 0.01, ^e*P* $\leq$ 0.05.

Metabolite	Level (μ mol/g wet wt.)	
	Control	Infused
Group A: freeze-clamped at the end of stimulation (30 min)		
Inosine	0.28 $\pm$ 0.07	0.38 $\pm$ 0.06 ^b
NAD	0.50 $\pm$ 0.03	0.42 $\pm$ 0.06
IMP	1.71 $\pm$ 0.41	2.09 $\pm$ 0.40
AMP	0.06 $\pm$ 0.02	0.06 $\pm$ 0.01
ADP	0.92 $\pm$ 0.10	0.88 $\pm$ 0.13
ATP	3.59 $\pm$ 0.19	2.28 $\pm$ 0.92 ^c
ATP/ADP	4.0 $\pm$ 0.6	3.2 $\pm$ 0.7
Total adenine nucleotides	4.56 $\pm$ 0.13	3.47 $\pm$ 1.05
Glucose	4.12 $\pm$ 0.49	4.21 $\pm$ 0.05
Lactate	7.31 $\pm$ 1.74	7.08 $\pm$ 1.17
Phosphocreatine	7.15 $\pm$ 0.60	< 0.05 ^a
Adenylate energy charge	0.89 $\pm$ 0.01	0.87 $\pm$ 0.02
Twitch tension (%)	56 $\pm$ 16	17 $\pm$ 6 ^a
Group B: freeze-clamped after 15 min recovery (45 min)		
Inosine	0.23 $\pm$ 0.03	0.41 $\pm$ 0.11 ^e
NAD	0.45 $\pm$ 0.08	0.45 $\pm$ 0.06
IMP	1.15 $\pm$ 0.32	2.43 $\pm$ 0.60 ^b
AMP	0.04 $\pm$ 0.00	0.05 $\pm$ 0.02
ADP	0.72 $\pm$ 0.05	0.89 $\pm$ 0.12
ATP	4.55 $\pm$ 0.96	3.21 $\pm$ 0.62
ATP/ADP	6.3 $\pm$ 1.1	3.7 $\pm$ 1.1 ^c
Total adenine nucleotides	5.28 $\pm$ 1.01	4.14 $\pm$ 0.58
Glucose	2.55 $\pm$ 0.84	2.63 $\pm$ 0.82
Lactate	4.11 $\pm$ 1.77	8.85 $\pm$ 3.32
Phosphocreatine	18.50 $\pm$ 3.12	9.50 $\pm$ 2.27 ^d
Adenylate energy charge	0.93 $\pm$ 0.01	0.89 $\pm$ 0.02 ^c
Twitch tension (%)	61 $\pm$ 9	47 $\pm$ 12

was evident following the recovery phase but the twitch response rapidly fatigued.

Biochemical studies after intra-arterial infusion of diphenyleneiodonium

Preliminary experiments established that intra-arterial infusion of diphenyleneiodonium into rat hind limb *in vivo* caused a dose-dependent inhibition of the oxidation of NAD-linked substrates as judged by the respiratory properties of mitochondria isolated from the affected muscles (results not shown).

Two groups of four animals were studied and infused with diphenyleneiodonium at a concentration of 2–4 mg/kg body wt. Control animals were infused with equal volumes of 5% (w/v) glucose. The gastrocnemius muscle of the first group (A, Table 1) was freeze-clamped after 30 min of infusion during which the muscle was stimulated at 5 Hz. In the second group (B, Table 1) the muscle

was freeze-clamped after allowing a 15 min recovery period following the 30 min of 5 Hz stimulation. Prior to freeze clamping of the muscles in group B, each muscle was stimulated twice and the isometric twitch tension was recorded.

