Some Biological Effects of Experimentally Induced Inhibition of Oxidative Phosphorylation in Rat Skeletal Muscle.

by

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AFFIDAVIT AND CONDITIONS OF WORK

1. This work was carried out in the laboratories of the University Department of Clinical Neurology at the Institute of Neurology, Queen Square, London, U.K. and in the Enzyme Unit, Department of Biochemistry, Oxford University, Parkes Road, Oxford, U.K.

2. The experimental work was done from February 1981 to May 1982.

3. The work was carried out under the general supervision of Dr. J.A. Morgan-Hughes, M.D., F.R.C.P., Senior Lecturer in the University Department of Clinical Neurology and Consultant Physician to the National Hospitals for Nervous Diseases, Queen Square, London.

4. During the period of this work I held a Muscular Dystrophy Group of Great Britain Research Fellowship (February 1981 - May 1982).

5. I hereby declare that the development of the methods used and all the experiments with results shown, were done by myself. This thesis was composed by myself.

Signed..... Edward Byrne ..... Date..1/7/1982....

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Summary

1) Selective defects in the mitochondrial respiratory chain and in the coupling of respiration to phosphorylation underlie weakness and exercise intolerance in certain myopathies (see Carafoli and Roman 1980; Morgan-Hughes 1981). In this study specific defects in these pathways have been reproduced in the rat using the respiratory chain inhibitors diphenyleneiodonium and antimycin A and the uncoupling agent dinitrophenol. These disease models have been characterised using electrophysiological, biochemical and morphological techniques and the findings have furthered the understanding of the pathophysiology of human mitochondrial myopathies. Mitochondrial inhibitors were either injected intraarterially into anaesthetised animals or given by repeated daily subcutaneous injection. Isometric twitch tension and compound muscle action potentials evoked by nerve or by direct muscle stimulation were recorded from the gastrocnemius muscle in vivo during various stimulus patterns. The gastrocnemius muscle was either freeze clamped for metabolite assay or fixed for histological study at various times after injection. In a second series of experiments, the biochemical changes were followed using 31 phosphorous nuclear magnetic resonance. The effects of acute ischaemia and blocked glycolysis were also studied in vivo for comparative purposes. In vitro intracellular recordings were made from individual soleus muscle fibres in separate experiments.

2) Intra-arterial injection of dinitrophenol resulted in irreversible failure of twitch tension and loss of muscle action potential associated with the development of an electrically silent contracture. In vitro intracellular recordings from soleus muscle fibres demonstrated a progressive broadening of the action potential, with a fall in amplitude and eventual failure of propogation. This was associated with a small reduction in resting potential. Force failure was accompanied by a rapid depletion of phosphocreatine and adenosine triphosphate (ATP) accumulation of inosine monophosphate (IMP) and a fall in intramuscular pH with lactate accumulation. Enlarged mitochondria with whorled, fractured or lysed cristae, myelin figures, and intracristal linear inclusions were seen 60 minutes after infusion.

3) Intra-arterial or repeated subcutaneous injection of diphenyleneiodonium which is known to inhibit NADH ubiquinone reductase resulted in a progressive fall in twitch tension with 1 and 5c/sec stimulation patterns accompanied by a marked prolongation of relaxation time. Failure of muscle excitation paralleled force failure. Contracture as determined by a rise in resting tension developed inconsistently as a late finding only in animals given large doses of DPI. Considerable recovery of force generation occurred with rest. After a single injection a pattern of rapid fatiguability followed by a partial recovery was established with repeated bursts of 5c/sec

stimulation. With DPI, force failure was associated with depletion of PCr but ATP levels were similar to those in control muscle exposed to the same stimulus pattern. A massive rise in muscle lactate concentrations with light work was seen in animals given subcutaneous DPI.

With DPI mitochondrial changes were minor after intra-arterial infusion. In animals given repeated subcutaneous injections, the changes were more marked and resembled those seen more acutely after dinitrophenol.

4) Abnormal force failure followed injection of antimycin A, but systemic toxicity limited the usefulness of this agent and only 5 experiments were undertaken.

5) Intra-arterial infusion of the glycolytic inhibitor iodoacetate resulted in a rapid failure of twitch tension and muscle exictability, associated with contracture. Severe ATP depletion and IMP accumulation were found in contractured muscle.

INTRODUCTION

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INTRODUCTION

i. Historical Review of the Human Mitochondrial Myopathies

Mitochondria were first recognised in the latter part of the ninteenth century as improved lenses became available. The term mitochondria was proposed by Benda (1903) (see Cowdry 1918) from the Greek mitos (thread) and chondros (grain). The ground work which lead to the concept that cellular respiration is dependent upon a highly organised system of mitochondrial membrane - bound electron carriers was largely carried out in the 1920's by Wieland, Warburg and Kielin (see Lehniger 1965). A more detailed picture of the respiratory chain emerged from the work of Chance and his colleagues (Chance 1954; Chance and Williams 1955a&b) who recorded oxidation reduction difference spectra of the electron carriers in isolated intact mitochondria using a double-beam spectrophometer. The key role of mitochondria in cellular respiration was finally established in experiments at the Rockafeller Institute at about this time (Kennedy and Lehniger 1948 & 1949).

Increased knowledge of mitochondrial structure paralleled advances in understanding mitochondrial function. Supravital dye techniques were developed in the early part

of the century which allowed mitochondria to be stained in fresh unfixed preparations (see Cowdry 1952). A modification of one of these by Cunningham and Engel has proved useful in the examination of frozen muscle sections (1963). The major ultrastructural features of mitochondria were defined independently by Sjöstrand (1953) and Palade (1952) who published the first high resolution images of mitochondria in ultrathin sections. These advances provided the basic methodology for a morphological and functional assessment of mitochondrial function in man and the scene was set for the detection of mitochondrial dysfunction in human disease.

This discovery fell to Luft and his co-workers (Ernster et al 1959; Luft et al 1962) when they investigated a 35 year old woman with hypermetabolism of non-thyroid origin, who presented with polydypsia and polyphagia, with moderate skeletal muscle weakness, wasting and arreflexia. The basal metabolic rate was elevated to 210% and detailed biochemical studies on isolated muscle mitochondria using Warburg manometry revealed defective respiratory control by ADP but a normal phosphorylative capacity. Large numbers of mitochondria with densely packed crista and occasional paracrystaline inclusions were seen in the muscle fibres. This case is of historic importance in that it represented a new application of scientific techniques to the study of human disease. Only one additional case of 'Lufts'

syndrome has been reported since then. Haydar et al (1971) reported a 19 year old girl with severe nonthyrotoxic hypermetabolism (BMR $^{+}$180%) in whom ultrastructural examination of muscle revealed an increased number of mitochondria with closely packed crista, some of which contained paracrystalline inclusions. Biochemical studies by Haydar et al (1971) and more detailed studies by Di Mauro et al (1976) again demonstrated loss of respiratory control with a normal phosphorylative capacity (loose coupling).

Striking hypermetabolism in the presence of normal thyroid function in these patients provided clues that detailed biochemical studies might be rewarding. Hypermetabolism was not a feature of subsequent cases of mitochondrial myopathy and the next phase in the development of this field was concerned with the identification of morphological abnormalities in skeletal muscle mitochondria although occasional cases continued to be studied by Warburg manometry.

Shy et al (1966) described two patients with myopathy and striking abnormalities of mitochondrial structure. One patient, (labelled megaclonial myopathy), was an 8 year old girl with marked proximal weakness dating

from infancy. Histochemical stains on a muscle biopsy showed high oxidative enzyme activity in some type I fibres and electron microscopy revealed large mitochondria, some of which were of unusual shape and contained abnormal cristae with inclusions. The second case (labelled pleoclonial myopathy) was a boy with salt craving and congenital weakness in whom muscle biopsy examination revealed a marked increase in the number of mitochondria, the bulk of which were structurally normal, although some contained concentric crista or electron opaque matrix masses. The terms pleoclonial and megaclonial myopathy are now of historic interest only. Several further cases of myopathy with unusual mitocondrial structure were reported soon afterwards. Van Wijngaarden et al (1967) reported the case of a 15 year old boy with progressive proximal weakness dating from the age of 4 years. Histological studies on a muscle biopsy showed subsarcolemmal deposits which stained intensely with oxidative stains and these were shown by electron microscopy to contain large aggregates of spherical, rod shaped or angular mitochondria. Warburg manometry on isolated mitochondria demonstrated "loose coupling" although the patient did not have a hypermetabolic state. Price et al (1967) described a myopathic patient whose muscle biopsy contained fibres with an excess of lipid and mitochondria containing intracristal inclusions. Fischer and Danowski (1969) reported

a 39 year old man with an 8 year history of proximal weakness in whom examination of a muscle biopsy revealed an excess of fat in many fibres and EM studies showed many large pleomorphic mitochondria with inclusions. Spiro and his colleagues (1970) saw a 13 year old boy with salt craving and mild weakness punctuated by one severe episode of adynamia in whom, EM studies on a muscle biopsy showed abnormal numbers of enlarged mitochondria. Schellens and Ossenjuk (1969) reported the case of a 20 year old man with undue fatiquability but no fixed weakness in whom the muscle biopsy showed increased oxidative staining in some type I fibres and EM showed mitochondria of abnormal shape and size with paracrystalline inclusions. Biochemical studies revealed loose mitochondrial coupling. A 2 year old girl with progressive weakness and biopsy evidence of giant muscle mitochondria with lamellar crista was reported by Hulsmann et al (1967). Biochemical studies again revealed loose coupling. Additional adult cases with myopathy and morphological evidence of a pre-eminent mitochondrial ultrastructural abnormality have also been reported (Shibaski et al 1973; Kamieneka 1976). These cases are clinically heterogenous, and contrast with the most frequently seen disorder of muscle with morphological mitochondrial abnormalities in which a characteristic clinical syndrome is apparent, namely chronic progressive external opthalmoplegia (CPEO). Large mitochondria with rectangular inclusions were identified in CPEO patients by Zintz (1966) and by Ketelsen et al (1968). Olson et al

(1972) coined the term occulocraniosomatic disease to describe cases with external opthalmoplegia in whom the modified Gomori trichrome stain of Cunningham and Engel showed a significant number of fibres with a ragged red rim, corresponding to subsarcolemmal mitochondrial aggregates. The association of complex central multi-system involvement in occasional cases with CPEO had long been recognised (Drachman 1968; Kearns and Sayre 1958) and mitochondrial abnormalities are most frequent in such cases. Intracristal parking lot inclusions are characteristically seen (Morgan-Hughes and Mair 1973) although giant mitochondria with concentric crista are also frequent (Adachi et al 1973; Dodson et al 1976). Some idea of the frequency of structural mitochondrial abnormalities in muscle is gained from Kamienaka's study in which abnormal mitochondria were seen in 17 of 135 consecutive biopsies. Nine of these patients had CPEO.

Abnormal lactic acidaemia either at rest or with exertion has been documented in several patients with CPEO and in other myopathies with structural mitochondrial abnormalities (Hackett et al 1973; Rawles and Weller 1974; Reske-Neilson et al 1976; Scarlato et al 1978; Sengers et al 1975; Sulaiman et al 1974; Tarlow et al 1973). This finding suggested the presence of a defect in the further metabolism of pyruvate by the mitochondria and provided some support for the view that the morphological mitochondrial abnormalities in these cases were functionally significant.

In most of the cases referred to, however, the contention that mitochondrial dysfunction was central to the pathophysiology rested solely on the morphological findings.

How reliable, then, are morphological indicators of "mitochondriopathy"? Unusual mitochondria are seen in small numbers in diverse myopathies including polymyositis (Chou 1967) myotonic dystrophy (Klinkerfuss 1967) hypothyroid myopathy (Norris and Panner 1966) and fascioscapulohumeral dystrophy (Kamienacka 1976). However in all these conditions, major alterations are also identifiable in other fibre components and it is only in the cases reviewed that the mitochondrial changes are preeminent. The range of morphological variation in "normal" mitochondria is wider than was initially appreciated. Normal mitochondria from heart (Moore 1957) and skeletal muscle (Leeson and Leeson 1969) may contain concentric cristae. Walter et al (1981) have recently presented evidence that electrical stimulation of rat muscle leads to the development of giant mitochondria with concentric cristae within sixty minutes. Paracrystalline inclusions have been identified in normal liver mitochondria (Wills 1965) and Hammersen et al (1980) have described two types of paracrystalline inclusion in normal human skeletal muscle biopsied at the time of knee surgery, either under non-ischaemic conditions or soon after the application of a tourniquet. The second type of inclusion described, closely resembles the parking lot inclusions in myopathic patients, with the exception that the mitochondria containing them were not greatly enlarged. The first and

commoner type of inclusion seen by Hammersen et al, an intracrystal quadrilamellar structure, has also been seen in myopathic patients.

In addition to the variability in normal mitochondrial morphology, ischaemia can produce marked morphological changes in this organelle. Resnik and Hansen (1969) observed mitochondria with irregular, sometimes concentric cristae, and a range of inclusions in ischaemic rabbit muscle and suggested that the mitochondrial changes may represent a compensatory process rather than a primary abnormality. Heffner and Barron (1978) noted ragged red fibres, giant mitochondria with abnormal cristal morphology, including crystalloids and myelin figures in ischaemic rat anterior tibial muscle. Large subsarcolemmal aggregations of abnormal mitochondria were visible after 12 to 18 hours of ischaemia and giant mitochondria, often larger than the myonuclei, with concentric crista were present after 24 hours. Heffner and Barron pointed out that most of the mitochondrial changes seen in patients with mitochondrial myopathies were also encountered in their ischaemic model and suggested that, as with other subcellular structures, the range of morphological response of mitochondria to insults of different types is restricted. No typical paracrystalline inclusions were observed in either ischaemic model and although Karpati et al (1974) have seen crista with trilaminar inclusions in experimental ischaemia, this appearance also differs from that seen in human disease.

In most mitochondrial myopathies the changes in mitochondrial structure have constituted the major or exclusive abnormality. These findings taken in conjunction with the presence of lactic acidaemia in selected cases provides strong indirect evidence for an underlying mitochondrial lesion.

The next major advance in understanding abnormal mitochondrial function in human cases came when similar but more advanced techniques to those used by Luft and Ernster were applied to the study of intact isolated skeletal muscle mitochondria obtained from selected patients with muscle biopsy changes pointing to a mitochondrial defect. The number of centres equipped to carry out such studies is small, and the amount of tissue required to provide an adequate yield of functionally intact mitochondria is large. Consequently, only a small number of patients have been studied. The biochemical procedure generally adopted in these cases has been the measurement of 1) O_2 uptake rates with various substrates 2) respiratory control indices (Chance and Williams 1955 a) 3) oxygen phosphorylation ratios and 4) spectrophotometric analysis of mitochondrial cytochromes (see Makinen and Lee 1968). Several mitochondrial lesions have been pin-pointed with these techniques including defects at different loci in the respiratory chain, impaired coupling of oxidation with phosphorylation and possible abnormalities of ATP synthesis or translocation.

The first cases with a probable defect in mitochondrial NADH oxidation were two mentally retarded siblings with lactic acidaemia. Muscle mitochondria were not examined but Senior and Jungas (1974) found that glutamate and β hydroxybutyrate oxidation was defective in hepatic mitochondria. In more extensive studies Morgan-Hughes et al (1978) and Land et al (1981) have demonstrated a defect in NADH - coenzyme Q reductase activity in muscle mitochondria in two patients. The first case, (whose sister had a similar illness) had severe exercise intolerance with marked lactic acidaemia. Both sisters had attacks of increased weakness which were precipitated by moderate exercise, fasting or alcohol. Studies on isolated mitochondria demonstrated markedly depressed rates of O_2 uptake with all NAD linked substrates tested but succinate oxidation and O_2 uptake rates with tetramethyl phenylenediamine (TMPD) and ascorbate were much higher. These findings indicated that the respiratory chain on the oxygen side of coenzyme Q - cytochrome b was intact. This observation was confirmed by measurement of the cytochrome oxidation reduction difference spectra. Supportive evidence for the presence of a functional mitochondrial defect in both sisters was obtained with the technique of 31 phosphorous nuclear magnetic resonance (31p NMR). An abnormally rapid fall in phosphocreatine levels (PCr) during aerobic forearm exercise was followed by extremely delayed PCr regeneration at rest (Radda et al 1982). In comparing the findings with these two different techniques it is interesting that the rate of NADH oxidation (determined in isolated mitochondria) and the rate of PCr regeneration

(determined by 31P NMR) were reduced by a similar factor (i.e. $\frac{1}{6}$ normal). In a second patient (Land et al 1981) the defect at NADH - coenzyme Q reductase was more severe in that oxygen uptake was not demonstratable utilising pyruvate and was only minimal with other NAD linked substrates (β hydroxybutyrate and glutamate). As in the sisters, succinate oxidation and O_2 uptake rates with TMPD and ascorbate were normal. The presence of high NADH ferricyanide reductase activity in this case suggested that the markedly impaired NADH oxidation was not due to a deficiency of NADH dehydrogenase or its associated flavin mononucleotide but may have resulted from a defect involving one of the non haem non sulphur centres (Fe - S).

Cytochrome b deficiency has been found in 4 patients. Morgan-Hughes et al (1977) reported a patient with muscular fatiguability, exercise induced lactic acidaemia and occasional episodes of adynamia. Mitochondrial respiratory rates were depressed with all NAD and flavoprotein linked substrates which were examined. Mitochondria with disordered crista and occasional intermembranal paracrystalline inclusions were prominent on electron microscopy. Spiro et al (1970) had earlier reported a father and son with a complex multisystem disease characterised by dementia, ataxia, arreflexia and proximal weakness. The fourth patient with cytochrome b deficiency presented in adult life with myoclonic epilepsy, progressive dementia, ataxia, and

proximal weakness (Morgan-Hughes et al 1982). Two patients with a combined deficiency of cytochrome b and cytochrome aa_3 have also been reported (Van Bliervliet et al 1977; Di Mauro et al 1978). These cases presented in infancy with severe lactic adicosis and the De Toni Fanconi Debre syndrome and died from respiratory insufficiency within a few weeks. Cytochrome aa_3 deficiency has also been reported in trichopoliodystrophy (French et al 1972) although this has not been a universal finding (Zellkowitz et al 1976) and also in a complex multisystem disorder in which pyruvate dehydrogenase activity was low (Monnens et al 1975).

Investigations have uncovered a possible ATPase deficiency in two patients. Schotland et al reported a 37 year old lady with proximal weakness in whom numerous intramitochondrial paracrystalline inclusions were visible in the muscle biopsy. Biochemical studies showed reduced respiratory rates with all substrates tested and mitochondrial ATPase activity, both basal levels and levels after stimulation with Mg^{2+} or DNP were very depressed (1978). Tomasi et al (1980) reported a 17 year old athlete with muscle pain and cramps with similar biochemical findings and pointed out that a defective adeninenucleotide translocator could also explain these biochemical results.

Although only two cases of Luft's syndrome have been reported, loss of respiratory control in the absence of hypermetabolism has been a more frequent finding (Hulsmann et al 1967; Spiro et al 1979; Van Wijngaarden et al 1967; and Worsfold et al 1973). Such reports must be treated with caution as poorly coupled mitochondria may result from damage encurred during the isolation procedure. Loss of respiratory control confined to α - glycerophosphate has been reported by Di Mauro et al (1973) in a patient with occular myopathy, but Land & Clark (1979) have questioned the biological significance of this finding. Loosely coupled mitochondria have also been identified by some workers in various neurogenic and myopathic disorders (Gimeno et al 1973). In Duchenne dystrophy loose coupling was associated with low oxygen uptake rates with NAD and FAD linked substrates (Scholte et al 1981). Other workers have reported normal oxidative phosphorylation in Duchenne dystrophy (Peter et al 1970). In cases where both the clinical assessment and the morphological features lend little or no support to the diagnosis of a mitochondrial myopathy, the significance of abnormal mitochondrial metabolism in vitro is uncertain and may reflect either degenerating mitochondria or organelles which are more easily damaged by the isolation procedure. The possibility that a biochemical lesion primarily affecting a structural protein or lipid component might also lead to secondary impairment of mitochondrial function, particularly in isolated samples, cannot be excluded.

Detailed biochemical investigations into cases with CPEO are limited. Di Mauro (1973) and Kark et al (1978) provide data pointing to a possible pyruvate translocase deficiency in 2 cases and Morgan-Hughes group have recently identified a possible defect in the vicinity of ubiquinone - cytochrome b in 3 cases. However no specific defect has been identified in other patients with CPEO and mitochondrial changes on muscle biopsy (Berenberg et al 1977; Carafoli et al 1980).

The spectrum of human mitochondrial myopathies therefore covers a substantial number of patients in which morphological findings, sometimes supported by the finding of abnormal lacticacidaemia, are suggestive but not conclusive pointers to a primary functional mitochondrial defect and a smaller number of patients in whom a well localised defect in mitochondrial respiration has been demonstrated biochemically. It is probable that with further studies both the number of cases with localised defects and the range of defects will increase.

ii Normal Muscle Energy Metabolism

Continuous resynthesis of adenosine triphosphate is essential for normal cross bridge cycling (Weber and Murray 1973) (Huxley 1974) for calcium reuptake by the sarcoplasmic reticulum (Hasselbach 1964) and for the maintenance of cellular organisation and resting membrane potential. ATP stores in muscle are limited and the pool of about 6ug/gm would be rapidly depleted (in approximately 1 sec of contraction) unless restored (Sahlin et al 1978). Phosphocreatine (PCr) stores are higher and rapidly restore ATP levels through the phosphokinase reaction (Essen and Kayser 1978). PCr becomes undetectable after exhaustive exercise (Radda personal communication) (Sahlin et al 1979) but plateaus out at intermediate levels during less severe exercise (Harris et al 1977). ATP is also resynthesised from two molecules of ADP by the adenylate kinase reaction with the formation of AMP. Rapid removal of AMP by myoadenylate deaminase (Chapman and Atkinson 1973; Lowenstein 1972) may serve to stabilise the adenylate energy change during periods of ATP hydrolysis. These immediately available energy stores have a limited capacity to buffer the ATP pool and even with a block to glycolysis and aerobic metabolism, frog muscle at 0°C can sustain several hundred twitches. (Fink and Luttgau 1976).

As in all tissues, muscle ATP synthesis is dependent upon anaerobic glycolysis and oxidative phosphorylation. During short bursts of intense activity ATP levels are largely maintained through glycolysis which may increase 1000 fold at the onset of contraction (Newsholme 1980). The anaerobic pathway however can supply only a limited amount of ATP and during sustained work oxidative phosphorylation becomes crucial (Felig and Wahren 1975).

Electrons generated by the Krebs cycle and by oxidation of fatty acids are transferred via NAD or FAD carriers into the respiratory chain. The respiratory chain components are situated in the inner mitochondrial membrane. Electrons are transferred down the chain as successive components became alternatively oxidised and reduced. The components are organised in order of increasing redox potential as shown in Figure 1 and the fall in the free energy of electrons is fractioned into 3 major steps which are each linked to ATP synthesis. The chain is probably organised in a zigzag manner with some carriers on the matrix side and some facing the outer surface (De Pierre and Ernster 1977). Many of the carriers in the transport chain have been investigated in great detail. The structure of each individual site is complex with many subcomponents (including flavoprotein and non haeme iron centres) interacting.cytochrome oxidase, for example, has at least 7 subunits (Eytan et al 1975).

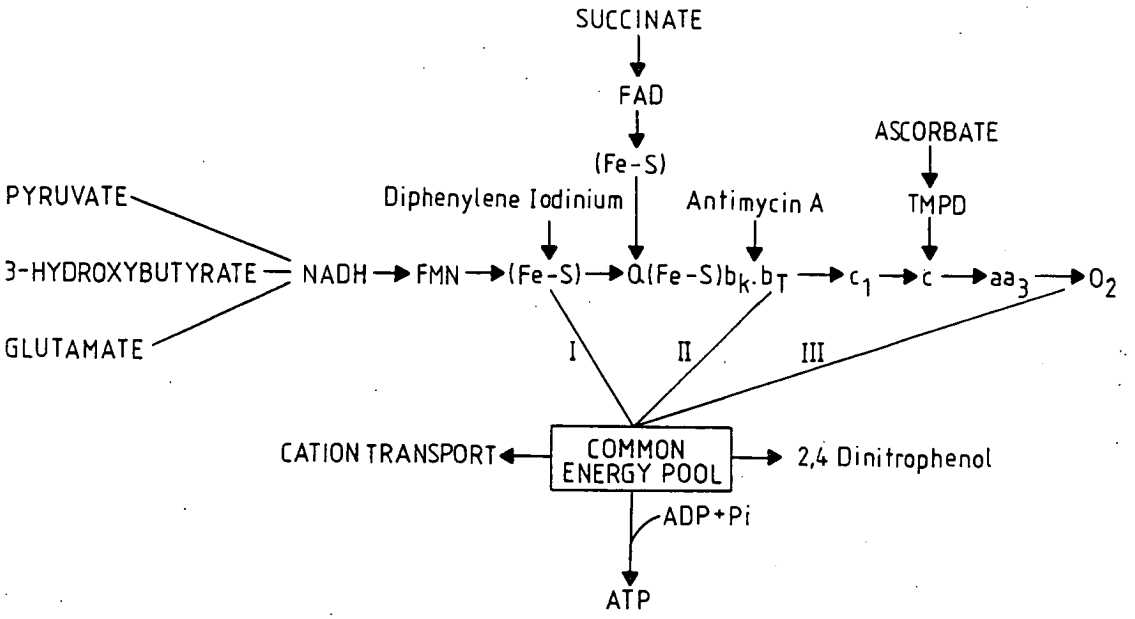
Mitchell's view that the respiratory chain generates a proton gradient across the inner membrane is now firmly established (Mitchell 1966&68). The inner membrane is impermeable to protons and inwards proton flux is channelled through a vectorial ATPase which spans the membrane. ATP is then probably transported into the intermembranous space from where it can diffuse freely into the cytosol by an adenine nucleotide translocase. The ATPase system is highly complex and contains at least 9 subunits (Pedersen 1975; De Pierre and Ernster 1977). Additional processes such as Ca^{2+} and Na^{+} transport are also dependant upon the proton gradient (Chance 1965).

The precise mechanism of transport of high energy phosphates from the mitochondria to the myosin filaments and to the sarcoplasmic reticulum is uncertain. ATP generated by the mitochondria is classically considered as diffusing directly to these sites (Mommaerts 1969). Recently, Saks and his co-workers (1976) have suggested that PCr may be crucial in the transport of high energy phosphates. They prepose that ATP generated during oxidative phosphorylation is used to synthesise PCr in the mitochondrial intermembrane space, only catalytic amounts of ADP being required. PCr may then diffuse to sites of ATP utilisation in the cytosol. This preposed transport function of PCr is still unproven.

Figure 1

Title

The mitochondrial respiratory chain.



Legend

Schematic representation of the mitochondrial respiratory chain showing the sites of action of the inhibitors investigated in this study.

iii Oxidative Phosphorylation Inhibitors

A number of chemical compounds have been identified which block or uncouple oxidative phosphorylation. Inhibitors of NADH oxidation include rotenone (Horgan et al) pericidin a (Gutman et al 1970) barbituates, rhein,(Kean 1968) and diphenyleneiodonium (Holland et al 1973). Succinate oxidation is selectively inhibited by 2 thenoyltrifluoroacetate (2 iegler 1966) and by carboxin (Urich and Mathrel 1972). Antimycin a and n-Heptylquidine N-oxide inhibit between cytochrome b and c, (Lightbrown and Jackson 1956). Cyanide ions and hydrogen sulphate bind to cytochrome oxidase (aa_3). The two respiratory chain inhibitors examined in this study, diphenyleneiodonium and antimycin a were used because they act at loci in the respiratory chain which have been shown to be defective in some patients with mitochondrial myopathies.

Interest in diphenyleneiodonium followed the observation that it has a potent hypoglycaemic effect in animals (Stewart and Hanley 1969); Holland and Sherratt (1972) subsequently demonstrated a complete inhibition of ADP linked and dinitrophenol induced stimulation of succinate oxidation by isolated rat liver mitochondria exposed to diphenyleneiodonium. This effect followed an acceleration in the rate of cl^- / OH^- ion interchange across the inner mitochondrial membrane. Holland et al (1973) showed that such a mechanism may not be operative in vivo as DPI had no effect upon the rate of cl^- uptake by the liver. The in vivo actions of

diphenyleneiodonium are now thought to be due to selective inhibition of the oxidation of NADH linked substrates (Holland et al 1973). Intraperitoneal injection of between 5 and 10 ug/gm body weight results in severe hypoglycaemia within 30 minutes and death within 2 hours. The agent is sequested in the liver when given by this route and a failure of oxidative phosphorylation leads to impairment of hepatic gluconeogenesis. This is associated with an increase in whole body lactate levels. Diphenyleneiodonium was quickly confirmed as a potent inhibitor of NADH - ubiquinone reduction in isolated liver mitochondria (Gatley and Sherratt 1974) and has a similar action to rotenone and pericidin. Using purified NADH - ubiquinone oxidoreductase from bovine heart mitochondria, Ragan and Bloxham (1977) showed that diphenyleneiodonium inhibited NADH oxidation, with a time dependent onset of inhibition following pseudo-first-order kinetics. Analysis by sodium dodecyl sulphate/ polyacrylamide-gel electrophoresis showed that inhibition was accompanied by specific co-valent modification of a 23, 500 Dalton polypeptide, suggesting that this protein is the binding site. It's precise role in the site I complex has not been established. DPI has the advantage over rotenone and pericidin of being water soluble, but it is relatively insoluble in the presence of chloride ions.

The second respiratory chain inhibitor studied, antimycin a inhibits the respiratory chain between cytochrome b and cytochrome c_1 (Lightbrown and Jackson 1956; Slater 1973).

Reducing equivalents from both NAD and FAD linked substrates are therefore prevented from passing down the respiratory chain.

Uncouplers are agents which dissociate mitochondrial respiration from phosphorylation. They produce a number of well defined effects on isolated mitochondria. Oxygen uptake rates are maximal in the absence of ADP and the activity of mitochondrial ATPase is greatly increased (Potter and Recknaqel 1951). They also produce osmotic swelling of mitochondria in vitro (Packer 1963). They are conventionally divided into classical uncouplers which are weak acids and non classic uncouplers which include agents like cadmium (Jacobs 1956) which disrupt mitochondria and crown ionphores such as valinomycin which form lipid soluble complexes with monovalent cations allowing equilibration across the inner mitochondrial membrane and short circuting the ion pump.

The uncoupler 2-4 dinitrophenol used in this study acts in a "classical" manner. Hotchkiss (1944) first demonstrated that DNP abolished aerobic ATP synthesis in bacterial preparations without impairing oxygen consumption. The significance of this uncoupling of respiration from phosphorylation was rapidly recognised and subsequently confirmed by a number of workers in mitochondrial preparations (Cross et al 1949); Lardy and Elvelijem (1945); Loomis and Lipmann (1948). Classical uncouplers are proton ionophores and act by increasing proton conductivity across the inner mitochondrial membrane (Cunarro and Weiner 1975). The effect is to dissipate the proton gradient upon which ATP synthesis is dependent in

Mitchell's view (1966).

Dinitrophenol in high concentrations also inhibits specific substrate dehydrogenases(Katyare et al 1971). In addition there is some evidence that sites I and III are more sensitive to the uncoupling action than site II (Katore et al 1971). These authors also showed that the concentration response curves of DNP induced ATPase activation and of the uncoupling effect are not precisely correlated.

In addition to its effects on mitochondrial function, DNP also has a general effect on ATP-binding sites. In particular DNP modifies the active site of myosin in such a way as to allow attack by water with resultant ATP hydrolysis. This differs from the hydrolysis of ATP associated with normal contraction in that it is not coupled to contraction. The interaction between DNP and myosin ATPase is complex and activation requires binding at more than one site. Unless the active site is protected however by the binding of an ATP molecule, at least 3 molecules of DNP bind to each active unit of myosin and result in an irreversible loss of enzymic activity (see Levy et al 1963).

In a comparative study, the effects of the putative glycolytic blocker, iodoacetate were also studied in vivo. This agent was initially thought to be a selective inhibitor of glyceraldexhyde 3 phosphate dehydrogenase (Cori et al 1948) but it was subsequently recognised to be less specific

in vitro where it also inhibited creatine phosphokinase. In vivo, however, this enzyme appears to be protected from its action (Carlson and Singer 1959).

iv Animal Studies

Loose mitochondrial coupling has been reported in dystrophic hamsters (Lockner and Brunch 1967; Wrogemans and Blanchaeur 1968; Wrogemans et al 1970), in dystrophic chickens (Ashmore and Doerr 1970) and in hypothyroid myopathy in rabbits (Meijer 1972). Respiratory chain defects have also been identified in dystrophic mice (Lee et al 1979). The significance of these changes in mitochondrial function is uncertain as in these animals there was severe disorganisation of muscle fibre structure.

Several attempts to produce an experimental mitochondrial lesion in vivo have been made. Melmed et al (1975) infused the uncoupling agents 2-4 DNP and CCH into the aorta of rats via a catheter in the iliolumbar artery. The hind limbs went rigid but no physiological studies were performed. Some animals recovered over 24 hours. The plantaris showed an increase in the number of "ragged red" fibres with the Gomori trichrome stain within 90 minutes of the onset of infusion. Ragged red fibres were less numerous in soleus where many necrotic fibres were identified. Ultrastructural studies confirmed the presence of mitochondrial aggregations with fractured crista and platelike intra-cristal inclusions, as well as showing an increase in the mitochondrial mass. The rapid mitochondrial proliferation was blocked by chloramphenicol, a known inhibitor of mitochondrial protein synthesis. The authors attributed these changes to a direct action

of the uncoupling agents rather than to other factors such as hypoxia, hyperthermia or intracellular acidosis. They postulated that differences from the mitochondrial structural changes observed in human mitochondrial myopathies could be accounted for by the acute nature of the experimental exposure and by species differences. Sahgal et al (1979) investigated the biochemical and morphological changes in rat hind limb muscles after intraarterial infusion of the uncoupler DNP, the respiratory chain inhibitor antimycin a and the ATPase inhibitor oligomycin. With the doses of oligomycin and antimycin a used, the rats died within 5 - 10 minutes. Ultra-structural mitochondrial changes with swelling and fracture of crista were seen but as the authors acknowledge, ischaemia due to cardio respiratory failure may have contributed to these changes. After dinitrophenol infusion, hind limb rigidity rapidly appeared. A large number of ragged red fibres were identified in soleus and plantaris after 15 minutes and enlarged mitochondria, some of which contained crystalloids, were seen with the electron microscope. A marked fall in muscle ATP and PCr levels and a rise in lactate concentrations was found in muscles analysed 1 hour after DNP infusion and the nucleotide and metabolite levels returned to normal over 24 hours. A gradual clinical improvement with disappearance of rigidity began 2 hours after infusion. Walter et al (1980) also studied the acute effects of dinitrophenol in rat muscle morphologically both after intraaterial injection and direct injection into the muscle.

Accumulations of enlarged mitochondria with proliferated crista were identified within 15 minutes and at 60 minutes there were many giant mitochondria containing electron dense bodies and linear intracristal inclusions similar to those seen by Melmed et al at 2 hours. The morphological findings in the studies by Melmed et al and Walter et al, are not identical to those seen in patients with mitochondrial myopathy in that no crystalloids were identified and the picture was one of acute mitochondrial degeneration. The findings of Sahgal et al bear a much closer resemblance to the morphological mitochondrial changes seen in patients. The reason for this discrepancy is not obvious as a similar protocol was followed in all 3 studies.

No attempt has been made to correlate physiological changes with biochemical or structural changes in muscle mitochondria after infusion of respiratory chain inhibitors or uncoupling agents.

v In Vitro Studies

The development of a contracture in vitro after exposure of isolated muscle to dinitrophenol was noted by Cori and Cori (1936) in frog muscle and by Weeks and Chenoworth (1952) in a rat diaphragm preparation. The action of dinitrophenol on an isolated, rat diaphragm preparation was studies by Barnes et al (1955). A progressive contracture and twitch tension failure followed exposure to dinitrophenol, but the resting tension gradually fell to its pre contracture level after 60 minutes even with continued exposure to DNP. The rate of contracture was noted to be concentration dependent. As contracture developed,a fall in phosphocreatine levels to less than 10% of that in controls, of ATP levels to a third and of glycogen levels to a half the control level was observed. No recovery in PCr or ATP concentrations was observed as the contracture wore off. DNP contracture was observed to develop in muscles depolarised with isotonic K_2so_4 when added after the depolarisation induced contracture had disappeared, thereby disproving the suggestion of Hajdu and Svent-Gyorgyi (1951) that DNP contracture is due to membrane depolarisation. A similar contracture follows acute metabolic exhaustion with other agents including iodoacetate and is not specific to dinitrophenol (Murphy 1966) (Sandow and Schneyer 1955).

A number of studies have been carried out more recently examining the effects of dinitrophenol on both muscle contractility and electrical activity. Beresford

et al (1979) using an isolated rat diaphragm preparation confirmed an 6 fold increase in O_2 uptake associated with twitch failure after addition of dinitrophenol to the chamber. DNP also produces contractile failure in isolated rabbit detrusor muscle (Paton 1968) where increasing the glucose concentration of the medium permits some recovery probably through glycolysis and in isolated myocardial strips where ATP depletion has been documented (McDonald et al 1971; McDonald and McLeod 1972). The fall in ATP levels demonstrated by McDonald et al with DNP was more rapid than that which developed during ischaemia or after exposure to sodium cyanide, suggesting an acceleration of ATP hydrolysis. An alteration in membrane excitability in cardiac and skeletal muscle also follows exposure to dinitrophenol. A decrease in action potential duration and amplitude associated with a partial depolarisation is seen in cardiac muscle (McDonald et al 1971; McDonald and McLeod 1972; De Mello 1959). A partial depolarisation has been observed in frog muscle in vitro (Goldsmith et al 1975). A similar depolarisation with very high concentrations of DNP was earlier demonstrated by Abood et al (1961) and was associated with a progressive fall in action potential amplitude progressing to inexcitability. Anodal hyperpolarisation, however, restored action potential propogation. The muscle membrane characteristics

in metabolically exhausted skeletal muscle was further evaluated by Fink and Luttgau (1976) who investigated frog muscle with a block to glycolysis and mitochondrial respiration. They found that a progressive fall in action potential amplitude with eventual inexcitability occurred. Although this was associated with a small initial membrane depolarisation from -75 to -69mV, a hyperpolarisation subsequently developed (to -83mV). These changes were associated with a massive increase in membrane K^+ conductance. Hyperpolarisation of the membrane resulted in a decline in potassium conductance. In metabolically exhausted muscle, therefore inexcitability is not always associated with depolarisation, changes in membrane excitability and in resting potential both reflecting increased K^+ conductance.

vi New Techniques - Nuclear Magnetic Resonance

The assay of metabolite levels in living tissues has traditionally been done by rapid freezing to the temperature of liquid nitrogen in a clamp (freeze clamping). Using this technique Davies et al (1959) were able to demonstrate depletion of phosphocreatine in a single contraction in vitro in muscle exposed to dinitrophenol, and Cain and Davis (1962) demonstrated utilisation of ATP during a single contraction in muscle poisoned with the creatine kinase inhibitor dintrofluorobenzene, thereby providing the first direct demonstration of the crucial role of ATP in muscle contraction. A similar technique was used to prepare muscle for biochemical analysis in many experiments in this project. In addition, however, biochemical changes were followed in vivo using 31 phosphorus nuclear magnetic resonance (see Ackerman et al 1980). This technique has the advantage that serial biochemical observations can be made non invasively in the same animal. The first successful NMR experiments were carried out in 1946 (Bloch et al 1946) and within a few years the technique was used to investigate living systems (Odeblad and Lindstrom 1955) although resolution in early proton NMR experiments was low. The development of high field super-conducting magnets and the emergence of fourier transformation NMR techniques revolutionised the biological applications of this approach. Moon and Richards (1973) using 31p NMR were able to detect signals from inorganic phosphate and ATP in intact erythrocytes.

A year later Hoult et al (1974) obtained high resolution 31p NMR spectra in intact recently excised rat hind limb muscle in which inorganic phosphate, phosphocreatine and 3 ATP peaks were identifiable and measureable.

The development of the surface radio frequency coil by Radda's group and the assembly of larger magnets enabled experiments to be carried out in vivo in which selected regions of live animals were examined. Ackerman, et al (1980) obtained excellent 31p NMR spectra with a surface coil placed over the hind limb of an anaesthetised rat and demonstrated complete depletion of phosphocreatine with ischaemia. Studies on working animal muscle in vivo and the effects of metabolic blockade on muscle in vivo using NMR has not been previously undertaken.

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Materials

1. Diphenyleneiodonium

2. Antimycin A

3. Dinitrophenol

4. Iodoacetate

MATERIALS AND METHODS I

1. Acute Experiments in Vivo

1.1 Physiological Methods: In Vivo Experiments with Intra-arterial Injection of Inhibitors

1.1.1 Animals

Approximately 250 adult male wistar rats between 190 and 340 grams in weight were used in these experiments. They were housed in batches of 4 in large wire mesh cages and fed a standard rat diet. (Rat and mouse maintenance diet no. 1 with water ad lib).

1.1.2 Anaesthesia

Rats were anaesthetised with an intraperitoneal injection of urethane (25% w/v). The initial dose was 0.5mls/100g body weight. Additional doses (0.1mls/100g body weight) were given as recquired during the course of the experiments. At the end of each experiment, the animal was killed with an overdose of pentobarbital given intraperitoneally (0.5mg/gm).

1.1.3 Preparation of the Experimental Animal

The left femoral artery was exposed through an oblique groin incision with the rat supine. Under a

Leitz dissection microscope, the femoral sheath was exposed and the artery, vein and nerve separated by blunt dissection. A clip was applied to the femoral artery near the abdominal wall and an arteriotomy made below this with iris scissors. A polyethyelene catheter (portex size 2 FG diameter 0.63mm) was advanced to the aortic bifurcation and tied into position, the latter having been exposed through a left flank incision to ensure cannula placement was optimal. The abdominal and groin incisions were then closed with black silk.

Muscles were exposed through a midline incision extending from the R calcaneus to above the R knee. The biceps femoris muscle was then divided and retracted exposing the gastrocnemius. In experiments using gastrocnemius the lateral popliteal and medial popliteal nerve distal to the branches to gastrocnemius were crushed and the soleus and plantaris muscles were divided inferiorly. The calcaneus was divided below the insertion of the tendo achilles and the lower part of the gastrocnemius freed with care to preserve blood supply. In a small number of experiments where the soleus was studied, this muscle was exposed under the lateral head of gastrocnemius and the gastrocnemius and plantaris muscles divided near the tendo achilles. The nerve to the medial head of gastrocnemius, the lateral popliteal and the anterior tibial nerves were divided and the lower soleus freed with preservation of innervation and blood supply.

The R sciatic nerve was exposed for 1-2cm beneath the gluteal muscles crushed proximally and gently hooked over fine silver stimulating electrodes, the cathode being placed distally. Mammalian ringer solution (37°C) was used to keep the nerve and muscle moist during each experiment. Teflon coated silver wires (25g AG10T clark electro medical) were bared for 2-3cms at one end and two loops applied to the muscle for direct stimulation, the anode being placed over the tendo achilles and the cathode over the body of the muscle, 1.5-2cms proximally.

Two steelpins, 2 cms apart were driven through the upper R tibia near the knee and the mid shaft of the R femur. These were used to fix the R leg rigidly in a stainless steel animal frame with the gastrocnemius muscle in a horizontal plane (Figure 2). The skin temperature of the rat's trunk was maintained between 34 and 37°C using a heating lamp throughout recording.

1.1.4 Nerve and Muscle Stimulation Parameters

A Devices stimulator (type 3072; voltage output range 0-400V; pulse duration range 50-500usec; Output impedance of less than 500ohms) was used to deliver rectangular pulses for nerve and muscle stimulation. Low frequency stimulus trains (up to 5c/sec) were triggered by a Medelec A56 stimulator control and tetanic pulses by a Devices

type 2521 gated pulse generator triggered by a Devices digitimer. Supramaximal pulses (50V, 50usec duration) were used for nerve stimulation. For direct muscle stimulation, 200V pulses, 200usec in duration were used. Preliminary experiments in curarised muscle showed that this stimulus strength produced a maximal response.

1.1.5 Tension Recording

To record tensions from gastrocnemius the achilles tendon was attatched to a statham UC3 strain gauge with a L4S accessory diaphragm by braided steel wire (Down's surgical diameter 36). The length of the tie was 2mm or less. Black silk thread was used in recordings from soleus. The strain gauge had a maximum displacement of 0.1mm, an undamped oscillation frequency of 1200Hz and a full scale non linearity and hysteresis of $\pm$ 0.25% over a 2.27Kg range. The response was linear over the force range examined in these experiments. The strain gauge output was amplified by a purpose built conventional strain gauge amplifier with variable gain setting (from X200-X1,000). The output was displayed on a 4 channel Medelec oscilloscope and recorded photographically. The optimum resting length (L_o) at which a maximal twitch response was evoked was determined at the beginning of each experiment. Single responses were recorded at intervals from 15 seconds to 5 minutes. Continuous

recordings at a slow paper speed were made for brief periods in selected experiments.

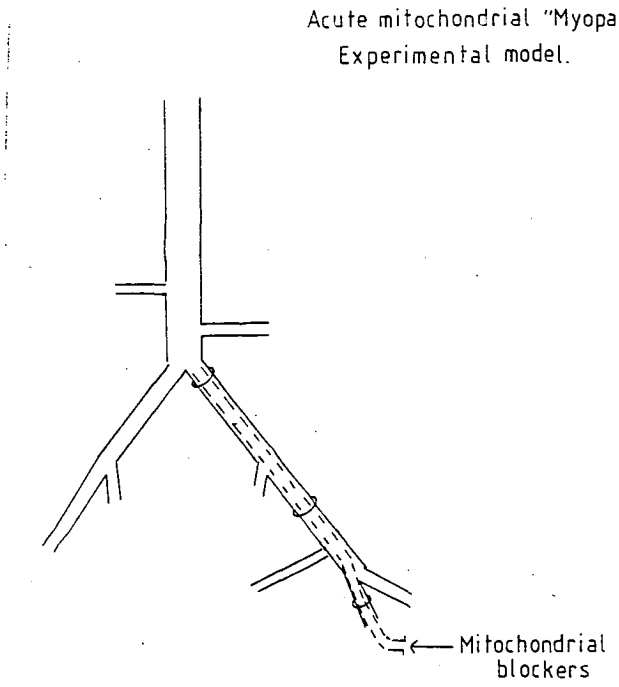
1.1.6 Muscle Action Potential Recording

The muscle action potential with nerve stimulation was recorded through a teflon coated silver wire, with the distal 5mm bared, inserted into the endplate zone through a 21 gauge needle which was then withdrawn. The indifferent electrode was inserted into the R foot and the earth into the tail. The action potential was displayed on a Medelec oscilloscope (Band width 10-16,000 H_3).

Figure 2

Title

Cannulation technique.



Legend

The tip of the catheter was carefully positioned at the aortic bifurcation without interference to the blood supply to the right hind limb.

Figure 3a

Title

Animal frame used for physiological studies.