The major metabolite differences between the control and diphenyleneiodonium-infused muscles which had been stimulated at 5 Hz were that the inosine levels were significantly higher in the experimental group whereas the ATP levels were lower and there was no detectable phosphocreatine in the infused muscles (see Table 1). However, there were several more differences after the 15 min recovery period. In control animals the rest phase allowed a resynthesis of ATP and phosphocreatine and lactate levels fell by about 56%. Although the diphenyleneiodonium-infused animals showed some increase in ATP levels after the recovery phase this gain failed to improve the ATP/ADP ratio significantly (see Table 1). How-

Table 2. *Metabolite levels in the stimulated and contralateral gastrocnemius muscle from rats which had been injected subcutaneously with diphenyleneiodonium*

Animals were injected on each of 2 consecutive days with diphenyleneiodonium (3 mg/kg body wt.) and were studied 12–15 h after the second injection. The gastrocnemius muscle was stimulated at 1 Hz for 15 min, followed by a 15 min recovery period, followed by restimulation at 1 Hz for a further 15 min, at which point both the stimulated and contralateral gastrocnemius muscle were freeze-clamped. The results for both groups are of duplicate estimations averaged, and the mean $\pm$ S.D. was calculated from these; *n* is number of animals used in each group. Student's *t*-test: ^a*P* $\leq$ 0.001, ^b*P* $\leq$ 0.002, ^c*P* $\leq$ 0.01, ^d*P* $\leq$ 0.02, ^e*P* $\leq$ 0.05.

Metabolite	Level (μ mol/g wet wt.)	
	Control (<i>n</i> = 3)	Injected (<i>n</i> = 5)
Contralateral (non-stimulated) muscle		
Inosine	0.22 $\pm$ 0.04	0.31 $\pm$ 0.15
NAD	0.49 $\pm$ 0.02	0.57 $\pm$ 0.13
IMP	0.52 $\pm$ 0.08	0.13 $\pm$ 0.08 ^c
AMP	0.004 $\pm$ 0.001	0.04 $\pm$ 0.01 ^a
ADP	0.78 $\pm$ 0.08	0.99 $\pm$ 0.19
ATP	7.60 $\pm$ 0.04	8.48 $\pm$ 1.37
ATP/ADP	9.77 $\pm$ 1.15	8.6 $\pm$ 0.28
Total adenine nucleotides	8.39 $\pm$ 0.13	9.58 $\pm$ 1.57
Glucose	2.12 $\pm$ 0.59	3.45 $\pm$ 0.76
Lactate	1.79 $\pm$ 0.64	3.27 $\pm$ 0.59 ^e
Phosphocreatine	27.74 $\pm$ 2.52	28.97 $\pm$ 6.42
Adenylate energy charge	0.95 $\pm$ 0.01	0.95 $\pm$ 0.01
Stimulated muscle		
Inosine	0.13 $\pm$ 0.04	0.43 $\pm$ 0.08 ^a
NAD	0.44 $\pm$ 0.03	0.5 $\pm$ 0.08
IMP	0.19 $\pm$ 0.06	1.77 $\pm$ 0.37 ^a
AMP	0.003 $\pm$ 0.001	0.11 $\pm$ 0.03 ^b
ADP	0.74 $\pm$ 0.06	1.28 $\pm$ 0.27 ^c
ATP	5.88 $\pm$ 0.79	5.54 $\pm$ 0.99
ATP/ADP	8.0 $\pm$ 0.9	4.4 $\pm$ 0.4 ^a
Total adenine nucleotides	6.62 $\pm$ 0.85	6.93 $\pm$ 1.28
Glucose	3.27 $\pm$ 0.38	3.77 $\pm$ 0.92
Lactate	3.89 $\pm$ 0.68	18.09 $\pm$ 2.76 ^a
Phosphocreatine	14.30 $\pm$ 2.18	5.27 $\pm$ 1.52 ^a
Adenylate energy charge	0.95 $\pm$ 0.01	0.89 $\pm$ 0.01 ^a
Twitch tension (%)	118 $\pm$ 10	9 $\pm$ 11 ^a

ever, a marked resynthesis of phosphocreatine was observed in the infused muscle after recovery as compared with the end of stimulation values and both lactate and IMP levels were also elevated. There was also a marked improvement in the twitch tension following the 15 min recovery period in the infused muscle (Table 1) but this response would fatigue rapidly upon restimulation (see Fig. 2).

Biochemical studies after subcutaneous injection of diphenyleneiodonium

Metabolite analysis. In these studies, animals were injected subcutaneously with diphenyleneiodonium (3 mg/kg body wt.) and both the stimulated and contralateral (non-stimulated) gastrocnemius muscles were freeze-clamped at the end of the second period of stimulation (see Table 2). Another group of rats, similarly injected, were used

to investigate mitochondrial parameters *in vitro*, e.g. oxygen uptake and enzymic assays.

The differences between the injected rat contralateral (non-stimulated) muscle metabolite status and the control contralateral muscle were limited to a significant decrease of IMP in the injected muscle together with a 10-fold increase in AMP and almost doubling in the lactate concentrations (Table 2). However, when the stimulated muscle was analysed, most metabolites differed from their respective control values following stimulation, only glucose and interestingly ATP remaining similar to the controls (Table 2).

However, despite this there was a significant decrease in the ATP/ADP ratio in the injected muscle, and the level of phosphocreatine was less than 40% of its control value, suggesting an impaired mitochondrial oxidative capacity. Significant increases in inosine, IMP, AMP and ADP

and lactate were also observed in the injected muscle (see Table 2).

Liver and skeletal muscle mitochondria studies. Liver and muscle mitochondria were isolated from

diphenyleneiodonium-injected animals. The mitochondria from both tissues from injected animals showed a marked inhibition of O_2 uptake with NAD-linked substrates, whereas that from

Fig. 3. ^{31}P -n.m.r. spectra of muscle from rats injected with diphenyleneiodonium

Rats were injected with diphenyleneiodonium (3 mg/kg body wt.) for two consecutive days. The animals were mounted in the n.m.r. spectrometer with a surface coil located over the gastrocnemius muscle. The muscle was stimulated at 1 Hz for 10 min and then allowed to recover for 70 min. (a) Representative spectra at various times indicated throughout the stimulation and recovery period of a typical experiment. Sugar-P, sugar phosphate (phosphomonoesters); P_i , inorganic phosphate; PCr, phosphocreatine; α , β , γ , α -, β -, γ -phosphates of ATP. (b) The metabolite profiles from (a) have been calculated and plotted as a percentage of the maximum value obtained during the particular experiment. □, Phosphocreatine; ○, P_i ; △, pH; ●, ATP.

ascorbate + tetramethyl-*p*-phenylenediamine-linked oxidation was unaffected. The oxidation of succinate was also unaffected in liver mitochondria (cf. Holland *et al.*, 1973), but was significantly lowered in muscle mitochondria. The reason for this differential effect on muscle and liver mitochondria by diphenyleneiodonium is at present unclear.

Subcutaneous injections of diphenyleneiodonium were also shown to have no significant effect on the total muscle creatine kinase activity (88% of control activity) or on the activity of the mitochondrially bound creatine kinase activity (results not shown).

³¹P-n.m.r. studies. The gastrocnemius muscle was stimulated at 1 Hz and spectra were collected before, during and after a 10 min period of stimulation. In control animals the phosphocreatine concentration fell to a level which was approx. 75% of its initial value (results not shown) and had returned to baseline (initial) values within 2 min of the stimulation train ceasing. The data from a typical diphenyleneiodonium-injected animal are shown in Fig. 3; the baseline spectrum and pH (7.10) were within normal limits (control 7.03 ± 0.04 , $n = 5$). However, the response to stimulation was abnormal, and the phosphocreatine signal decreased to less than 20% of its initial concentration by the end of the stimulation train. There also appeared to be a large anaerobic contribution to the energy metabolism of the diphenyleneiodonium-injected muscle as judged by the increase in lactate concentrations (Table 2) and decrease in intramuscular pH (from 7.1 to 6.3). Furthermore, the muscle pH remained around 6.4 throughout the experiment, whereas that of the control animals returned to pH 6.95–7.00 within 1 min of the end of stimulation. Interestingly, at the start of stimulation in the myopathic animals there was a sharp increase in the resonance in the phosphomonoester region, approximating to an increase in concentration in the region of $5 \mu\text{mol/g}$ wet wt. (assuming that the T_1 of this resonance is similar to that of ATP). This phosphomonoester resonance subsequently fell and remained constant (see Fig. 3). The chemical assignment of the phosphomonoester peak is uncertain. However, it seems unlikely to be AMP or IMP since the total ATP concentration measured remains constant during this period. The most likely candidates are hexose monophosphates (e.g. glucose 6-phosphate or fructose 6-phosphate) and this may reflect a transient inhibition of glycolysis/glycogenolysis, possibly at the level of phosphofructokinase.