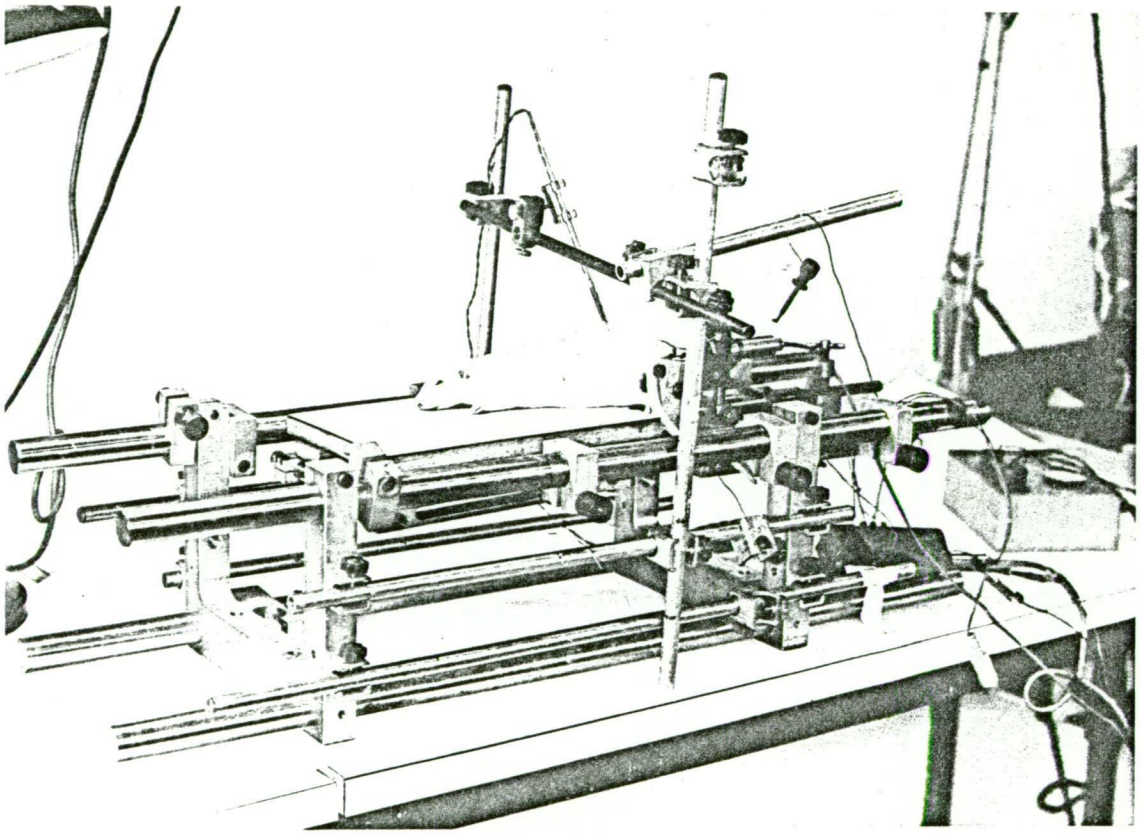
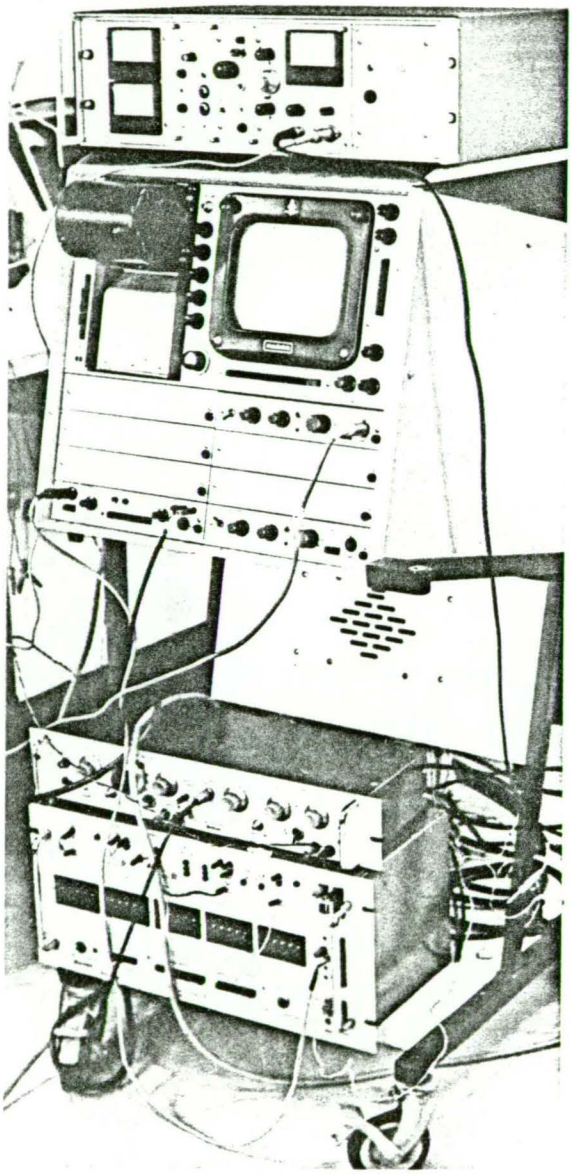


Figure 3b

Title

Rack used in physiological studies.



Legend

Medelec oscilloscope, tension transducer, stimulator unit and digitimer used in physiological experiments.

1.2 Morphology

1.2.1 Histology and Histochemistry

In experiments in which biochemical analysis was carried out, the whole muscle was removed for that purpose. Histological findings were investigated in separate experiments.

At the end of the experiment, muscles for histological examination were removed, trimmed and mounted on a block with gum traganth. The block was then frozen in isopentane cooled to the temperature of liquid nitrogen for 6-8 seconds and stored at -170°. Cryostat sections 7um thick were examined by the following methods: Gomori trichrome stain: succinic dehydrogenase stain and periodic acid Schiff stain (PAS) (see Appendix).

1.2.2 Electron Microscopy

Muscles for ultrastructural study were infiltrated in vivo with 3% gluteraldehyde in 1% cacodylate buffer using a 25 gauge needle. Fifteen minutes later the muscle was excised, divided into small blocks and fixed in 3% glutaraldehyde in cacodylate buffer overnight. After embedding in epoxy resin, 1um sections were cut and stained with Toluidine blue for light microscopy. Ultra thin sections were cut from selected areas and stained with uranyl acetate followed by lead citrate.

1.3 Biochemical Methods

1.3.1 Freeze Clamp Techniques

Brass plates were bolted to towel forceps. Ten minutes before completion of physiological observations, the forceps were emersed in liquid nitrogen. To prepare gastrocnemius for biochemical analysis, the body of the muscle (either at rest or while contracting) was clamped between the cooled brass plates and immediately emersed in liquid nitrogen. The frozen muscle was stored at -170°C prior to analysis which usually took place within two weeks of the experiment.

For biochemical analysis, muscle was thawed and homogenised. Specimens were analysed for ATP, ADP, AMP, IMP, glycogen lactate and creatine phosphate. The methods used for assay of glycogen, lactate, and creatine phosphate are similar to those described in methods of Enzyme Analysis (Bergmeyer, M. Ed. 1974). Nucleotide extraction was carried out by the method of Lush et al (1979) and nucleotides were assayed by an H.P.L.C. method (Knox et al 1981). The much larger gastrocnemius muscle was selected for all of these experiments.

1.3.2 Nuclear Magnetic Resonance

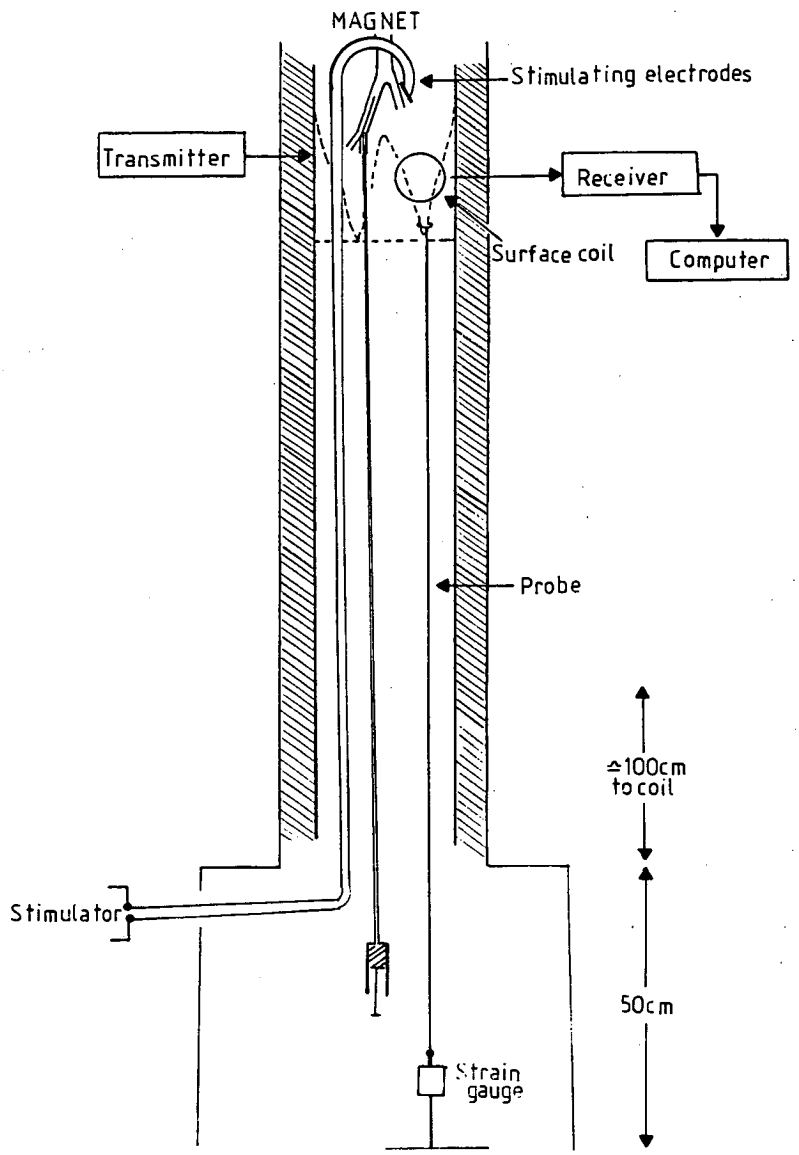
NMR observations were made in 10 experiments.

Rats were anaesthetised with intra peritoneal urethane. The left femoral artery was cannulated by the technique already described. As it was not possible to keep the gastrocnemius moist in the NMR spectrometer, the dissection technique was modified and the skin over the R hind limb left intact. The R achilles tendon was dissected free, the calcaneus divided and a heavy black silk thread (6/0 surgical silk) used to attatch the tendo achilles to the strain gauge. Metal threads of lower compliance were found to be unsuitable because of interference with the magnetic field. The extensor tendons were divided over the ankle. The R sciatic nerve was exposed and hooked over fine silver electrodes 1cm apart. The skin was then closed. The rat was fixed in a cylindrical perspex chamber using binding tape. Copper wires were soldered onto the silver stimulating electrodes and brought out of the probe. The stimulating electrodes were shielded with voltage traps. After great care to ensure that the field was free of all moisture, a surface radio frequency coil 1cm in diameter was closely applied to the R gastrocnemius muscle (Figure 4) similar to that used by Ackerman et al (1981). A surface radio frequency coil will detect signals from a disc shaped area immediately in front of it of radius and depth about equal to the radius of the coil, and by using this method, the signal from gastrocnemius was selectively examined. The chamber containing the animal was then placed into the centre of an NMR spectrometer (Oxford Instruments) with a bore of 10cms (Figure 4). The ligature from the achilles

Figure 4

Title

NMR spectrometer.



Legend

Schematic representation of the NMR spectrometer showing the position of the animal during recording.

tendon was attatched to a statham strain gauge bolted to the floor beneath the magnet. The later could not be fixed closer to the animal because metallic components interfered with the uniformity of the magnetic field. NMR spectra were recorded at 1sec intervals during application of a radio frequency pulse (fourier transform NMR), and sequential responses (over 1sec periods) were automatically added in a computer until an adequate signal to noise ratio was obtained. Periods of between 36 seconds and 300 seconds were used to group data. PCr and Pi peaks were adequately defined in the shorter period but the longer period was necessary to adequately distinguish ATP peaks from background noise.

1.4 Blood Flow Studies

Muscle blood flow was recorded in 2 experiments. Teflon coated platinum electrodes (100u diameter) were placed in the gastrocnemius at different sites (2-5) polarised to $^{+}$400mV with reference to a silver/silver chloride electrode and allowed to stabilise for 1 hour. 2% hydrogen gas in oxygen was then delivered via a face mask. Clearance of H_2 was recorded from each site after cessation of flow. Muscle blood flow was calculated by the initial slope method (Rockard et al 1980). This has mainly been used in cerebral studies and has the advantage that tissue perfusion in several sites is followed rather than whole muscle blood

flow so that patchy ischaemia should be apparent. Two sets of observations were made before and 3 sets at 10 minute intervals after administration of inhibitors. As any movement altered the electrode placement, muscle blood flow was only followed at rest. This method gave similar baseline blood flow levels at any one site but there was considerable inter-site variation.

1.5 Arterial Blood Gas Analysis

Aliquots of 250ul of arterial blood were collected from the arterial cannula in a heparanised syringe in selected animals, either before or after contractile failure. Specimens were analysed for PaO_2, $PaCO_2$ and arterial blood pH using an automatic blood gas analyser (ABL2 VA Hare, London).

2. Subcutaneous Injection of Metabolic Blockers.

In an attempt to produce a chronic disease model, metabolic blockers were injected subcutaneously in small doses on 4 or 5 days a week in 44 animals. Weak animals were studied electrophysiologically using similar techniques to those used after intraarterial infusions. The gastrocnemius was freeze clamped for biochemical assay after stimulation in most experiments and the unstimulated gastrocnemius was freeze clamped immediately afterwards. Morphological studies were carried out in separate animals.

3. In Vitro Studies

In vitro experiments were carried out on the rat soleus. Rats were anaesthetised as described and the soleus removed intact with preservation of a nerve stump 1-2mm long. The soleus was washed with Krebs ringer solution and emersed rapidly in a perspex bath containing oxygenated Krebs ringer. (22°C) One end of the soleus muscle was tied to a steel pin with black silk thread. In control experiments, 5 shocks via nerve (50us 12V) and 5 shocks via direct stimulation muscle (100us 80V) were given at 5 minute intervals for 90 minutes. In experiments with DNP, after 2 sets of baseline observations 5 minutes apart, DNP was added to the bath to produce a concentration between 0.1 and 1mM. Twitch tensions and resting tensions were recorded through a Grass paper recorder. For measuring resting potentials in individual

fibres and propogated muscle fibre action potentials the muscle was fixed at each end with steel pins through the tendon. Direct muscle activation was used and intracellular recordings made with a filamented glass recording electrode containing 3M KCL (resistance 5MΩ). Resting membrane potential and propagated action potential (if present) were recorded in all fibres entered. The responses were measured from the oscilloscope screen using a storage oscilloscope. After addition of DNP (concentration 0.3mM), observations were made in as many fibres as possible and the results were collated in five minute periods. Only superficial fibres were studied and the muscle was transversed circumferentially to ensure that no fibre was entered twice.

Materials

1. Diphenyleneiodonium (DPI)

Diphenyleneiodonium was prepared as a sulphate salt and stored in a crystalline form. Bis (diphenyleneiodonium) sulphate was prepared from 2 aminobiphenyl and sodium iodide by a two stage procedure involving the conversion of 2 - iodobiphenyl to bis (diphenyleneiodonium) sulphate as described initially by Collette et al (1956). DPI sulphate precipitates out as the chloride salt in solutions containing chloride ions and was therefore dissolved in 5% dextrose. The pH of the resultant solution was 6.9. DPI was administered in volumes of 200-500ul in initial experiments, the cannula being filled with 5% dextrose prior to the infusion. 200ul volumes were given as a bolus and larger volumes as an infusion over 1 minute. 5% dextrose in equivalent volumes was used in control experiments.

2. Antimycin A

Antimycin A was obtained from sigma chemicals (no A 2006). This compound is insoluble in water and was dissolved in pure alcohol and stored at $-4^{o}C$. Aliquots were mixed with saline, after which the antimycin A pre-

cipitated out to produce a uniform colloidal suspension, and the final concentration was adjusted to 4mg/ml in 10% ethanol. 10% ethanol in normal saline was used in control experiments.

3. Dinitrophenol

2-4 Dinitrophenol was obtained from sigma chemicals (no D 7004). This was dissolved in 1 N NaOH by heating at 60^{o} for 30 minutes with frequent stirring. The solution was then adjusted to pH7.4 with 1ON HCL. The final concentration was 10mg/ml, and solutions were stable for up to 24 hours.

4. Iodoacetate

Iodoacetatic acid was obtained from sigma chemicals (no 1-6250). This was dissolved in water in a concentration of 100mg/ml and the solution pHed to 7.4 with sodium hydroxide.

RESULTS

1. Intraarterial Administration of Metabolic Inhibitors

1.1 Physiological and clinical observations

1.1.1 Control Experiments

1.1.2 Ischaemic Experiments

1.1.3 Dinitrophenol Experiments

1.1.4 Diphenyleneiodonium Experiments

1.1.5 Antimycin a Experiments

1.1.6 Iodoacetate Experiments

1.2 Histology

1.2.1 Control Experiments

1.2.2 Dinitrophenol Experiments

1.2.3 Diphenyleneiodonium Experiments

1.2.4 Iodoacetate Experiments

1.3 Electron Microscopy

1.3.1 Dinitrophenol Experiments

1.3.2 Diphenyleneiodonium Experiments

1.4 Biochemical Observations

1.4.1 Control Experiments

1.4.2 Ischaemic Experiments

1.4.3 Dinitrophenol Experiments

1.4.4 Diphenyleneiodonium Experiments

1.4.5 Antimycin a Experiments

1.4.6 Iodoacetate Experiments

2. Subcutaneous Injection of Inhibitors. Physiological, Morphological and Biochemical Findings.

2.1 Diphenyleneiodonium Experiments

2.2 Dinitrophenol and Antimycin Experiments

3. In Vitro Experiments. Twitch Tension and Single Fibre Electrical Recordings in Soleus.

3.1 Control Experiments

3.2 Dinitrophenol Experiments

RESULTS AND DISCUSSION

1.1 Clinical and Physiological Observations After Intra-arterial Infusion of Metabolic Inhibitors

1.1.1 Control Experiments

The right hind limb of anaesthetised animals was fixed in a rigid frame and the gastrocnemius muscle attached to the strain gauge as described in the methods. Supramaximal sciatic nerve stimulation at either 1Hz or 5Hz was carried out in 17 experiments. Animals stimulated at 1Hz were given 500ul of either 5% dextrose or normal saline via the cannula at 60, 70 and 80 minutes of stimulation. Animals stimulated at 5Hz were given 250ul of isotonic dextrose or saline at 6, 8, 10 and 12 minutes of stimulation. These volumes were identical or greater than those used to infuse the active agents in the experimental group. With these volumes of infusant, the physiological responses of the gastrocnemius were unaltered and the results with both solutions are grouped together.

Clinical Observations

The animal's general state remained stable for upto 6 hours of recording. No respiratory irregularity, tachyproea (respiratory rate 40 - 60) or muscular rigidity developed..

Measurement of the Mechanical and Electrical Responses

The isometric twitch tension developed in response to a single supramaximal stimulus at the beginning of the experiments ranged from 300-620g (mean 448 $\pm$ SD 87). The magnitude of the twitch response showed some correlation with body weight, the linear regression line having a slope of 190g twitch tension per 100g body weight and a correlation co-efficient of 0.864 ($P > 0.001$).

a) 1c/sec Stimulation (6 experiments)

Twitch Tension Amplitude

The amplitude of the isometric twitch response with nerve stimulation (5 experiments) began to fall within 2-3 minutes (120-180 stimuli) of the onset of stimulation. No staircase enhancement was seen. Twitch tension stabilised out after 5-10 minutes (300-600 stimuli) and then remained constant for upto 4 hours of stimulation (Figure 5). The steady state evoked tension measured 68-85% (mean 74%) of the tension evoked by the first stimulus of the train. In one animal, the gastrocnemius was activated with direct muscle stimulation. A similar time course for the decline in isometric tension was observed, the steady state tension being 83% of the initial response.

Twitch Characteristics

Half relaxation time of the first twitch in the stimulus train ranged from 21-36msec (mean 26msec) and remained relatively stable over the first 120 stimuli (after 2 minutes stimulation, range 21-37msec, mean 27msec). Thereafter half relaxation time steadily shortened, stabilising out at 14-19msec (mean 16msec) after 300-600 stimuli. The time course of these changes closely followed the time course of the changes in twitch tension. Half relaxation times then remained stable for upto 60 minutes of stimulation but a slight prolongation developed between 60 and 120 minutes (after 120 minutes stimulation, range 15-24msec, mean 19.2msec).

The rise time shortened progressively as the evoked tension fell. Values for the initial responses ranged from 20-27msec (mean 24msec). The rise time for steady state responses ranged from 16-21msec (mean 19msec). Values remained stable for several hours of stimulation (mean 20 msec after 120 minutes).

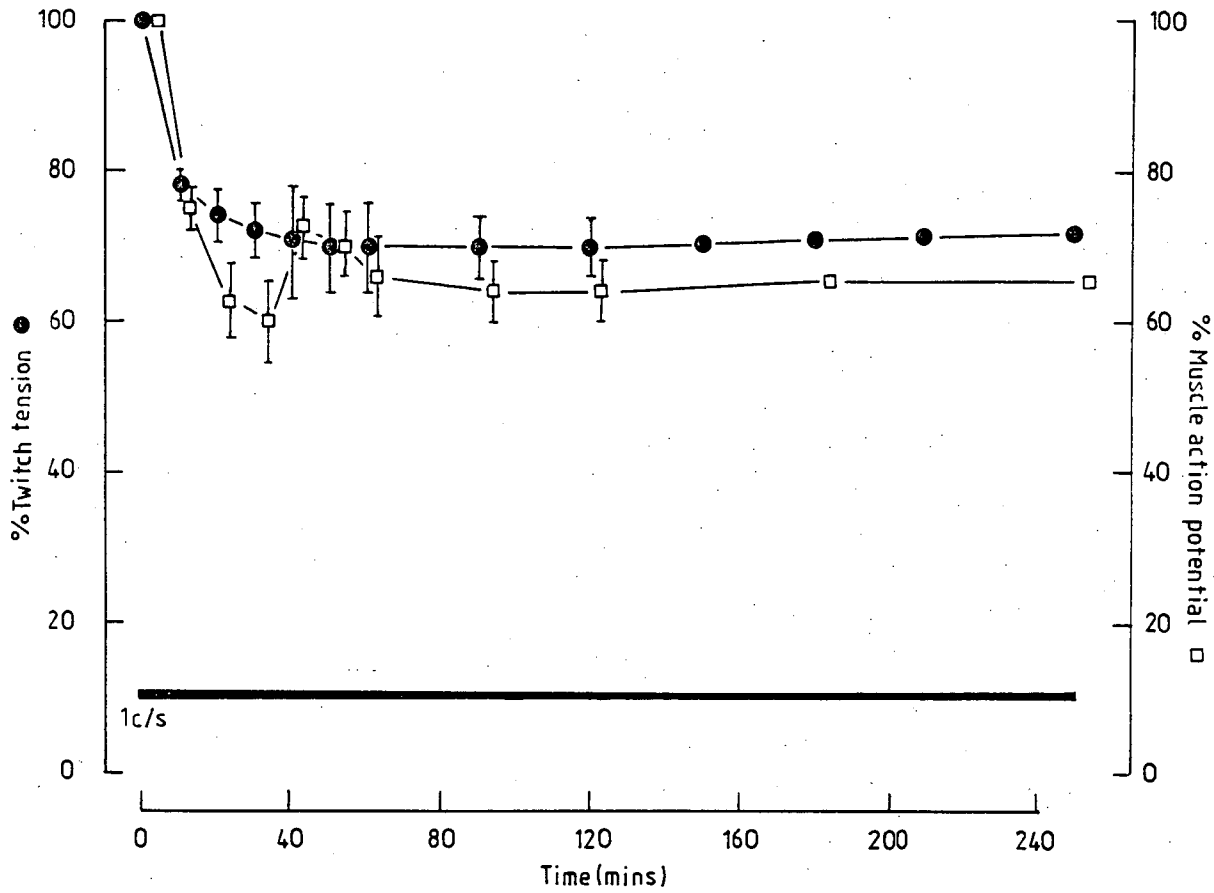
Compound Muscle Action Potential

The decline in the amplitude of the compound muscle action potential at 1c/sec stimulation closely paralleled the fall in isometric tension. With the first stimulus, values ranged from 34-59mV (mean 45 $\pm$ SD 9). Over 300-600 stimuli, the voltages fell to 56-81% (mean 63%) of initial values. Thereafter action potential amplitudes remained constant for up to 4 hours.

Figure 5

Title

Control experiments: twitch tension and muscle action potential amplitude during continuous 1c/sec stimulation.



Legend

Ordinate - % of initial amplitude isometric twitch tension ● and compound muscle action potential □ . Abscissa - time in minutes. Bars indicate standard error of mean (SEM). For points from 0 to 120 minutes N=5 and from 120 to 250 minutes N=2. The mean initial twitch tension was 517g and the mean initial compound action potential amplitude was 44mV.

b) 5c/sec Stimulation (N12)

Twitch Tension Amplitude

With sciatic nerve stimulation at 5c/sec (12 animals) there was a rapid rise in evoked tension during the first 75-150 stimuli to reach values 10-19% (mean 14%) above these obtained with the first response. This early rise in tension represented the staircase phenominon (Krarup 1981). Thereafter isometric tension fell steadily and stabilised out after 1200-1800 stimuli. The steady state twitch tension ranged from 50-76% (mean 61%) of the first response. This tension remained relatively constant for upto 30 minutes at this frequency (Figure 6). In one animal stimulated for 2 hours, there was no further decline in twitch tension. In this animal, isometric twitch tension was 58% of the initial value after 30 minutes and after 2 hours of stimulation. In these experiments, there was no recovery of isometric tension within 15 minutes of stopping stimulation (Figure 6). Most animals showed a small initial fall in twitch tension in response to a single stimulus during rest. In one animal studied 2 hours after stopping stimulation, isometric tension had recovered to 89% of its initial value. With repeated trains of stimuli in 2 animals, interrupted by upto 2 hours of rest, the steady state tension remained stable.

In 5 experiments, a single isometric twitch response to direct muscle stimulation recorded at the beginning of

the experiment was followed by repetitive nerve stimulation (5c/sec) for 25 minutes and then by 5 minutes of direct muscle stimulation at the same frequency (5c/sec) without interrupting the stimulus train. Initial isometric twitch tension in response to direct muscle stimulation was 10-30g (2-6%) greater than with nerve stimulation. After 25 minutes of nerve stimulation, the mean twitch tension had stabilised at 51% of the initial value. On switching immediately to direct muscle stimulation, however, the tension developed to the first stimulus was significantly greater (mean 62% of the initial value with direct muscle stimulation). With continued repetitive direct muscle stimulation, the isometric tension fell to that evoked by nerve stimulation after 300-600 stimuli.

Twitch Characteristics

Half relaxation time began to lengthen after the first few contractions. The mean value for the first response in 12 animals was 26.8msec ($\pm$ SD 3.7). At the peak of the staircase effect this had prolonged to 39.3 msec ($\pm$ SD 10.0). As the evoked tension fell to its steady state level, the half relaxation time progressively shortened and plateaued out at a mean of 17msec $\pm$ (SD 3.7). The rise time fell steadily with the fall in tension and unlike the half relaxation time showed no initial prolongation.

With 15 minutes of rest, after 30 minutes of repetitive nerve stimulation, the half relaxation time had prolonged markedly (Table 1). The integral of the relaxation

and contraction phases of the twitch response, calculated by measuring the areas under each part of the curve, ($\frac{R}{C}$) are shown in Figure 7 for the first 10 minutes of stimulation. Absolute values of rise time and half relaxation time are dependent in part upon peak height. The expression of the integrals of the relaxation phase/contraction phase removes peak tension as an important variable, and confirms the significance of the early prolongation in relaxation time at this stimulus frequency.

Muscle Action Potential Amplitude

The mean muscle action potential amplitude to the first stimulus was 42.2mV ($\pm$ SD 11.4). This fell steadily and stabilised at 21-49% (mean 31%) of the initial amplitude after 1200-1500 stimuli. The mean amplitude after 30 minutes stimulation was 13.1mV ($\pm$ SD 3.6). With rest, the action potential recovered rapidly to a mean level of 35.8mV ($\pm$ SD 10.8)(N=6) after 15 minutes. The electrical recovery was therefore more rapid than the mechanical recovery (Figure 6).

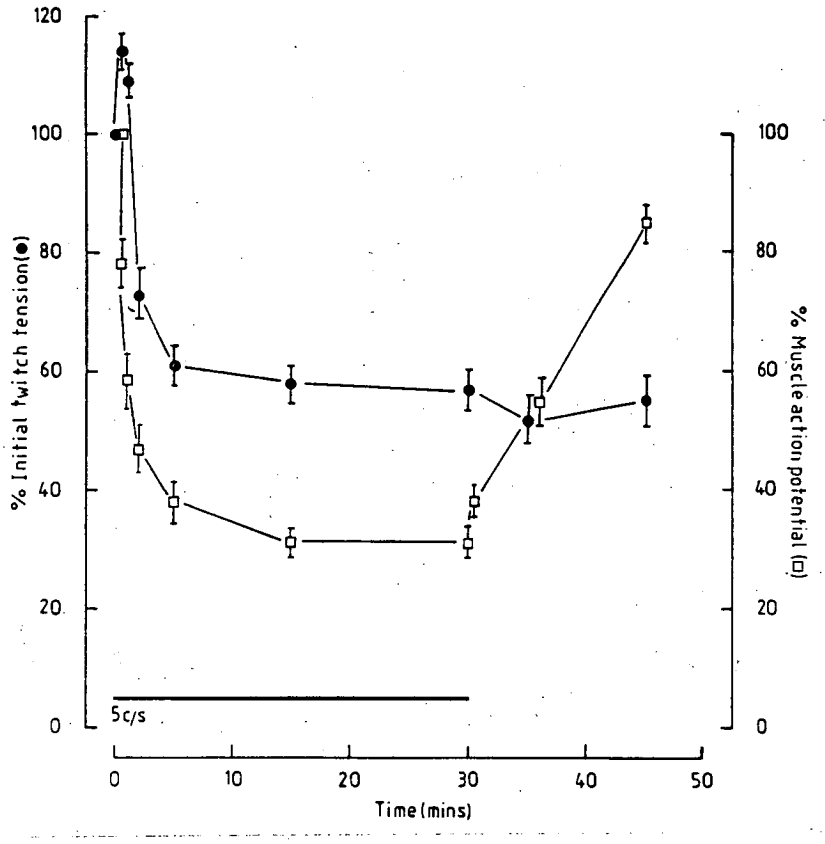
c) Tetanic Stimulation

In 2 animals, the sciatic nerve was stimulated tetanically (100c/sec) for 2 seconds every minute for 30 minutes. Peak tetanic tension (gastrocnemius) remained unchanged over this period (30 X 2 sec stimulus trains). Mean peak tetanic tension with the first and last trains were 2040 and 2020g. Tetanic tensions with nerve and with direct muscle stimulation were identical.

Figure 6a

Title

Control Experiments: twitch tension and muscle action potential amplitude with 5c/sec stimulation.



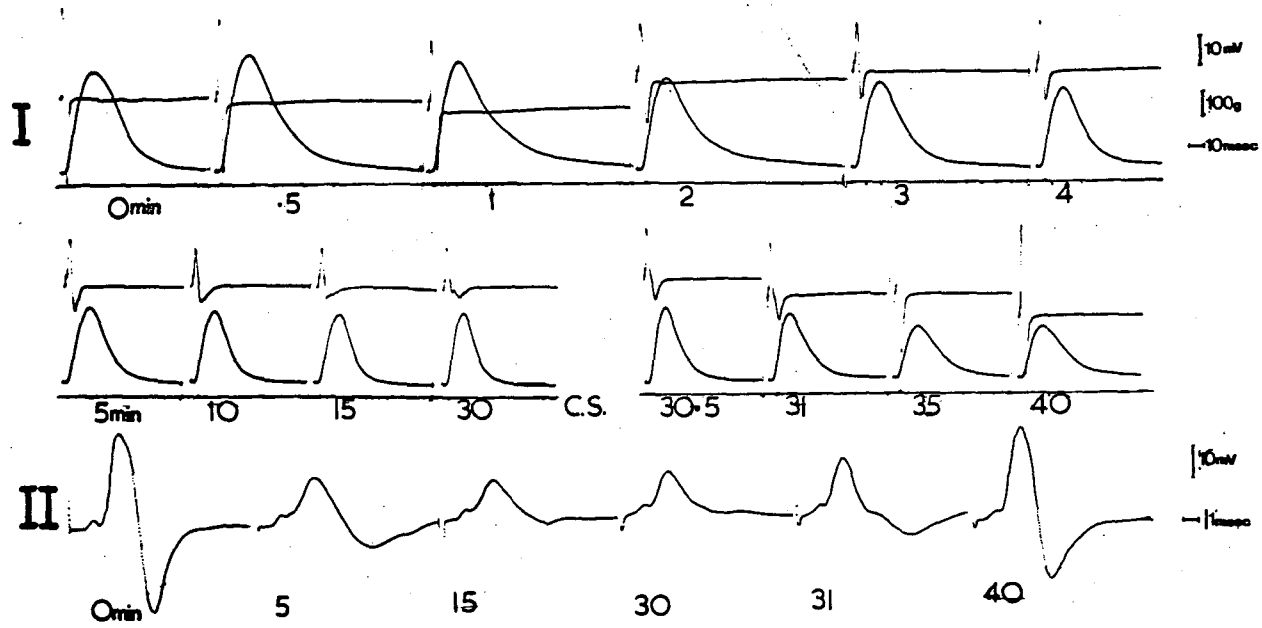
Legend

Ordinate - % of initial amplitude, isometric twitch tension (nerve stimulation) ● , muscle action potential □ . Abscissa - time in minutes. Bars indicate standard error of mean. For points from 0 - 30 minutes N=12 and for points from 30 - 45 minutes N=6. 5c/sec nerve stimulation from 0 - 30 minutes. Injections of 200ul of either normal saline of 5% dextrose were given at 6, 8, 10 and 12 minutes. Note the more rapid recovery of action potential amplitude with rest.

Figure 6b

Title

Control experiment: 5c/sec stimulation



Legend

Selected isometric twitch responses (nerve stimulation) and compound muscle action potential recordings during and after a 5c/sec stimulus train. Continuous 5c/sec stimulation was carried out from 0 to 30 minutes. I. twitch and action potential responses at the indicated time in minutes. II. action potential responses recorded on a faster sweep. Note the rapid plateauing out of twitch tension amplitude during stimulation and the absence of early recovery during rest.

TABLE 1

Title

Control Data: 5c/sec Stimulation: Twitch Characteristics.

DURATION STIMULATION 5c/sec	RISE TIME (MEAN & SD) (msec)	HALF RELAXATION TIME (MEAN ± SD) (msec)
1st Response	25.0 ± 3.5	26.3 ± 6.0
30 sec	23.2 ± 4.3	39.3 ± 10.0
5 min	19.0 ± 2.1	22.5 ± 7.0
15 min	17.0 ± 3.5	17.3 ± 3.7
30 min	15.5 ± 2.1	16.1 ± 3.2
Recovery		
1 min	17.0 ± 3.0	22.5 ± 7.6
5 min	17.6 ± 4.2	29.1 ± 9.0
15 min	19.8 ± 5.6	32.3 ± 10.0

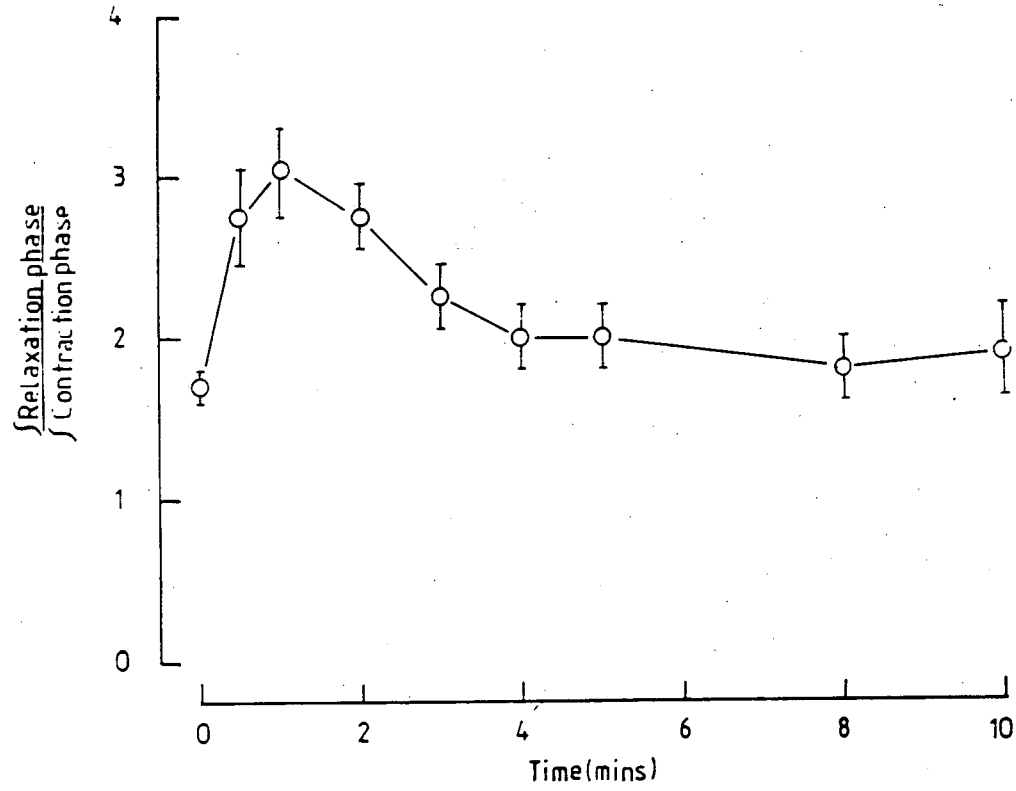
Legend

Response measurements in 6 animals during 30 minutes of stimulation followed by 15 minutes rest. Note the early prolongation of the half relaxation time after the onset of stimulation and during rest.

Figure 7

Title

Control experiments: Twitch characteristics during 5c/sec stimulation.



Legend

Ordinate - integral of the rise and relaxation phases of the isometric twitch response calculated graphically and expressed as a ratio during 5c/sec stimulation. Abscissa - time in minutes. N=8. Bars indicate standard error of mean.

1.1.2 Effects of Aortic Occlusion on Twitch Tension and Muscle Action Potential Amplitude

In 4 experiments, the aorta was occluded with a ligature or clamp after the twitch and action potential responses had reached steady state values during 5c/sec stimulation. The steady state twitch tension (after 6 minutes stimulation) was 50-72% (mean 62%) of the amplitude of the first response. After aortic ligation, isometric twitch tension fell rapidly and became unrecordable within 180-210 seconds. The action potential fell more slowly but had disappeared within 600 seconds of aortic ligation (Table 2).

Half relaxation time prolonged following aortic occlusion reaching a mean of 160% of the steady state value after 90 seconds.

In one experiment, the aortic clamp was released after complete loss of the twitch response. With continued stimulation twitch tension recovered to 78% of the pre-ischaemic steady state value within five minutes (Figure 8).

In the remaining 3 experiments, stimulation was continued at 5c/sec for 24 minutes after aortic ligation and the muscle then rested for a further 15 minutes. Twitch tension did not recover. Resting tension began to increase 7-13 minutes after complete loss of the evoked muscle response.

By 24 minutes (at end of stimulus train) baseline tension had risen by a mean of 78g and continued to increase after stopping stimulation (i.e. mean increase 115g after 37 minutes of ischaemia). The progressive increase in baseline tension was due to electrically silent contracture.

TABLE 2

Title

The Effects of Aortic Occlusion on Twitch Tension and Muscle Action Potential Amplitudes.

TIME AFTER LIGATION	TWITCH TENSION (% STEADY STATE LEVEL)	MAP (% STEADY STATE LEVEL)
15 sec	67.0 ± 6.5	84.0 ± 8.0
30 sec	53.0 ± 5.0	72.0 ± 12.0
60 sec	37.0 ± 8.0	56.0 ± 9.0
90 sec	23.6 ± 8.0	53.0 ± 8.0
120 sec	12.0 ± 5.0	44.0 ± 8.0
180 sec	2.0 ± 3.0	34.0 ± 10.0
240 sec	0.0 ± 0.0	22.0 ± 4.0
300 sec	0.0 ± 0.0	15.0 ± 3.0
600 sec	0.0 ± 0.0	0.0 ± 0.0

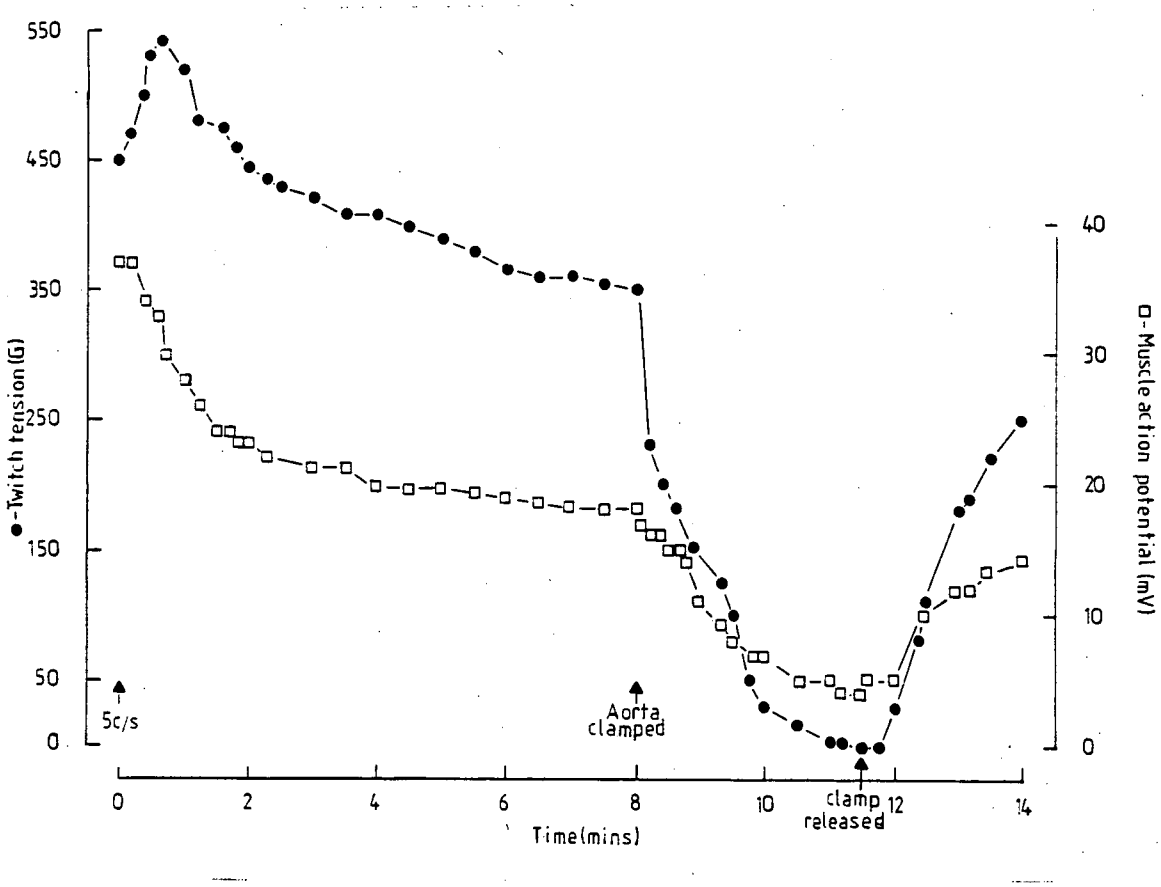
Legend

Changes in isometric twitch tension and compound muscle action potential (mean and SD) in 3 animals at different times after closing an aortic ligature during a 5c/sec stimulus train. The values are given as a percentage of the stable (or steady state) levels immediately prior to aortic ligation.

Figure 8

Title

Twitch tension and action potential changes during ischaemia.



Legend

Experiment 87. Aortic occlusion during continuous 5c/sec stimulation. Ordinate - isometric twitch tension ● (g) and compound muscle action potential amplitude □ (mV). Abscissa - time in minutes. The aorta was occluded between 8 and 11.5 minutes.

DISCUSSION

Control and Ischaemic Experiments

Twitch tensions recorded from gastrocnemius were similar to those reported previously (Walker 1951), the inter-experiment variability being accounted for by differences in body weight. The staircase effect was present with 5c/sec stimulation but not with 1c/sec stimulation. The magnitude of the staircase effect is known to be influenced by muscle temperature, being much less at 30°C than at 37°C (Close and Hoh 1968; Krarup 1981b). Surface temperatures of the muscles in this study were maintained between 26°-30°. The mean maximum staircase enhancement of 14% at 5c/sec stimulation is similar to that recorded by Krarup at 30°C in extensor digitorum longus (Krarup 1981 a & b).

Krarup (1981a) identified two concurrent processes in describing the changes in twitch tension during a 5c/sec stimulus train (Krarup 1981a). He showed that the staircase enhancement continued to operate but is overtaken by muscle fatigue. In the present study, isometric twitch tensions with 1 & 5 Hz stimulation stabilised after an initial fall and remained relatively constant for several hours repetitive stimulation, With 5c/sec stimulation prolongation of the duration of tension relaxation preceded a fall in evoked tension. As the latter stabilised out relaxation times

rapidly shortened. Prolonged relaxation is known to accompany muscle fatigue (Edwards et al 1972) and probably reflects a deterioration in the energy status of the fibre, although the exact biochemical mechanism is disputed (Dawson et al 1980; Edwards et al 1975).

The prolongation in relaxation times found at the peak of the staircase effect therefore suggests that some fibres were beginning to fatigue. The shortening of the half relaxation times observed as twitch tensions fell to a stable level suggests that the fibres which had developed prolonged relaxation have ceased to contract and that the fall in tension is due to a fallout of these fibres. Indirect evidence for this hypothesis comes from the work of Kugelberg and Edstrom (Kugelberg and Edstrom, Edstrom and Kugelberg 1968). They observed a similar fall in twitch tension to a stable level in rat extensor digitorum longus with 5-10c/sec stimulation associated with glycogen depletion in type A fibres. With a split ventral root preparation they demonstrated that after approximately 1500-2000 twitches (5c/sec) the type A (fast twitch glycolytic units) failed to contract and were depleted of glycogen. The initial fall in twitch tension in this study in gastrocnemius was therefore probably due to a selective failure of fast twitch glycolytic fibres. Further support for this hypothesis comes from histochemical studies which have shown that about 50% of muscle fibres in rat gastrocnemius have the histochemical characteristics of fast twitch glycolytic fibres (Ariano et al 1973). The stable twitch

tension of between 50 and 70% of the initial tensions with 5c/sec stimulation would be compatible with a failure of most of these fibres.

Muscle action potential amplitude also fell rapidly during 5c/sec stimulation and stabilised at 21-49% of its initial value. The steady state action potential responses (related to the initial value) was consistently lower than the twitch tension. Fibre type distribution is patchy in gastrocnemius and it is possible that fast twitch glycolytic units were contributing substantially to the initial action potential. The rapid recovery in action potential amplitude during rest, at a time when no twitch tension recovery has developed, strongly suggests that muscle fatigue in these experiments is due to a failure of contraction or excitation contraction coupling, rather than of excitation. In experiments with 5c/sec stimulation, the twitch amplitude was transiently slightly greater with direct muscle activation, when this was commenced after 25 minutes of nerve stimulation. The difference in amplitude was small, however, and neuromuscular failure is not important in fatigue at these frequencies.

Stimulation frequencies of 1 and 5c/sec were used in most of these experiments because: - (a) At these frequencies muscle blood flow is maximal upto 5c/sec but declines at higher stimulus frequencies due to compression of intramuscular blood vessels (Aruep and Saulfield 1935; Kjellner 1964).

(b) at these frequencies the steady state tension is probably generated by motor units with a high dependence on energy generated by oxidative phosphorylation and should reflect impairment of this process rapidly; (c) the steady state responses maintained for long periods at these frequencies provides a stable baseline against which the effects of inhibitors can be assessed; and (d) continuous stimulation at low frequencies results in a greater work load over a given number of stimuli than with higher frequencies where failure of muscle membrane excitation tends to protect the contractile apparatus (Krnjevic and Miledi 1958)

Ischaemic Experiments

The isometric twitch tension fell very rapidly with 5c/sec stimulation under ischaemic conditions. The muscle action potential amplitude also fell, but at a slower initial rate, both parameters disappearing within 10 minutes of the onset of ischaemia. An electrically silent contracture began to evolve 7-13 minutes after complete twitch failure. Ischaemic contracture is well recognised in rigor muscle (Bendall 1951) and can involve cardiac as well as skeletal muscle (Bing and Fishbein 1979). Contracture will be discussed further in the next section.

1.1.3 Dinitrophenol Experiments

i Initial Observations

In 10 experiments, a single bolus of 8-16mg/Kg body weight of dinitrophenol was injected by the intra-arterial route 60 minutes after the onset of stimulation at 1c/sec. At this time twitch tension had stabilised at a mean of 80.4% of the initial value. Stimulation (1c/sec) was continued until the twitch response failed.

Clinical Observations

within 10-15 seconds of injection, the respiratory pattern altered. Transient apnoea lasting several seconds was followed by sustained hyperventilation (rate 90-120/min). One animal died within 5 minutes of injection and 4 further animals died within one hour. Progressive rigidity developed in the tail and hind limb muscles 5-15 minutes after injection. In animals which died, rigidity spread to involve the forelimbs prior to death.

Measurement of the Mechanical and Electrical Responses.

A typical experiment is illustrated in Figure 8 (Exp.53). Isometric twitch tension began to fall within one minute of injection, to reach 7% of the pre-injection steady state value after 12 minutes (T.C. 6.5 minutes). Muscle action potential amplitude fell at a slower rate initially but by

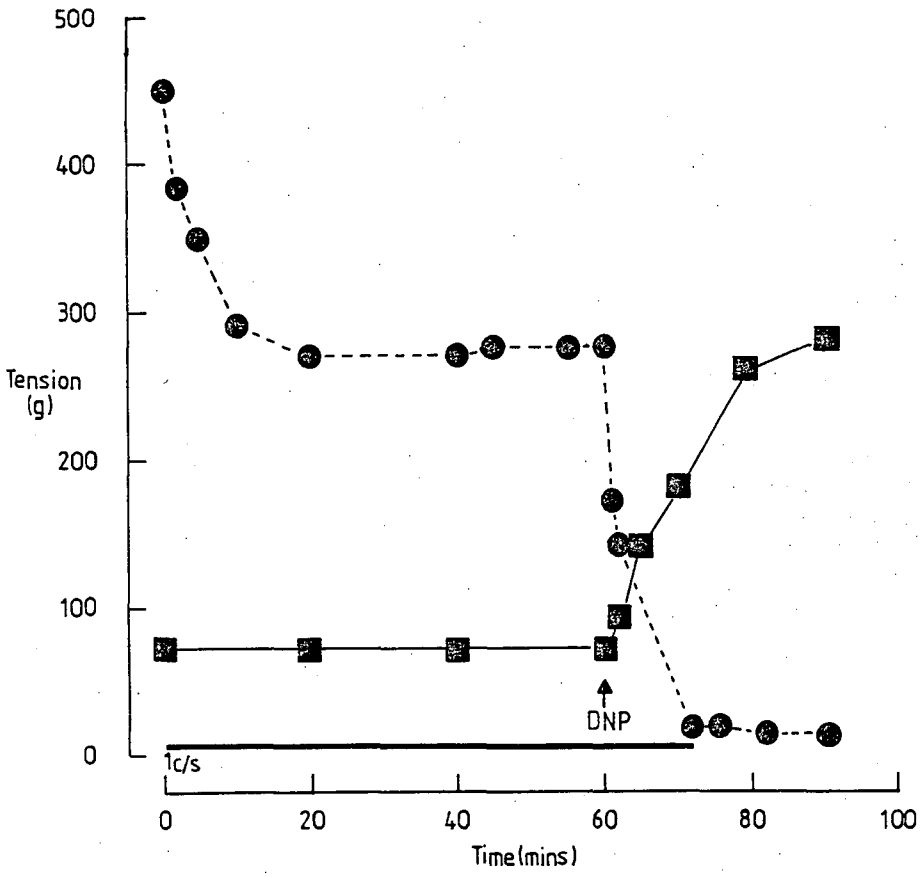
12 minutes it had reached 8% of the steady state value. Resting tension began to rise 2 minutes after injection at a time when isometric twitch tension was 48.8% of the steady state value. Resting tension then progressively increased to approximately 50% of the tension generated by the first response in the train prior to injection. There were no significant differences between the pattern of twitch failure with nerve and with direct muscle stimulation (Figure 9b). No recovery of action potential or twitch tension occurred for up to 2 hours after stopping stimulation even when the muscle was released so as to restore resting tension to the pre-injection level. The relationship between the changes in twitch tension and the development of contracture for 9 experiments is shown in Figure 10. Contracture (increase in resting tension) is expressed as a percentage of the amplitude of the first twitch in the train in order to standardise the data from animals of different weights. Contracture began when the twitch tension had fallen by a mean of 32% of the initial value and progressed most rapidly in the later stages of twitch failure. The final contracture ranged from 38-77% of the initial twitch tension amplitude (mean 56.1% SD 13.3). Contracture continued to increase for several minutes after stopping stimulation in most experiments. The length/tension curve in contractured muscle was shifted to the left (Figure 11). The % fall in compound muscle action potential has been plotted against % fall in twitch tension in Figure 12. The results suggest a biphasic relationship which can be described by two linear regression lines. The first regression line, which includes

all points upto a 50% fall in steady state tension has a slope of 0.75 giving a correlation co-efficient of 0.99 ($p > 0.001$). Points beyond a 50% fall in tension can be described by a second regression line having a slope of 1.35 and a correlation co-efficient of 0.98 ($p > 0.001$).

Figure 9a

Title

Dinitrophenol infusion during 1c/sec stimulation.



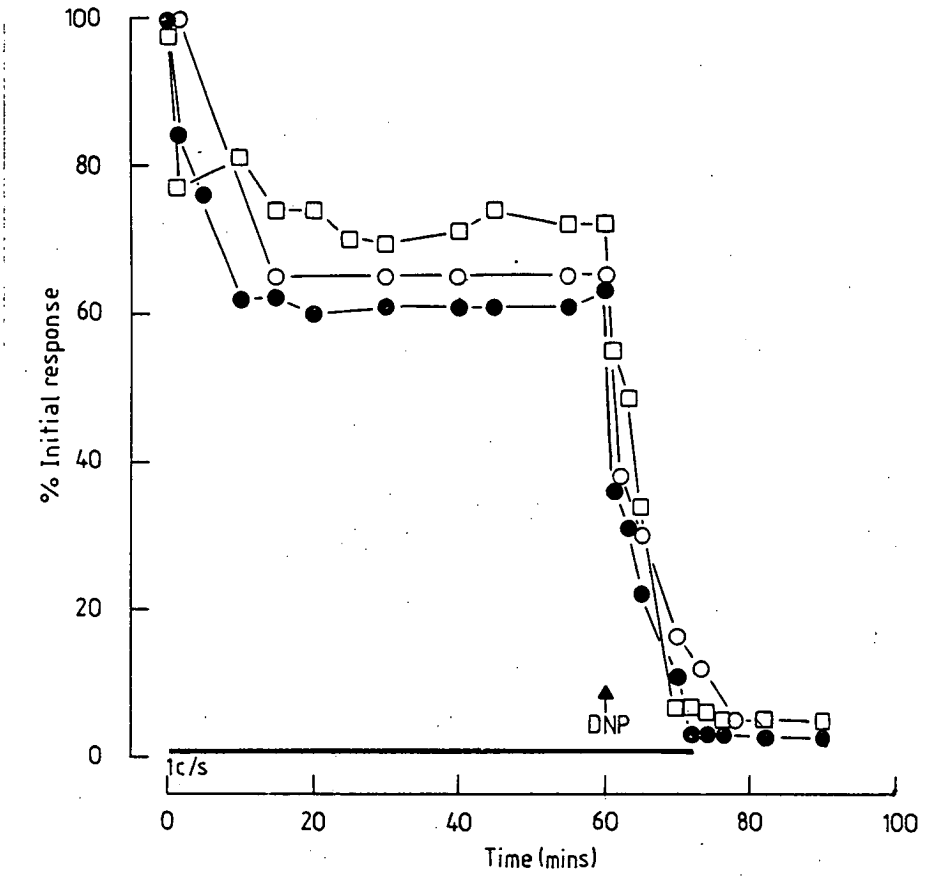
Legend

Experiment 53. Ordinate - isometric twitch tension nerve stimulation ● and resting tension ■ in grams. Abscissa - time in minutes. 1c/sec stimulation indicated by solid line. 12mg/Kg body weight dinitrophenol injected at 60 minutes.

Figure 9b

Title

Dinitrophenol infusion during 1c/sec stimulation.



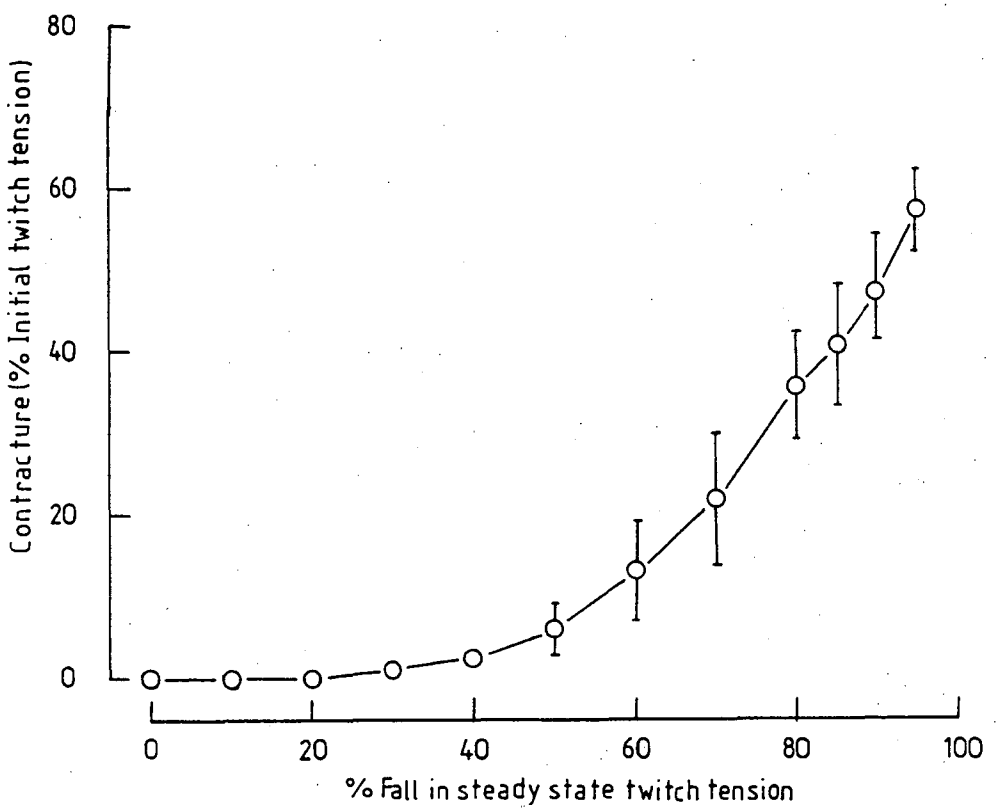
Legend

Experiment 53. Ordinate - % of initial amplitude isometric twitch tension nerve stimulation ● , isometric twitch tension direct muscle stimulation ○ , and compound muscle action potential □ . Abscissa - time in minutes. 1c/sec nerve stimulation was carried out from 0 to 72 minutes. Single shocks with direct muscle stimulation were interposed in the stimulus train. Dinitrophenol was injected after 60 minute stimulation at which time the isometric twitch tension and compound muscle action potential had stabilised.

Figure 10

Title

Dinitrophenol infusion during 1c/sec stimulation: the relationship between contracture evolution and isometric twitch tension failure.



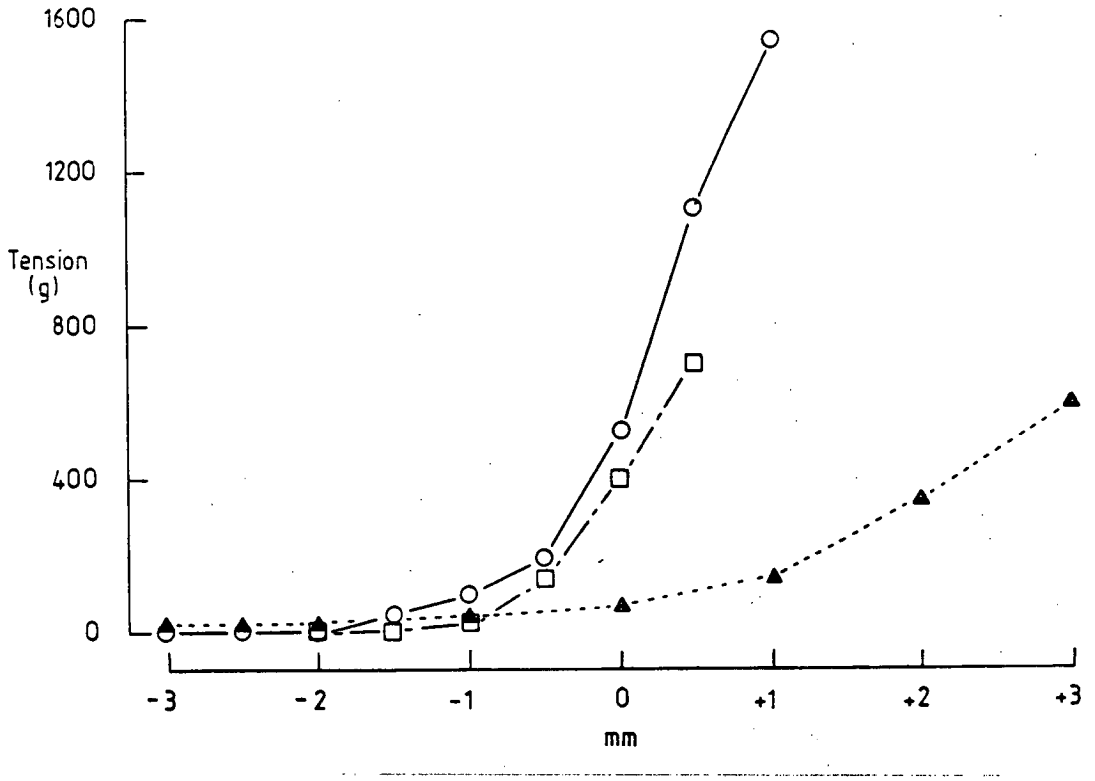
Legend

Ordinate - contracture tension (i.e. increase in resting tension expressed as a % of the initial twitch tension amplitude). Abscissa - % fall in steady state twitch tension amplitude. N=9. Bars indicate standard error of mean. Dinitrophenol (8-16mg/Kg) was injected after 60 minutes preliminary stimulation at 1c/sec at which time the twitch response had stabilised (steady state value). Stimulation was continued as twitch tension failed.

Figure 11

Title

The relationship between resting tension and muscle length after DNP induced contracture.



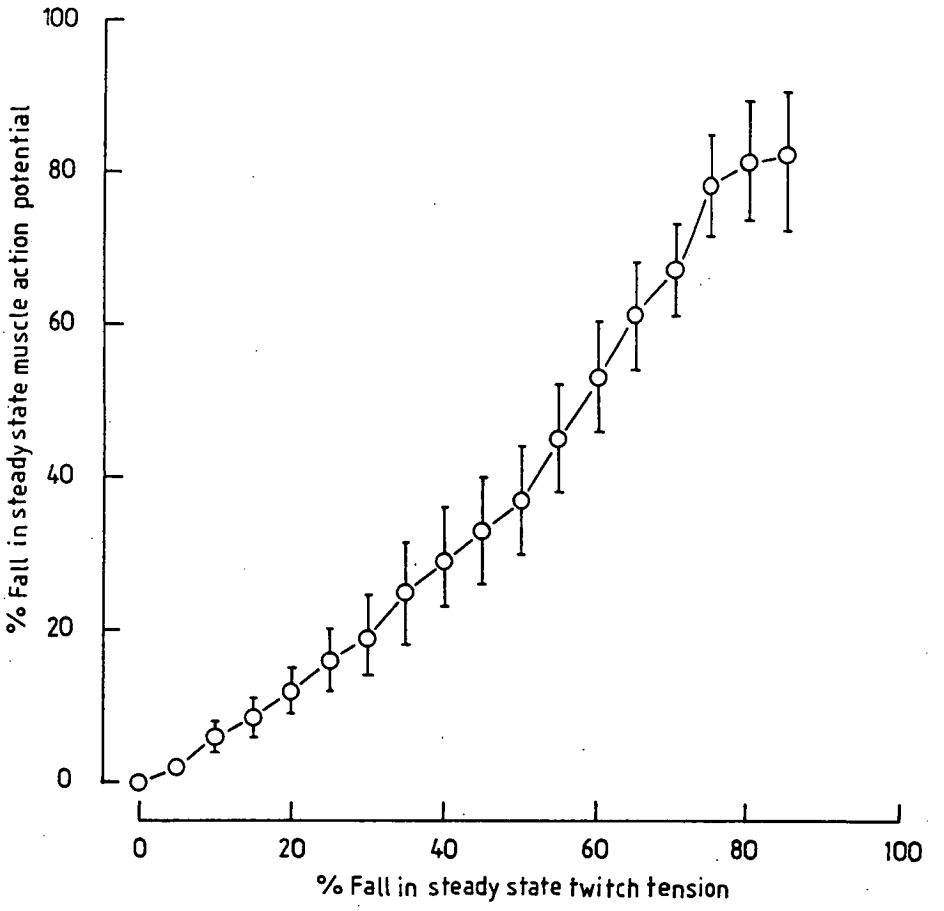
Legend

Ordinate - resting tension in grams. Abscissa - muscle length. 0 indicates the length at commencement of the experiment. 2 experiments in which contracture was induced ○, □ are compared with length tension changes in a control experiment ▲ .

Figure 12

Title

Dinitrophenol infusion during 1c/sec stimulation: the relationship between twitch tension and action potential failure.



Legend

Ordinate - fall in steady state muscle action potential amplitude. Abscissa - fall in steady state isometric twitch tension. N=9. Bars indicate standard error of mean.

ii Experiments with 2c/min stimulation

As contracture developed in unstimulated as well as in stimulated muscles in preliminary experiments, force generation was followed at very low stimulus frequencies in a further group. In 23 animals the gastrocnemius muscle was activated by one stimulus via sciatic nerve stimulation and one stimulus via direct muscle stimulation each minute. In view of the fatal result with larger bolus doses in some of the earlier experiments, the dosage regimen was modified, 4 smaller boluses of 4mg/Kg in 100ul volumes being given at 2 minutes intervals. This regimen was also followed in subsequent experiments.

Clinical Observations.

Persistent tachypnoea with hyperventilation began soon after the first injection of DNP but the ataxic respiratory pattern seen after larger bolus doses was not evident. Rigidity in the tail and hindlimb muscles again developed 10 - 20 minutes after the beginning of infusion. Two animals died within 10 minutes of the first injection of DNP and are excluded from the grouped results. Arterial blood sugar levels after the onset of muscle contracture averaged 7.2mMol/L (N4). Arterial blood gas estimations were performed on 200ul aliquots of blood in 6 experiments after the development of a severe twitch tension failure. Mean arterial PaO_2 was 12.32 Tor and arterial $PaCO_2$ 4.88 Tor. No significant systemic acidosis developed (Figure 13).

Blood Flow Studies

Muscle blood flow was measured at rest in one experiment. A mean flow of 15.0mls/100 grams muscle (N5) was obtained at rest prior to infusion of DNP. A four fold increase in muscle blood flow developed after infusion of dinitrophenol and persisted for 30 minutes (Figure 14).

Mechanical and Electrical Responses

The gastrocnemius muscle was freeze clamped in 4 experiments soon after the onset of twitch failure and these are not included in the grouped data. Of the remaining 17 experiments in this group, 4 were followed for 15 minutes after the onset of DNP infusion, 5 for 20 minutes, 7 for 30 minutes and 1 for 60 minutes. In most experiments the muscle was rapidly frozen for biochemical analysis at these times.

Initial twitch tension ranged from 364-625g (mean 477g) and initial resting tension ranged from 50-85g (mean 65g). Typical experiments are illustrated in Figure 15a-c. A fall in twitch tension was seen soon after the commencement of DNP infusion (mean time of onset 5.3 mins($\pm$ SD 2.3)after first injection) and this was followed by muscle contracture (mean time of onset 10.6 mins ($\pm$ SD 2.7). Isometric twitch tensions were followed to levels less than 5% of the initial value in 5 experiments. The rate of tension failure in these experi-

ments was linear and ranged from 4.4-7.6% fall in initial twitch amplitude/minute, with a mean of 6.3% (correlation co-efficients ranged from 0.91-0.99: $P>0.001$). The remaining experiments were terminated by freeze clamping while the tension was still falling. The rate of twitch failure was identical with nerve and with direct muscle activation.

Twitch characteristics remained fairly stable as the amplitude fell. The rise time of the first response ranged from 20-31msec (mean 25.6 $\pm$ SD 4.6). Ten minutes after commencement of DNP infusion, the rise time ranged from 18-30msec (mean 21.5 $\pm$ SD 4.8). The initial half relaxation time varied from 19-34msec (mean 24.4 $\pm$ SD 4.6). After 10 minutes, this value ranged from 16-56msec (mean 25 $\pm$ SD 11) and after 20 minutes from 20-70msec (mean 28.4 $\pm$ SD 15). In most experiments, the ½ relaxation time remained unchanged as the tension failed, a major prolongation being seen in only 1 experiment.

Twitch tensions fell by a mean of 34% (range 20-66%) before contracture began to evolve. The changes in twitch tension and resting tension with time are shown in Figure 16 and the relationship between twitch failure and contracture in Figure 17.

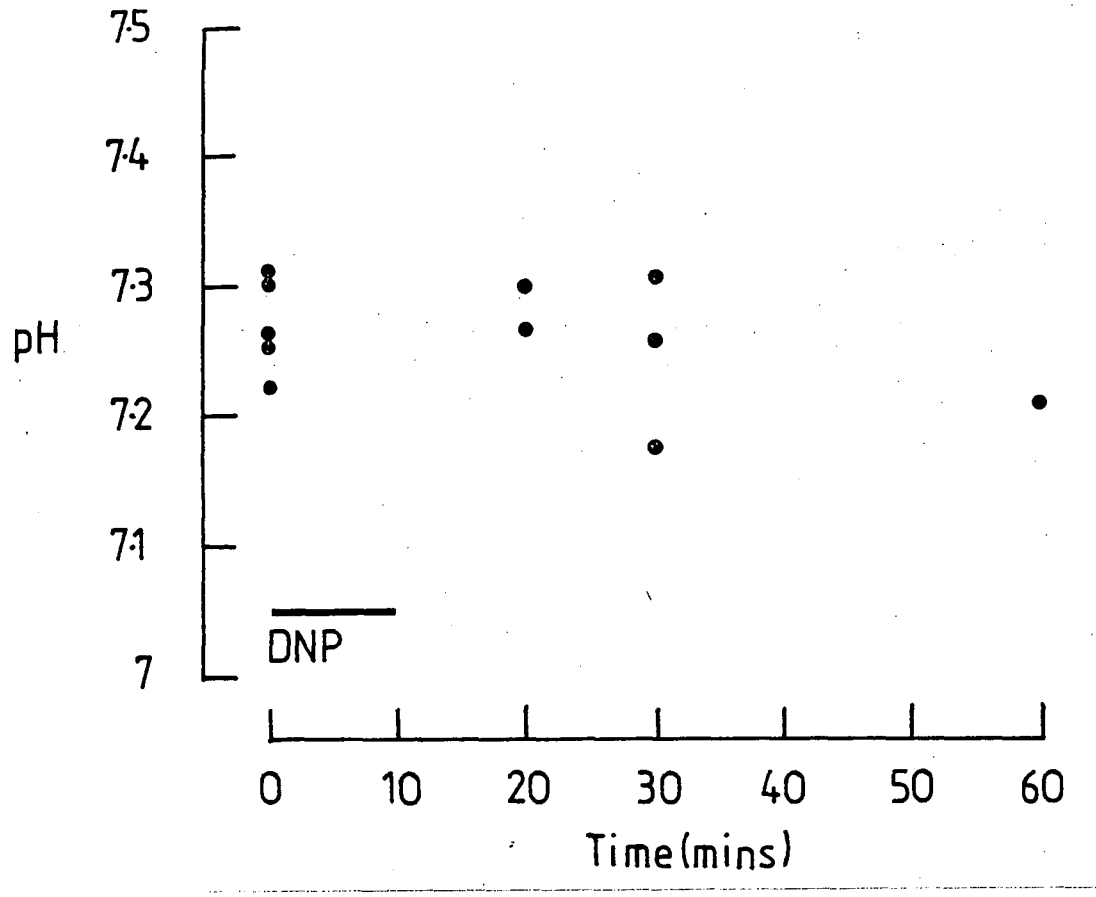
Compound muscle action potentials (with nerve stimulation) were recorded in 15 experiments. The amplitude fell steadily after dinitrophenol injection but at a slower initial rate than twitch tension (Figure 18). This relationship had

a biphasic relationship similar to that found in experiments with 1c/sec stimulation (0.6% fall in MAP per 1% fall in twitch tension for points up to 60% fall in initial twitch amplitude, (cc 0.99: $P < 0.001$) and 1.5% fall in MAP per 1% fall in tension for points from 60-95% (cc 0.97: $P<0.001$). In the latter stages of twitch failure, there was slight prolongation in the latency from the stimulus artefact to the take off of the evoked muscle action potential with nerve stimulation, suggesting either slowing of nerve conduction or neuromuscular delay (Figure 19). These relatively minor abnormalities, however, did not contribute to force failure as the changes in twitch tension were identical with direct muscle stimulation. Muscle surface temperature remained stable during these observations.

Figure 13

Title

Arterial blood pH levels after infusion of dinitrophenol.



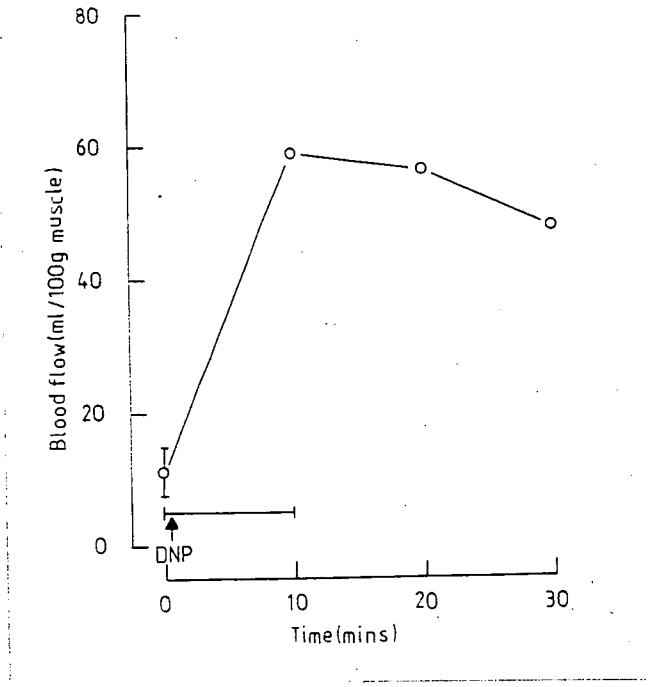
Legend

Ordinate - arterial blood pH. Abscissa - time in minutes 16mg/Kg DNP injected between 0 and 10 minutes. 5 observations were made prior to infusion and 6 observations after the onset of a severe force failure. Only 1 sample was analysed in each experiment to minimise blood loss.

Figure 14

Title

Muscle blood flow after dinitrophenol infusion.



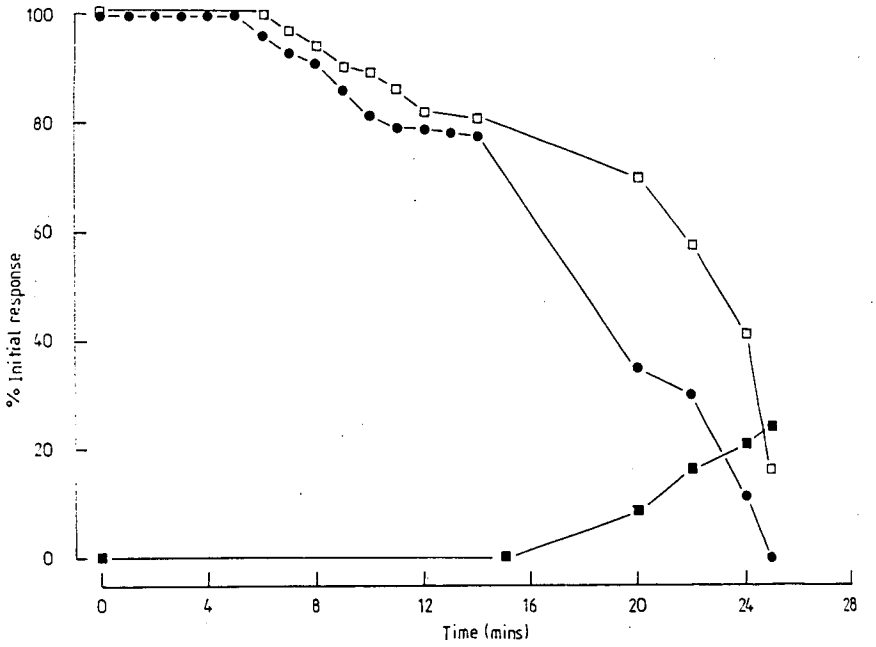
Legend

Ordinate - blood flow in mls/100g muscle. Abscissa - time in minutes. 16mg/Kg of DNP was injected between 0 and 10 minutes. These observations were made in one experiment. The initial point represents an average of 5 observations (bars indicate range) and subsequent points the mean of 2 observations at separate sites.

Figure 15a

Title

Dinitrophenol infusion with 2c/min stimulation.



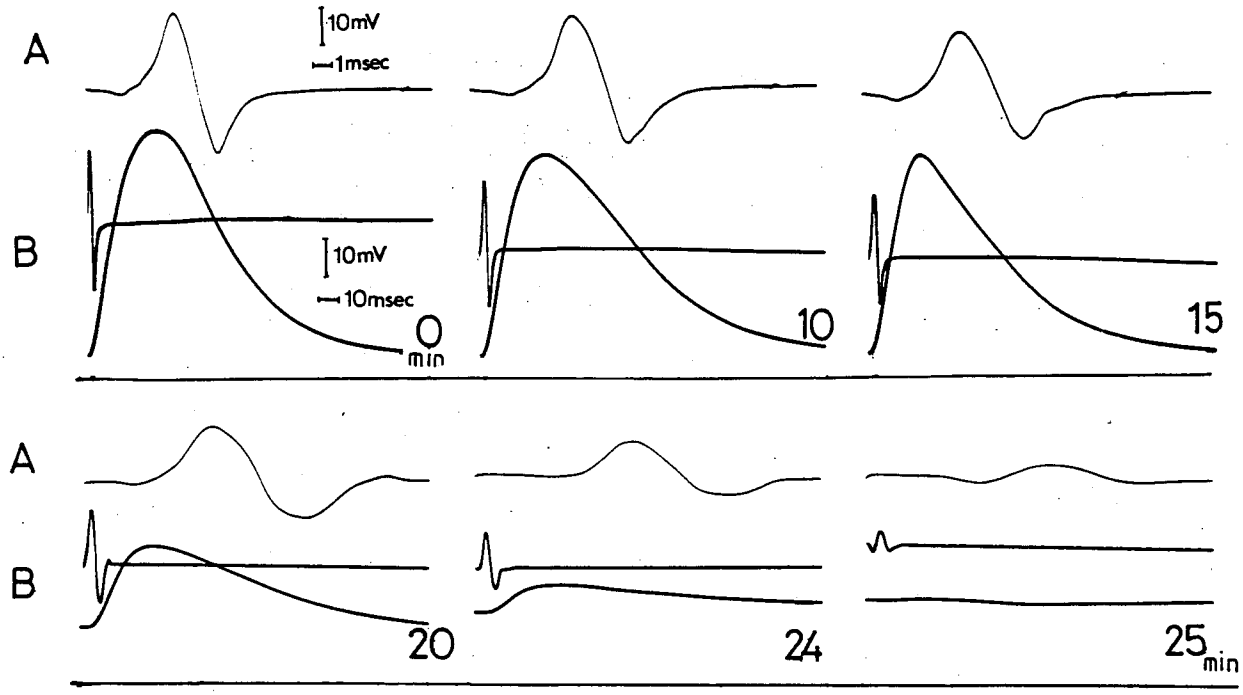
Legend

Experiment 207. Ordinate - % of initial response, isometric twitch tension nerve stimulation ● and compound muscle action potential amplitude □ . Contracture tension (increase in resting tension expressed as % of the initial twitch tension amplitude) ■ . Abscissa - time in minutes. 16mg/Kg DNP injected between 0 and 10 minutes.

Figure 15b

Title

Dinitrophenol infusion with 2c/min stimulation:



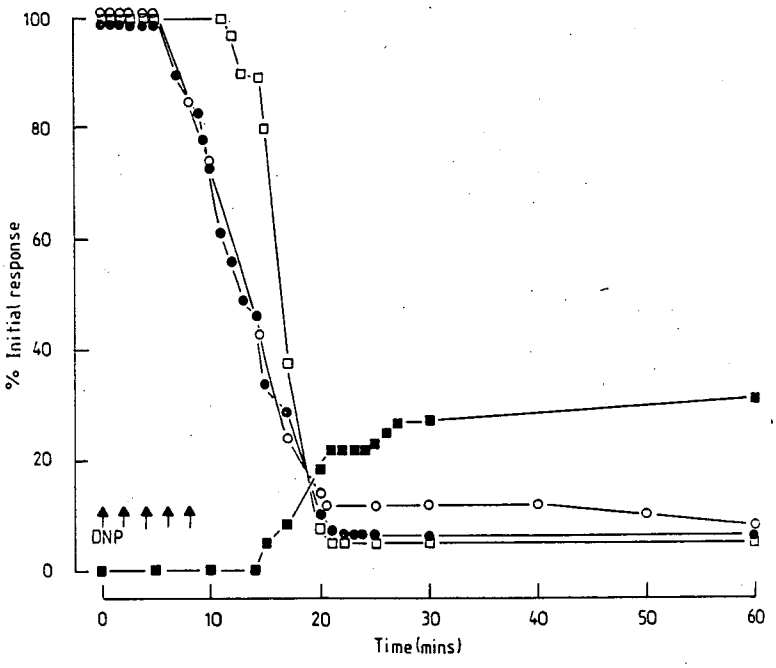
Legend

Experiment 207. Selected isometric twitch tension and compound muscle action potential responses (B) and muscle action potential responses recorded on a faster sweep speed (A) at the times indicated in minutes. The experiment was terminated by freeze clamping at 30 minutes.

Figure 15c

Title

Dinitrophenol infusion with 2c/min stimulation: typical experiments.



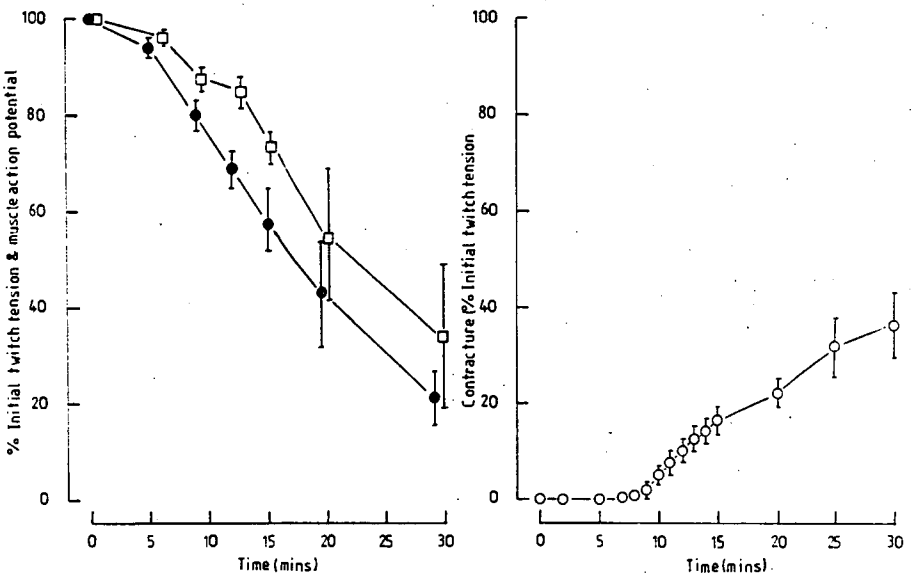
Legend

Experiment 234. Ordinate - % of initial amplitude. isometric twitch tension nerve stimulation ● , isometric twitch tension direct muscle stimulation ○ , and compound muscle action potential □ . Contracture tension ▣ (i.e.) rise in resting tension as a % of the initial twitch tension amplitude). 16mg/Kg DNP injected between 0 and 10 minutes.

Figure 16

Title

Dinitrophenol infusion 2c/min stimulation: twitch tension and action potential amplitude in all experiments.



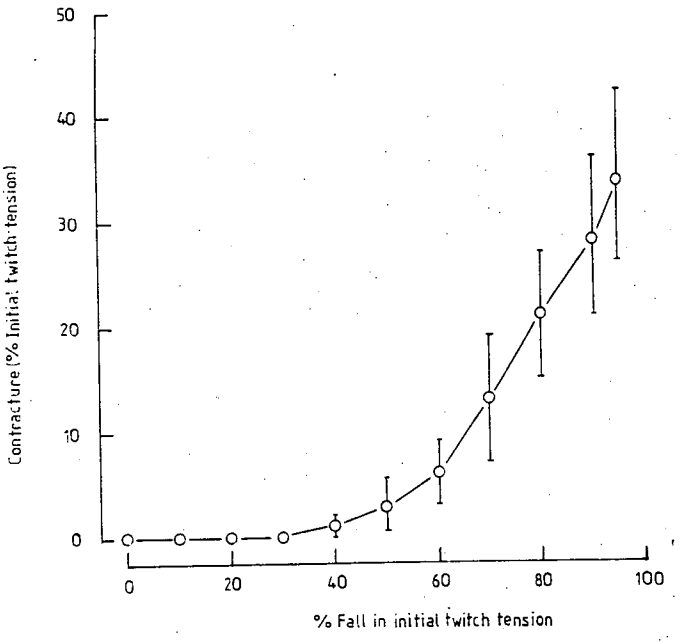
Legend

Ordinate - (left hand graph) % of initial isometric twitch tension ● and muscle action potential amplitude □ . (right hand graph) Contracture tension ○ (i.e.) increase in resting tension as a % of the initial twitch tension amplitude). Abscissa - time in minutes. Bars indicate standard error of mean. For points from 0 to 15 minutes N=15, for 15 to 20 minutes N=12 and for 20 to 30 minutes N=7. Note the wide range in the rate of tension failure in animal given an identical dose of dinitrophenol and the slower rate of action potential failure.

Figure 17

Title

Dinitrophenol infusion, 2c/min stimulation: the relationship between contracture and twitch tension failure.



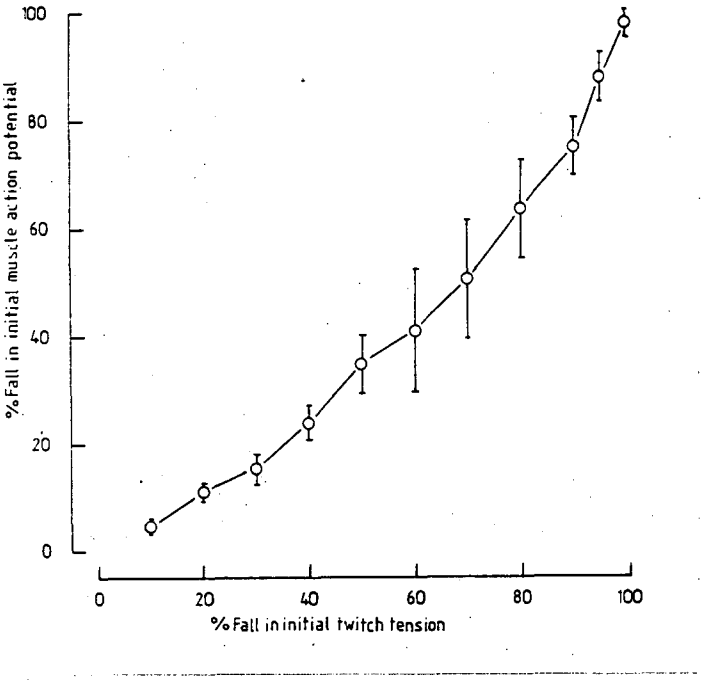
Legend

Ordinate - contracture (i.e. increase in resting tension as a % of the initial twitch tension amplitude). Abscissa - % fall in initial twitch tension. Bars indicate standard error of mean. This data is from 5 experiments in which the twitch tension fell to 5% or less of the initial value.

Figure 18

Title

Dinitrophenol infusion, 2c/min stimulation: the relationship between twitch tension and action potential failure.



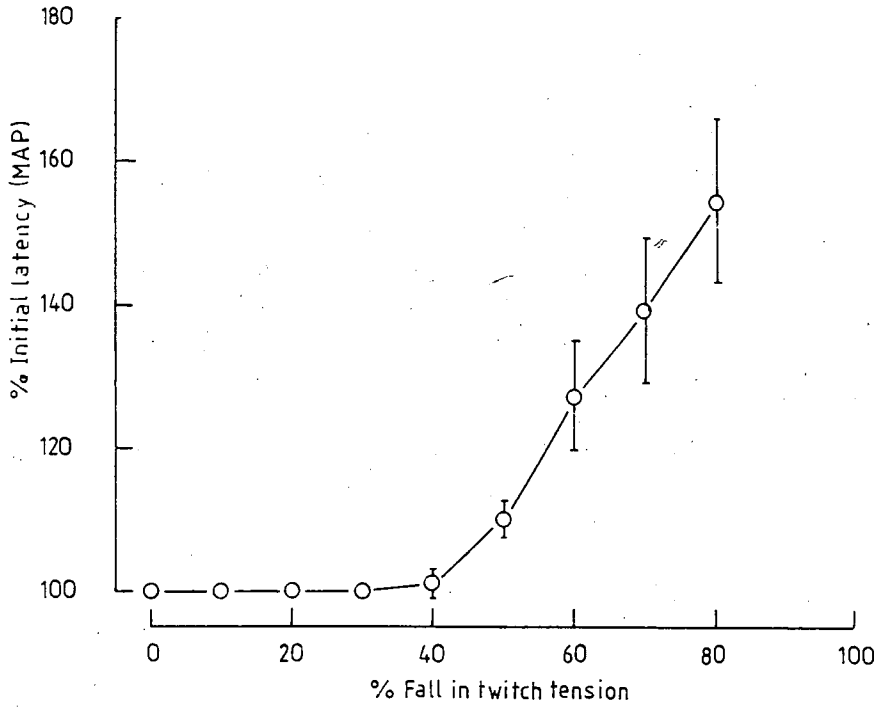
Legend

Ordinate - % fall in initial muscle action potential amplitude. Abscissa - % fall in initial twitch tension. Bars indicate standard error of mean. For points between 0 and 50% twitch tension failure N=15, and from 50 to 95% failure N=5. Linear regression line 0 to 60% twitch failure, slope 0.60, correlation co-efficient 0.94, 60 to 95% twitch failure, slope 1.50, correlation co-efficient .99.

Figure 19

Title

Dinitrophenol infusion, 2c/min stimulation: relationship between electrical latency and twitch tension failure.



Legend

Ordinate - % of the initial latency to take off of the muscle action potential (mean initial latency 0.9msec). Abscissa - % fall initial twitch tension amplitude. Bars indicate standard error of mean. For points from 0 to 50% twitch failure N=10 and from 50 to 80% failure N=5. Recordings on a rapid time base suitable for latency measurements were not made in 5 experiments.

iii Experiments with 5c/sec Stimulation Commencing at Onset of DNP Infusion.

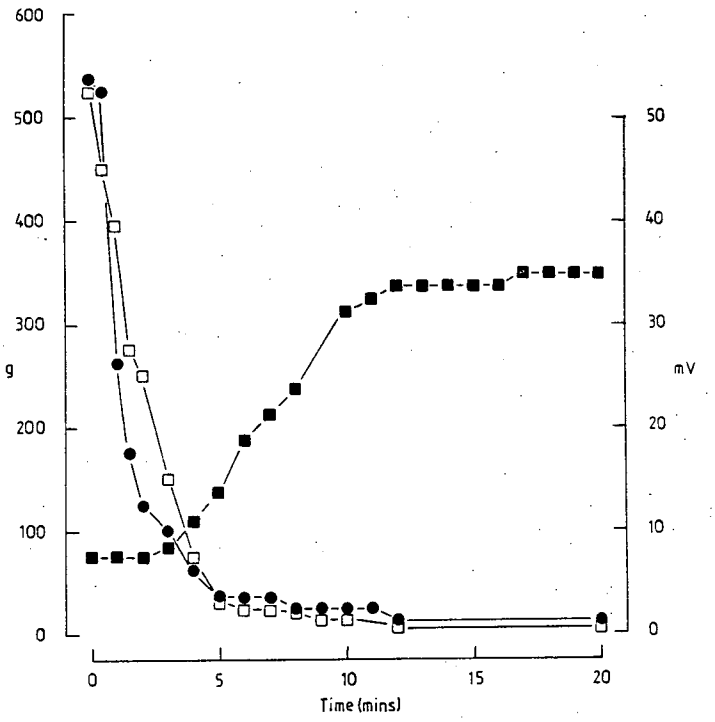
In 6 animals, the gastrocnemius muscle was stimulated at 5c/sec from the commencement of DNP injection. The dose and timing of DNP injections was identical to that in experiments where 2c/min stimulation was employed. A typical experiment is shown in Figure 20 and the changes in twitch and resting tension for all 6 experiments are shown in Figure 21. Twitch amplitude fell rapidly to very low levels, contrasting with the findings in controls where a steady state response 50 - 70% of the initial amplitude was maintained.

The resting tension began to increase between 3.5 and 10 minutes (mean 6.9mins) after the first bolus of DNP. Contracture evolved more rapidly than in muscles stimulated at 2c/min (Figure 26). The compound muscle action potential at this stimulus frequency fell at a similar rate to the twitch tension amplitude (Figure 22).

Figure 20

Title

Dinitrophenol infusion with 5c/sec stimulation starting at the onset of infusion: typical experiment.