Discussion

Injection of diphenyleneiodonium, either acutely or chronically, into rats caused abnormalities in

skeletal muscle function. Stimulation of the gastrocnemius muscle produced a typical fatigue response with a rapid decline in twitch tension coupled with a prolongation in the muscle half-relaxation time, which increased by almost 3-fold (results not shown, but see Edwards *et al.*, 1975a,b). The concentration of ATP was maintained at control levels but at the expense of phosphocreatine (Tables 1 and 2, Fig. 3).

The precise mechanism(s) which give rise to muscular fatigue are unclear (Porter & Whelan, 1981). A number of parameters have been implicated, three of which may be usefully discussed with respect to the chronic diphenyleneiodonium model (see Table 2). These are: elevated free ADP concentrations, a low pH coupled with a lactic acidosis, and a reduced rate of energy supply. Estimates of the free ADP concentrations in the muscle may be calculated from the n.m.r. spectra and the chemically determined ATP concentration (see Shoubridge & Radda, 1984) using the creatine kinase reaction and a total muscle creatine pool (phosphocreatine + free creatine) of 30.1 ± 2.2 (means $\pm$ s.d., $n = 28$) $\mu\text{mol/g}$ wet wt. Under resting conditions the chronically injected animals (Fig. 3) had similar free ADP concentrations to controls (18 and $12 \mu\text{M}$ respectively). At the end of the stimulation protocol (see Fig. 3), the free ADP content had risen to $68 \mu\text{M}$ (at pH 6.4) and $24 \mu\text{M}$ (at pH 6.98) for diphenyleneiodonium injected animals and controls respectively. Although free ADP concentrations of that order or higher are not uncommon in rat muscle at pH values around 6.4, they normally only occur at higher work rates and the pH fall is transient, unlike that seen in the chronically injected animal model (Shoubridge & Radda, 1984). However, in control animals where the pH falls and the free ADP content increases, there is no prolongation in the half-relaxation time of the muscle (Shoubridge & Radda, 1984; D. J. Hayes & E. A. Shoubridge, unpublished data). ADP is known to be a competitive inhibitor of the myosin ATPase reaction (Green & Mommaerts, 1950) and its binding relative to ATP is enhanced at low pH and in the presence of Ca^{2+} ions (Kielley, 1961). It seems possible therefore that, although the concentration to which ADP rises in those experiments is only just into what might reasonably be considered as the effective inhibitory range (Green & Mommaerts, 1950), the increased ADP might well be a contributory factor in the establishment of muscle fatigue. The low intramuscular pH coupled with lactate production may also serve to disrupt muscle function both directly and indirectly by shifting the kinetic properties of enzymes with pH optimum around pH 7 (e.g. phosphofructokinase).

The last factor, namely energy supply as related

Table 3. *Respiratory activities of mitochondria isolated from rats injected with diphenyleneiodonium*

Respiratory activities were measured polarographically at 25°C (see Morgan-Hughes *et al.*, 1977, for details) in the presence of 0.1 µM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The results are expressed as means ± s.d. for the average of duplicate estimations with the number of animals in parentheses. Control mitochondrial incubations had respiratory control ratios (i.e. state-3/state-4 respiratory rates ≥ 5 with pyruvate and malate or ≥ 3 with succinate). **P* ≤ 0.001 by Student's *t*-test. Abbreviation used: TMPD, *NNN'*-tetramethylphenylenediamine.