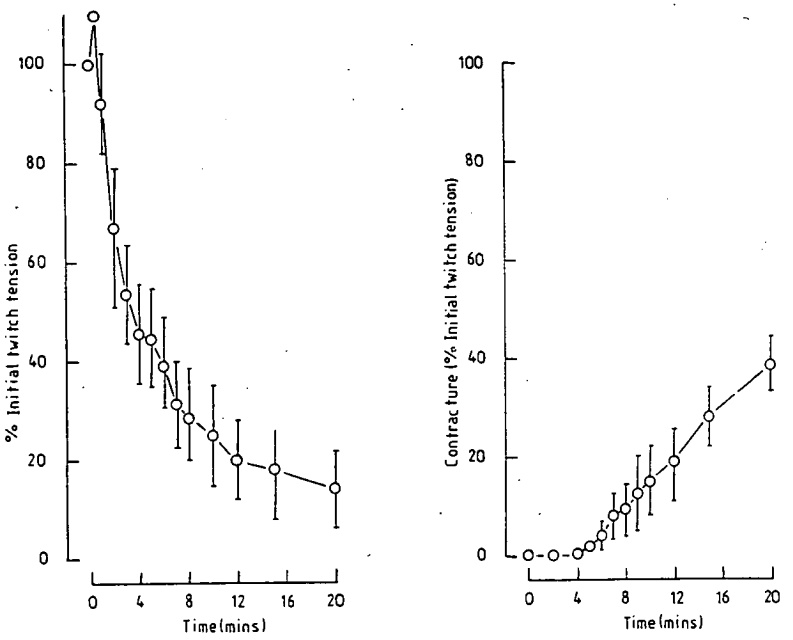
Legend

Experiment 238. Ordinate - isometric twitch tension nerve stimulation ● , resting tension ■ , and compound muscle action potential □ . Abscissa - time in minutes. 16mg/Kg bodv weight dinitrophenol injected between 0 and 10 minutes. 5c/sec stimulation 0 to 20 minutes.

Figure 21

Title

Dinitrophenol infusion with 5c/sec stimulation starting at the onset of infusion: group data.



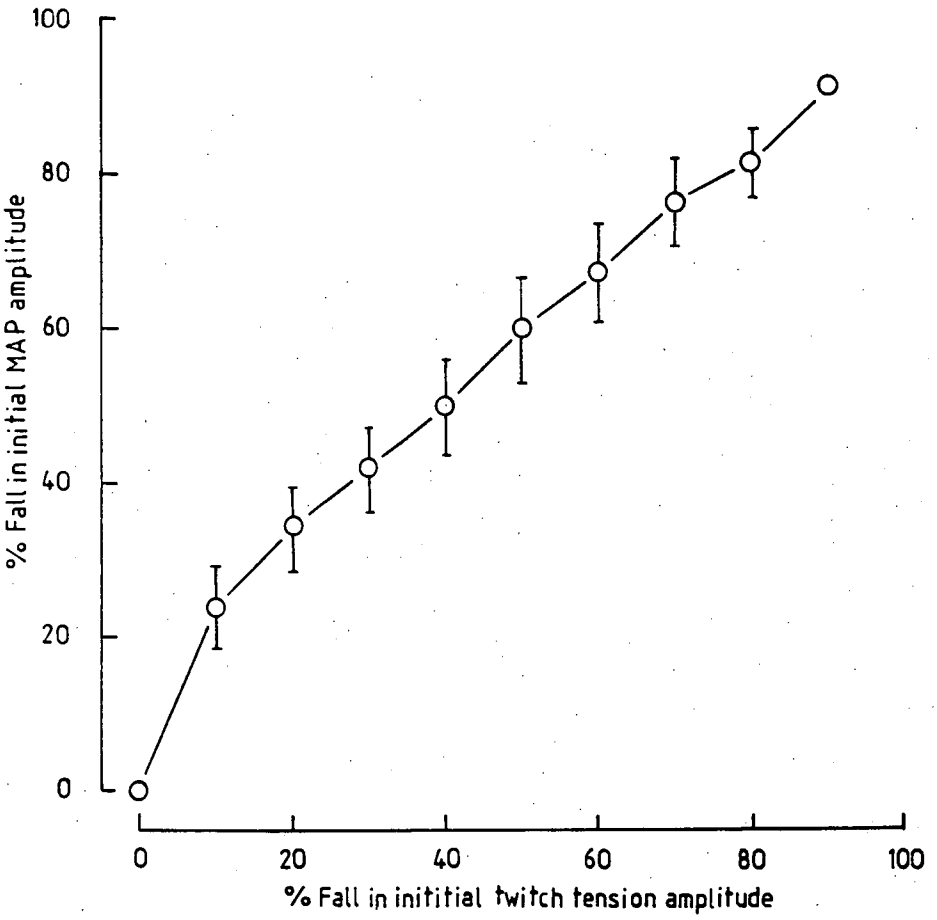
Legend

Ordinate - (left hand graph) % of initial twitch tension. (right hand graph) contracture (i.e. increase in resting tension as a % of the initial twitch tension.) Abscissa - time in minutes. N=6. Bars indicate standard error of mean. Continuous 5c/sec nerve stimulation and 16mg/Kg dinitrophenol infused between 0 and 10 minutes in all experiments.

Figure 22

Title

Dinitrophenol infusion with 5c/sec stimulation starting at the onset of infusion: relationship between action potential and twitch tension failure.



Legend

Ordinate - % fall in initial action potential amplitude. Abscissa - % fall in initial twitch tension amplitude. N=6. Bars indicate standard error of mean.

iv Dinitrophenol Infusion After Stabilisation of Twitch Tension with 5c/sec Stimulation

In a group of 12 experiments (7 with nerve, 5 with direct muscle activation) injection of dinitrophenol was delayed until a steady state twitch response was obtained, 6-8 minutes after the onset of stimulation. Either 3 or 4 boluses of 4mg/Kg DNP were given at 2-4 minute intervals. This protocol was designed firstly to assess the effect of preliminary depletion of energy stores (PCr, glycogen) on the rate of contracture development and secondly to investigate the effects of the uncoupler on muscle physiology in a stable preparation with a high dependency on energy obtained through oxidative phosphorylation. These animals were heavier than those used in earlier experiments (280-340g) and the mean initial twitch tension was 560 grams (SD 31.6g). Prior to injection of DNP this had stabilised at between 49 and 76% (mean 64%) of the initial level. Typical experiments with nerve and with direct muscle stimulation are shown in Figures 23a-f. After each bolus of uncoupler, the isometric twitch tension began to fall rapidly within 10 seconds. The time constant of this acute drop was fairly stable between different experiments and after successive boluses of DNP in the same experiment (mean 10.7sec SD 1.9 after the first bolus; 11.0sec SD 1.8 after the second bolus and 11.5 after the third bolus) N10. Twitch tensions rapidly stabilised at a lower level after the first bolus and then either remained constant or showed a slight recovery. With each subsequent injection

of DNP, the twitch tension amplitude plateaued out at successively lower levels and eventually failed completely. In 2 of the 12 experiments, this pattern was not seen, twitch tension falling steadily after the first injection of DNP.

The compound muscle action potential was unaffected by the first bolus in 2 experiments and fell by approximately one third of the fall in twitch tension (as a % of steady state level) in the remaining 5 experiments where continuous recordings of both parameters were made. After subsequent injections, the action potential amplitude steadily declined, but the time course differed from that seen with twitch failure in that the fall was steady rather than steplike. In experiments in which direct muscle activation was used, interpolated nerve stimuli revealed a similar pattern of twitch tension failure (Figure 23b).

Electrically silent contracture developed in all experiments. As at lower stimulus frequencies, this was preceded by a major fall in steady state twitch tension and it evolved most rapidly in the later stages of fatigue. The fall in twitch tension prior to the onset of contracture was greater than that in experiments where lower stimulus frequencies were employed. The mean twitch tension at the onset of contracture was 29.5% of the initial twitch tension (SD 12.3) and 44.5% of the steady state twitch tension (SD 16.6). Contracture evolved more rapidly than in muscles stimulated at the same frequency where DNP was injected at the commencement of the stimulus train (Figure 26). The final extent of

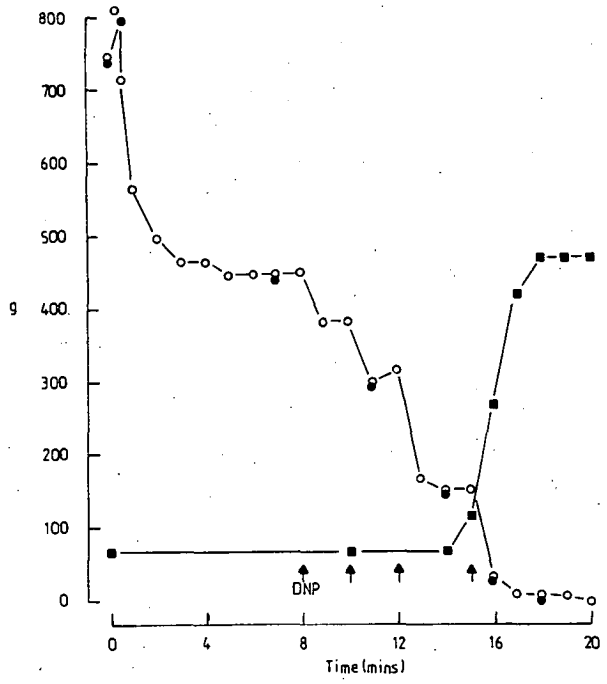
contracture tension was variable ranging from 50-95% of the initial twitch amplitude. In one experiment (Figure 23d) a further bolus of dinitrophenol given at a time when a twitch response was almost unobtainable (3% steady state value) and contracture tension had been stable for 10 minutes, resulted in an immediate acceleration of contracture.

A similar steplike pattern of twitch tension failure was seen in 3 experiments with soleus (Figure 27) and one with soleus and plantaris mounted together (Figure 28), where the same protocol was followed.

Figure 23a

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.



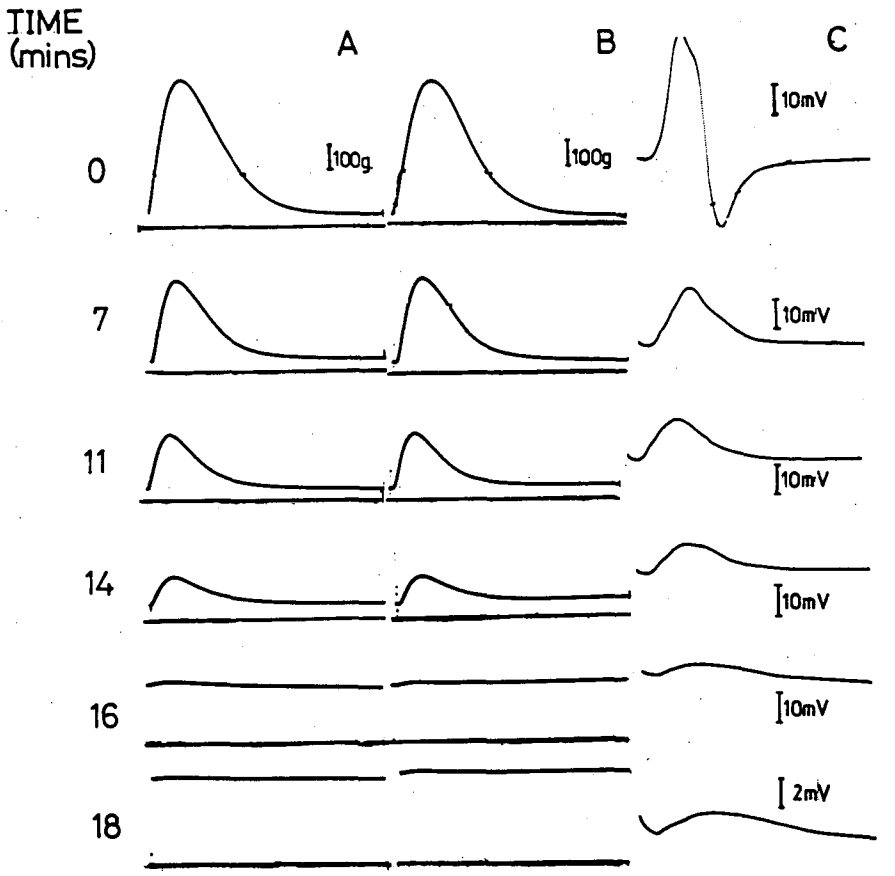
Legend

Experiment 244. Continuous 5c/sec muscle stimulation with single shocks via nerve stimulation interposed in the stimulus train. Ordinate - isometric twitch tension muscle stimulation ○ , isometric twitch tension nerve stimulation ● , and resting tension in grams. Abscissa - time in minutes. Dinitrophenol given in 4mg/Kg boluses as indicated by arrows.

Figure 23b

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.



Legend

Experiment 244. Selected isometric twitch tension responses with muscle stimulation (left), nerve stimulation (centre) and compound muscle action potential with nerve stimulation (left) recorded at the indicated times in minutes. The baseline position indicates the resting tension.

Figure 23c

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.

Legend

Experiment 244. Continuous recordings with slow scan speed showing changes in isometric twitch tension and in resting tension immediately after each bolus of dinitrophenol.

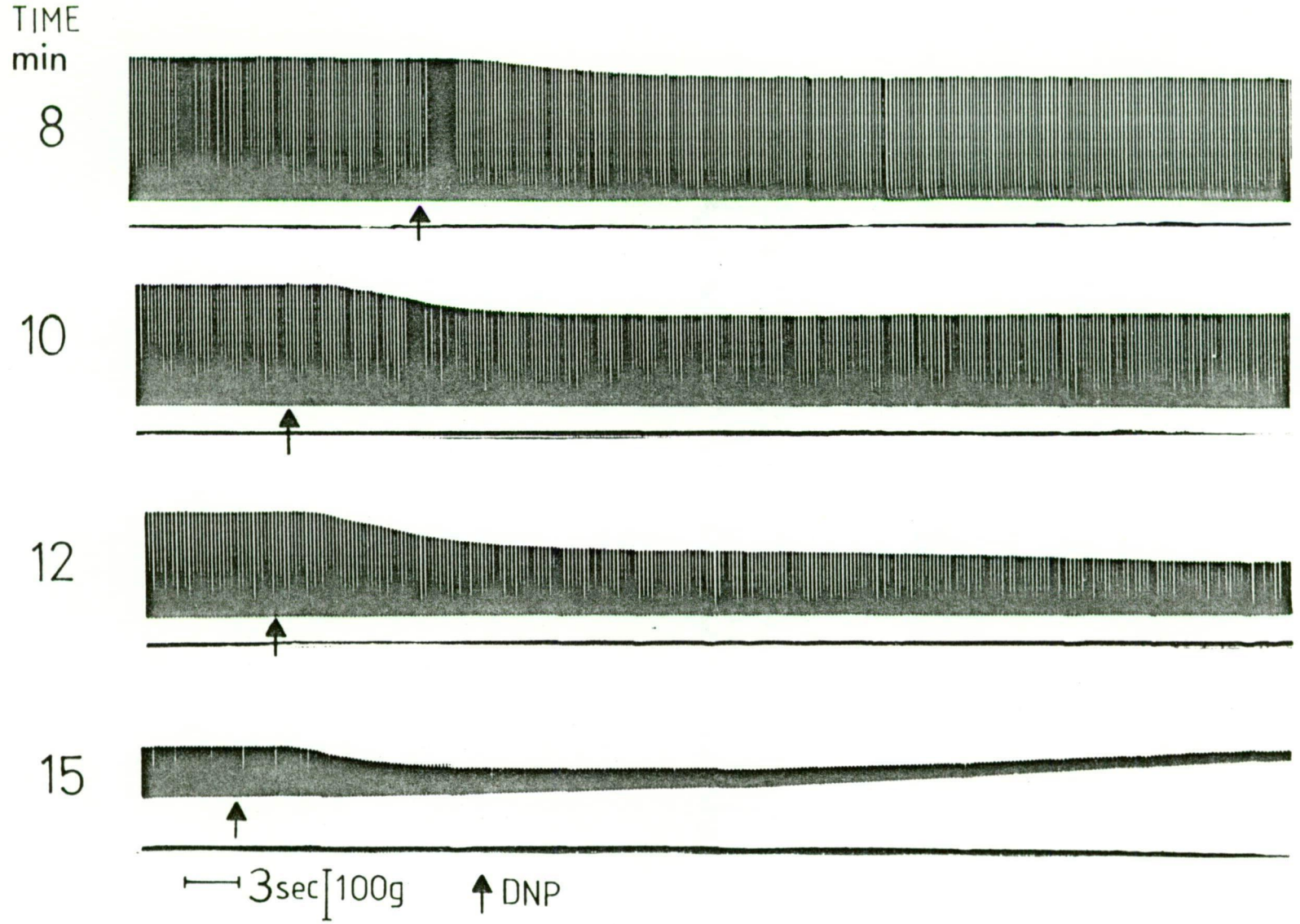
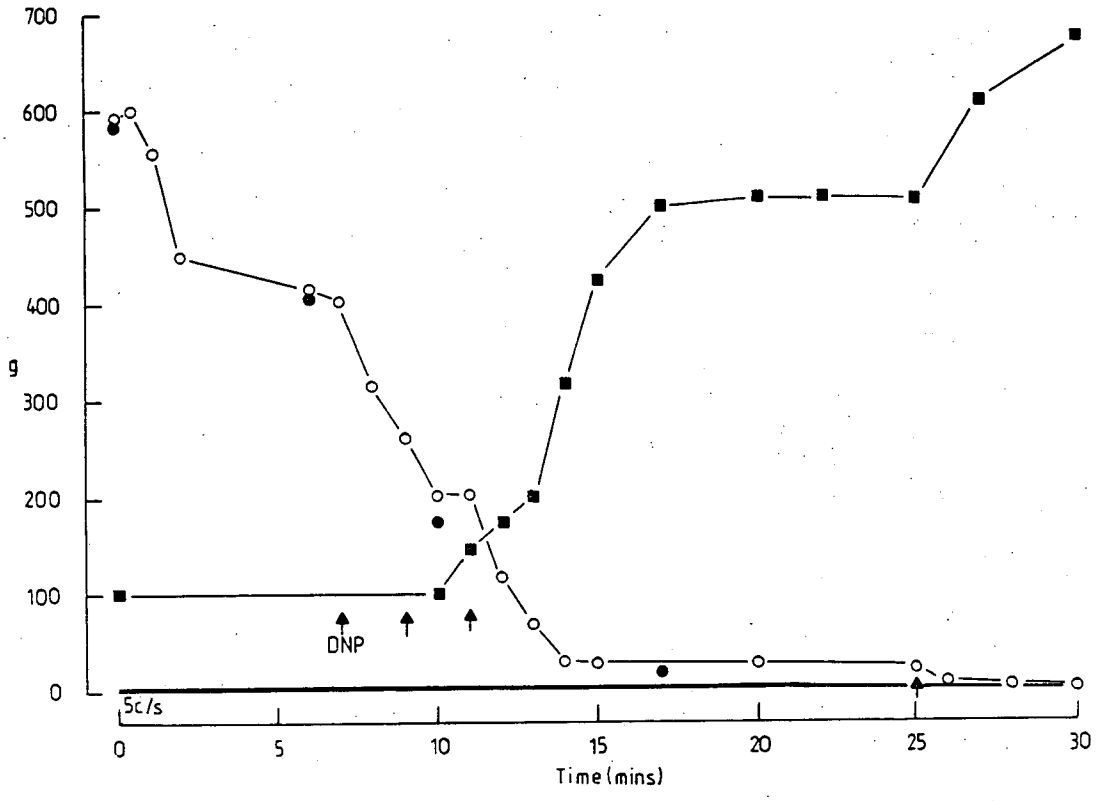


Figure 23c

Figure 23d

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.



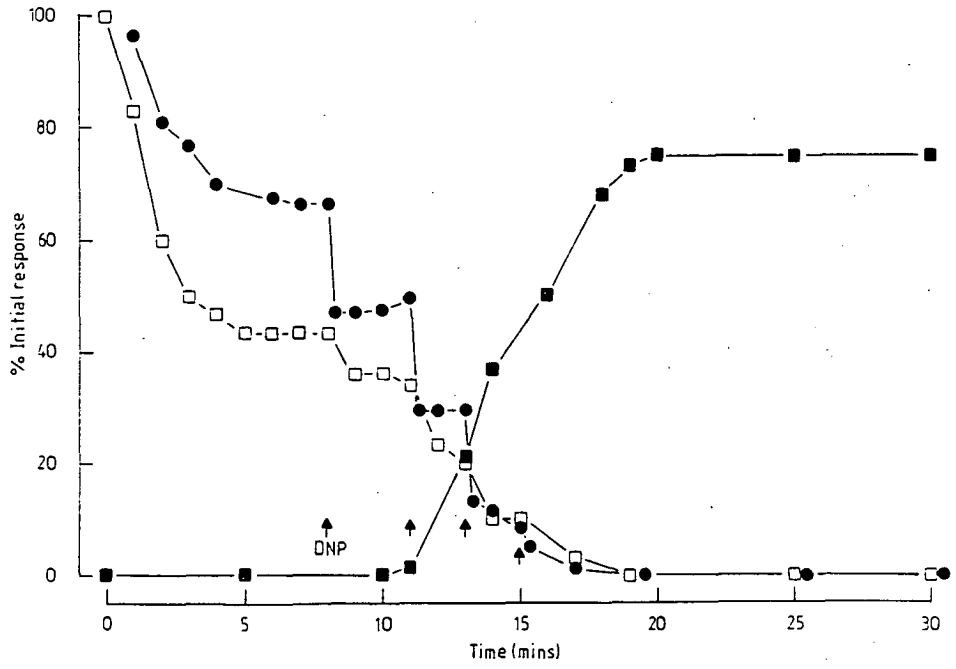
Legend

Experiment 247. Continuous 5c/sec muscle stimulation. Ordinate - isometric twitch tension muscle stimulation ○ , twitch tension nerve stimulation (with interpolated shocks) ● and resting tension in grams. Abscissa - time in minutes. 4mg/Kg doses of dinitrophenol are indicated by the arrows. Note the further acceleration of contracture development after the 4th bolus of dinitrophenol which was given at a time when the contracture tension appeared to have stabilised.

Figure 23e

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.



Legend

Experiment 249. Continuous 5c/sec nerve stimulation. Ordinate - % of initial isometric twitch tension ● , % of initial compound muscle action potential amplitude □ and increase in resting tension as a % of the initial twitch tension amplitude ■ . Abscissa - time in minutes. 5c/sec nerve stimulation from 0 to 30 minutes. Injections of 4mg/Kg of dinitrophenol are indicated by the arrows.

Figure 23f

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.

Legend

Selected recordings from experiment 249. Continuous records of the muscle action potential amplitude (A) and isometric twitch tension (B) are shown after each injection of dinitrophenol. (Each bin is approximately 45 seconds). The position of the baseline indicates the resting tension. Selected twitch and action potential responses recorded on a faster time base are shown in the lower part of the record. The time in minutes is indicated on the left of each bin.

Figure 23f

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: typical experiments.

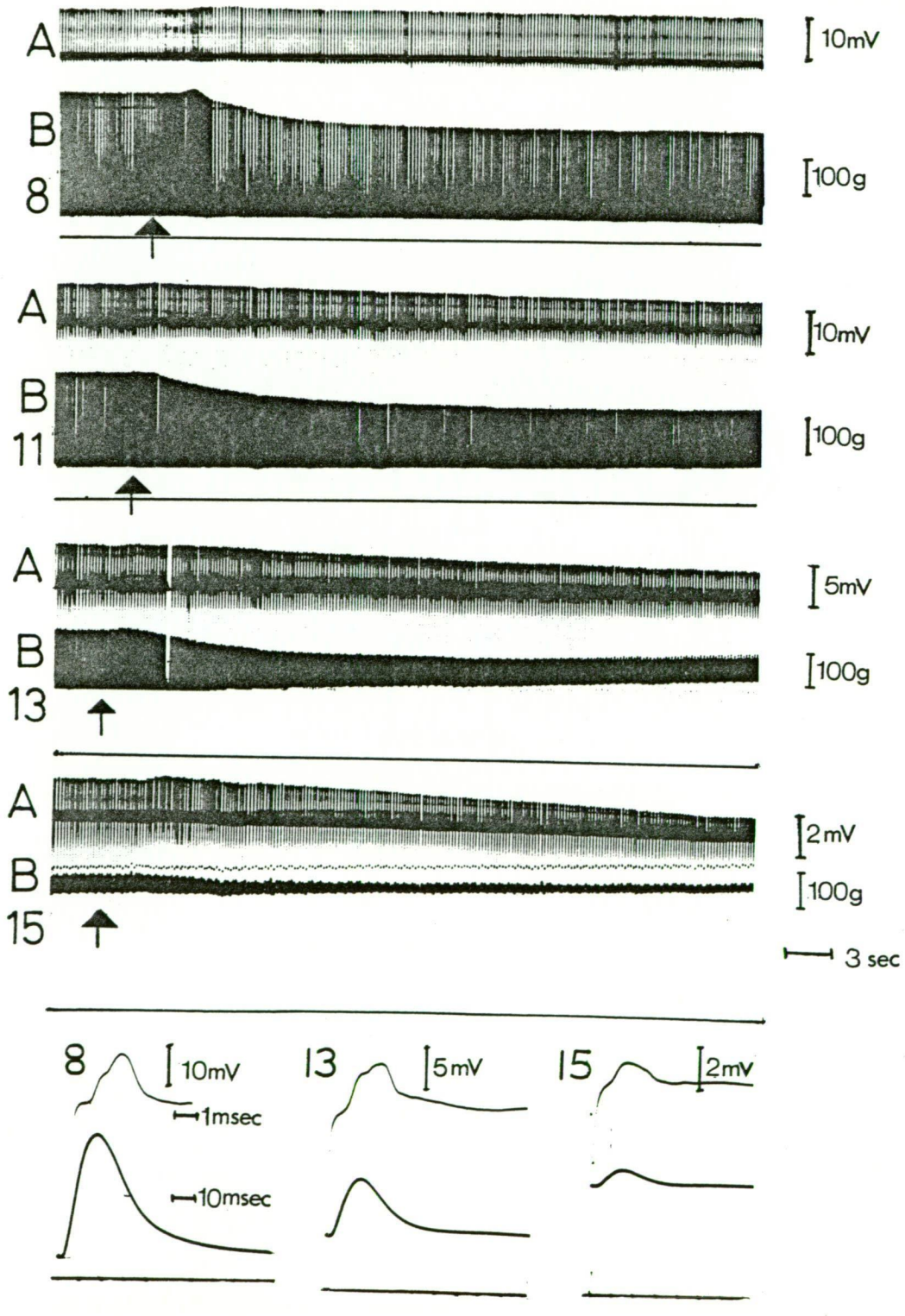
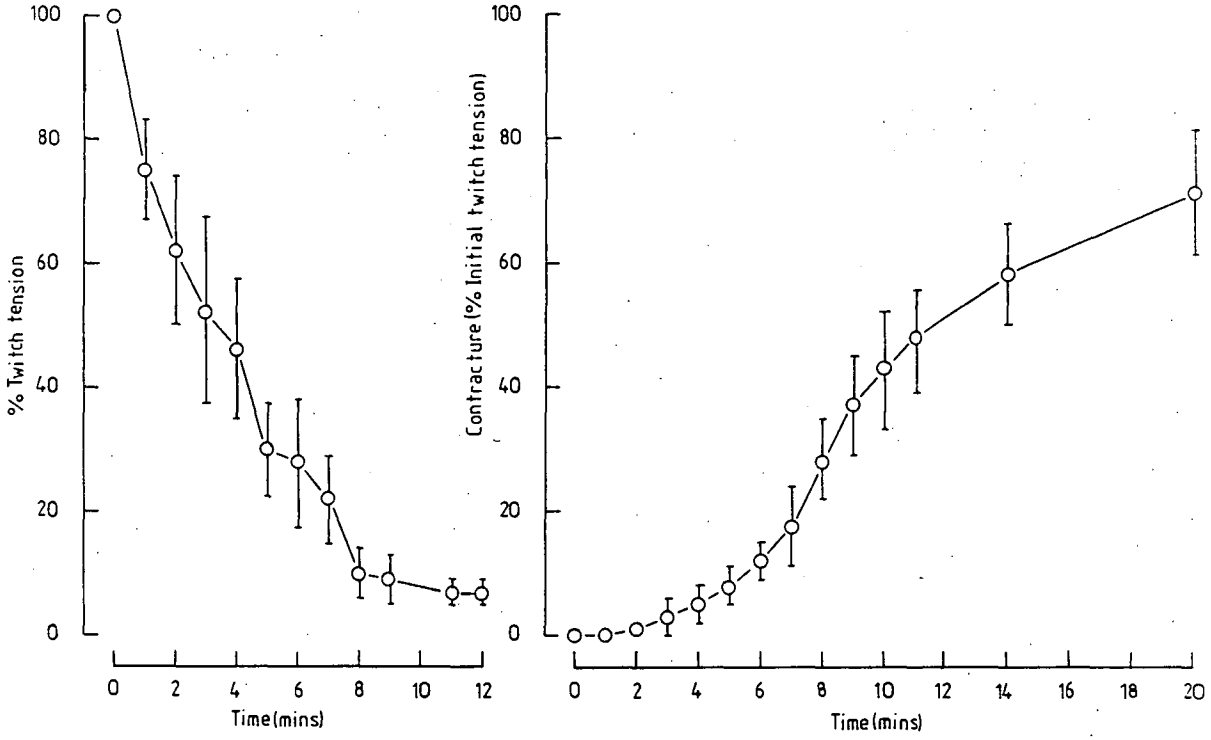


Figure 24

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: grouped results.



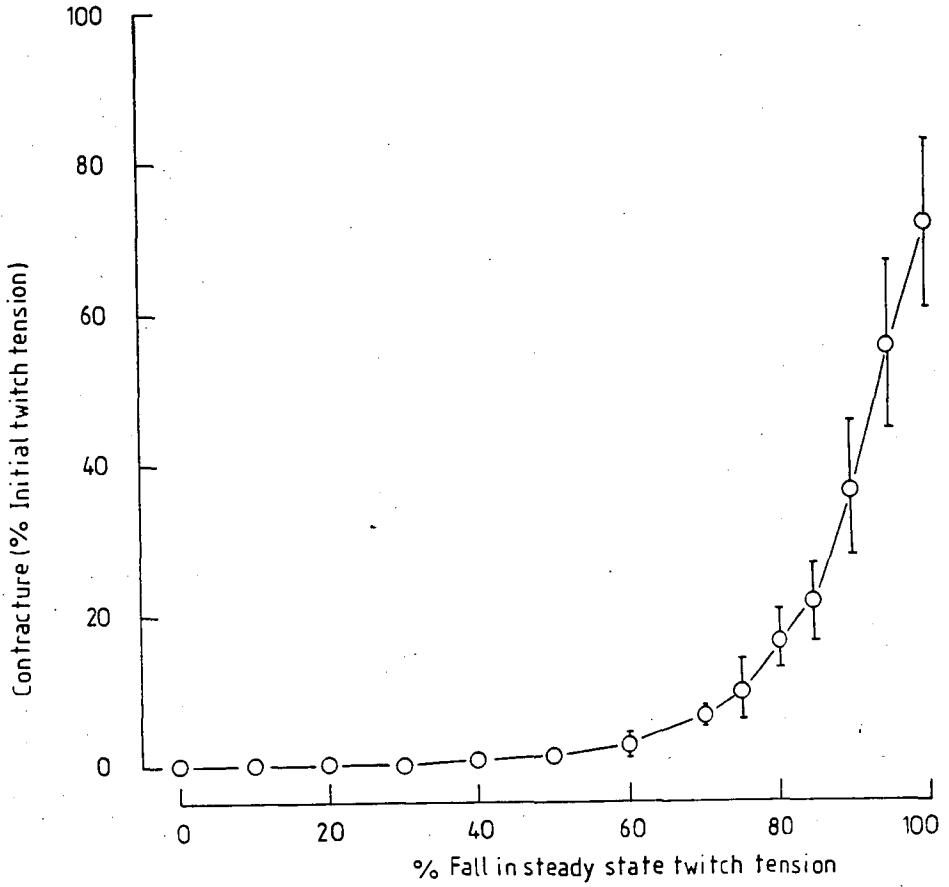
Legend

Ordinate - (left hand graph) % steady state twitch tension (right hand graph) increase in resting tension as a % of initial twitch tension. Abscissa - time in minutes. Bars indicate standard error of mean. The results represent group data from 5 experiments in which 16mg/Kg of dinitrophenol was infused over 10 minutes commencing 6 to 8 minutes after the onset of 5c/sec direct muscle stimulation at which time the twitch tension had stabilised (steady state value).

Figure 25

Title

Infusion of dinitrophenol after stabilisation of the twitch tension amplitude during 5c/sec stimulation: relationship between contracture evolution and twitch failure.



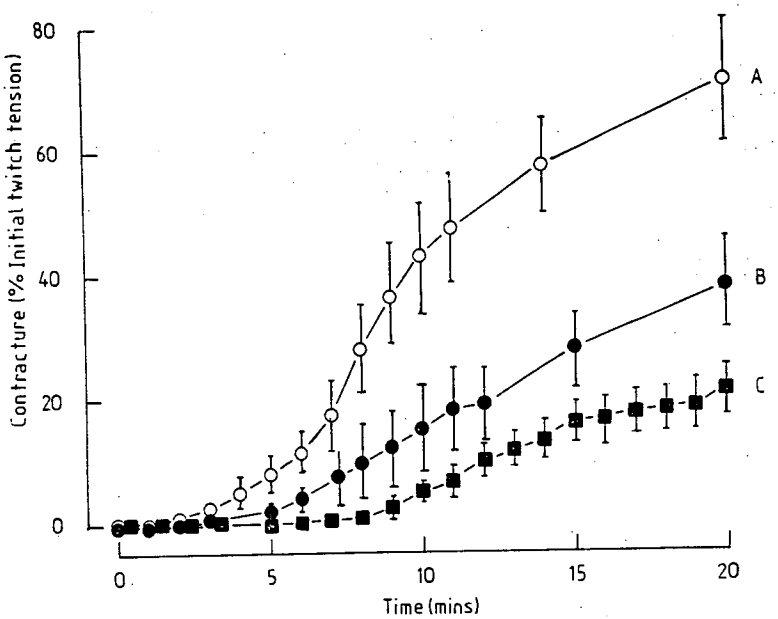
Legend

Ordinate - contracture tension (increase in resting tension as a % of the initial twitch tension). **Abscissa -** % fall in steady state twitch tension. **Bars** indicate standard error of mean. Data for 5 experiments in which 16mg/Kg of dinitrophenol was infused during 5c/sec muscle stimulation.

Figure 26

Title

Contracture evolution after dinitrophenol infusion during different stimulus paradigms.



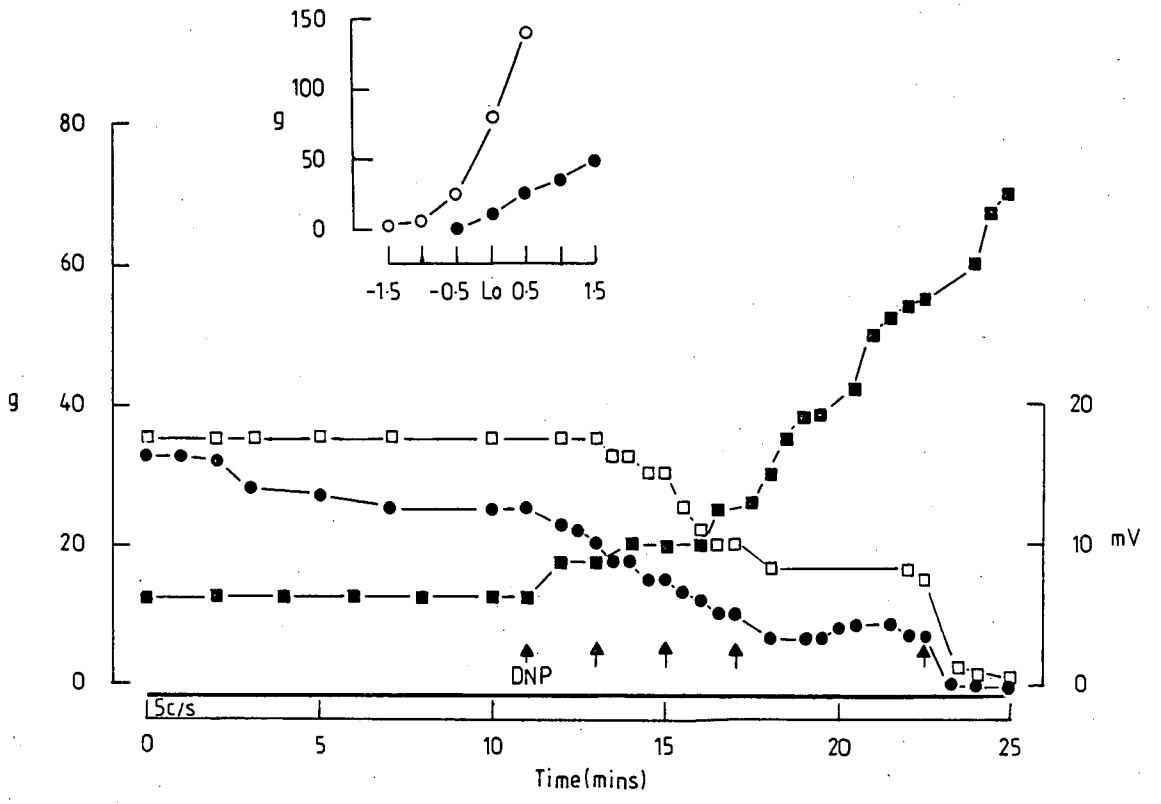
Legend

Ordinate - contracture tension (increase in resting tension as a % of the initial twitch tension). A. Delayed infusion of dinitrophenol 6 to 8 minutes after commencement of 5c/sec stimulation. (N=5). B. 5c/sec stimulation starting at the same time as dinitrophenol infusion. (N=6). C. 2c/min stimulation. (N=15). The bars indicate standard error of mean. 16mg/Kg of dinitrophenol was injected over 10 minutes in all experiments. The difference between A & B, and B & C is significant ($P > 0.001$) at 10 minutes (t test).

Figure 27

Title

Dinitrophenol infusion during 5c/sec stimulation: recording from soleus.



Legend

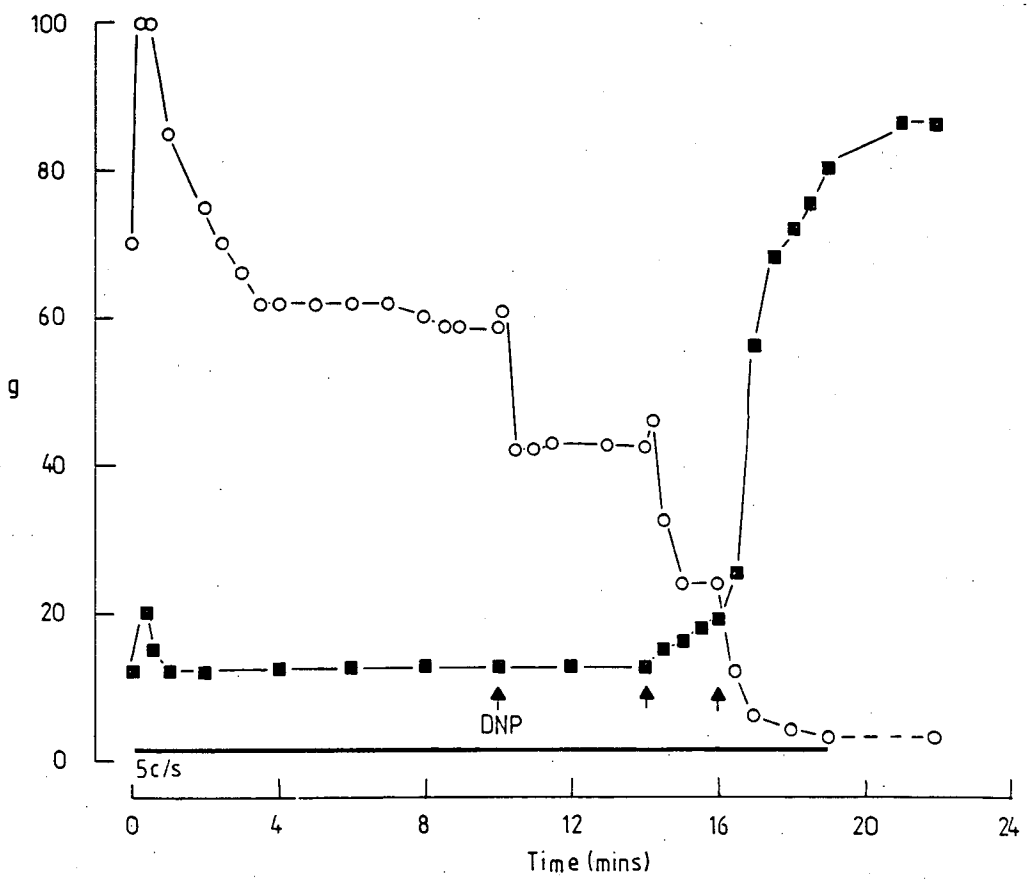
Experiment 107. Ordinate - isometric twitch tension nerve stimulation ● , resting tension ■ grams, and compound muscle action potential □ mV. Abscissa - time in minutes. 4mg/Kg doses of dinitrophenol are indicated by the arrows.

Insert. Resting tension length curves at the beginning ● and at the end of the experiment ○ . Lo indicates the initial resting length. In contracture the length tension curve is moved to the left.

Figure 28

Title

Dinitrophenol infusion during 5c/sec stimulation: recordings from the soleus and plantaris muscles.



Legend

Experiment 105. Ordinate - isometric twitch tension ○ and base line tension in grams ■ . Soleus and plantaris muscles recorded together. Dinitrophenol injected in 4mg/Kg boluses as indicated by the arrows. 5c/sec stimulation from 0 to 19 minutes. The initial rise in resting tension between 0 and 2 minutes is due to fusion. Fusion was excluded by brief interruption of the stimulus train as contracture evolved.

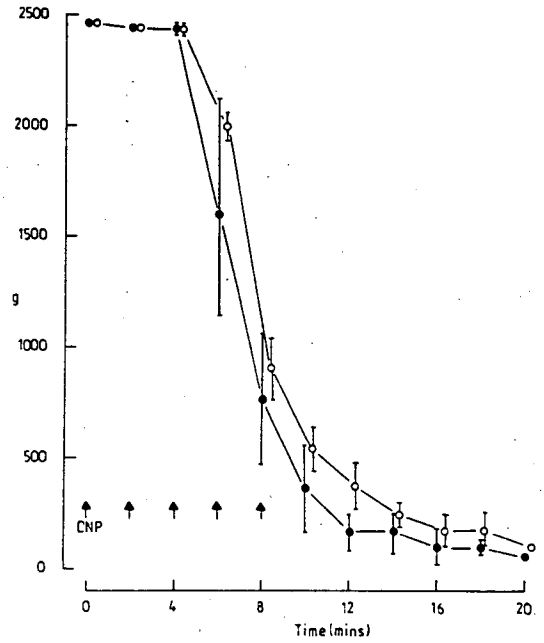
v Dinitrophenol. Tetanic Stimulation

The gastrocnemius was activated by brief tetanic stimulations (100c/sec for 2 secs) in 4 animals. In 2 experiments, a nerve tetanus followed after 5 secs by a direct muscle tetanus was given every 2 minutes and in 2 experiments, nerve and muscle tetanic stimulations were carried out at alternate 1 minute intervals. A fall in tetanic tensions was seen after DNP infusion with both stimulus paradigms (Figures 29a-d). Peak tetanic tension fell more rapidly with nerve than with direct muscle stimulation. In addition tension during individual stimulus trains were better maintained during direct muscle activation than with nerve stimulation.

Figure 29a

Title

Dinitrophenol infusion: tension changes during brief tetanic stimulations.



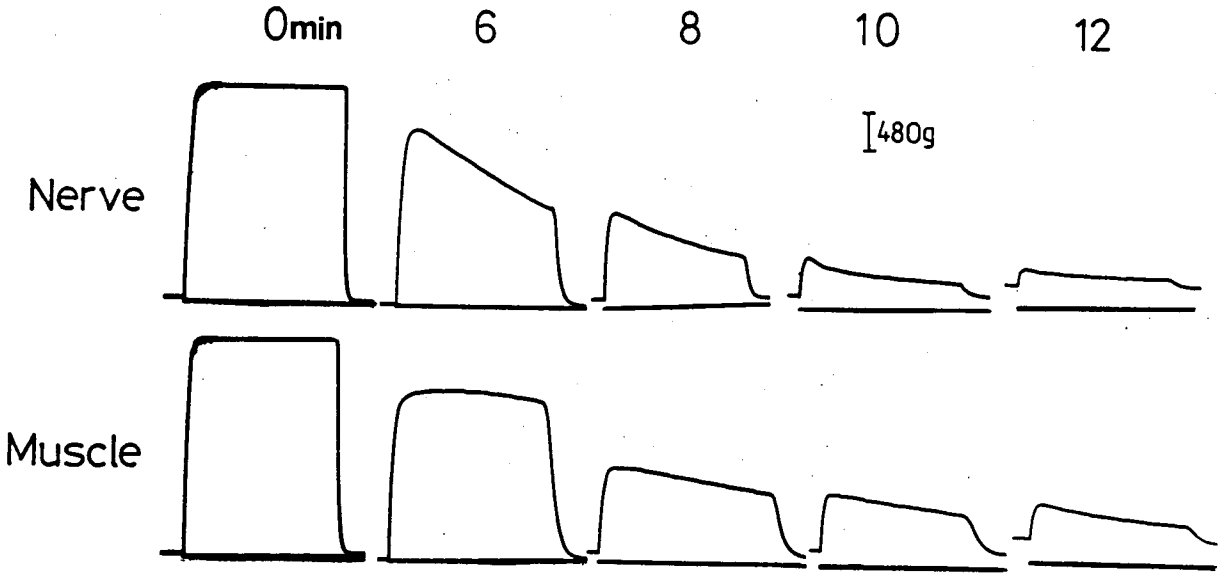
Legend

Experiment 247. Ordinate - tetanic tension with nerve ● and with direct muscle stimulation ○ . The bars indicate peak and end tetanic tensions. Stimulation at 100c/sec for 2 seconds every 2 minutes by nerve stimulation was followed 5 seconds later by a similar tetanic stimulation by direct muscle stimulation. Boluses of 4mg/Kg of dinitrophenol are indicated by the arrows.

Figure 29b

Title

Dinitrophenol infusion: tension changes during brief tetanic stimulations.



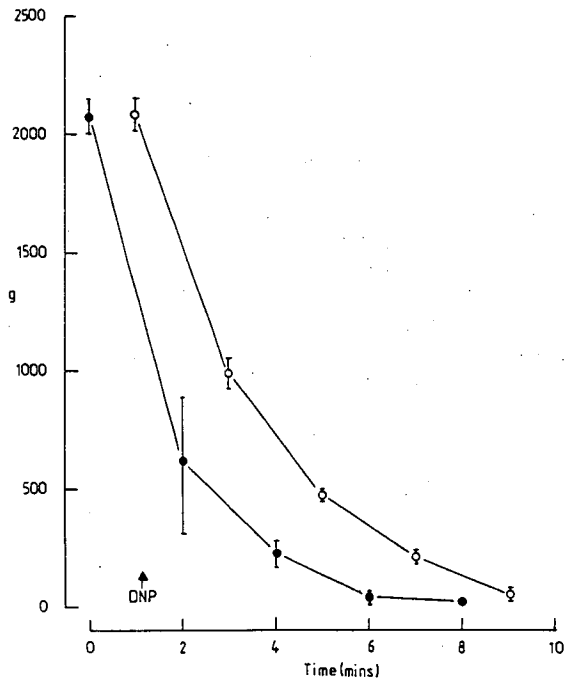
Legend

Experiment 247. Isometric tetanic tension records with nerve stimulation (upper traces) and with direct muscle stimulation (lower traces) at the indicated times in minutes. Note rise in baseline tension as tetanic tensions fail.

Figure 29c

Title

Dinitrophenol infusion: tension changes during brief tetanic stimulations.



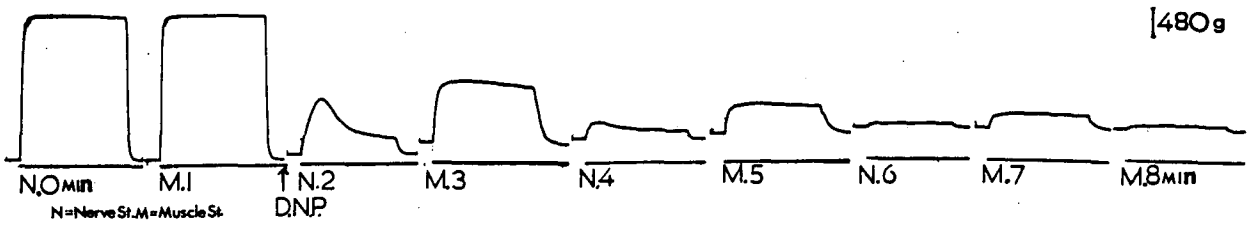
Legend

Experiment 250. Ordinate - tetanic tensions with nerve ● and with direct muscle stimulation ○ . The bars indicate the peak and end tetanic tensions. Nerve and direct muscle stimulation (100c/sec for 2 seconds) were carried out alternatively at 1 minute intervals.

Figure 29d

Title

Dinitrophenol infusion: tension changes during brief tetanic stimulations.



Legend

Experiment 250. Tetanic tension record with nerve and muscle stimulation at 1 minute intervals. 12mg/Kg dinitrophenol injected as a single bolus at 1 minute.

DISCUSSION

The physiological effects of dinitrophenol on rat gastrocnemius in vivo are complex. The biphasic relationship between the fall in twitch tension and muscle action potential amplitude at very low frequencies (see Figures 12 & 18) and the different patterns of mechanical and electrical failure observed when uncoupler was injected during steady state 5c/sec stimulation (see Figure 23) suggest a primary failure of muscle contractility or defective excitation contraction coupling. Considerable overlap between the fall in twitch tension and action potential amplitudes was found, however, and sarcolemmal inexcitability may contribute to the later stages of twitch failure.

The steplike fall in twitch tension observed with each bolus of DNP at 5c/sec suggests that there is a considerable range of muscle fibre impairment, some fibres continuing to contract normally after others have failed. The rapid post injection drop in twitch tension reflects the high dependancy of the steady state twitch response on energy provided through oxidative phosphorylation. The rate of ATP synthesis by functionally intact mitochondria may determine the level at which the twitch tension amplitude eventually stabilises after each bolus. Glycolysis is activated by dinitrophenol in vitro (Barnes et al 1955) and addition of very high concentrations of glucose to a bath in experiments with smooth muscle has resulted in some functional recovery (Patton 1969).

Accelerated glycolysis is likely to be of only transient importance however, expecially in a muscle partially depleted of glycogen reserves by preliminary stimulation.

An alternative hypothesis can be invoked to explain the steplike fall in twitch tensions observed. It ia possible that subsarcolemmal mitochondria are more susceptible to the action of the uncoupler both by virtue of their superficial anatomical location, and also because in normal fibres they are probably more loosely coupled than intermyofibrillar mitochondria (Hulsmann 1962, 1970). Each injection of DNP can then be envisaged as producing a wide spectrum of mitochondrial involvement in each fibre. Fractional force failure might then follow a selective failure of peripheral myofibrils while centrally placed contractile units are still functional. As free ATP appears to diffuse readily throughout the fibre (Podolski 1966), this hypothesis is less attractive.

The half relaxation times either remained stable or prolonged slightly as the twitch amplitude fell in all but one experiment where a marked prolongation was seen. Prolongation in tension relaxation times, is an established finding in muscle fatigue (Feng 1936; Mosso 1915). This phenomena has been studied in vitro in mouse muscle poisoned with metabolic inhibitors by Edwards et al (1975), who presented evidence that it is closely related to a fall in ATP levels, and follows a reduction in the rate of cross bridge

cycling. Dawson et al (1980) found a closer relationship with the free energy of ATP hydrolysis available for calcium reuptake by the sarcoplasmic reticulum. It is surprising, therefore, that a greater prolongation in relaxation times was not seen in DNP poisoned muscle. This finding can be explained if the time interval between the onset of a state of energy depletion and complete twitch tension failure in individual fibres is very short, with the result that at any one time only a small number of compromised fibres are contributing to the twitch response.

Dintirophenol induced contracture in isolated skeletal muscle in vitro has long been recognised (Barnes et al 1955; Cori and Cori 1936; Weeks and Chenworth 1952). A similar evolution of contracture was seen in vivo in these experiments. Confinement of contracture to the hind limbs and tail suggests that intraarterial administration is effective in producing high local tissue concentrations. The clear differentiation between the onset of isometric twitch tension failure and contracture in all experiments, with the former falling by up to 80% of the initial level before contracture began, implies that in any one fibre of myofibril the processes of twitch failure and contracture are not intimately related. Failure of the twitch response is probably followed by a period in which the fibre or myofibril is relaxed before contracture evolves. The final extent of contracture was quite variable, (a similar finding to that noted by Barnes

et al in diaphragm preparations in vitro), suggesting that either a variable number of fibres enter contracture or that there is a considerable range in the number of rigor complexes formed in each fibre (see Weber and Murray 1974). The rate of contracture development is accelerated by increasing doses of dinitrophenol, by increased work loads and by antecedent depletion of energy stores. These observations confirm the close relationship of this phenomena to the energy status of the fibre.

A small prolongation in the latency to take off of the action potential was seen late in the course of twitch tension failure. It was not defined further whether this was due to slowing of nerve conduction, delayed neuromuscular transmission or slowed muscle membrane conduction. However, as similar twitch tensions were obtained with nerve and with direct muscle stimulation at low stimulus frequencies, it is clear that neuromuscular failure did not influence the pattern of twitch decay.

With tetanic stimulations the rate of peak tension failure in successive tetani was more rapid with nerve than with muscle stimulation. In addition the force generated during each individual tetanus with nerve stimulation fatigued more rapidly. These findings suggest a neuromuscular transmission failure is unmasked at tetanic stimulus frequencies.

1.1.4 Experiments with Intra-arterial Injection of Diphenyleneiodonium

Diphenyleneiodonium was injected after the twitch response had stabilised during 1 or 5c/sec stimulus patterns.

Clinical Observations

The respiratory pattern was regular and no rigidity was evident in unstimulated muscles over recording periods of up to 5 hours. In early experiments 60-90 minutes after injection of DPI the trunk surface temperature dropped several degrees. This was prevented in subsequent animals by external heating. The muscle surface temperature was maintained between 26° and 30°C. The venous blood sugar level (tail vein) was estimated at 15-20 minute intervals after injection of drug using an ames strip. In preliminary experiments hypoglycaemia (<1.4mMol/L) developed between 60-90 minutes after injection of 2mgs of DPI. This was prevented by dissolving the inhibitor in 5% dextrose and by injecting 50ul boluses of 5% dextrose at approximately 20 minute intervals. The general state of the animals remained stable throughout recording. In 3 preparations arterial blood gas estimations were carried out 90 minutes after injection of DPI. The mean pH was 7.17, mean P_aO_2 17.3 Tor and mean P_aCO_2 4.3 Tor. As the mean pH level in control specimens was 7.26, a severe systemic acidosis did not develop.

Blood Flow Studies

Muscle blood flow using the hydrogen washout technique was followed in resting muscle in one experiment after injection of 2mg of DPI. The mean initial blood flow was 34.6 $\pm$ 10.9mls/100g muscle, (average of 2 readings at each of 5 sites). Ten minutes after injection of 500ul of DPI washed through with 100ul of 5% dextrose, the blood flow was 28.0 $\pm$ 9.5 mls (N5) and after 30 minutes, it was 27.5 $\pm$ 9.8mls (N5). These differences are not significant with this technique.

Recording of the Mechanical and Electrical Responses

I. 1c/sec Stimulation (N15).

1c/sec stimulation was continued for 60 minutes prior to infusion of DPI. A protocol of direct muscle activation interposed with single nerve stimuli was used in 6 experiments. In 9 animals nerve activation interposed with single muscle stimuli was employed. The same sequence of twitch tension and muscle action potential amplitude failure was seen with both stimulus protocols and the results are considered together. Mean initial twitch tension amplitude was 468 grams (SD 69) and this stabilised at between 67 and 85% of the initial value (mean 76%). Two mg of DPI in 500ul of 5% dextrose was infused over 1 minute. Isometric twitch tension fell within 2-5 minutes of injection. The rate of twitch failure after DPI

was very variable. In 6 preparations, the twitch tension amplitude fell rapidly after the first injection of DPI, whereas in 6 other experiments, by contrast, the average fall in twitch amplitude was less than 20% of the steady state value 30 minutes after injection. In these experiments injection of a further 1mg (in 250ul) bolus resulted in a marked acceleration of twitch fatigue (Figure 30). In the remaining 3 experiments a third 1mg injection after a further 10 minutes was necessary to induce a fall in twitch tension to less than 20% of the steady state value. Typical experiments are illustrated in Figure 31a-d. Marked prolongation in the relaxation phase of the twitch response accompanied force failure. The mean half relaxation time of 19msec pre-injection, (steady state value) more than doubled as the twitch amplitude fell to 20% of the steady state value (Figure 32). A small prolongation in twitch tension rise time was also seen (Figure 32). Substantial recovery in twitch tension amplitude developed with rest. In 5 experiments, in which the muscle was rested for between 60 and 75 minutes, mean twitch tension at cessation of stimulation was 74 grams (range 10-140) having fallen from a steady state value of 324 grams (range 250-380). In these animals twitch tension recovered to a mean of 160 grams (range 60-280). The extent of recovery was greater in muscles in which the twitch response had been less severely depressed. Restimulation rapidly led to tension failure (Figure 31).

Resting tension levels were not recorded in 2 experiments. In 3 others followed for between 30 and 90 minutes after injection of iodonium no rise in resting tension

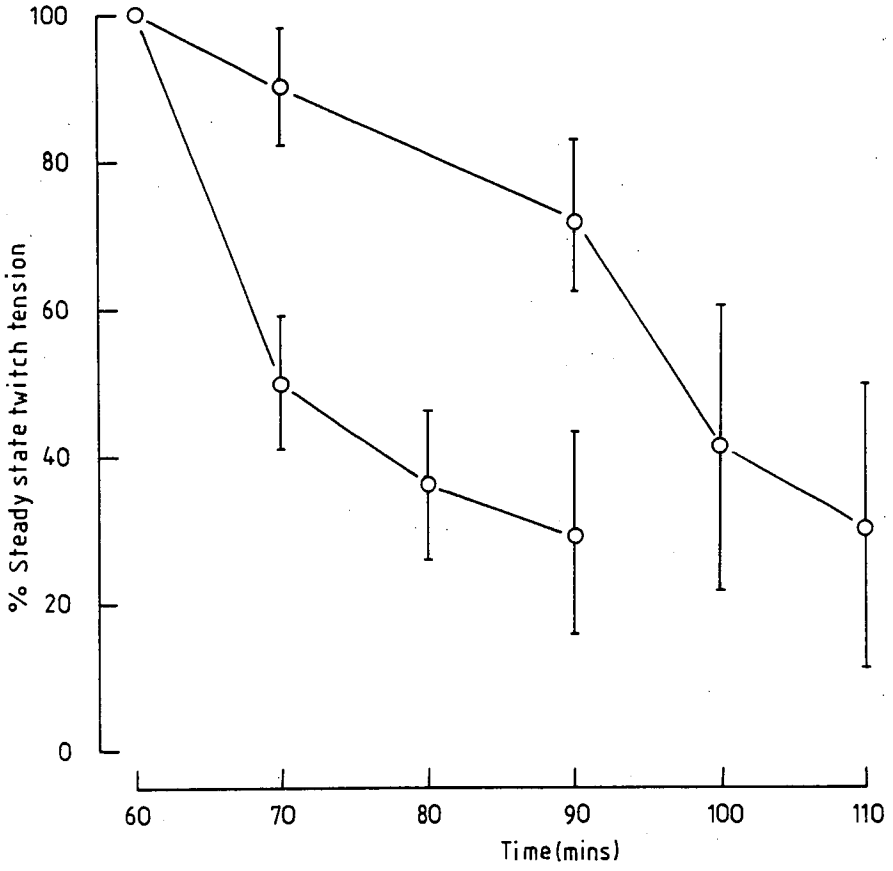
developed despite a fall in twitch tension to a mean of 20% of the steady state value. Complete force failure without contracture was seen in one experiment. An electrically silent rise in resting tension developed eventually in the remaining 10 experiments in this group (Figures 33 & 34), although there was no precise correlation with the dose of DPI administered. Evolution of contracture in all 10 experiments is shown in Figure 33 and in selected individual experiments in Figure 34. Mean twitch tension at the onset of contracture was 25.9% of the steady state value. Contracture evolution was less closely related to twitch failure than in DNP experiments, twitch tension stabilising at lower levels before the resting tension began to rise Contracture evolved in resting muscle in 4 experiments at a time when the twitch tension was slowly recovering (Figure 31).

Compound muscle action potential amplitude fell in parallel with twitch tension (Figure 35). It was never possible to record an action potential in any experiment after disappearance of the evoked mechanical response.

Figure 30

Title

Isometric twitch tension failure after injection of diphenyleneiodonium after stabilisation of the twitch response during a 1c/sec stimulus pattern.



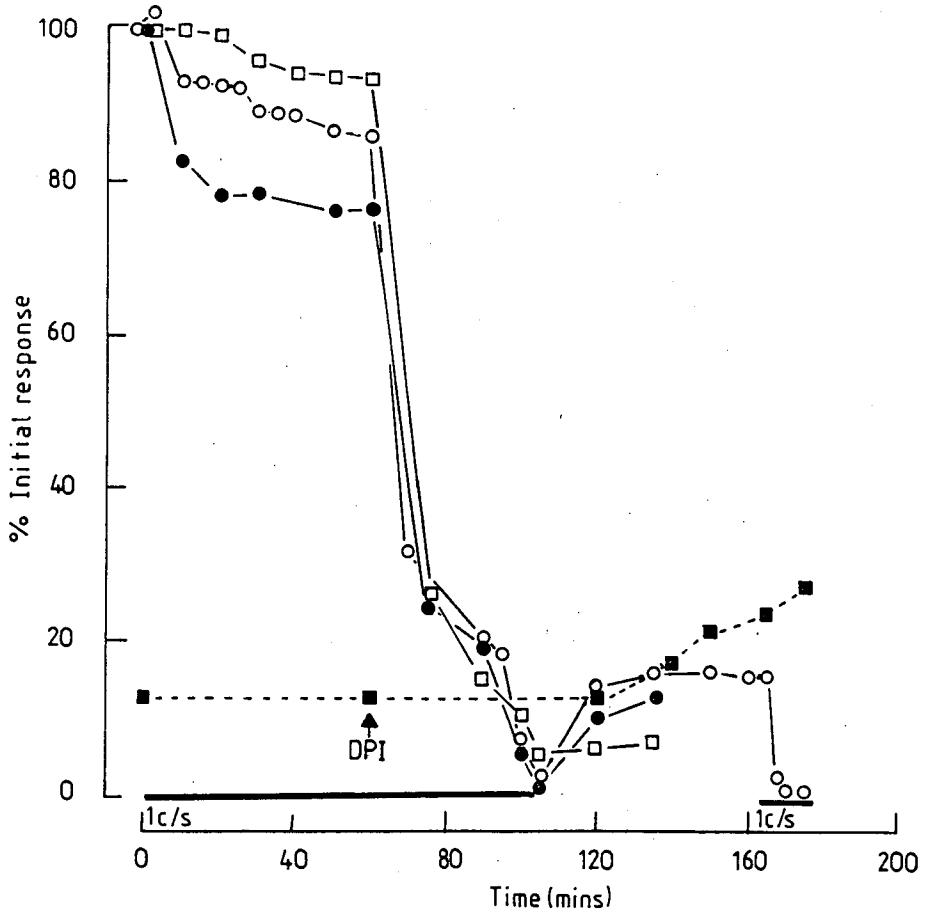
Legend

Ordinate - % steady state twitch tension (stable twitch tension after 60 minutes 1c/sec stimulation). Abscissa - time in minutes. The lower line represents force failure in a group of 6 experiments given a single injection of 2mg of diphenyleneiodonium at 60 minutes. The upper line represents twitch changes in a group of 6 experiments in which the injection of 2mg of diphenyleneiodonium at 60 minutes led to a less severe force failure and a second injection of 1mg was given at 90 minutes. The bars indicate standard deviation. There is considerable variation in the rate of force failure after a similar dose.

Figure 31a

Title

Infusion of diphenyleneiodonium during a 1c/sec stimulus pattern.



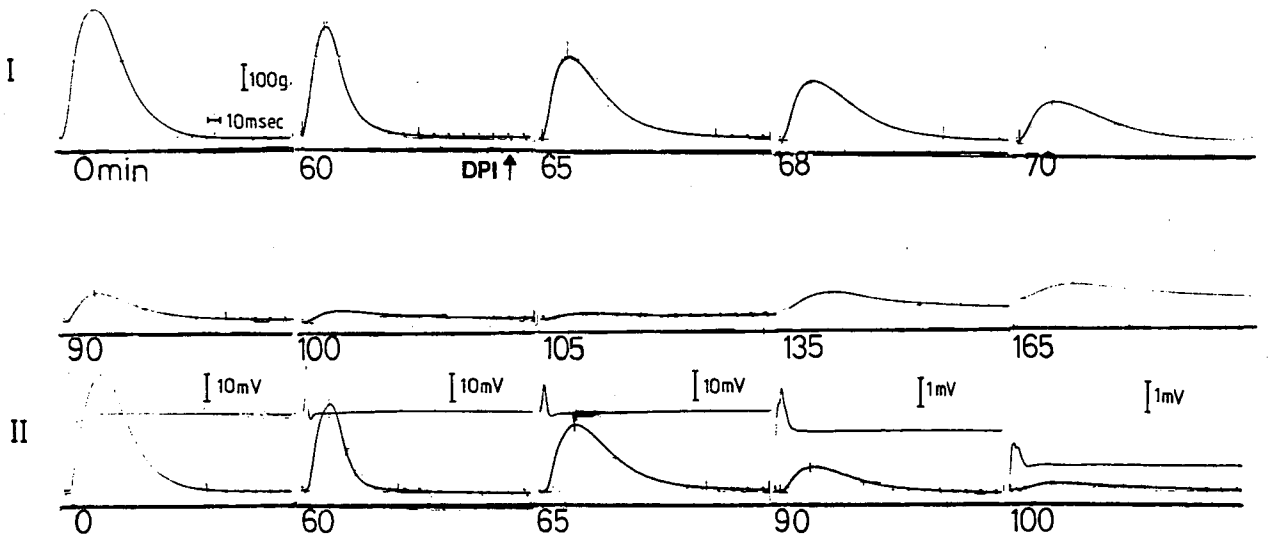
Legend

Experiment 49. Ordinate - % of initial amplitude, isometric twitch tension nerve stimulation (interpolated shocks) ● , isometric twitch tension direct muscle stimulation ○ , muscle action potential (nerve stimulation) □ and resting tension (expressed as a % of initial twitch tension amplitude) ■ . 1c/sec direct muscle stimulation from 0 to 105 and 165 to 175 minutes. 2mg of diphenyleneiodonium were infused between 60 and 61 minutes.

Figure 31b

Title

Infusion of diphenyleneiodonium during a 1c/sec stimulus pattern.



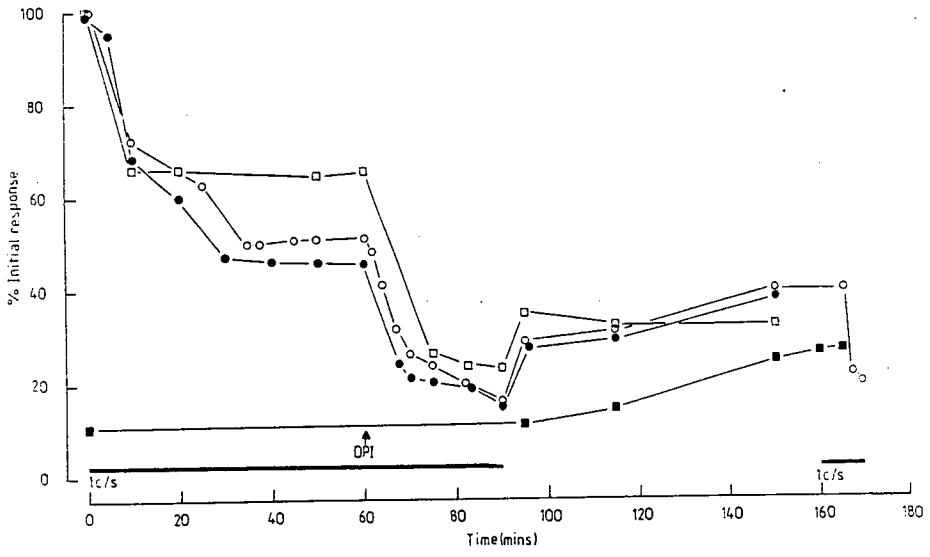
Legend

Experiment 49. Selected isometric twitch tension responses with muscle stimulation (I) and twitch and muscle action potential responses with interpolated nerve stimuli(II) recorded at the times indicated in minutes. The baseline position indicates the resting tension.

Figure 31c

Title

Infusion of diphenyleneiodonium during a 1c/sec stimulus pattern.



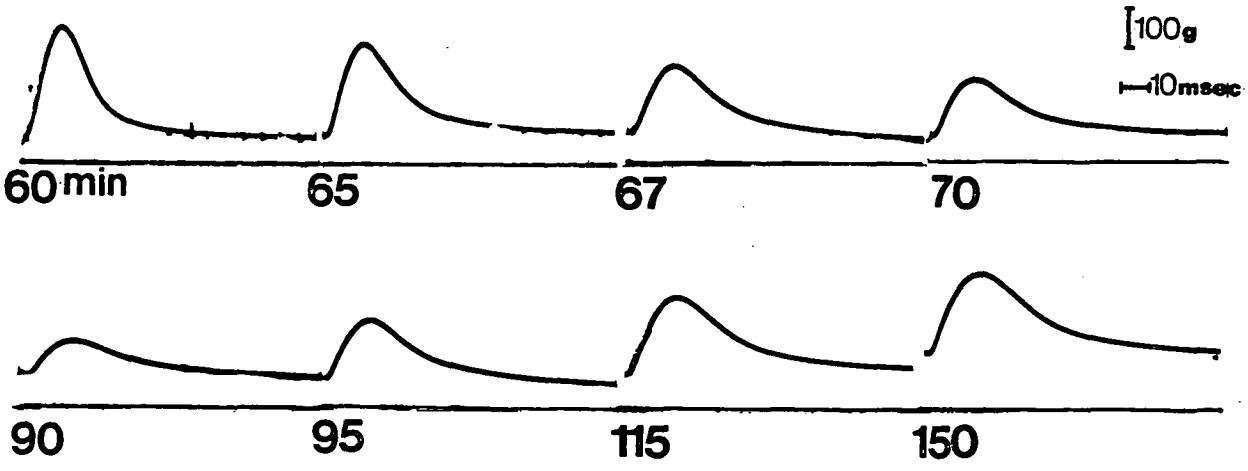
Legend

Experiment 35. Ordinate - % of initial amplitude, isometric twitch tension nerve stimulation (interpolated shocks) ● , isometric twitch tension direct muscle stimulation ○ , muscle action potential (nerve stimulation) □ and resting tension (expressed as a % of initial twitch tension amplitude) ■ . Continuous 1c/sec muscle stimulation from 0 to 90 and from 160 to 170 minutes with single shocks via nerve stimulation interposed. 2mg of diphenyleneiodonium was infused between 60 and 61 minutes.

Figure 31d

Title

Infusion of diphenyleneiodonium during a 1c/sec stimulus pattern.



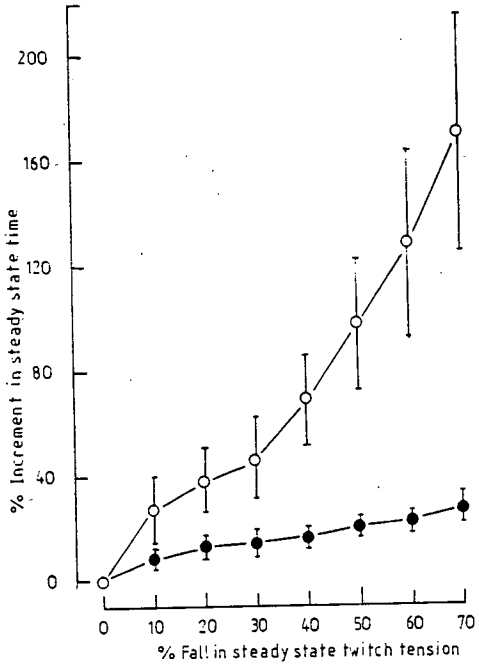
Legend

Experiment 35. Selective isometric twitch responses with direct muscle stimulation recorded at the times indicated in minutes. Note the progressive fall in amplitude and prolongation of twitch relaxation after injection of diphenyleneiodonium at 60 minutes. After cessation of stimulation at 90 minutes there is a partial recovery of twitch tension amplitude associated with an increase in resting tension.

Figure 32

Title

Infusion of diphenyleneiodonium during a 1c/sec stimulation: twitch characteristics.



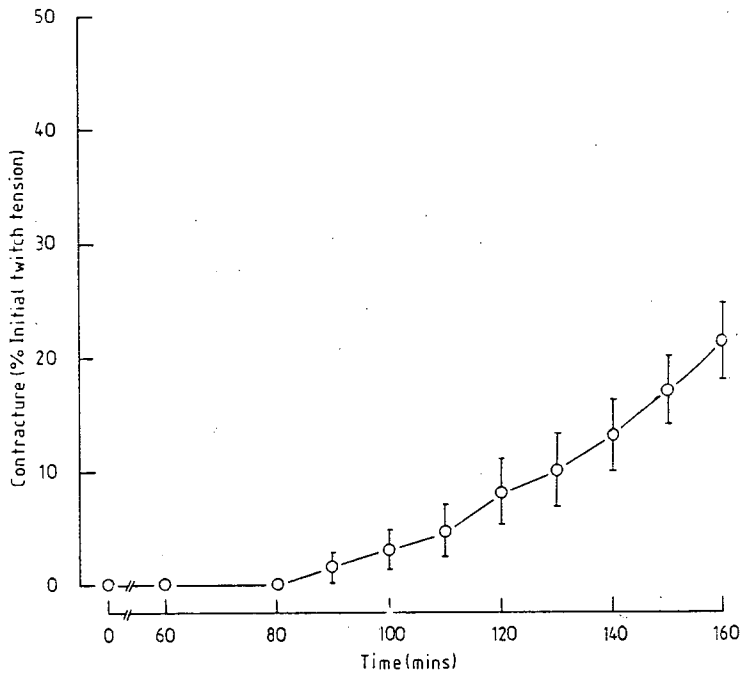
Legend

Ordinate - % increase in steady state half relaxation time ○ and peak time ● . Abscissa - % fall in steady state twitch tension. N=12. Bars indicate standard error of mean. (steady state refers to the value after 60 minutes 1c/sec stimulation prior to injection of diphenyleneiodonium). (Twitch characteristics are for the experiments shown in Figure 30).

Figure 33

Title

Contracture evolution after diphenyleneiodonium injection during 1c/sec stimulation.



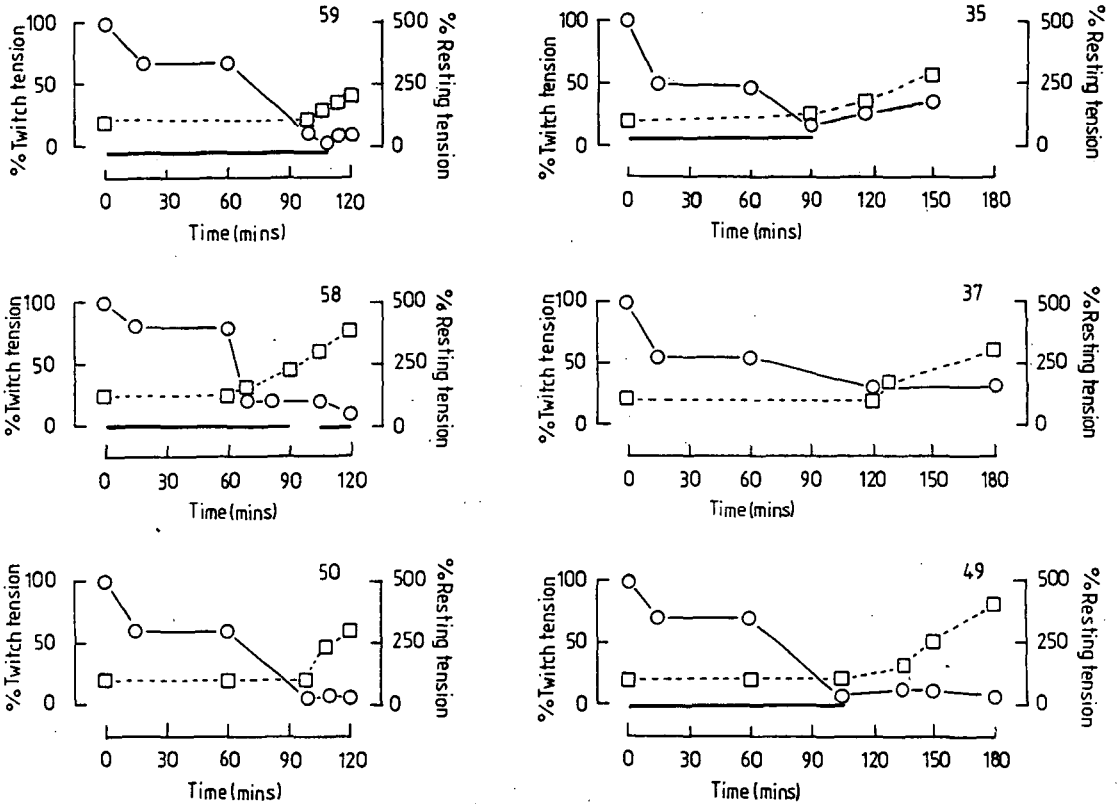
Legend

Ordinate - % contracture (expressed as a% of initial twitch tension amplitude). Abscissa - time in minutes. Bars indicate standard error of mean. Data for 10 animals given between 2 and 4mg of diphenyleneiodonium (see text).

Figure 34

Title

Contracture evolution after diphenyleneiodonium: illustrative experiments.



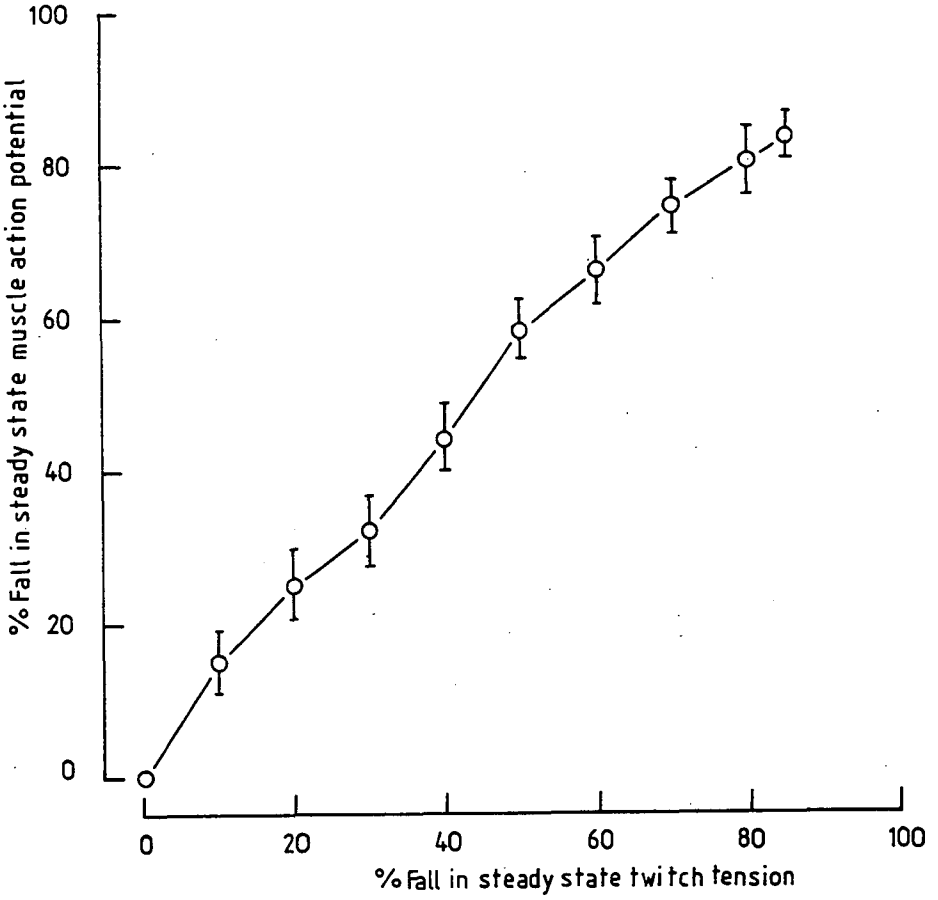
Legend

Ordinate - % of initial twitch tension ○ and % of initial resting tension □ . Abscissa - time in minutes. Continuous 1c/sec stimulation unless intermittent stimulation shown by solid line.

Figure 35

Title

Diphenyleneiodonium infusion with 1c/sec stimulation: relationship between action potential and force failure.



Legend

Ordinate - % fall in steady state muscle action potential amplitude. Abscissa - % fall in steady state twitch tension. N=10. Bars indicate standard error of mean. MAP was not recorded in 5 experiments.

II. 5c/sec Stimulation (N9).

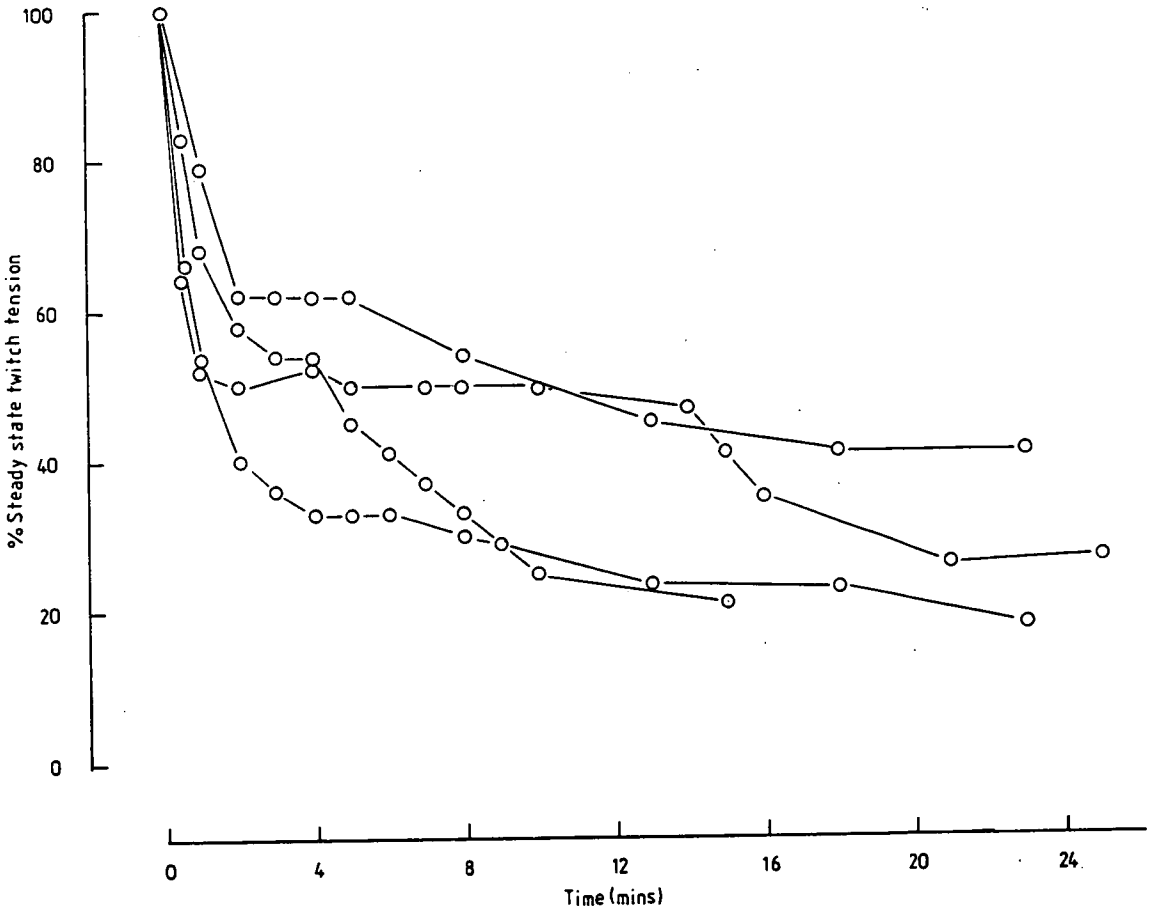
In 9 animals, the gastrocnemius was activated by nerve stimulation at 5c/sec. Prior to injection of DPI, (after 6-8 mins stimulation) isometric twitch tension had stabilised at 42-76% (mean 60.2 $\pm$ SD 6) of the initial value, and muscle action potential amplitude had stabilised at 19-46% (mean 31.8 $\pm$ SD 8.5) of the initial level. 1mg of DPI (in 250ul of 5% dextrose) was then injected as a bolus. As in experiments with 1c/sec stimulation, there was considerable variation in the rate of tension failure after the same dose of inhibitor. Rapid initial fall in isometric twitch tension amplitude followed by a slower decline to very low levels was seen in 4 experiments (Figure 36a). In the remaining animals after injection of 1mg of DPI the twitch either stabilised at an intermediate tension or fell only transiently. In these animals either 1 or 2 additional 1mg boluses were required to induce a severe force failure (Figure 36b). Considerable recovery in twitch tensions developed with rest (see Figure 38). Marked prolongation in half relaxation times, similar to that with 1c/sec stimulation, accompanied force failure (mean half relaxation time at 50% of steady state twitch tension had prolonged to 172%($\pm$ SD 26) of the steady state time immediately before injection of DPI). Contracture was not observed in these animals.

Muscle action potential amplitude fell steadily with the isometric twitch tension (Figure 37). Latency to take off of the action potential was unaltered. Typical experiments are illustrated in Figure 38. In one of these (Exp. 303) (Figure 38a-c) injection of 2 X 1mg boluses of DPI after the twitch tension had stabilised at 58% of the initial level led to a fall to 18% of the initial amplitude over 15 minutes. The muscle action potential also fell from 40 to 27% of the initial value over the same period. After rest for 60 minutes, considerable recovery in twitch tension and action potential amplitude had developed. A pattern of fatigue and recovery was then established with 2 further short stimulus runs, at 5c/sec and the time constant of the fall in tension (TC 120secs) and in muscle action potential (TC 90secs) was more rapid than that seen during the first period of stimulation. A similar fall in twitch tension amplitude developed with nerve and with direct muscle activation.

Figure 36a

Title

Diphenyleneiodonium infusion with 5c/sec stimulation: twitch tension failure.



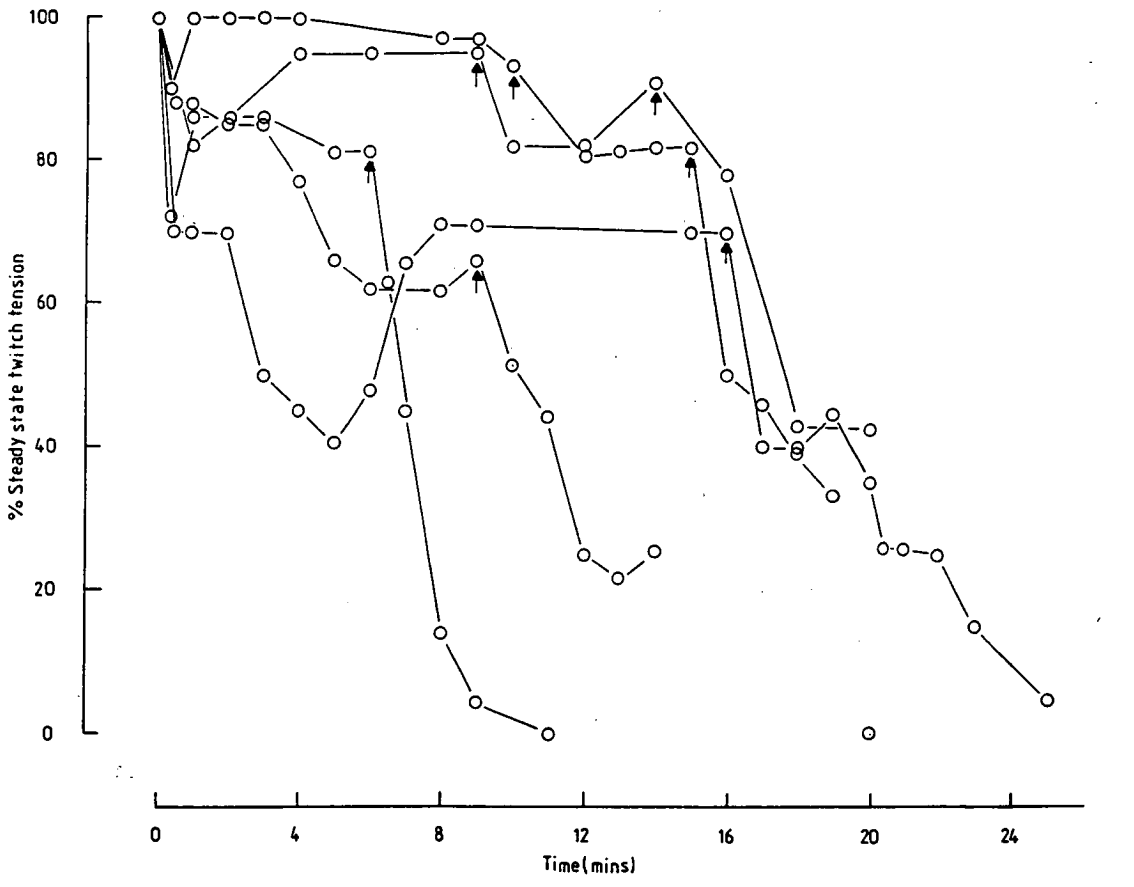
Legend

Twitch tension failure in animals given 1mg of diphenyleneiodonium as a single bolus after stabilisation of twitch tension. The time scale refers to time after injection of DPI.

Figure 36b

Title

Diphenyleneiodonium infusion with 5c/sec stimulation: twitch tension failure.



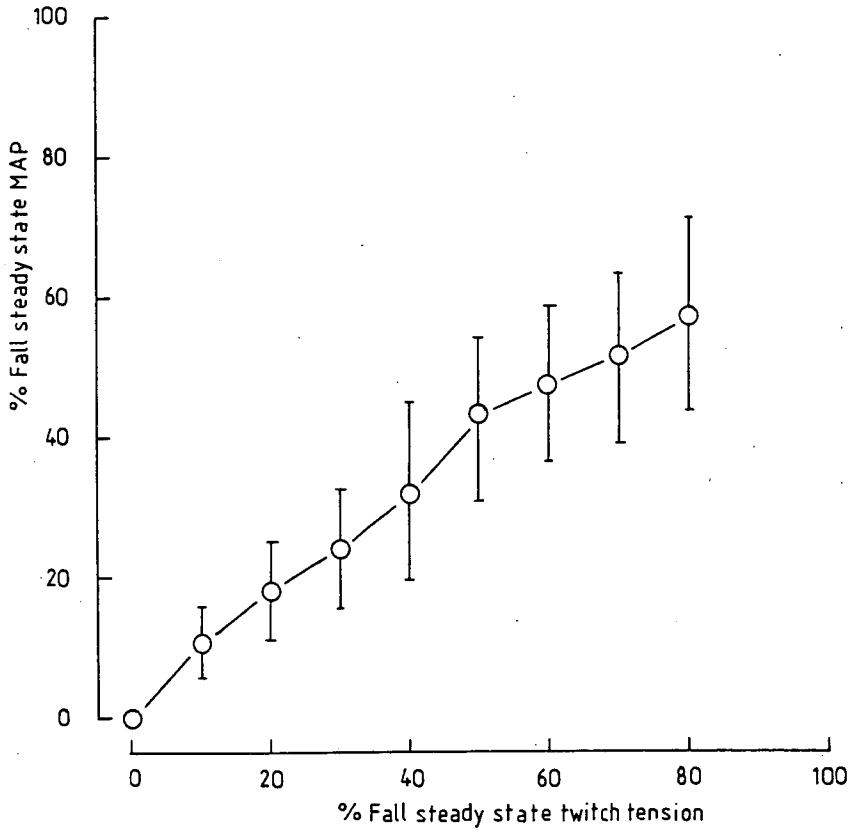
Legend

Twitch tension failure in animals given several boluses of diphenyleneiodonium. Each arrow indicates a 1mg injection. The time scale refers to time after the first injection.

Figure 37

Title

Diphenyleneiodonium infusion with 5c/sec stimulation: relationship between action potential and force failure.



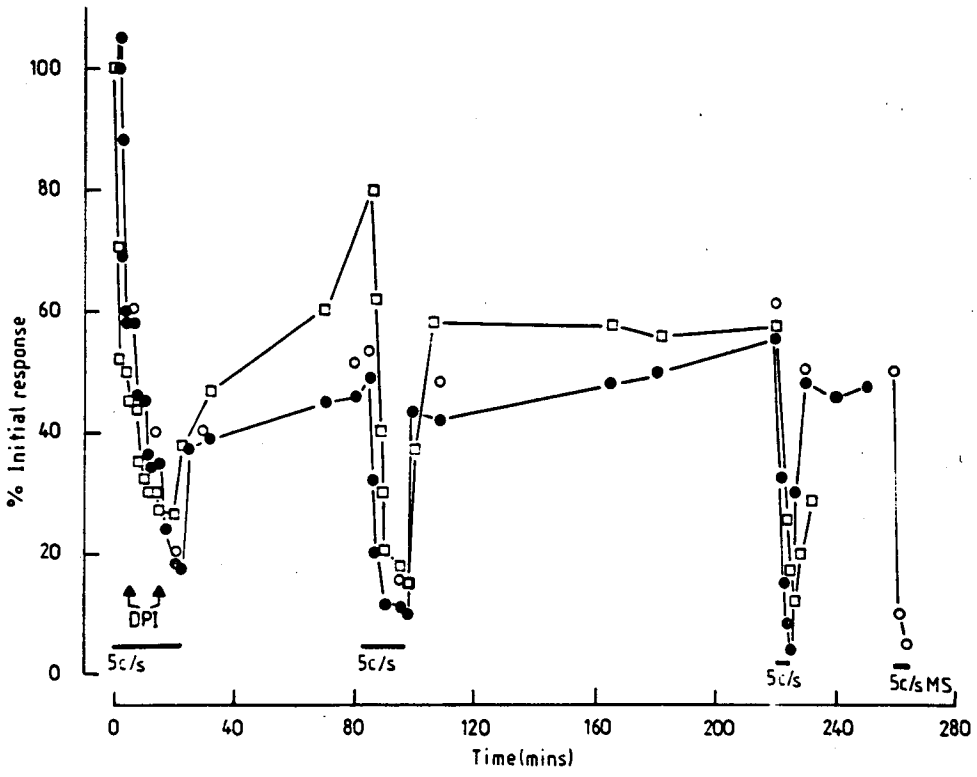
Legend

Ordinate - % fall steady state muscle action potential.
Abscissa - % fall steady state twitch tension. N= 9.
Bars indicate standard deviation.