Tissue	Substrates	Mitochondrial respiration (nmol of O/min per mg of mitochondrial protein)	
		Control	+ Diphenyleneiodonium
Skeletal muscle	5 mM-Pyruvate + 2.5 mM-malate	176 ± 18 (3)	46* ± 3 (3)
	10 mM-Succinate + 0.1 µM-rotenone	253 ± 19 (3)	51* ± 8 (3)
	40 mM-Ascorbate + 50 µM-TMPD + antimycin A (10 mg/ml)	240 ± 23 (3)	223 ± 25 (6)
Liver	10 mM-Glutamate + 2.5 mM-malate	70 ± 4 (6)	4 ± 0 (3)
	10 mM-Succinate + 0.1 µM-rotenone	49 ± 9 (3)	57 ± 8 (3)
	40 mM-Ascorbate + 50 µM-TMPD + antimycin A	52 ± 6 (6)	50 ± 6 (3)

to demand, clearly dominates the situation reported in the present paper. The chronically injected animals have depressed oxidative capacity (Table 3) and try to maintain supply via anaerobic glycolysis, resulting in a low pH and increased lactate production. In the myopathic animals stimulation gave rise to muscle glycogen depletion (Byrne, 1983) and it is well documented that force failure is associated with muscle glycogen depletion (Edstrom & Kugelberg, 1968; Kugelberg & Edstrom, 1968). However, when control studies are considered reduced energy supply as such cannot explain fatigue, since both this study and others have shown that fatigue occurs despite the apparent availability of millimolar concentrations of 'high energy' phosphate (Spande & Scholtelius, 1970; Edwards *et al.*, 1975a,b; Dawson *et al.*, 1980; Sahlin *et al.*, 1981; Wiles *et al.*, 1981; Wilkie, 1981). Perhaps the mechanism which underlies fatigue in these situations is the rate of substrate delivery, which is unable to keep pace with demand due to glycogen depletion. Thus force failure under these circumstances acts as a protective mechanism to prevent total cellular ATP depletion.

A main objective of this study was to establish an animal model for the human condition of 'mitochondrial myopathy'. The chronically administered diphenyleneiodonium model reported here closely resembles patients suffering from myopathy on whom ³¹P-n.m.r. studies were performed following light aerobic exercise (Edwards *et al.*, 1982; Radda *et al.*, 1982; Eleff *et al.*, 1984). These patients showed a marked reduction in the rate at which phosphocreatine levels were restored after exercise. However, two patients (with NADH: coenzyme Q reductase deficiency) studied by Radda *et al.* (1982) restored their intramuscular

pH within the control time despite the fact that the exercise provoked a lactic acidosis (Morgan-Hughes *et al.*, 1979). It is possible that they have established an adaptive mechanism which allows them to control intramuscular pH in spite of high lactate concentrations, unlike the situation in the chronic animal model where the intramuscular pH fell to 6.4 and showed no recovery. Animals maintained on a lower daily dose of diphenyleneiodonium for several weeks are currently being studied; preliminary results (D. J. Hayes & R. Petty, unpublished work) indicate that intramuscular pH does recover after exercise. This regime will clearly provide a much better animal model for the human myopathic condition.

This work has been supported by the Brain Research Trust (D. J. H.) and the Muscular Dystrophy Group of Great Britain (E. B.). E. A. S. was supported by a Post-doctoral Fellowship from NSERC (Canada). Running expenses and some equipment were provided by the Brain Research Trust, Muscular Dystrophy Group and the Mason Medical Foundation. The authors acknowledge with thanks the n.m.r. facilities provided by Dr. G. K. Radda (Oxford). Excellent technical assistance was provided by Miss M. Ellison, Miss S. D. Nurse and Miss E. A. Wilkie.

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