Figure 38a

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



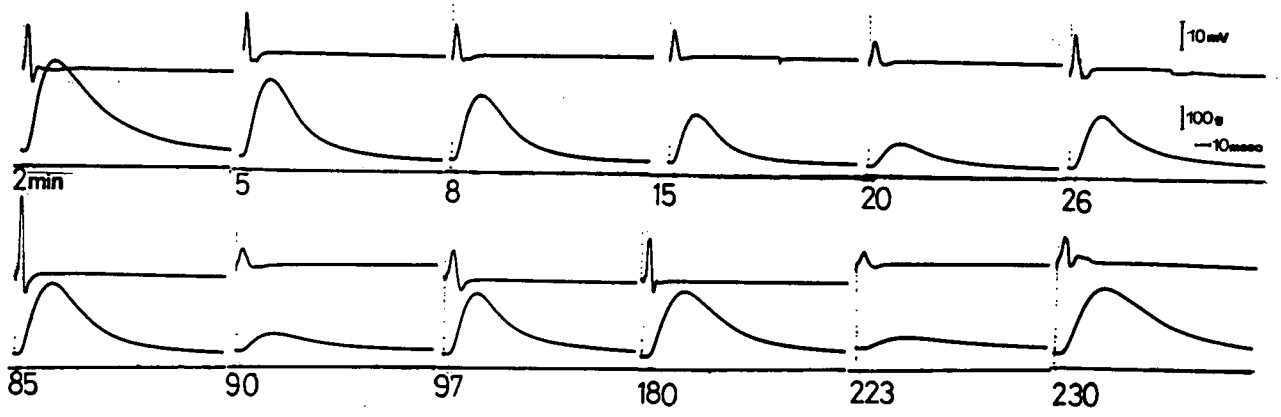
Legend

Experiment 303. Ordinate - % of initial isometric twitch tension nerve stimulation ● . isometric twitch tension muscle stimulation ○ , and muscle action potential □ . 5c/sec nerve stimulation from 0 to 22 minutes, 85 to 95 minutes and 220 to 225 minutes. 5c/sec muscle stimulation from 260 to 262 minutes. 1mg injections of diphenyleneiodonium were given at 6 and 15 minutes.

Figure 38b

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



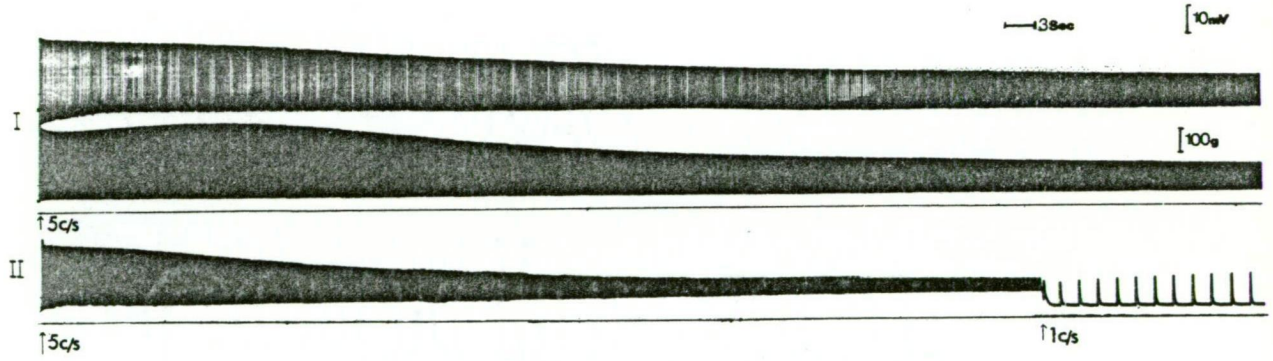
Legend

Experiment 303. Selective isometric twitch tension and muscle action potential responses at the indicated times in minutes, illustrating the pattern of severe fatigue followed by recovery with successive stimulus runs. Note the increase in baseline tension at 223 minutes is due to fusion and not contracture.

Figure 38c

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



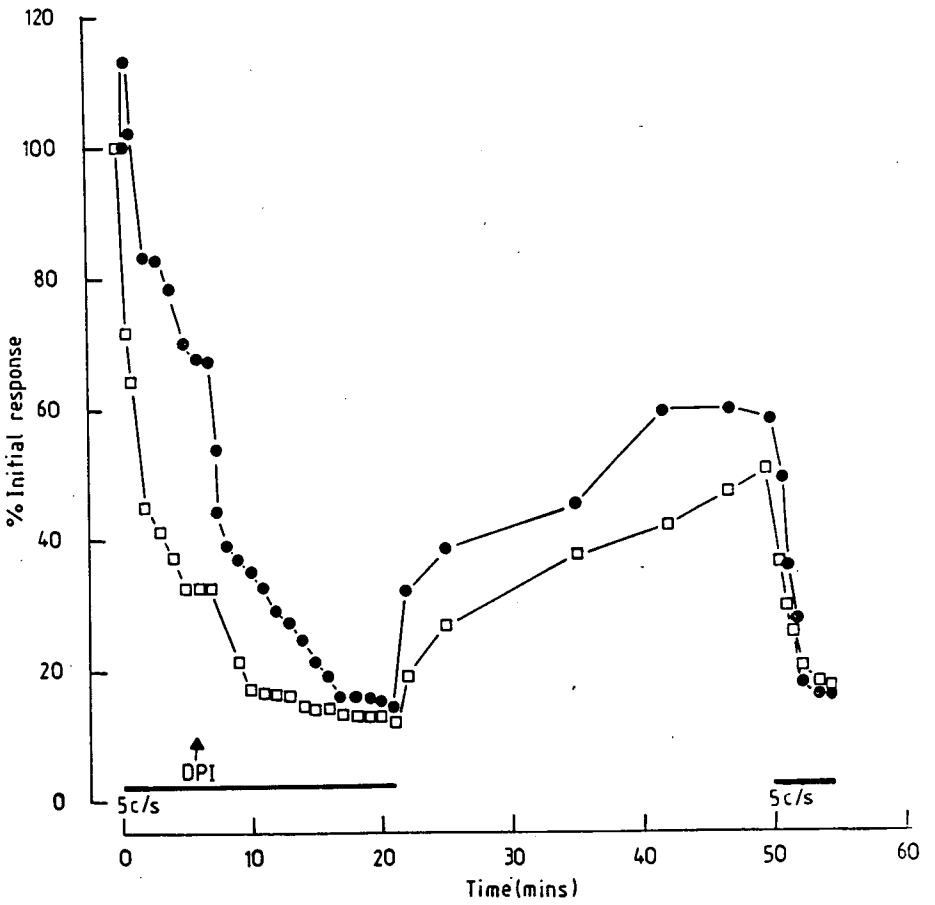
Legend

Experiment 303. I. Continuous recording of the beginning of the stimulus run at 85 minutes (nerve stimulation). Muscle action potential is shown by the upper trace and the isometric twitch tension by the lower trace. The baseline position indicates the resting tension.
II. Direct muscle stimulation run at 260 minutes. Note fusion of tension in the latter part of this stimulus pattern due to prolongation of the relaxation phase.

Figure 38d

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



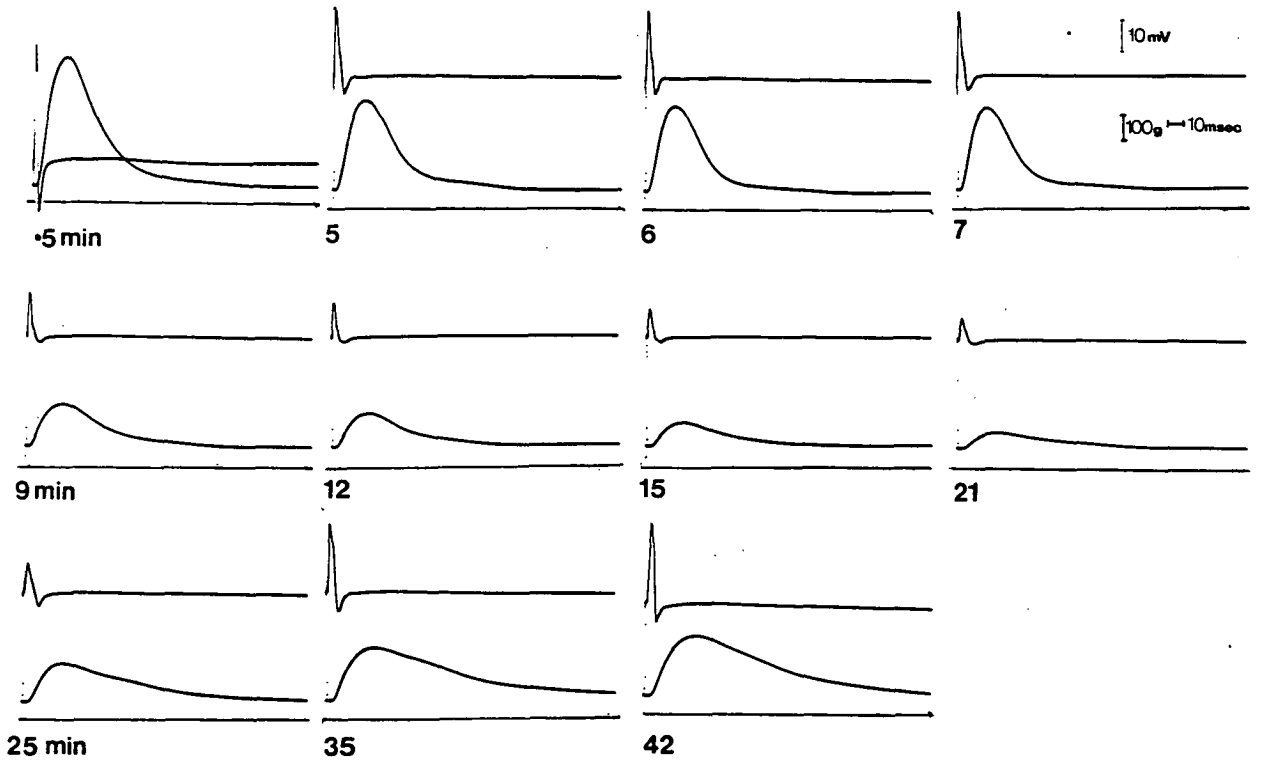
Legend

Experiment 302. Ordinate - % of initial response isometric twitch tension (nerve stimulation) ● , and muscle action potential □ . 5c/sec stimulation from 0 to 21 minutes and from 50 to 54 minutes. 1mg diphenyleneiodonium injected at 7 minutes.

Figure 38e

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



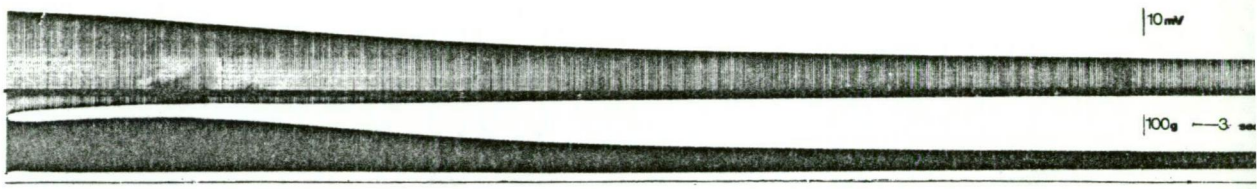
Legend

Experiment 302. Selected twitch tension and muscle action potential responses at the times indicated in minutes.

Figure 38f

Title

Typical experiments illustrating isometric twitch tension and muscle action potential responses after injection of diphenyleneiodonium during a 5c/sec stimulus pattern.



Legend

Experiment 302. Continuous recording of the beginning of the stimulus run at 50 minutes showing rapid fatigue of twitch tension (lower trace) and muscle action potential (upper trace).

DISCUSSION

Injection of diphenyleneiodonium led to an abnormal twitch tension fatigue during 1 and 5c/sec stimulus patterns. The fall in twitch tension amplitude seen with nerve and with direct muscle stimulation was similar. A fall in muscle action potential amplitude coincided with the fall in twitch tension.

A pattern of rapid fatiguability with delayed recovery was established with repeated 5c/sec stimulus patterns. These findings resemble the clinical presentation in some patients with mitochondrial myopathy where exercise intolerance is the major symptom.

In these experiments, force failure could reflect either a primary failure of excitation, coupling or contraction. The very close temporal relationship between mechanical and electrical failure makes it impossible to determine the precise fatigue mechanism.

Twitch tension declined at a much slower rate after injection of DPI than after DNP. In addition, a much greater prolongation in the duration of twitch relaxation was seen in DPI experiments. These findings suggest that fibres with a compromised energy status which have a delay in relaxation times as a result contribute to the twitch response for some minutes before failing to contract.

Unlike DNP, contracture was an inconsistent finding with DPI and occured much later in relationship to the onset of twitch failure. The development of partial contracture at a time when the twitch tension was recovering in several experiments again suggests that there is a considerable variation in the severity of muscle fibre involvement. Some fibres still have a potential for rapid recovery while others are entering irreversible contracture. In several experiments a pattern of repeated fatigue without contracture was seen.

Diphenyleneiodonium precipitates out of solution in the presence of physiological concentrations of chloride ions and forms a fine colloidal suspension. Because of this it was dissolved in and washed through with 5% dextrose. It is possible however that some of DPI precipitated out, and this may account for the variable response in different animals. Ischaemia also causes a rapid force failure in muscle and the possibility must be considered that a drug induced vasospasm is responsible in whole or in part for these findings. This is a very remote possibility for 3 reasons. Firstly, considerable recovery developed in fatigued muscle with rest after DPI, whereas in ischaemic muscle stimulated until complete force failure developed, no recovery was seen. Secondly, blood flow levels in resting muscle were not signicantly depressed by iodonium. Thirdly, acute force failure in striated muscle was also produced with subcutaneous injections of DPI (vide infra).

1.1.5 Antimycin A

In 5 animals, antimycin A was injected after the isometric twitch tension had stabilised during 5c/sec stimulation (6 - 8 minutes after onset). A single bolus of either 1.6 or 3.2mg/Kg body weight (in 100 or 200 uls 10% ethanol in normal saline) was administered in four experiments and in one experiment, 3 boluses each of 0.8mg/Kg were given. Injection of 100ul of 10% ethanol (the vehicle in which antimycin A was dissolved) in 2 experiments had no effect on the evoked mechanical response.

Clinical Observations.

Between 10 and 20 minutes after the injection of antimycin A respiration slowed (10 - 15 minute) and became shallow. The surface body temperature tended to fall at this time but was maintained with external heating. The rat given a larger dose died after 30 minutes. Animals given smaller doses survived for an average of one hour. Arterial blood gas and blood flow studies were not carried out.

Measurement of the Electrical and Mechanical Responses.

Isometric twitch tension fell rapidly to reach 8% of the steady state level within 7 minutes of injection of 3.2mg/Kg (TC 3mins). Muscle action potential amplitude fell to 38%

of the steady state level over the same interval. The fall in both parameters was steady with no sudden drop immediately post injection. In this animal, no recovery was seen with rest but the animal's cardio-respiratory status was clearly compromised.

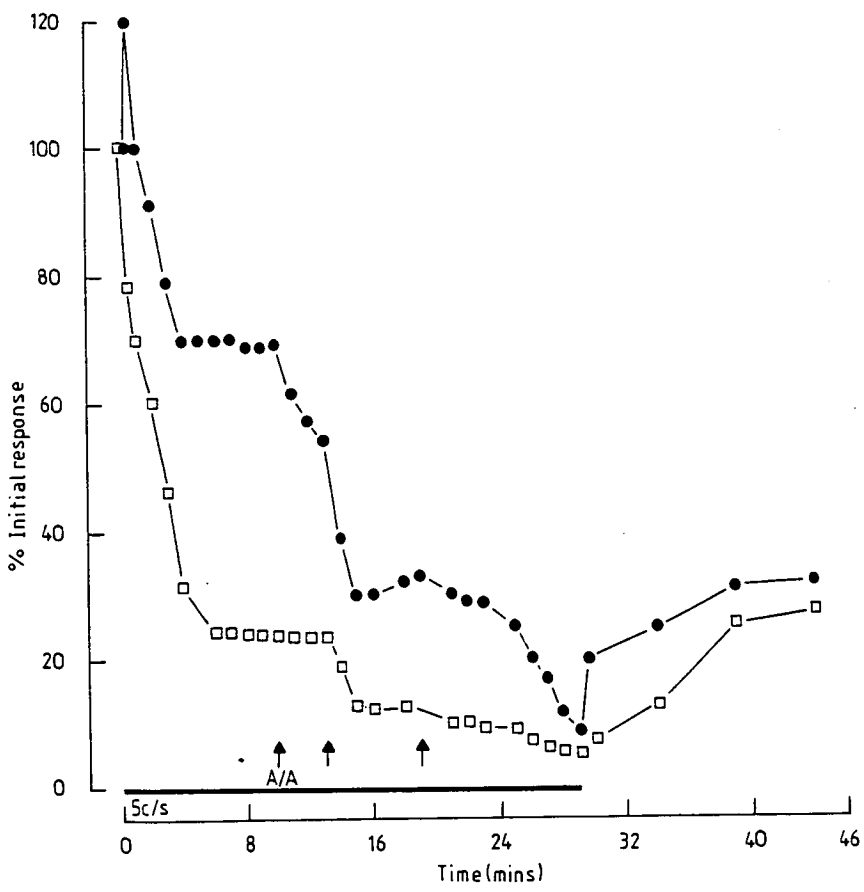
In 3 animals injection of 1.6mg/Kg antimycin A lead to a steady fall in twitch tension to a mean value of 14% (range 4 - 20%) of the steady state value within 200 - 500 seconds (TC 120 - 190 seconds). A progressive fall in muscle action potential amplitude to reach a mean of 40.6% of the steady state value paralleled force failure.

Considerable recovery was seen over 30 minutes of rest in 2 of these 3 experiments. Twitch tensions recovered from 9% of the initial value to 50% and the action potential from 24% of the initial value to 100% in one experiment and in the second the twitch tension increased from 16.5% of the initial value to 52% and the action potential from 17% to 44% of the initial value. A similar sequence of electrical and mechanical failure with partial recovery with rest was seen in the fifth experiment with antimycin A in which 3 smaller bolus doses were administered (Figure 39).

Figure 39a

Title

Typical experiment showing twitch and action potential failure after injection of antimycin A.



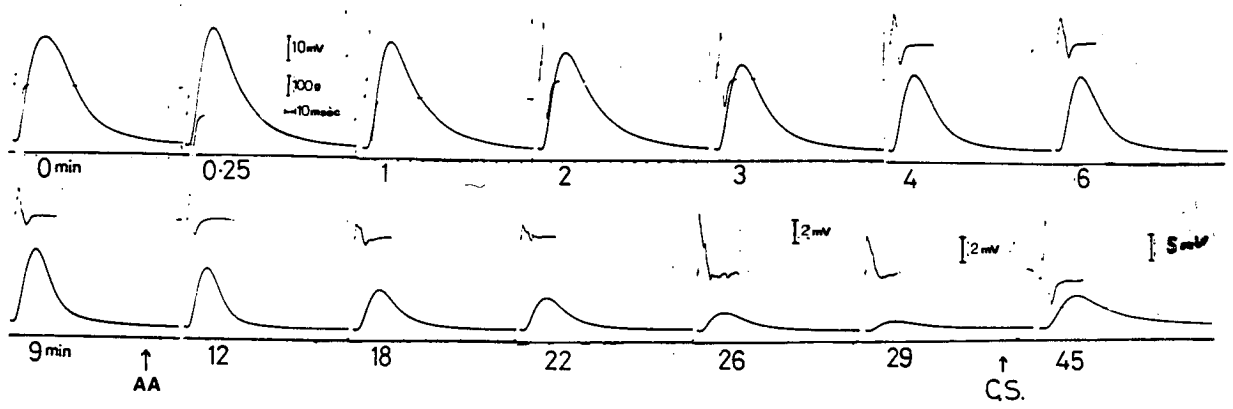
Legend

Experiment 253. Ordinate - isometric twitch tension ● , and compound muscle action potential amplitude □ , expressed as a % of the initial response. 5c/sec nerve stimulation from 0 to 29 minutes. 0.8mg/Kg body weight antimycin A injected at 10, 13 and 17 minutes.

Figure 39b

Title

Typical experiment showing twitch and action potential failure after injection of antimycin A.



Legend

Experiment 253. Isometric twitch tension and action potential responses recorded(at the times indicated)at the times indicated in minutes.

DISCUSSION

Antimycin A caused a similar force failure to that seen with diphenyleneiodonium. The animals' cardio respiratory status was more compromised than with the other agents used and it is possible that central hypoxaemia influenced tension failure. Although the smaller doses used in this study, were not so rapidly fatal as the doses used by Sahgal et al (1979), high systemic toxicity limits the usefulness of this agent.

1.1.6 Iodoacetate Experiments

A series of experiments was carried out in which the effects of the glycolytic blocker, iodoacetate, were examined in a similar preparation. In 8 experiments this agent was infused after twitch tension amplitude had reached a steady state level 40 - 60 minutes after beginning 1c/sec nerve stimulation. Either 1 or 2 boluses of 40mg/Kg iodoacetate (in NaCL pH 7.4, volume 100 - 150ul) were injected. In additional experiments, 5c/sec nerve stimulation was commenced immediately after injection of 40mg/Kg iodoacetate. A second bolus of 20mg/Kg was injected after 15 minutes in 2 animals where the twitch response had not failed completely.

Clinical Observations

Irregularity in the rate and depth of respiration developed within 30 seconds of injection and lasted for several minutes. Contracture was seen in the tail and hind limbs 10 - 20 minutes after injection. Two animals died within 30 minutes of injection and developed severe generalised rigor mortis almost immediately. Blood flow studies and arterial blood gas analysis was not carried out.

Measurement of the Electrical and Mechanical Responses. 1c/sec Stimulation.

Isometric twitch tension fell rapidly after the first (5 animals) or second (3 animals) bolus to reach levels between 0 and 5% of the steady state value. In experiments in which a single bolus was given, the time constant of the fall in tension varied from 4-8 minutes. Compound muscle action potential amplitude fell with the twitch response. No recovery in either parameter was seen with periods of rest of up to 2 hours. A typical experiment is shown in Figure 40. Contracture began to evolve following a major fall in isometric twitch tension and continued to progress after interruption of the stimulus train. The relationship between twitch failure and contracture closely resembled that seen in experiments with DNP (Figure 41). Prolongation in half relaxation times was again closely associated with twitch failure, (mean ½RT 181 $\pm$ SD 23.3% of duration immediately prior to injection of IA when the twitch tension had fallen to 20% of steady state level). The relationship between fall in twitch tension and in muscle action potential amplitude was also similar to that seen in experiments with dinitrophenol where 1c/sec stimulation was used (Figure 42). The slope of this graph for points from 0 to 50% fall in twitch amplitude is well described by a line with a slope of 0.58% fall in steady state muscle action potential per 1% fall in steady state twitch tension (correlation co-efficient 0.99 $P<0.001$) and the slope for points from 50-100% fall in twitch tension by a line with a slope of 1.62% fall in steady state muscle

action potential per 1% fall in twitch tension (correlation co-efficient 0.99 P 0.001). The rapid stage of action potential failure coincided with the onset of contracture.

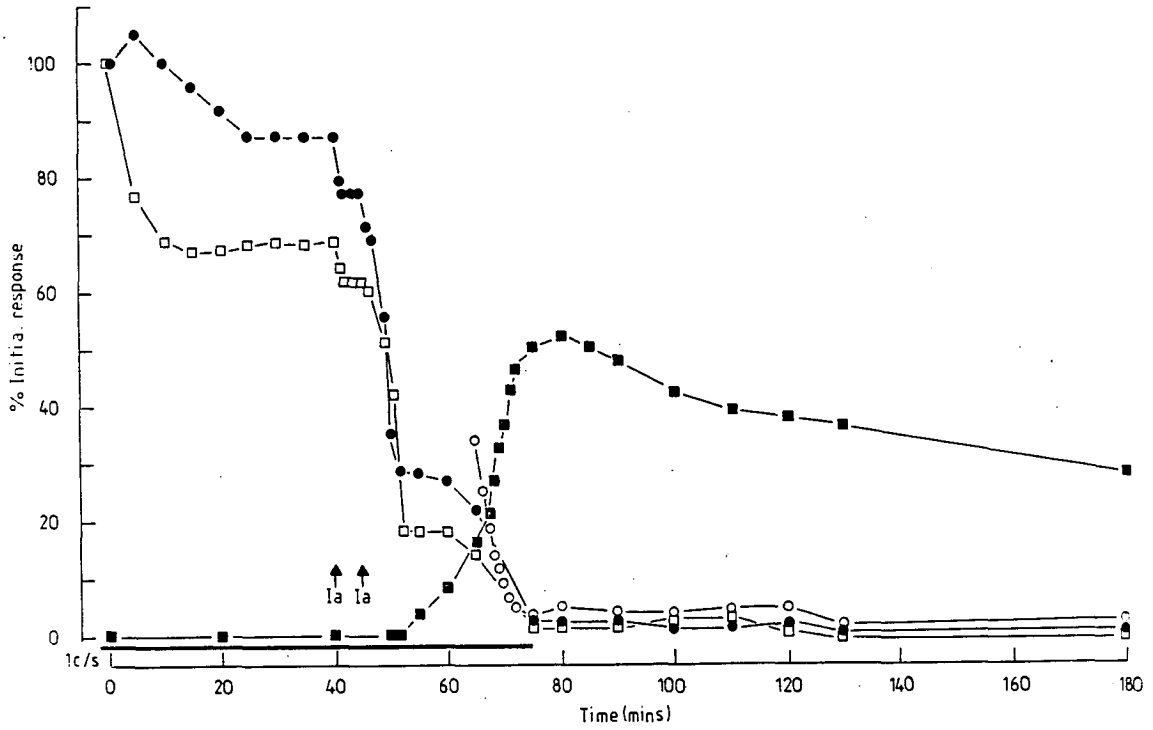
5c/sec Stimulation

The rate of twitch tension failure in these experiments was variable. In 4 animals a fall in twitch tension to less than 10% of the initial level developed rapidly TC 0.5-5mins). In the remaining 2 animals, twitch tension stabilised at an intermediate level after the first injection but failed rapidly after a second bolus. A train of stimuli was given with direct muscle stimulation when the mean twitch tension with nerve stimulation had fallen to 9.9% of the initial value (SD 6.1) without any break in the stimulus train and slightly higher tensions were obtained transiently (mean 26.4% initial value, SD 7.5) but rapidly fatigued (Figure 43).

Figure 40a

Title

Infusion of iodoacetate during a 1c/sec stimulus pattern: typical experiment.



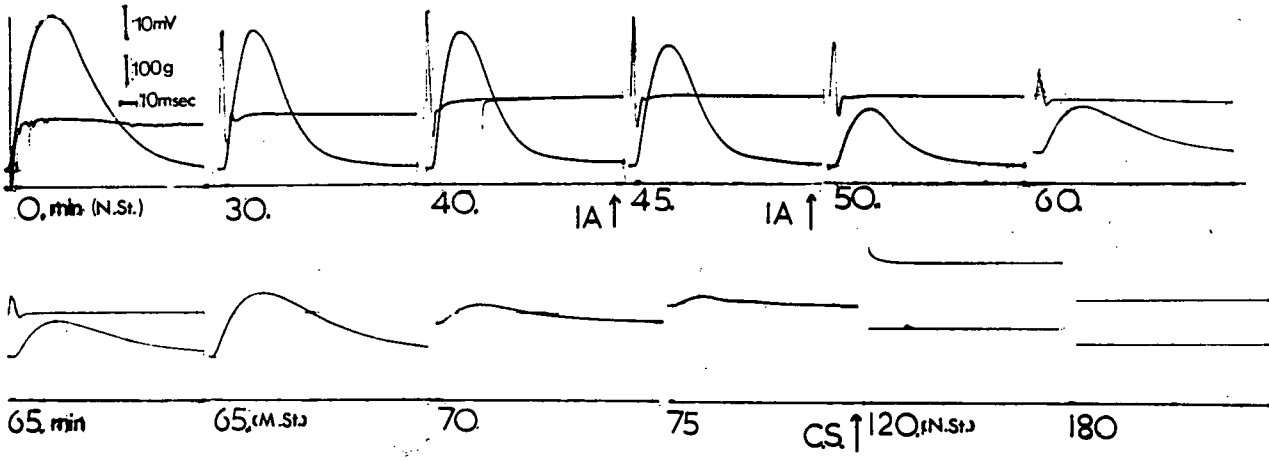
Legend

Experiment 53. Ordinate - % of initial response, isometric twitch tension nerve stimulation ●, isometric twitch tension muscle stimulation ○, compound muscle action potential □, and increase in resting tension (expressed as a % of the initial twitch tension amplitude) ■. Nerve stimulation (1c/sec) from 0 to 65 minutes, muscle stimulation (1c/sec) from 65 to 75 minutes. Boluses of 40mg/Kg body weight iodoacetate were injected at 40 and 45 minutes.

Figure 40b

Title

Infusion of iodoacetate during a 1c/sec stimulus pattern: typical experiment.



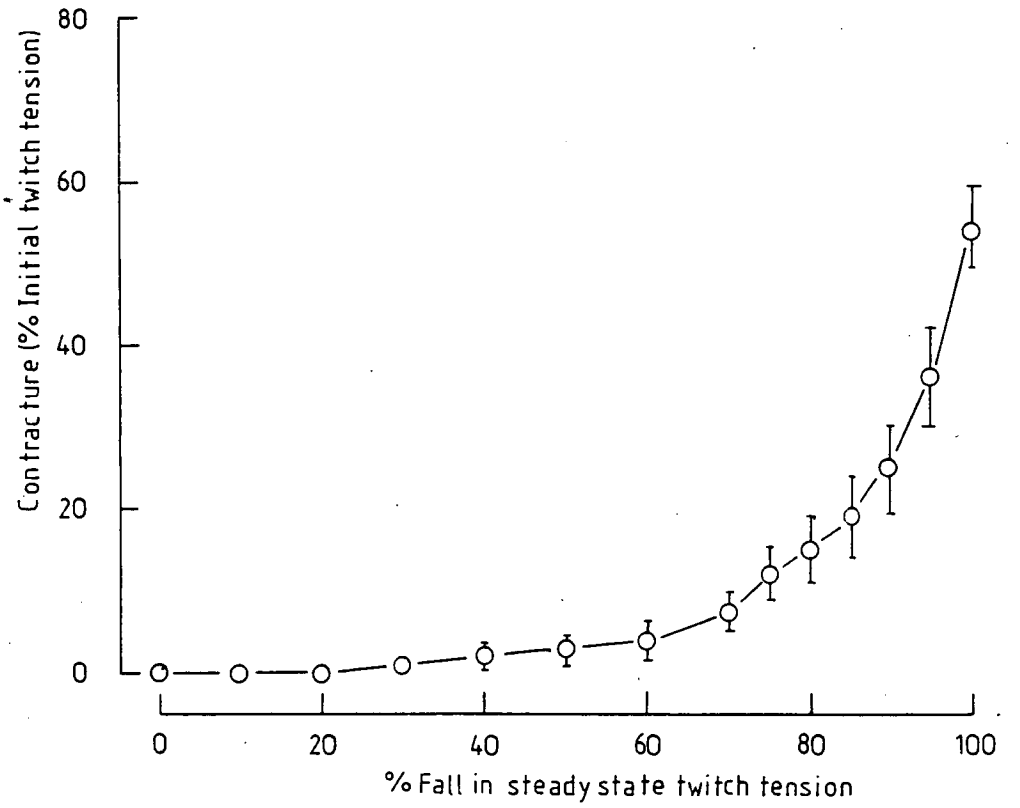
Legend

Experiment 53. Selected twitch tension and muscle action potential responses. The position of the baseline indicates the resting tension.

Figure 41

Title

Iodoacetate infusion during 1c/sec stimulation: relationship between contracture evolution and twitch tension failure.



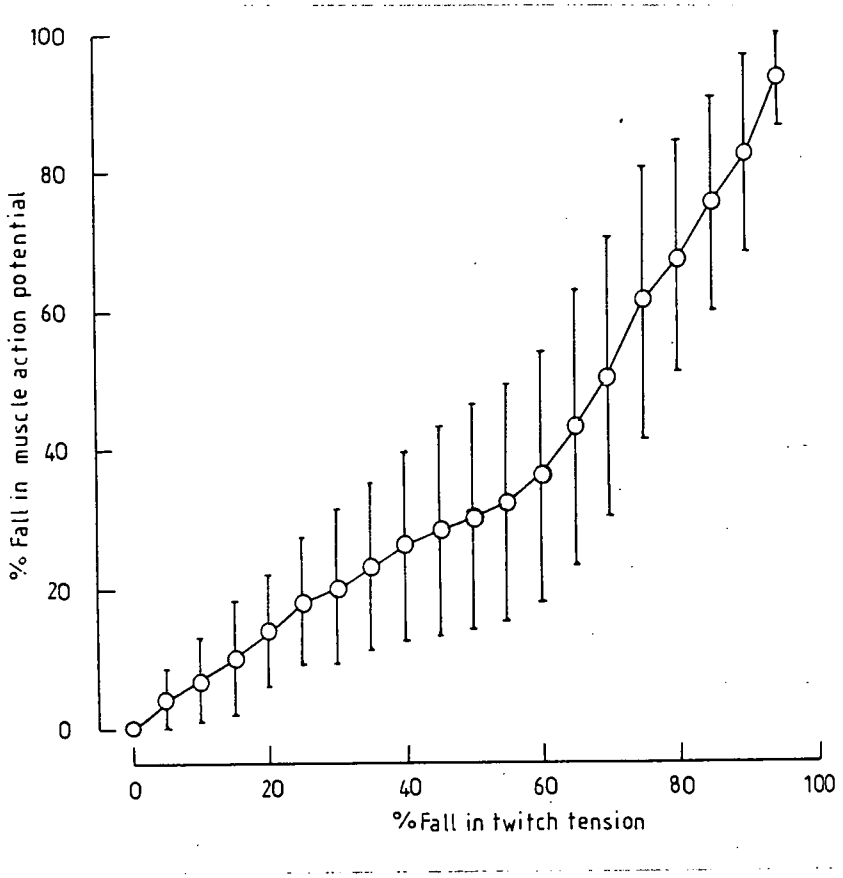
Legend

Ordinate - contracture (i.e. increase in resting tension as a % of the initial twitch tension amplitude). Abscissa - % fall in steady state resting tension. N=8. Bars indicate standard error of mean.

Figure 42

Title

Iodoacetate infusion during 1c/sec stimulation: relationship between muscle action potential and twitch tension failure.



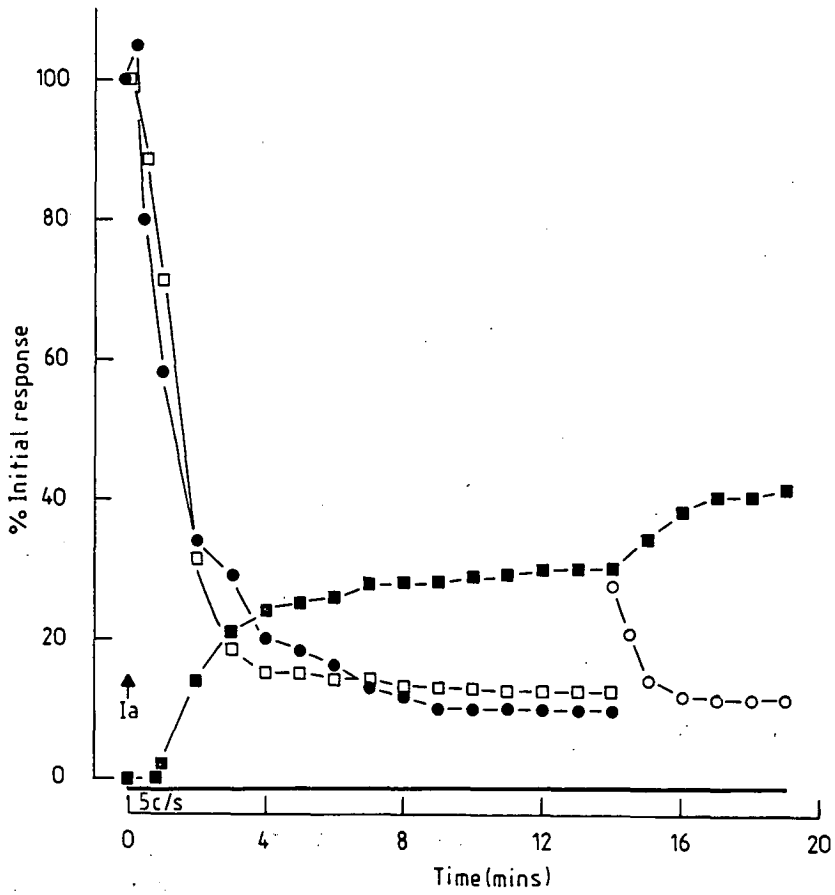
Legend

Ordinate - % fall in steady state muscle action potential amplitude. Abscissa - % fall in steady state twitch tension. N=8. Bars indicate standard deviation.

Figure 43a

Title

Iodoacetate infusion at rest followed by a 5c/sec stimulus pattern: typical experiment.



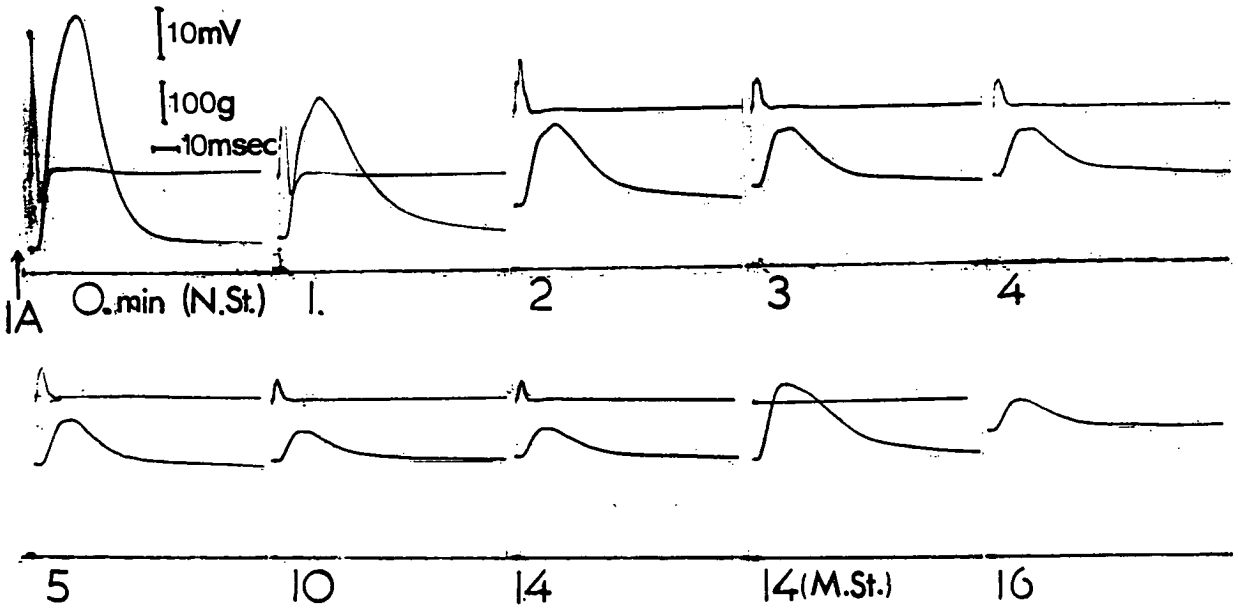
Legend

Experiment 81. Ordinate - % of initial response, twitch tension nerve stimulation ● , twitch tension muscle stimulation ○ , action potential □ , and increase in resting tension as a % of the initial twitch tension ■ . 5c/sec nerve stimulation from 0 to 14 minutes, 5c/sec muscle stimulation from 14 to 19 minutes. Iodoacetate 40mg/Kg injected prior to stimulation. Note acceleration of contracture when direct muscle stimulation is instituted.

Figure 43b

Title

Iodoacetate infusion at rest followed by a 5c/sec stimulus pattern: typical experiment.



Legend

Experiment 81. Selected isometric twitch tension and muscle action potential responses at the indicated times in minutes. Nerve stimulation 0 to 14 minutes. Muscle stimulation 14 to 16 minutes. The baseline position indicates the resting tension.

DISCUSSION

The induction of skeletal muscle contracture in vitro by iodoacetate was first recognised by Lundsgaard in a classic paper (1930) in which he reported contracture evolution under both aerobic and anaerobic conditions. This observation was rapidly confirmed (Henniques and Mawson 1932; Lundsgaard 1931). Iodoacetate inhibits the glycolytic enzyme glyceraldehyde 3 phosphate dehydrogenase (Cori et al 1946). It was investigated in this study to see if any differences could be established between its effects and those of mitochondrial inhibitors in vivo. More recently Brumbach (1980) injected iodoacetate into the aorta of rats in an attempt to produce an animal model of McArdles disease. He found that the animals became weak while swimming and that the isometric twitch tension in hind limb muscles fatigued abnormally rapidly during ischaemia. Contracture was observed but not measured. The precise in vivo relationship between twitch failure and contracture has been determined for the first time in the present study. The sequence of isometric twitch failure, sarcolemmal inexcitability and progressive contracture observed was identical to that seen with DNP. Twitch failure and contracture evolved much more rapidly with these agents than with respiratory chain inhibitors. Complete twitch failure developed after only 600 stimuli in the most severely effected muscles. This finding suggests that ATP production by oxidative phosphorylation as well as by glycolysis is compromised. Fatty acid oxidation provides a major

additional energy source to carbohydrate fuels in man during prolonged work which is not effected by iodoacetate (Felig and Warren 1975; Newsholm 1977; Paul 1975). In less protracted exercise however fatty acid oxidation is less important and under these conditions, pyruvate from glycolysis is the major energy substrate for cellular respiration Bergstrom and Hultman 1967; Wahren 1977). By blocking glycolysis iodoacetate will prevent the utilisation of carbohydrate substrates by the Krebs cycle and may therefore lead to a more severe immediate energy crisis than that which is induced by the respiratory chain inhibitors alone. The similarity in the physiological effects of a glycolytic blocker and an uncoupler suggests that twitch tension failure and contracture are the end result of an acute and severe energy shortage of any cause.

The finding that loss of force in iodoacetate poisoned muscle is paralleled by a decline in the amplitude of the evoked muscle action potential closely resembles physiological observations in patients with McArdles disease (Dyken et al 1967). Edwards and Wiles (1981) also demonstrated a parallel fall in electrical and mechanical activity in a patient with McArdles disease and also showed that no recovery of action potential amplitude developed under anaerobic conditions whereas in normals there is substantial anaerobic action potential recovery.

1.2 Histological Observations after Intraarterial Infusion of Metabolic Inhibitors.

Blocks from the gastrocnemius muscle were appropriately fixed for histological examination in 4 control experiments, in 10 experiments each after injection of diphenyleneiodonium and dinitrophenol, and in 2 experiments with iodoacetate. Muscle was not fixed for histology in Antimycin A experiments.

1.2.1 Control Experiments.

With the modified Gomori trichrome stain a peripheral smooth red ring was seen around many fibres in control experiments. No red staining material was seen in the central areas of fibres (Figure 44). An intense oxidative reaction was also visible around the periphery of many fibres with the NADH dehydrogenase and succinic dehydrogenase stains. No necrotic or hyper contracted fibres were seen.

1.2.2 Dinitrophenol Experiments.

Similar structural changes were seen in gastrocnemius 1 hour after infusion of dinitrophenol, regardless of the stimulus pattern used. Hypercontracted fibres were prominent. With the Gomori trichrome stain, many type I fibres showed a peripheral red staining zone. This was irregular, greater in extent than in the control group, and was associated with red staining accumulations in the central zone of many fibres

(Figure 44). The same areas stained intensely with oxidative enzyme reactions. A total depletion of glycogen was seen from all fibres (PAS stain).

1.2.3 Diphenyleneiodonium.

No necrotic or hypercontracted fibres were seen in muscles frozen between 2 and 3 hours after injection of DPI. Although several fibres with red staining marginal clumps were seen with the Gomori trichrome stain, no true ragged red fibres were present. The appearance seen with oxidative stains did not differ from that in control preparations. A total glycogen depletion was again evident.

1.2.4 Iodoacetate.

Hypercontracted fibres were visible in muscles sampled 60 to 90 minutes after injection of iodoacetate. With the Gomori trichrome stain, the appearance did not differ in other respects from that seen in controls.

Figure 44

Title

Muscle Histology

Legend

44a. Control experiment. Block from medial head of gastrocnemius fixed after 120 minutes stimulation at 1c/sec. Modified Gomori trichrome stain. Note smooth peripheral red staining in occasional fibres and red staining adjacent to a capillary.

44b. Dinitrophenol experiment. Block from medial head of gastrocnemius fixed after 60 minutes stimulation at 2c/min. Modified Gomori trichrome stain. Note fibres with ragged red rim and red staining material centrally.

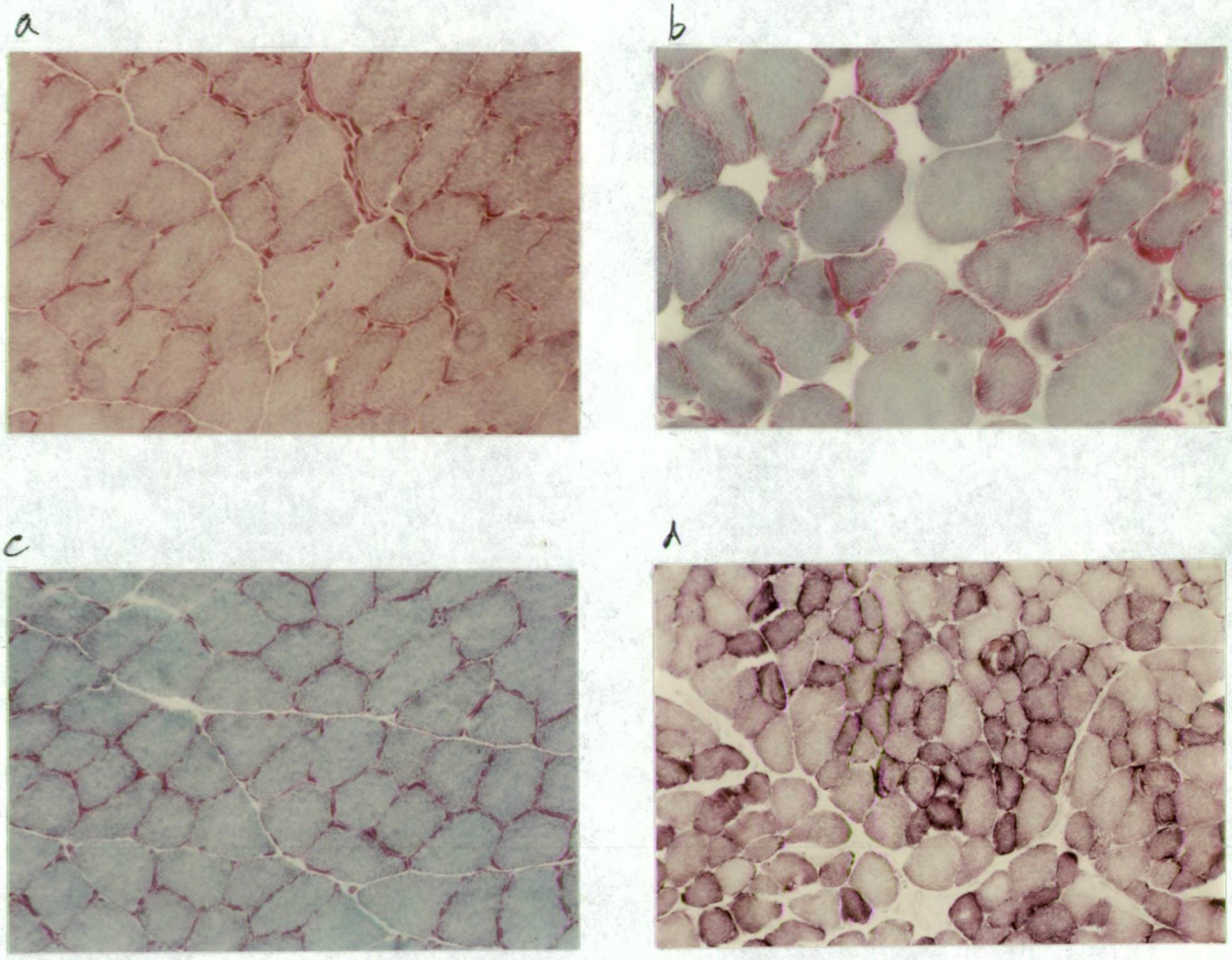
44c. Diphenyleneiodonium experiment. Block from medial head of gastrocnemius fixed after 120 minutes stimulation at 1c/sec. Modified Gomori trichrome stain. Note the absence of ragged red fibres.

44d. Dinitrophenol experiment. Succinic dehydrogenase stain. Note peripheral enhancement of oxidative staining and irregular staining in central areas in many fibres.

Figure 44

Title

Muscle Histology



1.3 Electron Microscopy.

1.3.1 Dinitrophenol.

The gastrocnemius muscle was fixed for electron microscopy in 4 animals, 60 - 90 minutes after infusion of 16mg/Kg body weight of DNP. The muscles were either not stimulated or stimulated at very low frequencies (2c/min) and contracture had developed in the R hind limb in all animals prior to fixation. Qualitatively similar changes were seen at both times but were more pronounced in muscles fixed at 90 minutes. Occasional necrotic fibres, with a disintegration of the myofibrillar network were scattered throughout the muscle. These contained large numbers of swollen mitochondria with fracture or lysis of crista. Abnormal mitochondria were present in all fibres. Subsarcolemmal aggregations containing mitochondria with concentric crista, mitochondria with straight crista not extending the width of the matrix space, some with linear intracristal inclusions and mitochondria containing membranous profiles were seen (Figure 45a-d). Phospholipid membranous structures surrounded many degenerating mitochondria. Some mitochondria contained osmophilic bodies. Qualitatively similar but less marked abnormalities were seen in the intermyofibrillar mitochondria.

1.3.2 Diphenyleneiodonium.

Sections were examined from the gastrocnemius in 2 animals after injection of DPI (2 - 3mg). Stimulation was continued at 1c/sec until isometric twitch tension had fallen to less than 10% of the initial level. In one experiment the gastrocnemius was fixed at 60 minutes and in the second experiment 120 minutes after injection.

The muscle architecture was intact and no necrotic fibres were identified. The great majority of mitochondria were normal in size, shape and internal morphology. Occasional, unusually rounded mitochondria were seen in the subsarcolemmal and intermyofibrillar areas but these were only slightly enlarged. No subsarcolemmal aggregations of abnormal mitochondria were identified. No intracristal or matrix inclusions were seen.

Figure 45

Title

Electron Micrographs.

Legend

45a. Section from medial head of gastrocnemius fixed 90 minutes after onset of DNP infusion. Note subsarcolemmal aggregate of abnormal mitochondria. Many of these are enlarged and have fractured crista. Some crista contain platelike inclusions. Degenerating mitochondria containing osmophilic bodies and myelin figures are prominent.

45b. High magnification view of abnormal mitochondria containing fractured crista with linear inclusions and myelin figures from a similar experiment.

45c. Section from medial head of gastrocnemius fixed 60 minutes after onset of DNP infusion. Note rounded mitochondria with cristal membrane whorling.

45d. Section from medial head of gastrocnemius fixed 90 minutes after onset of DNP infusion. Note abnormal intermyofibrillar mitochondria.

45e. Section from medial head of gastrocnemius fixed 2 hours after infusion of diphenyleneiodonium. Note unusually rounded mitochondria. No degenerating forms were seen.

Bars = 1um

Figure 45 a

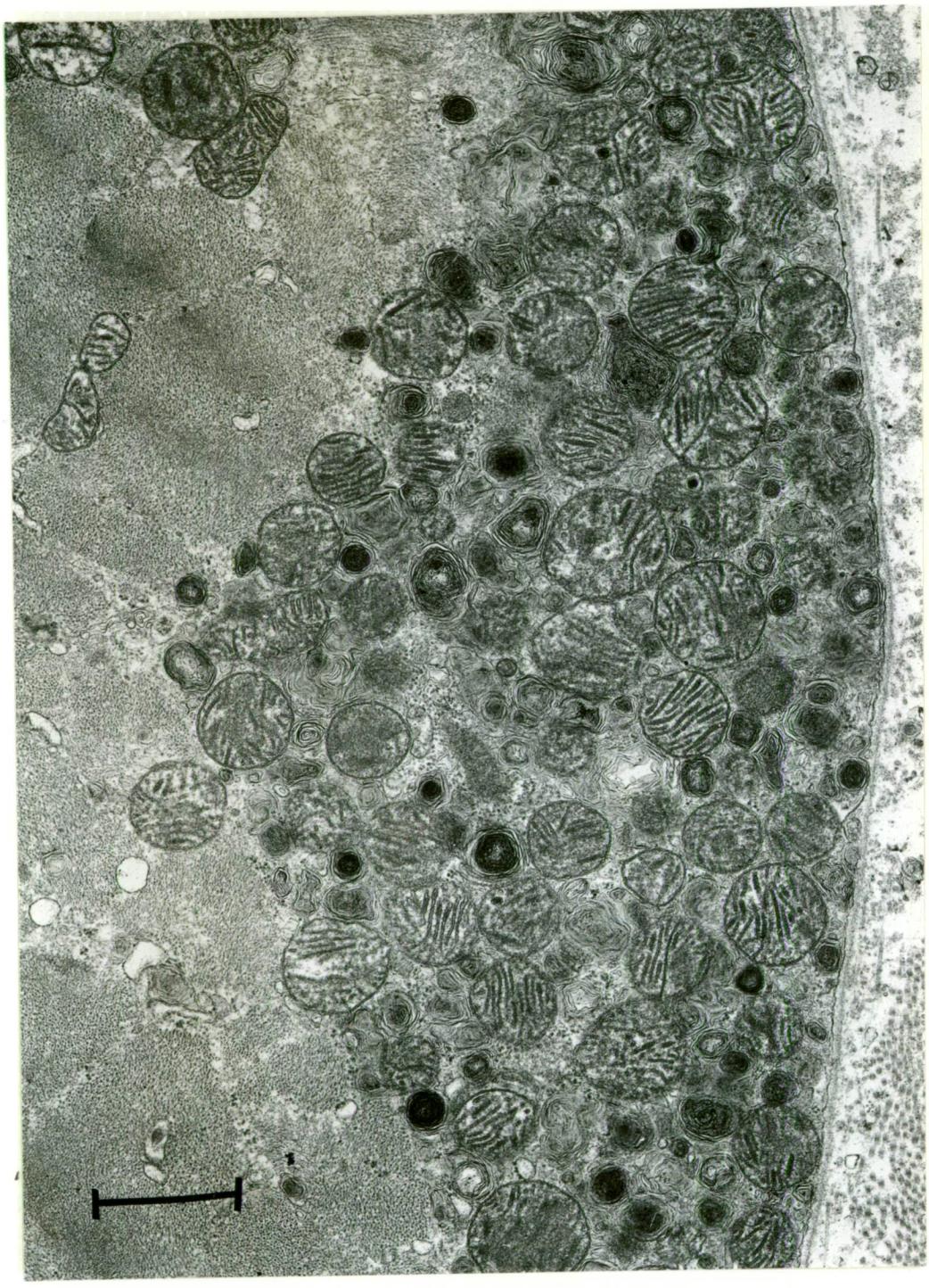


Figure 45b

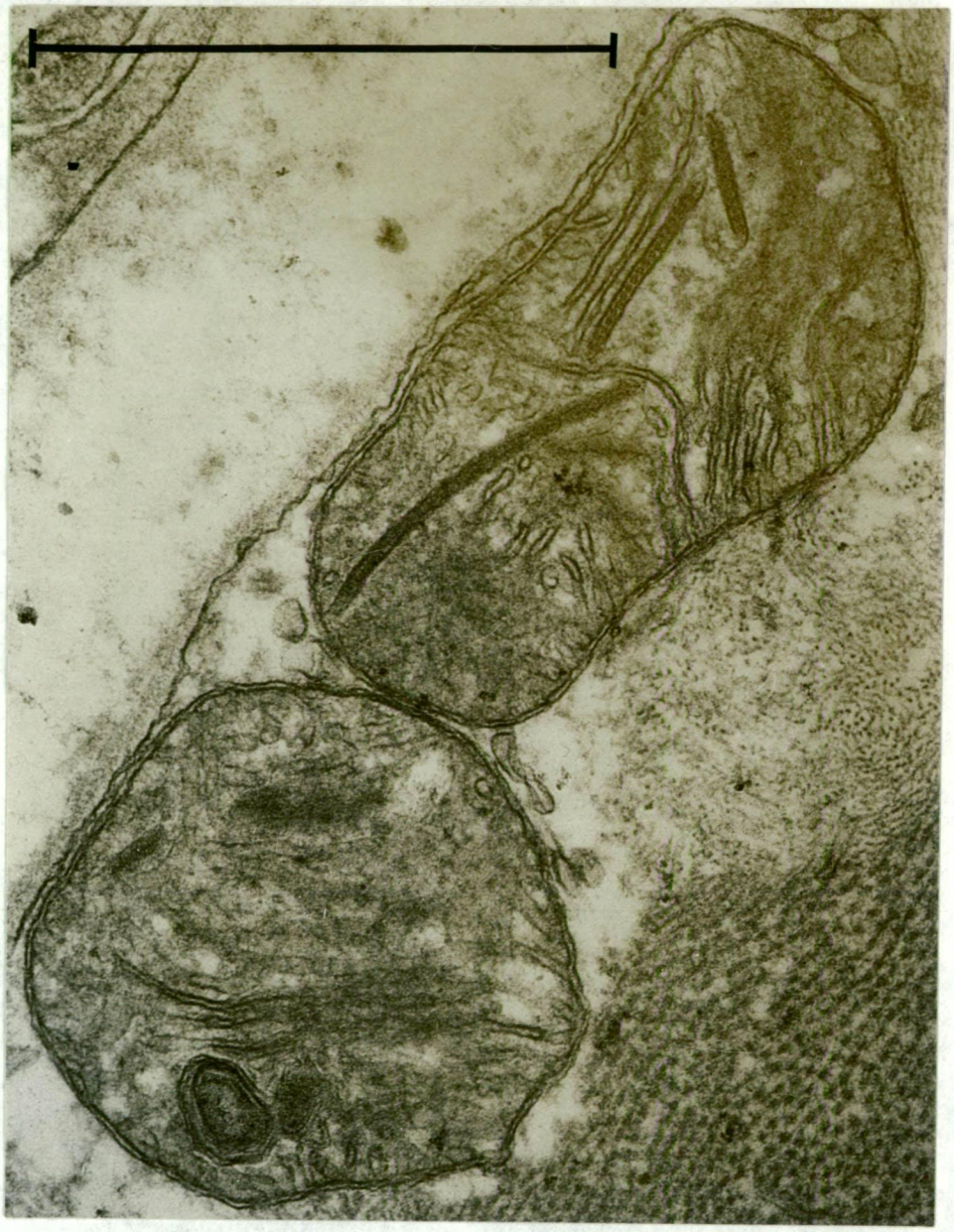


Figure 45c

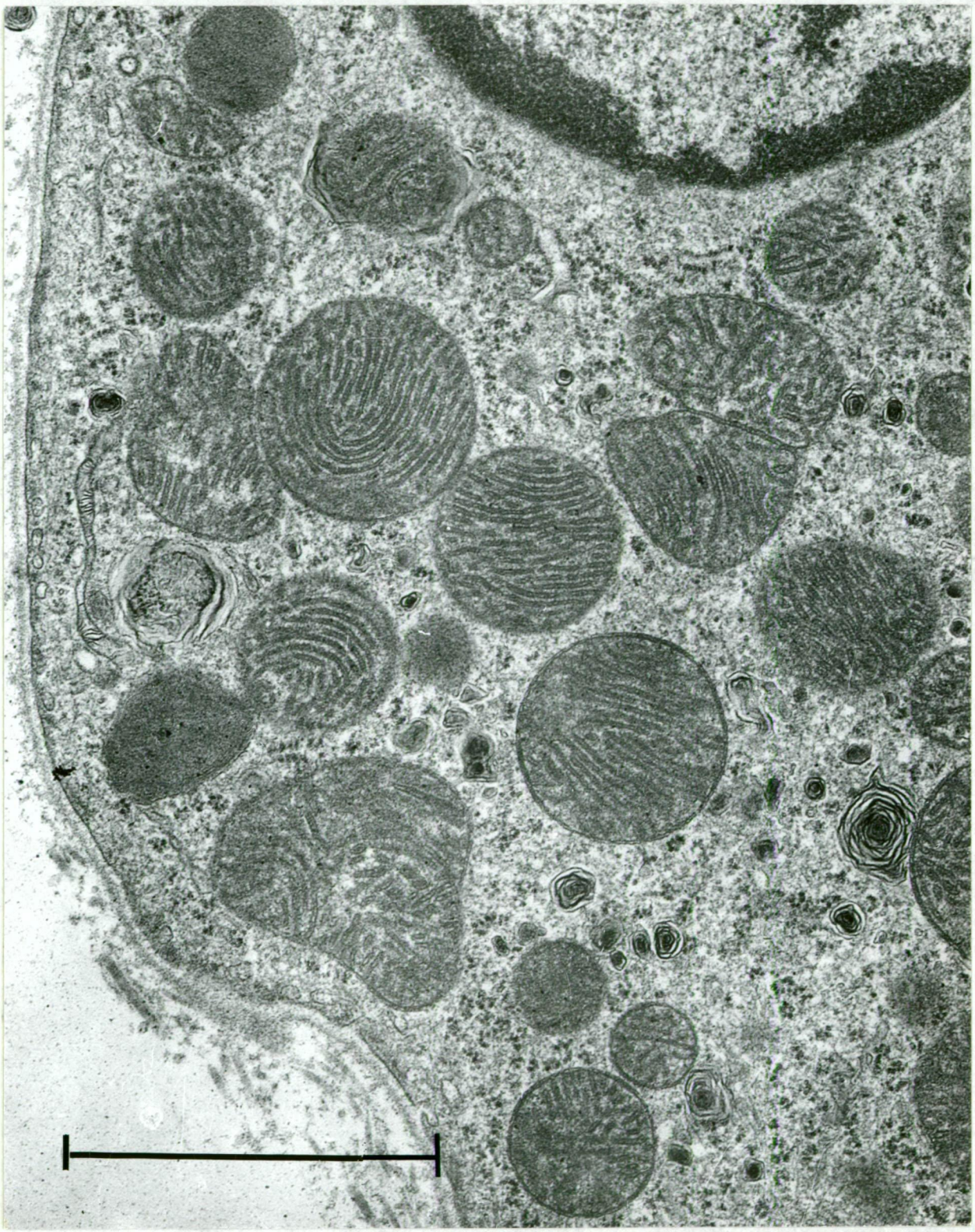


Figure 45d

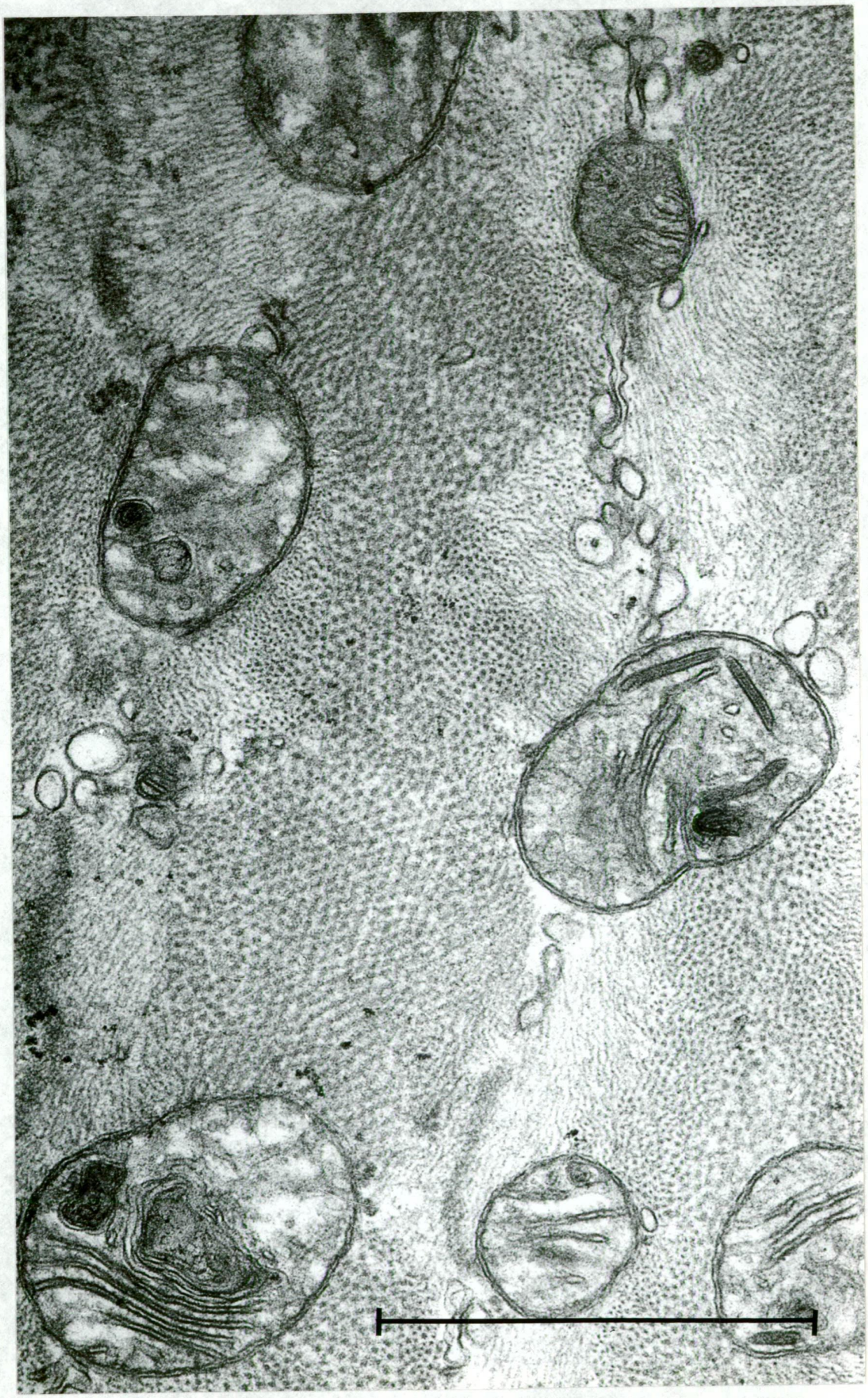
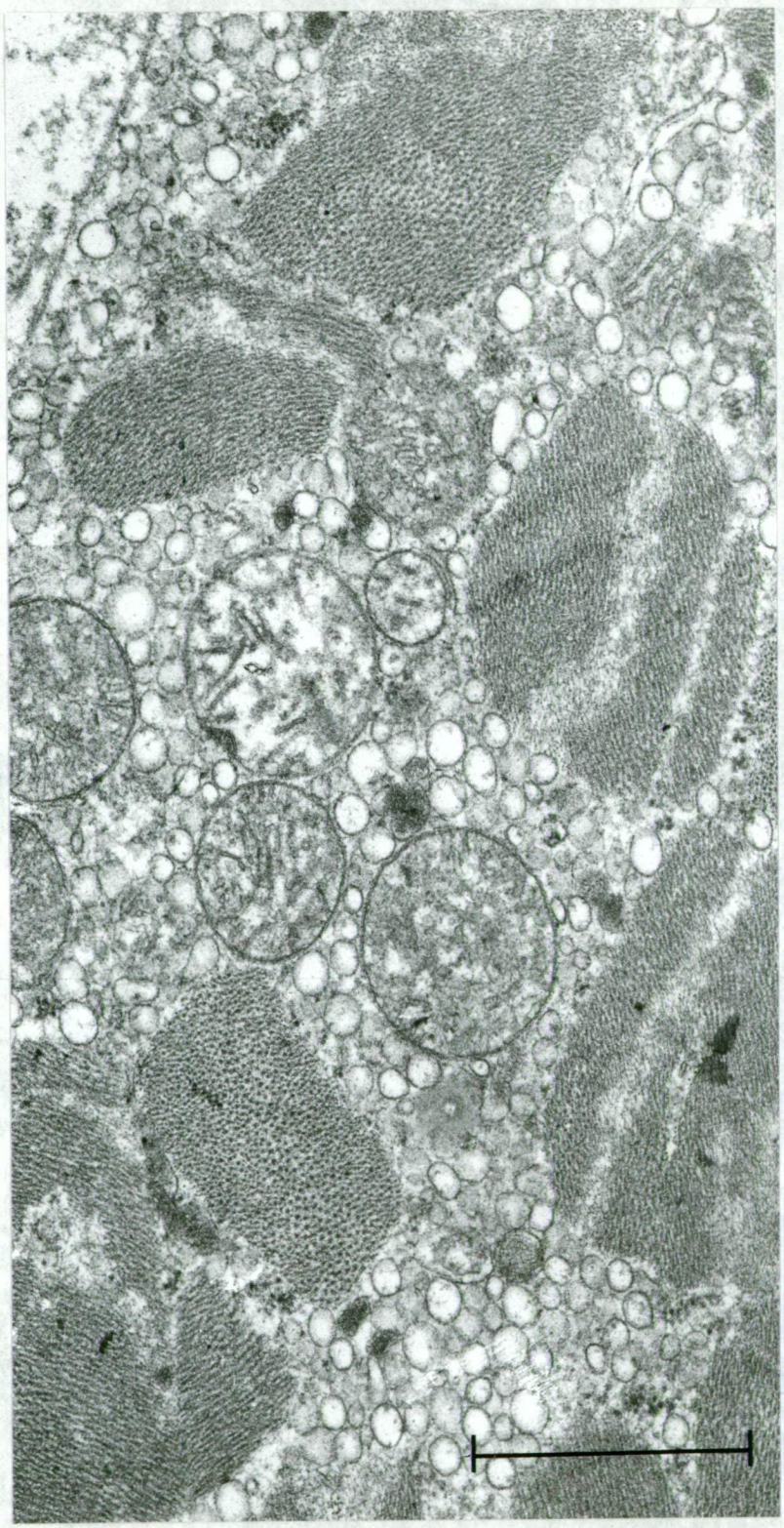


Figure 45e



DISCUSSION

Rat muscle has a greater mitochondrial mass than human muscle and many type I fibres show intense subsarcolemmal staining with oxidative reactions and have a peripheral smooth red ring with the modified Gomori trichrome stain. Histochemical pointers to mitochondrial disease are therefore more difficult to identify in rats than in humans.

Muscle fibres with a ragged red margin and internal accumulations of red staining material are not found in normal rat muscle, however, and the histological and histochemical changes seen after DNP are very similar to those in human mitochondrial myopathies.

The histological findings were identical to those found by Melmed et al (1975) and Sahgal et al (1979) in rat plantaris and soleus. The fact that ragged red fibres were less numerous in this study reflects the low proportion of oxidative fibres in the gastrocnemius muscle (Ariano et al 1973). A marked depletion of glycogen seen in DNP poisoned muscle stimulated at very low frequencies suggests that glycogenolysis and glycolysis are accelerated. A similar glycogen depletion was observed by Melmed et al (1975).

The ultrastructural findings after dinitrophenol poisoning were identical with those reported by Melmed et al and by Walter et al. No paracrystalline inclusions, similar to those produced by Sahgal et al were identified reflecting the shorter maximum delay between drug exposure and muscle sampling in this study. Melmed et al contrasted the appearance of the single line plate like intracristal inclusions produced by dinitrophenol with intracristal inclusions seen in ischaemic mitochondria (Karpati et al 1974) pointing out that in the latter the intracristal plates are typically larger and more elaborate with 2 rather than 1 dense lines (so called trilaminar inclusions). This differentiation is a tenuous one however, as Heffner and Barron identified single line intra cristal inclusions in ischaemic skeletal muscle.. The appearances of the mitochondria after dinitrophenol in this study have considerable similarities to those seen with ischaemia (Heffner and Barron 1978). The finding of numerous degenerating mitochondria with fractured or lysed crista and or myelin figures suggests that DNP induces more severe morphological changes than those seen in human mitochondriopathies.

Morphological alterations in diphenyleneiodonium poisoned muscle were much less striking. No definite ragged red fibres were seen with the trichrome stain and in most blocks the histochemical changes were marginal. Electron microscopy showed occasional rounded intermyofibriller and subsarcolemmal mitochondria but the great

majority of mitochondria were structurally normal. No degenerating forms were seen.

These observations suggest that mitochondrial structural alterations with swelling, development of intracristal plates and alterations in cristal orientation occur very rapidly after dinitrophenol poisoning, and parallel force failure. With intraarterial injection of the respiratory chain blocker, diphenyleneiodonium, however, a severe force failure was accompanied by only minor structural mitochondrial changes.

1.4 Biochemical Observations.

1.4.1 Control Experiments.

i. Analysis of Freeze Clamped Specimens

ATP, ADP, IMP, PCr, and lactate levels in the gastrocnemius muscle in control experiments are shown in Table 4. After stimulation at 5c/sec for 30 minutes mean PCr level was 14.9% of the mean concentration in unstimulated muscle. Mean PCr had recovered to 58% of initial mean value in muscles frozen after 15 minutes of rest. Mean ATP concentration was 47% of initial value in muscles frozen after 30 minutes stimulation, and recovery after 15 minutes rest was incomplete (to mean 60.5% of unstimulated level). The adenylate deaminase reaction is not readily reversible and the conversion of significant amounts of AMP to IMP probable accounts for the delayed ATP recovery post stimulation. A four fold rise in muscle lactate levels was seen after 30 minutes stimulation but the concentrations had almost returned to basal values after 15 minutes rest.

ii. Nuclear Magnetic Resonance

Separate NMR experiments were carried out as indicated in the methodology section. Tension recordings were not made under strict isometric conditions because of the need to avoid metallic structures inside the magnet, and as a result twitch

tension amplitudes recorded were only 20 - 25% of those recorded in the animal frame. Individual twitch tensions were stable in any one preparation however and serve as an indication of the working capacity of the muscle. 2 control experiments were carried out with 5c/sec stimulation and 1 with 1c/sec stimulation. The changes in ATP and PCr levels with work were similar to those found with conventional biochemical techniques. The PCr fell to 85% of the initial level after 10 minutes of 1c/sec stimulation and was then stable for upto 60 minutes of stimulation. 5c/sec stimulation resulted in a drop in PCr to 20 - 30% of the initial level but it again stabilised at a new plateau level for upto 30 minutes. A small fall in ATP levels paralleling observations in freeze clamped muscle was also seen during 5c/sec stimulation. In 1 experiment, ATP levels did not recover over 15 minutes rest, inspite of a marked increase in PCr. This finding again suggests that a significant amount of AMP had been converted to IMP and was not immediately available for ATP resynthesis (see Figure 54a).

TABLE 3

Title

5c/sec Stimulation: Control Biochemical Data

	Baseline Levels (N3)	5c/sec stimultn. 30 minutes (N4)	15 minutes rest post stimultn. (N5)
Lactate	1.79 ± 0.64	7.27 ± 1.70	2.64 ± 1.20
PCr	8.90 ± 0.81	1.33 ± 1.22	5.18 ± 0.55
ATP	7.60 ± 0.40	3.60 ± 0.19	4.62 ± 1.01
ADP	0.78 ± 0.08	0.92 ± 0.09	0.75 ± 0.30
AMP	0.004 ± 0.002	0.06 ± 0.20	0.008 ± 0.002
IMP	0.52 ± 0.08	1.71 ± 0.40	1.01 ± 0.20
Twitch Tension (% initial level)	100	55 ± 16	60 ± 7.5

Legend

Metabolic changes with 5c/sec stimulation (mean and SD, levels in umol/g wet weight of muscle). The gastrocnemius was frozen after 30 minutes stimulation in 4 experiments and 15 minutes post-stimulation for 30 minutes in 5. The baseline data represents unstimulated muscle. Note the rapid but still incomplete recovery in PCr and lactate levels. ATP recovery is also incomplete after 15 minutes rest and considerable amounts of IMP remain in the muscle.

1.4.2 Metabolic Changes with Ischaemia

The gastrocnemius muscle was freeze clamped after 39 minutes of ischaemia in 2 of the experiments shown in Table 3. At the time of freeze clamping, these muscles were unable to generate a twitch response and the resting tension had risen by a mean of 23% (22 - 24%) of the initial twitch tension amplitude. A marked fall in PCr and ATP levels was found, in association with a massive rise in IMP levels. Lactate levels were eight times those seen in control preparations frozen at a similar time.

TABLE 4

Ischaemic Experiments: Metabolite Levels (umol/g wet weight)

Lactate	16.20
PCr	0.06
ATP	0.67
AMP	0.12
IMP	5.17

Legend

Mean metabolite changes in 2 experiments in which the aorta was occluded during a 5c/sec stimuli train. Stimulation was continued for 30 minutes and the specimens frozen 15 minutes later.

1.4.3 Dinitrophenol.

i. Freeze Clamped Muscle

In experiments in which 2c/min stimulation was carried out, the muscle was frozen at present times after injection of dinitrophenol. Specimens were later analysed for metabolite and nucleotide levels as described in the methodology secion. The fall in isometric twitch tension was closely related to a fall in ATP levels (Figure 46) with a correlation co-efficient of 0.90. A massive rise in IMP levels also correlated fairly well (correlation co-efficient 0.89) with twitch failure. The relationship between contracture development and ATP depletion was less well defined. A considerable variation in the level of contracture in different experiments was seen at similar levels of ATP depletion (Figure 46). A severe depletion of phosphocreatine and an accumulation of lactate was also found in contractured muscle (Table 5). Similar changes in nucleotide levels were seen in muscles stimulated at 5c/sec, freeze clamped after contracture development (Table 6). These closely resembled the findings in ischaemic muscle.

ii. Phosphorus Nuclear Magnetic Resonance

The biochemical events in 2 muscles at rest were followed after intraarterial infusion of 16mg/Kg of DNP. A steady depletion of phosphocreatine was seen. This

observation confirms the suggestion that DNP actively accelerates ATP hydrolysis in vivo (Figure 47).

In 3 further experiments, 12mg/Kg of DNP was administered in 4mg/Kg bolus after 20 minutes of stimulation at 1c/sec. Prior to infusion the twitch tension, PCr and Pi levels had stabilised (Figure 51). The mean steady state twitch tension was 73.6% of the initial value, the mean steady state PCr/Pi + PCr ratio 81% of the resting value and the mean steady state ATP level (total α, β, γ peaks) 83% of the resting level. Following infusion of DNP a progressive failure of twitch tension, associated with a pathological contracture developed. The eventual contracture tension reached (mean 260g) equated fairly well with tensions recorded in the animal frame. Analysis of nuclear magnetic resonance spectra showed that these tension changes were associated with a steady depletion of PCr, followed by ATP depletion and with a fall in intramuscular pH (Figure 48a,b). Considerable amounts of PCr were still present, however, some minutes after the onset of contracture, although when rigor was complete, no PCr or ATP could be detected with this technique. The fall in PCr/Pi + PCr ratio and the less rapid fall in total ATP levels in relation to twitch failure are shown in Figure 49. Phosphocreatine depletion is well advanced before contracture begins (Figure 50b). ATP depletion is more closely related to contracture development than in experiments in which muscle was analysed by HPLC techniques (Figure 50a).

This apparent discrepancy probably reflects the fact that serial observations in the same muscle are only made in nuclear magnetic resonance studies. The fall in PCr/Pi + PCr ratio is related to the fall in total ATP levels in Figure 51. A moderate fall in intracellular muscle pH develops in the latter stages of twitch failure (Figure 52).

Figure 46

Title

Analysis of freeze clamped muscle: dinitrophenol infusion. 2c/min stimulation.

Legend

The upper graphs show the correlation between the fall in muscle ATP concentrations and twitch tension failure (left hand graph) and contracture expressed as a % of the initial twitch tension (right hand graph). Correlation co-efficient for ATP depletion and twitch failure 0.88 and for ATP depletion and contracture 0.60. The lower graphs show the relationship between IMP accumulation, twitch failure (on the left) and contracture (on the right). The correlation co-efficient for the linear regression line describing IMP accumulation and twitch failure is 0.92 and that for the line describing IMP accumulation and contracture is 0.61. Note that in contractured muscle, almost all the ATP is converted to IMP.

Figure 46

Title

Analysis of freeze clamped muscle: dinitrophenol infusion. 2c/min stimulation.

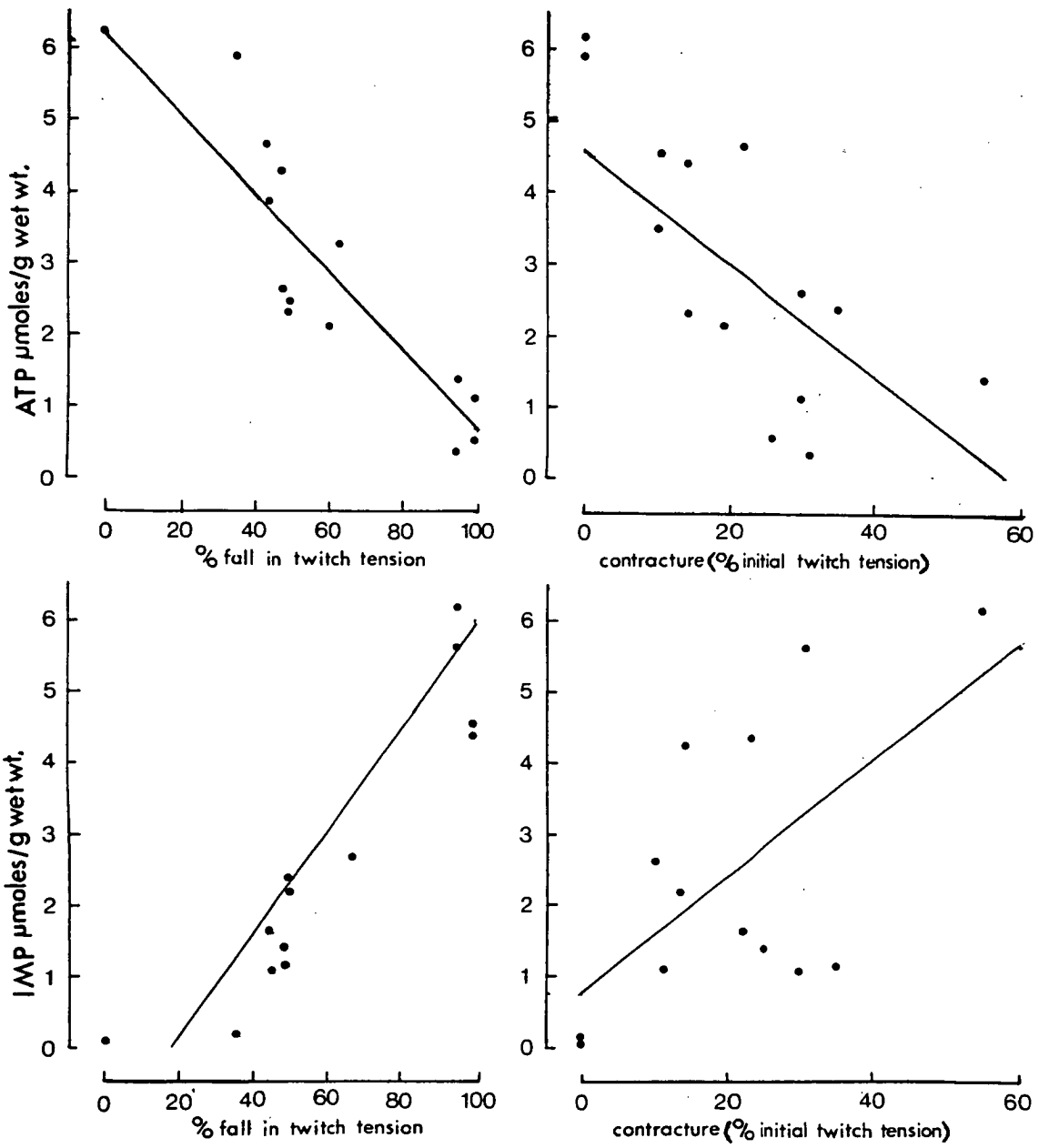


TABLE 5

Title

Nucleotide and metabolite levels: dinitrophenol infusion, 2c/min stimulation (levels in umol/g wet weight).

	TIME OF FREEZE CLAMPING			
	BASELINE	15MINS (N4)	20MINS (N4)	30MINS (N3)
Lactate	1.79±0.64	10.15±3.00	11.6±7.50	14.4±4.90
PCr	8.90±0.80	1.59±0.53	1.4±0.42	0.03±0.05
ATP	7.60±0.40	3.60±1.70	2.0±0.58	1.53±1.00
ADP	0.78±0.08	0.91±0.38	0.94±0.41	0.73±0.15
AMP	0.004±0.002	0.05±0.02	0.16±0.10	0.15±0.02
IMP	0.52±0.08	0.85±0.29	2.22±1.54	4.00±2.40
Twitch Tension	100	49	38	19
Contraction	0	13	25	35

Legend

16mg/Kg DNP was infused between 0 and 10 minutes. Results show mean and SD. Twitch tension are given as a % of initial amplitude and contracture tension as increase in resting tension as a % of initial twitch tension. Note the marked increase in lactate, and IMP levels and severe depletion of PCr after only 60 twitches.

TABLE 6

Title

Metabolite Levels: Dinitrophenol Infusion: 5c/sec Stimulation (N4)

Lactate	16.20 ± 1.30
PCr	0.06 ± 0.06
ATP	0.67 ± 0.31
ADP	0.63 ± 0.10
AMP	0.12 ± 0.08
IMP	5.17 ± 0.97
Twitch Tension	0.75 ± 0.95
Contracture	49.50 ± 4.80

Legend

Metabolite levels for 4 experiments in which 16mg/Kg DNP was infused over 10 minutes during a 5c/sec stimulus pattern. The gastrocnemius was frozen during stimulation after almost complete twitch failure. Twitch tension is expressed as a % of initial amplitude and contracture tension as increase in resting tension as a % of initial twitch tension amplitude. Nucleotide and metabolite levels (mean & SD) are in umol/g wet weight.

Figure 47

Title

31 phosphorus nuclear magnetic resonance (NMR) analysis: dinitrophenol injection: spectra recorded over resting muscle.

Legend

Spectra recorded through a surface radio frequency coil closely applied to the body of the right gastrocnemius muscle. 16mg/Kg of dinitrophenol was infused in 4 boluses at 2 minute intervals. NMR spectra are collated over 4 minute periods. Note the progressive depletion of phosphocreatine and accumulation of inorganic phosphate induced by dinitrophenol in resting muscle. The pH value is given by the distance between the Pi and PCr peaks.

Figure 47

Title

31 phosphorus nuclear magnetic resonance (NMR) analysis: dinitrophenol injection: spectra recorded over resting muscle.

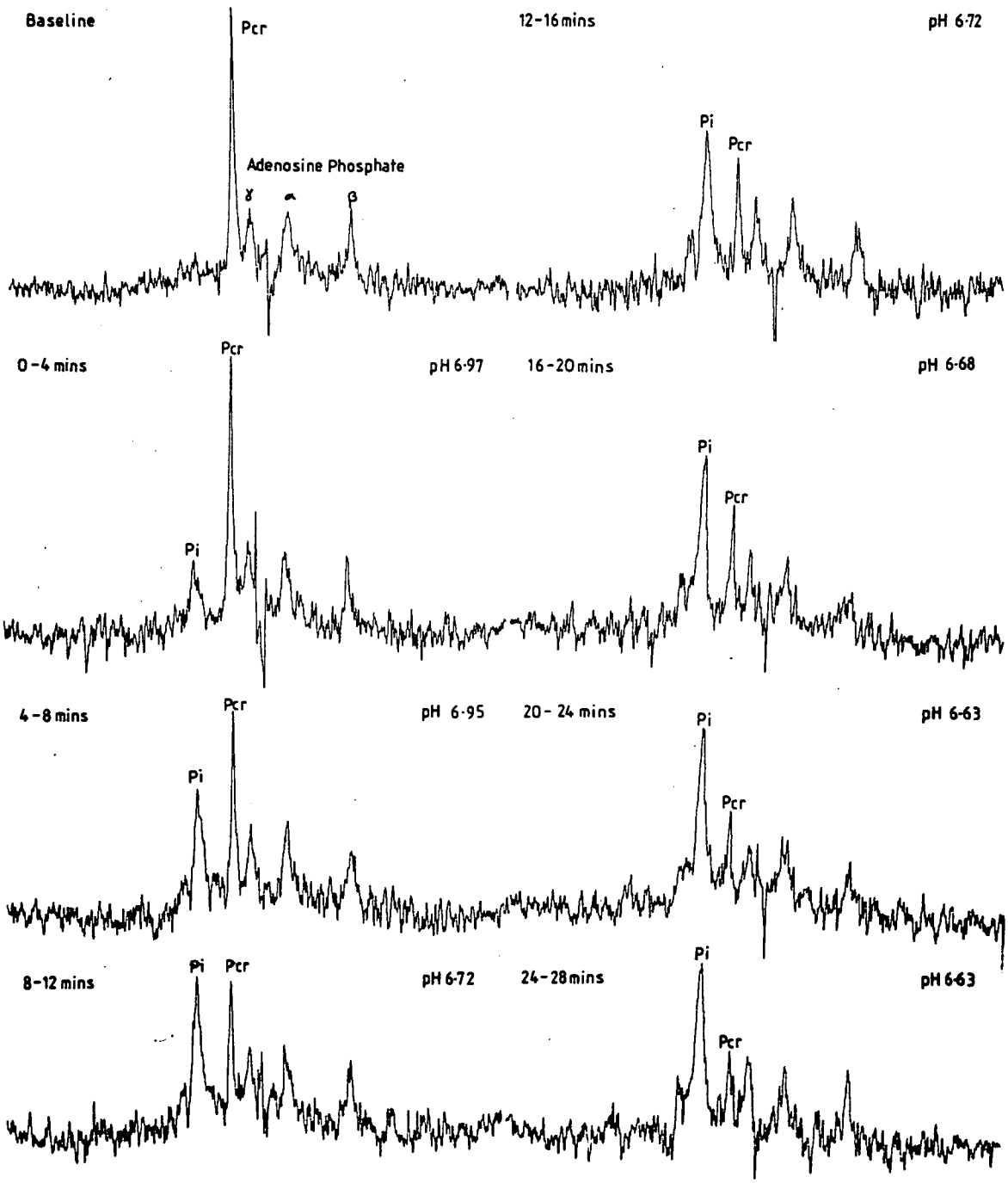
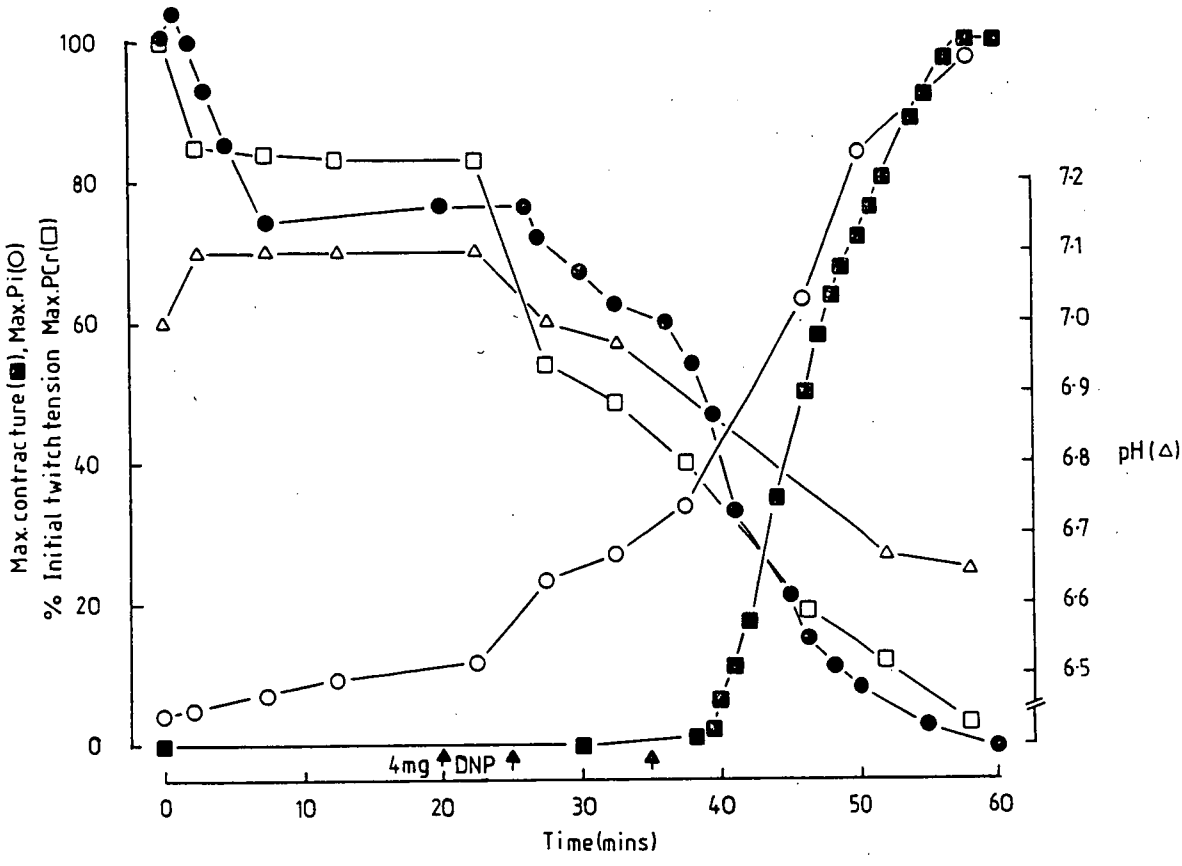


Figure 48a

Title

31 phosphorus nuclear magnetic resonance analysis: dinitrophenol infusion during 1c/sec stimulation: typical experiment.



Legend

Ordinate - % of initial twitch tension ● , % of maximum contracture tension ■ , % of initial phosphocreatine concentration □ , % maximum inorganic phosphorus concentration ○ and intramuscular pH △ . Abscissa - time in minutes. Boluses of 4mg/Kg DNP indicated by the arrows. NMR spectra were summated over 5 minute periods. Selected spectra from this experiment are illustrated in Figure 48b.

Figure 48b

Title

31 phosphorus nuclear magnetic resonance analysis: dinitrophenol infusion during 1c/sec stimulation: typical experiment.

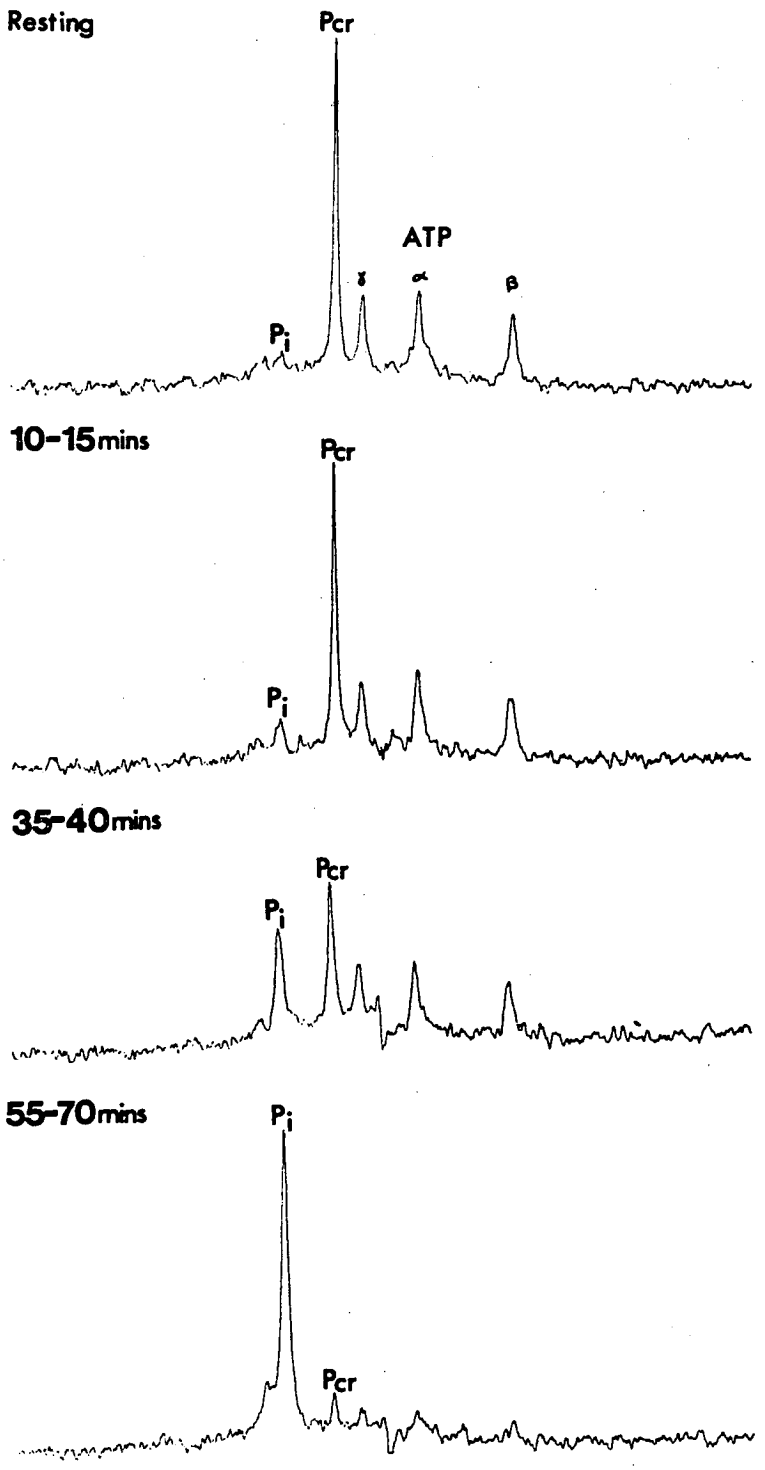
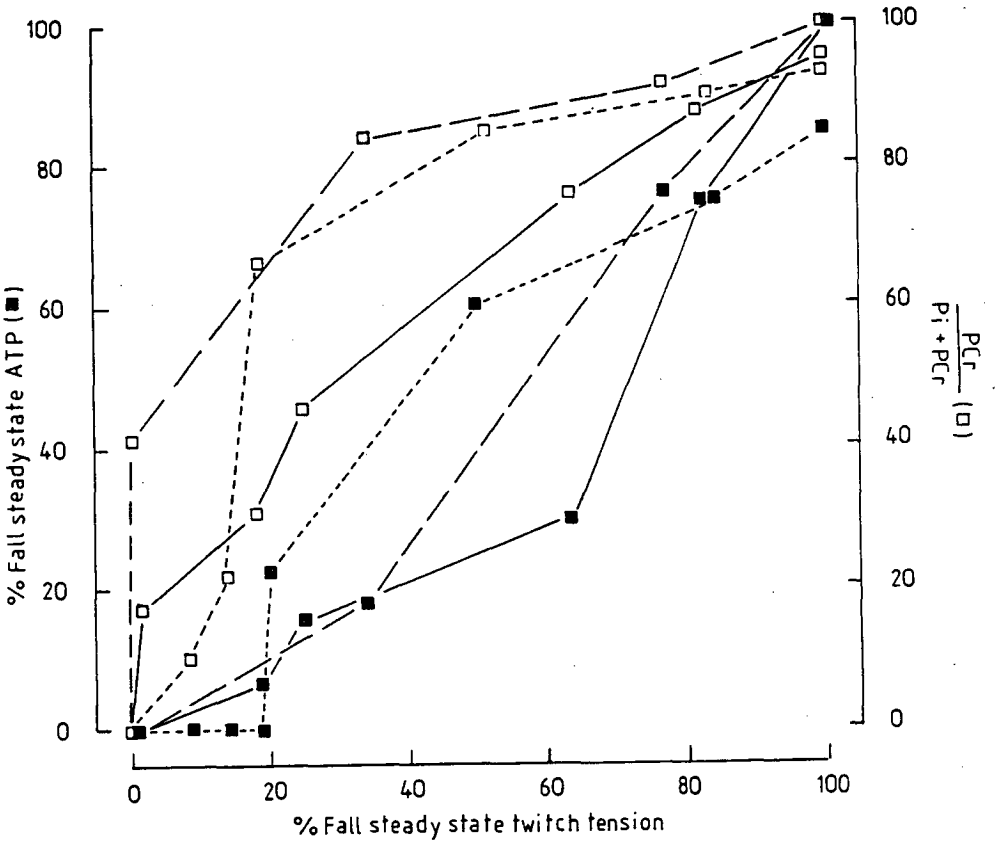


Figure 49

Title

31 phosphorus NMR analysis: dinitrophenol infusion during 1c/sec stimulation: relationship between fall in PCr/Pi + PCr ratio and in ATP concentration with twitch tension failure.



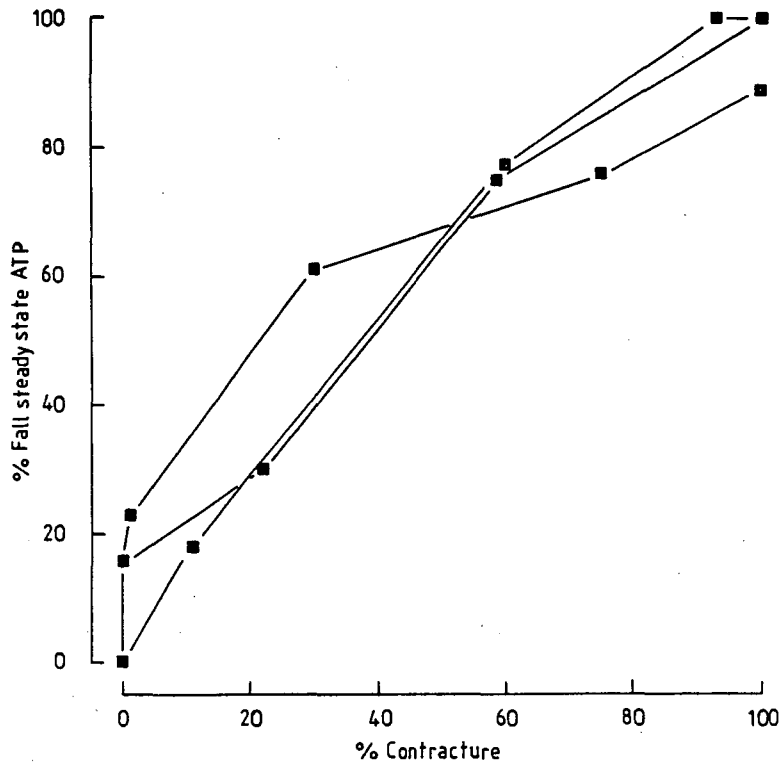
Legend

Ordinate - % of steady state PCr/Pi + PCr ratio □ and of steady state ATP concentration ■ in 3 experiments. Abscissa - fall in steady state twitch tension as a %. Each experiment is denoted by the same line pattern. The steady state value is that recorded after 20 minutes stimulation at 1c/sec prior to DNP injection. 12mg/Kg of dinitrophenol was injected in 3 boluses in all experiments. No recovery in PCr or ATP levels developed with rest. Note that although PCr is depleted more rapidly than ATP in the early stages of twitch failure there is considerable overlap in the rates of depletion.

Figure 50a

Title

31 phosphorus NMR analysis: dinitrophenol infusion during 1c/sec stimulation: relationship between fall in ATP concentrations and contracture.



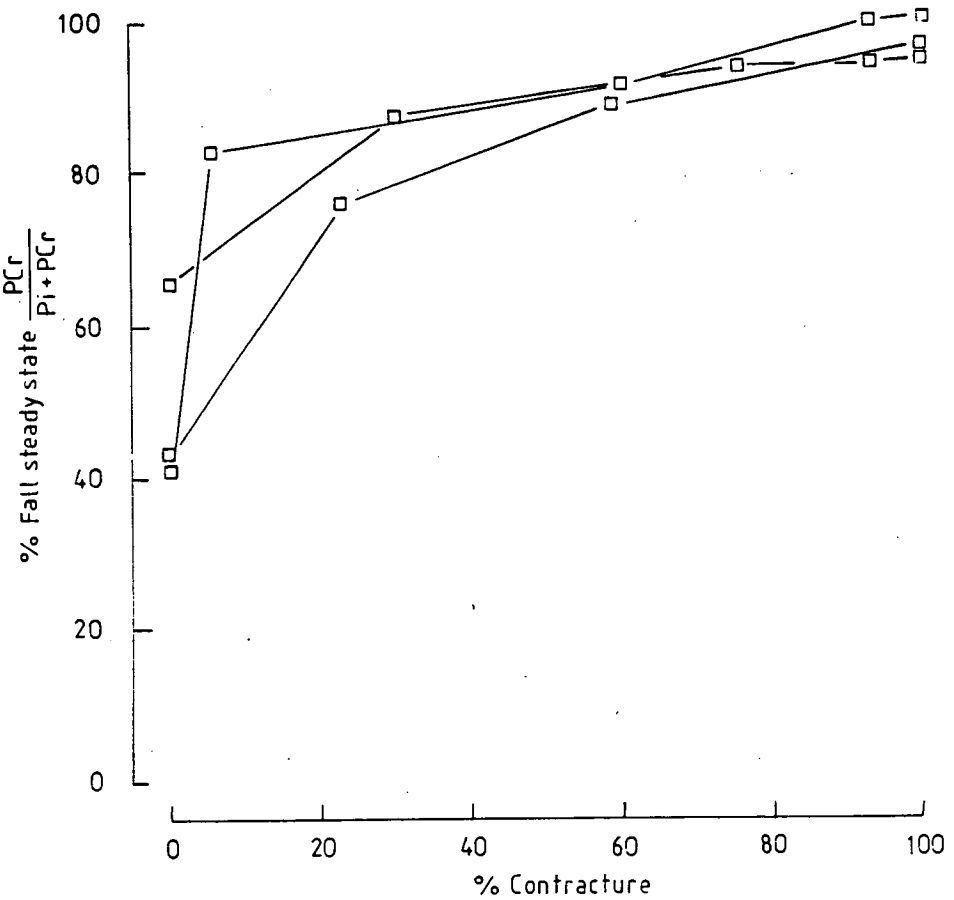
Legend

Ordinate - % of steady state ATP concentration (summation of alpha, beta and gamma peaks). Abscissa - % of maximum contracture tension. Data for 3 experiments with continuous 1c/sec stimulation.

Figure 50b

Title

31 phosphorus NMR analysis: dinitrophenol infusion during 1c/sec stimulation : relationship between fall in PCr/Pi + PCr ratio and contracture.



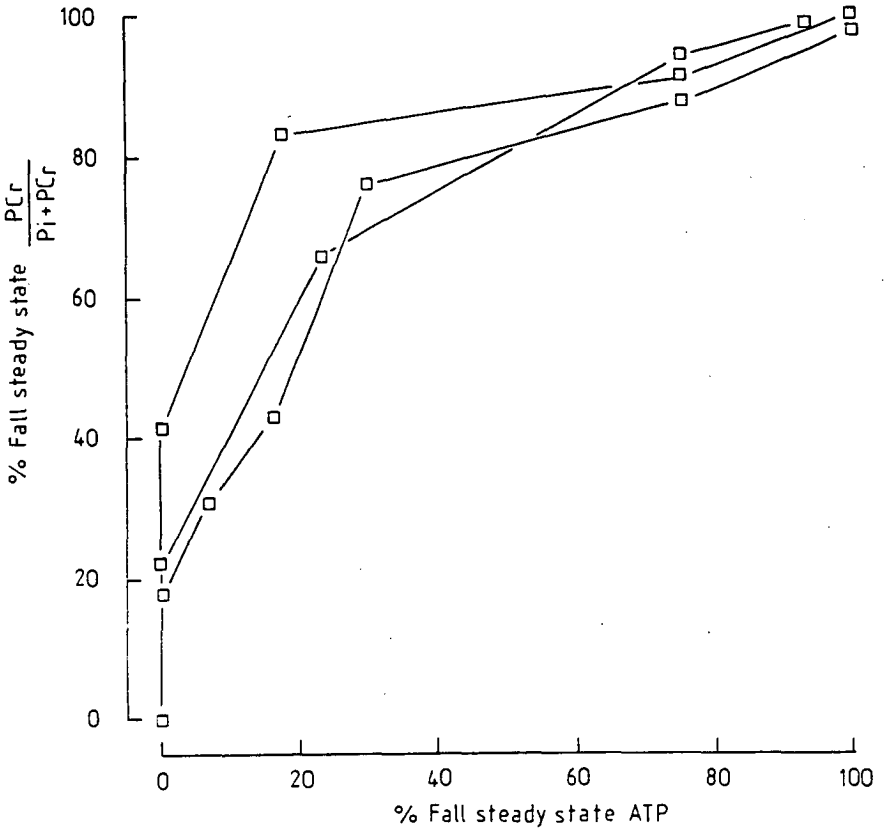
Legend

Relationship between the fall in $\frac{PCr}{Pi + PCr}$ ratio (as a % of the steady state level) and contracture as a % of the maximum levels reached. Data for the same 3 experiments.

Figure 51

Title

31 phosphorus NMR analysis: relationship between PCr and ATP depletion in working muscle after infusion of dinitrophenol.



Legend

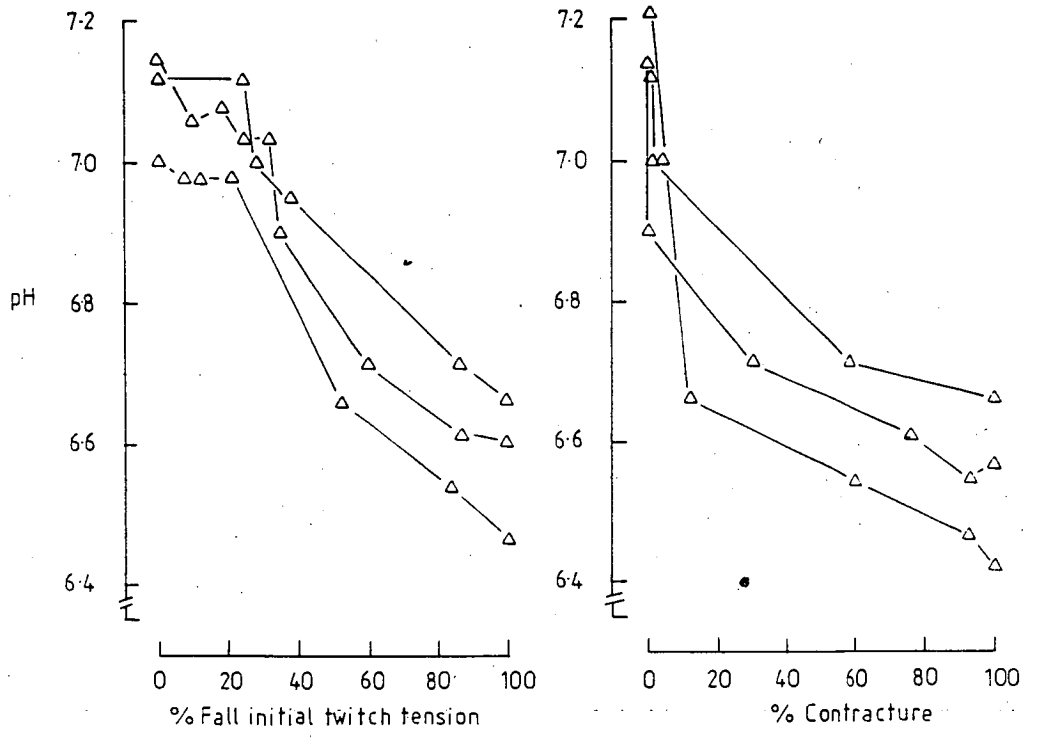
Ordinate - % fall steady state $\frac{PCr}{Pi + PCr}$ ratio.

Abscissa - % fall in steady state ATP concentration.
Data for 3 experiments in which dinitrophenol was infused after ATP and PCr levels had stabilised during a 1c/sec stimulus train.

Figure 52

Title

31 phosphorus NMR analysis: changes in intramuscular pH after dinitrophenol infusion in working muscle.



Legend

pH changes in the experiments illustrated in Figures 54 to 56 are correlated with % fall in initial twitch tension (left) and % maximum contracture (right).

1.4.4 Diphenyleneiodonium Experiments.

i. Freeze Clamped Muscle

In 4 experiments the gastrocnemius was frozen for biochemical analysis 22 minutes after injection of DPI during a 5c/sec stimulation train (30 minutes after the onset of stimulation). The isometric twitch tension had fallen to a mean of 16.6% (SD 5.6) of the initial value at the time of freezing. A further series of muscles were frozen for analysis after 15 minutes rest. Nucleotide and metabolite levels are shown in Table 7. Lactate accumulation in muscles frozen after 30 minutes stimulation was identical to the levels seen in controls. In control experiments, concentrations rapidly returned towards basal levels, whereas in the DPI group no fall was observed in specimens frozen after 15 minutes rest. The mean fall in ATP levels in the DPI group after 30 minutes stimulation was only slightly greater than in controls and there was considerable overlap in the range of individual values inspite of a major difference in isometric twitch tension (see Tables 3 & 7). No PCr was detected in the DPI group after 30 minutes stimulation with this technique, and the recovery in PCr concentrations after 15 minutes rest was less than that in the control group (see Tables 3 & 7) IMP concentrations were approximately double the control values after 30 minutes stimulation and 15 minutes recovery. Only one muscle was analysed in which contracture had began to evolve (Exp. 351). The twitch tension was only 5% of the initial amplitude and the resting tension had increased by 14%

of the initial twitch tension when the muscle was freeze clamped 140 minutes after injection of DPI. The ATP concentration was 1.84umol/g wet weight, IMP 2.51umol/g wet weight, and AMP 1.0umol/g wet weight. ATP depletion was therefore more severe than in non contractured muscle.

ii. 31P Nuclear Magnetic Resonance

2 experiments were carried out using phosphorus nuclear magnetic resonance to follow the biochemical changes. In one experiment, 4 X 1mg boluses of DPI were injected during a 1c/sec stimulus train and the changes in nucleotide levels followed serially. A progressive twitch failure was not associated with pathological contracture. The twitch tension fatigued completely and negligible amounts of PCr remained in the muscle at that time (Figure 53a & b). The extent of ATP and PCr depletion was as severe as that with DNP infusion at the time of onset of contracture.

In the second experiment, 5c/sec stimulus runs were carried out before and after administration of DPI. In the control 5c/sec stimulus run, the PCr/Pi + PCr ratio fell rapidly, stabilising at 29% of the initial value. Rapid recovery to 75% of the initial value within 4 minutes was seen with rest. The total ATP level stabilised at 49% of the initial level and recovery was slower (Figure 54a). Following infusion of diphenyleneiodonium (1mg X 2) during a second 5c/sec stimulation run, the PCr/Pi + PCr ratio fell

to 18.5% of the initial value. Recovery to 40% of the initial level was seen after 4 minutes rest but no further recovery developed over one hour. Recovery in twitch tension amplitude and in ATP levels was also incomplete (Figure 54b). Restimulation led to a fall within 3 minutes to a $\frac{PCr}{Pi + PCr}$ ratio of 6% of the initial value and a twitch tension of 12% of the initial value, the rapid fall in the latter closely resembling that seen in experiments in the animal frame.

TABLE 7

Title

Metabolite Changes: Intra-arterial injection of diphenylene iodonium during a 5c/sec stimulus pattern.

	Unstimulated Muscle (N3)	5c/sec stimultn. 30 minutes (N4)	15 minutes rest post stimultn. (N3)
Lactate	1.79 ± 0.64	7.07 ± 1.16	7.90 ± 3.30
PCr	8.90 ± 0.81	<0.40 ± 0.00	2.26 ± 1.60
ATP	7.60 ± 0.40	2.80 ± 0.92	3.35 ± 0.68
ADP	0.78 ± 0.08	0.87 ± 0.12	0.88 ± 0.14
AMP	0.004 ± 0.002	0.057 ± 0.01	0.050 ± 0.02
IMP	0.52 ± 0.08	2.13 ± 0.32	2.39 ± 0.73
Twitch Tension (% initial level)	100	16.6 ± 5.60	47 ± 12

Legend

Data for 7 experiments in umol/g wet weight,(mean & SD) in which DPI was infused during a 5c/sec stimulus pattern. The dose of DPI was between 1 and 3mg. Four muscles were freeze clamped after 30 minutes stimulation and 3 were freeze clamped 15 minutes after the end of a similar stimulus pattern. Twitch tension is given as a % of the initial amplitude. Note that mean ATP levels, both after 30 minutes stimulation and 15 minutes recovery, are only slightly less than in the control group (Figure 3). PCr was undetectable

in muscles exposed to DPI after 30 minutes stimulation and recovery in PCr was less than half that seen in controls after 15 minutes rest. The increase in lactate concentrations after 30 minutes stimulation was identical in controls and DPI experiments, but whereas lactate concentrations had almost returned to basal levels after 15 minutes rest in controls, in DPI experiments no fall was seen in lactate levels over this period.

Figure 53

Title

31 phosphorus NMR analysis: infusion of diphenyleneiodonium after stabilisation of twitch tension during a 1c/sec stimulus train.

Figure 53a

Legend

Ordinate - % of initial twitch tension ● , % of initial phosphocreatine concentration □ , % maximum inorganic phosphate concentration ○ and intramuscular pH △ . Abscissa - time in minutes. This graph shows the results in an experiment with 1c/sec nerve stimulation for 120 minutes. Diphenyleneiodonium was injected in 1mg boluses as indicated by the arrows. No recovery in nucleotide levels or in twitch tension was seen with rest. Contracture did not develop.

Figure 53b

Legend

Selected NMR spectra from the same experiment. Observations were collated over 5 minute periods.

Figure 53a

Title

31 phosphorus NMR analysis: infusion of diphenyleneiodonium after stabilisation of twitch tension during a 1c/sec stimulus train.

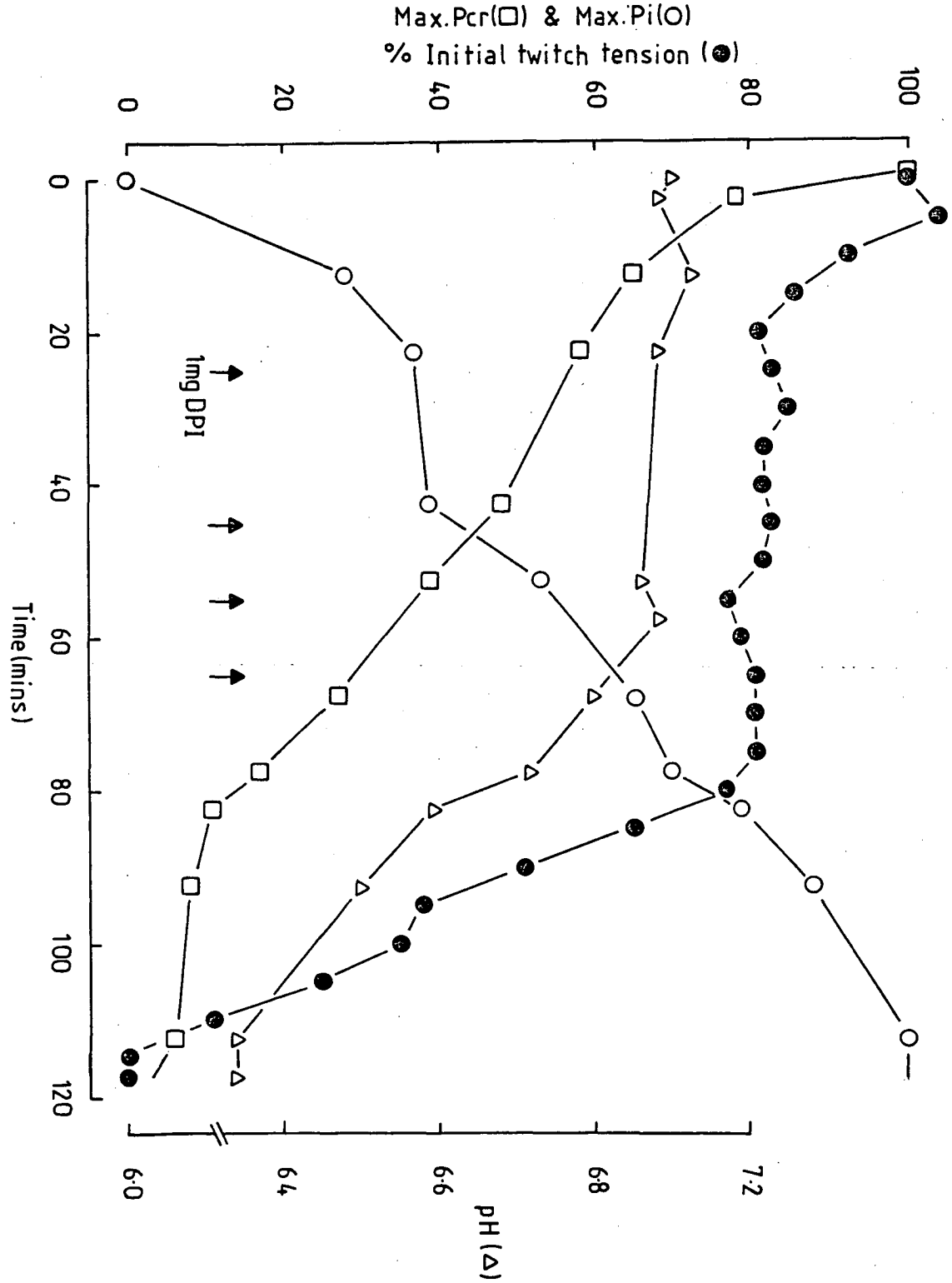


Figure 53b

Title

31 phosphorus NMR analysis: infusion of diphenyleneiodonium after stabilisation of twitch tension during a 1c/sec stimulus train.

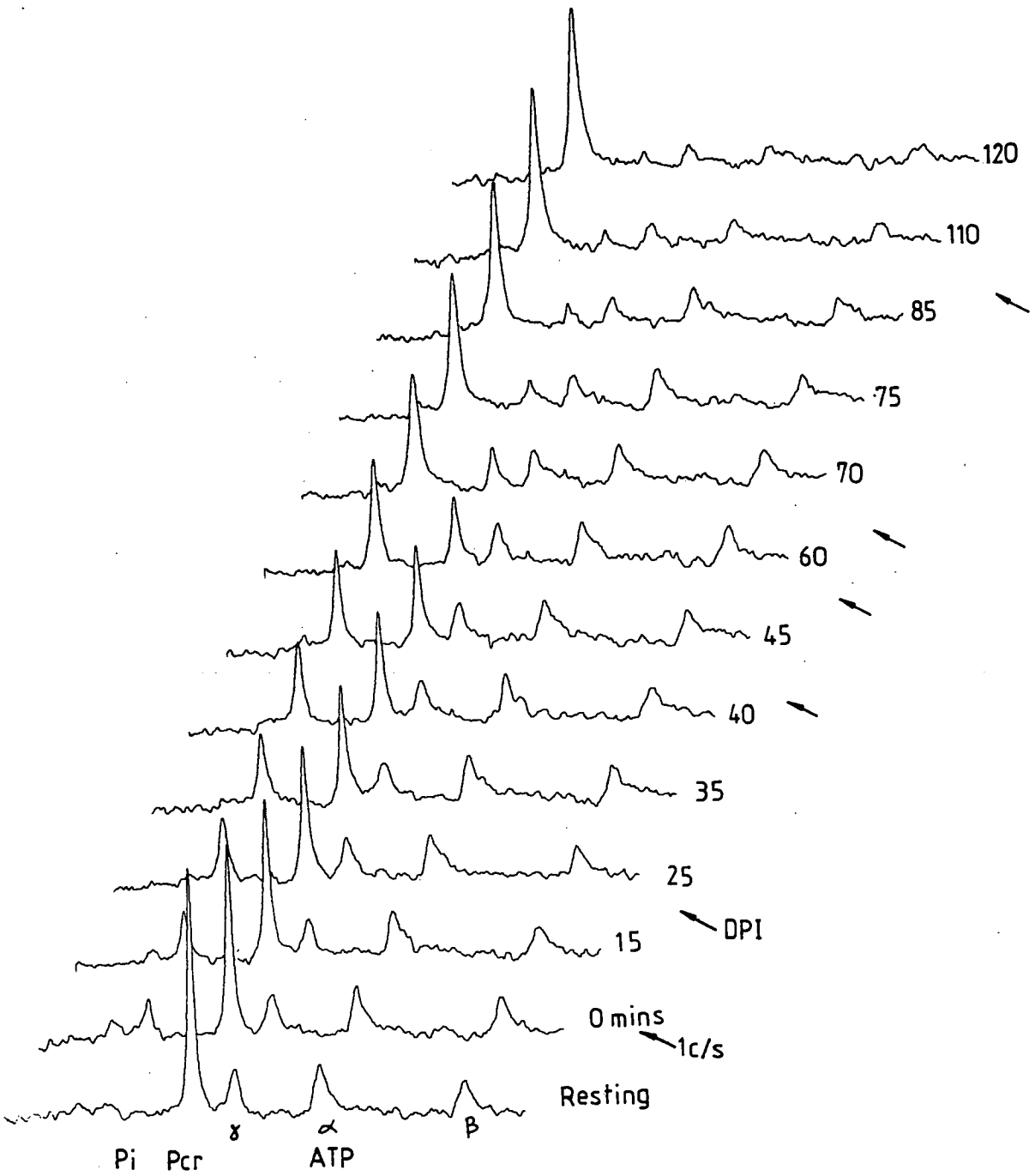
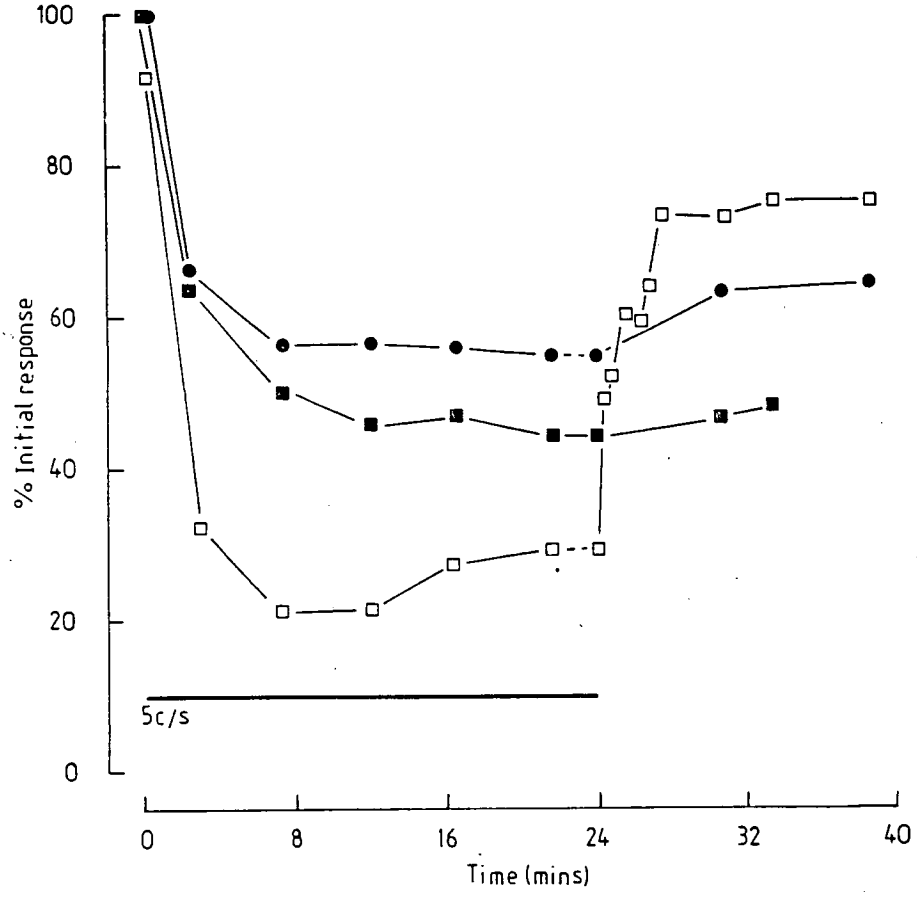


Figure 54a

Title

31 phosphorus NMR analysis: recordings during 5c/sec stimulus patterns before and after injection of diphenyleniodonium.



Legend

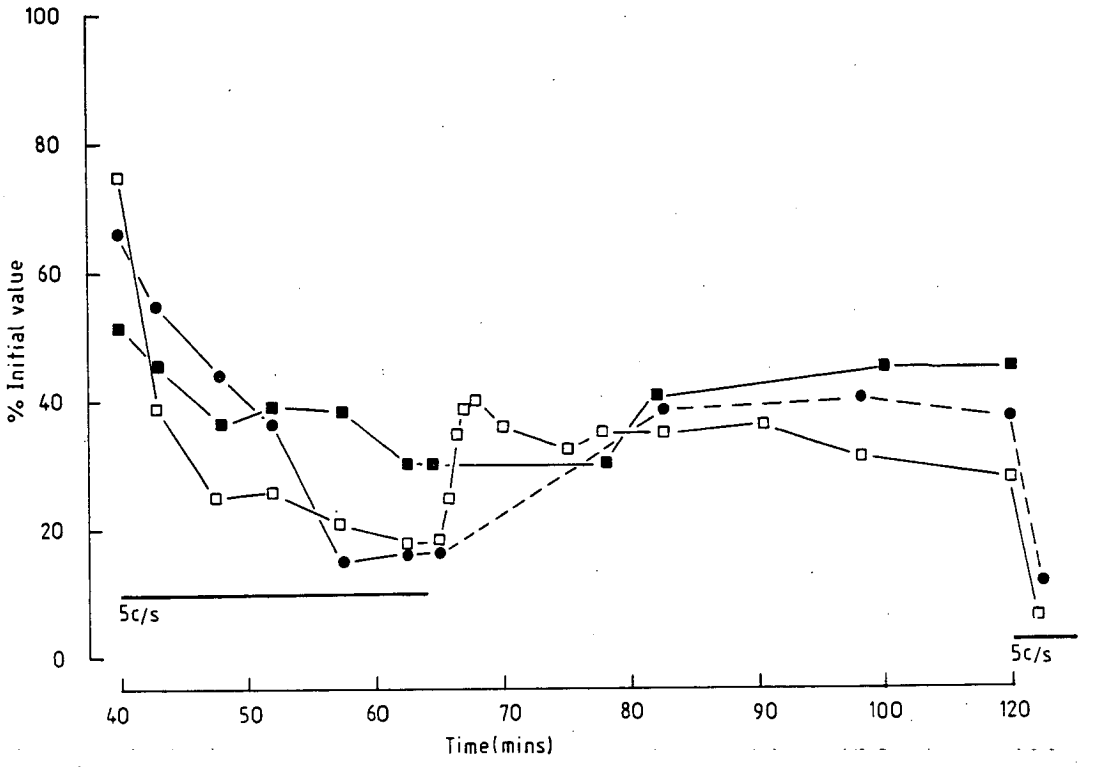
Control stimulus run before injection of diphenyleneiodonium. Ordinate - % of initial twitch tension ● , % of initial ATP concentration ■ , and % of initial $\frac{PCr}{Pi + PCr}$ ratio. □

Abscissa - time in minutes. 5c/sec stimulation from 0 to 24 minutes. Spectra were collated in periods of 4 minutes 48 seconds during stimulation and 36 seconds during early recovery. Note the delay in ATP recovery at this work rate (Paralleling recent observations in patients (Radda, personal communication).

Figure 54b

Title

31 phosphorus NMR analysis: recordings during 5c/sec stimulus patterns before and after injection of diphenyleneiodonium.



Legend

Data from the same experiment recorded during and after a second stimulus run (shown by a solid bar). 1mg boluses of diphenyleneiodonium were injected at 45 and at 50 minutes. Ordinate - % of initial value twitch tension ● , ATP ■ , and $\frac{PCr}{Pi + PCr}$ ratio □ . Abscissa - time in minutes.

Note that recovery in the $\frac{PCr}{Pi + PCr}$ ratio is initially rapid but plateaus out at an abnormally low level.

1.4.5 Antimycin A Experiments.

The gastrocnemius was freeze clamped in one experiment after injection of antimycin A during a 5c/sec stimulus pattern. The isometric twitch tension had fallen to less than 5% of the initial value at the time of freeze clamping. The biochemical findings resembled those seen in DPI poisoned muscle, with the exception that lactate levels were higher. No contracture had developed in this experiment.

TABLE 8

Title

Metabolite Levels after Injection of Antimycin A.

Lactate	17.93
PCr	0.93
ATP	2.45
ADP	1.0
AMP	0.09
IMP	2.88

Legend

Metabolite levels in one experiment in umol/g wet weight in which the gastrocnemius was freeze clamped 15 minutes after the end of a 5c/sec stimulus train. The twitch response had fallen to 4% of initial level after injection of 3.2mg/Kg of antimycin A and no recovery was seen prior to freeze clamping. Note appreciable ATP concentrations in a muscle with almost complete twitch failure but no contracture.

1.4.6 Iodoacetate Experiments

The gastrocnemius muscle was freeze clamped in 4 experiments in which almost complete twitch tension failure and a severe contracture developed after injection of iodoacetate during a 1c/sec stimulus pattern. A severe ATP depletion and IMP accumulation resembled that seen in DNP induced contracture.

TABLE 9

Nucleotide levels after injection of iodoacetate.

ATP	0.54 ± 0.29
ADP	0.51 ± 0.07
AMP	0.24 ± 0.15
IMP	2.43 ± 0.70
TWITCH TENSION	0.85% ± 1.2%
CONTRACTURE	57.2% ± 20.6%

Legend

Data (umol/g wet weight, mean and SD) for 4 experiments in which almost complete twitch failure and contracture developed after injection of iodoacetate during a 1c/sec stimulus pattern. Contractured muscle was freeze clamped in 1 experiment during stimulation and in 3 experiments after periods of rest of upto 2 hours.

DISCUSSION

Control Experiments

A considerable fall in PCr levels was seen after stimulation at 5c/sec for 30 minutes in samples analysed after freeze clamping. Nuclear magnetic resonance studies confirmed that the PCr/Pi + PCr ratio plateaued out at successively lower levels with increases in the work rate. This is in keeping with earlier observations in man that phosphocreatine is completely depleted after severe exercise (Sahlin et al 1979) and plateaus out at intermediate levels during continuous work of lesser severity (Harris et al 1977).

Analysis of freeze clamped material and NMR observations also revealed that ATP levels fell during 5c/sec stimulation to 60% of resting values. ATP recovery was incomplete after 15 minutes rest, because a considerable amount of AMP was converted to IMP and therefore was not immediately available for ATP resynthesis.

The rate of PCr recovery estimated by nuclear magnetic resonance after 5c/sec stimulation had a half time of 3 minutes, a similar time to that found with NMR studies in human subjects by Radda et al after exhaustive forearm exercise (Radda 1982 personal communication). The finding of substantial but incomplete recovery in PCr levels in freeze clamped muscle after 15 minutes rest parallels observations in human muscle (Essen & Kayser 1978).

Dinitrophenol Experiments.

A severe depletion of phosphocreatine was seen in muscle exposed to 2-4 dinitrophenol both at rest when examined by 31P nuclear magnetic resonance, and during stimulation at 2c/min, when examined by analysis of freeze clamped m scle. These observations parallel the in vitro findings of Davies et al (1959) and Fleckenstein et al (1954) many years ago when they observed rapid PCr depletion in resting frog muscle placed in a chamber containing DNP, and are in keeping with a stimulation of myosin and mitochondrial mg ATPase activity (see Levy et al 1963).

Muscle lactate concentrations in the gastrocnemius freeze clamped after intra-arterial injection of DNP in experiments with 2c/min and 5c/sec stimulation were much higher than the levels found in controls after 30 minutes stimulation at 5c/sec. This finding suggests either accelerated glycolysis as a compensatory mechanism or a defect in the further metabolism of pyruvate. It is paralleled by the exercise induced lactic acidaemia reported in several patients with mitochondrial myopathies (see Introduction).

Some differences were seen in the correlation of nucleotide levels when estimated by 31P NMR and by HPLC methods with mechanical events. The fall in ATP levels measured with both techniques correlated well with twitch tension failure. In freeze clamped specimens, however, a less linear relationship was found between contracture and ATP depletion.

Maximum contracture tension realised with DNP was variable between different experiments, and this data probably gives a misleadingly poor picture of the precise correlation between contracture and ATP depletion in any one experiment. This is supported by the finding of a linear relationship between ATP depletion and contracture in animals followed with 31P NMR. In severely contractured muscle almost all the ATP had been converted to IMP confirming the role of the adenylekinase and adenylate deaminase reactions in maintaining ATP levels for as long as possible (Chapman and Atkinson 1973).

Dinitrophenol induced contracture is unlikely to be related to changes in any single nucleotide or metabolite. Dinitrophenol blocks mitochondrial calcium reuptake and releases intra-mitochondrial calcium stores (Carafoli et al 1971; Lehniger et al 1967). A fall in ATP concentrations further raises cytosolic calcium levels by preventing calcium reuptake by the sarcoplasmic reticulm. The resultant rise in cytosolic calcium and ATP depletion may both be related to contracture. ATP estimations in other situations such as in rigor mortis or in skinned fibre preparations in contracture do not support ATP depletion as the sole immediate biochemical basis, as the ATP levels measured during contracture evolution was sufficient to prevent spontaneous rigor complex formation (Bendall 1951; Reuben et al 1971; Weber and Herz 1963). If however, ATP is not uniformly distributed throughout the cytoplasm, a controversial point (see Hill 1964; Podolski 1969) then more severe focal deficits in ATP concentrations could induce rigor. Ebashi and Lipmann (1962) found that in a rabbit

muscle suspension, very low levels of ATP (10^{-5}m) are adequate for calcium binding if ATP is continuously replenished by a phospho-enol pyruvate/pyruvate kinase system. If ADP is allowed to accumulate however, this inhibits calcium binding and much higher concentration of ATP (10^{-3}m) are necessary to maintain relaxation. Accumulation of cytosolic calcium, and ATP depletion therefore probably have a synergistic effect in contracture evolution.

The molecular basis of contracture has been explored in some detail. Xray data siggest that most myosin molecules are bound to actin during rigor (Huxley 1968; Huxley 1972; Moore et al 1970) and electron microscopy studies in insect flight muscle have confirmed this (Reedy 1967; Reedy et al 1965). Weber and Murray (1973) have coined the term rigor complex to describe the complex which forms between actin and myosin in the absence of ATP. It is probable that formation of rigor complexes underlies the contracture induced by metabolic blockers. Activation of Mg linked myosin ATPase by DNP could by reducing the number of myosin ADP.P complexes (see Weber and Murray 1973) accelerate the formation of rigor complexes. As an equally severe contracture is seen with iodoacetate, however it is not essential to invoke a direct interaction of DNP with myosin ATPase in the genesis of this phenomina.

Diphenyleneiodonium Experiments.

Although a marked fall in the isometric twitch tension followed intra-arterial DPI, analysis of freeze clamped muscle revealed that ATP levels were comparable with those in a control group. ATP depletion per se is not therefore responsible for tension failure. A fall in PCr to undetectable levels was seen during 5c/sec stimulation. If ATP is compartmentalised and PCr has a crucial transport role as Saks et al (1976) suggest, then this fall in PCr might contribute to tension failure (see general discussion).

The rise in muscle lactate concentration during work was no greater than that seen in the control group but fall towards basal levels with rest was delayed. This may reflect either on going glycolysis in the recovery period or impaired pyruvate metabolism.

NMR studies confirmed that with large doses of DPI, a severe energy depletion develops during 1c/sec stimulation with almost total depletion of PCr and a fall in ATP levels. The metabolic status of the muscle was worse than that seen at the onset of DNP contracture. As contracture did not develop, this finding lends some support to the hypothesis that activation of myosin ATPase activity with focal energy defects near the myofibril might explain the rapidity of onset of DNP contracture. Partial recovery of twitch tension was seen over 1 hours rest and this was paralleled by an incomplete recovery in PCr and in ATP levels. Recent studies in 2

patients with a proven site I defect using 31P NMR (Radda et al 1982) have demonstrated a very slow PCr repletion after exhaustive work. The findings in the experimental situation were not identical, however, in that a rapid initial recovery in PCr followed by a plateauing out at an abnormally low level was observed rather than a delayed recovery to normal levels. This difference may reflect a different topographical pattern of mitochondrial involvement. If in the experiments all the mitochondria in some fibres were blocked with no recovery with rest, and in other fibres most of the mitochondria were functional with rapid recovery of energy stores with rest, a rapid but incomplete PCr recovery would result. By contrast, if the extent of mitochondrial involvement was more uniform, with no fibre so severely effected that recovery does not eventually develop, then a delayed but complete recovery, such as that seen in the patients, would ensue.

Iodoacetate Experiments.

Analysis of freeze clamped contractured muscle revealed a severe ATP depletion and marked IMP accumulation. This observation apparently contrasts with the findings in McArdles disease, where both NMR analysis (Ross et al 1981) and analysis of muscle biopsies have suggested that ATP depletion does not occur (Rowland et al 1965). The reason for this discrepancy between the biochemical findings in experimental and patient muscle contracture due to a glycolytic block probably reflects the extent of contracture produced. In the animal model almost all the fibres probably enter contracture whereas in patients, only a small % of contractured fibres may be sufficient to cause severe symptoms.

Biochemical observations in contracture in McArdles disease, with both NMR and conventional techniques, therefore face the potential problem that many or most of the fibres analysed are not in contracture.

2. Experiments with repeated Subcutaneous Injection of Metabolic Blockers.

2.1 Diphenyleneiodonium Experiments.

2.1.1 Clinical and Physiological Observations

Twenty two rats were injected with either 1.5 or 3ugm/gram diphenyleneiodonium subcutaneously on 4 or 5 days a week. The rats were carefully examined each day for signs of unsteadyness or weakness. Nine animals died overnight without any preceding evidence of weakness being detected. Three rats given 1.5ugm/gram daily developed signs of unsteadyness with splayed limbs between 10 and 35 days after the commencement of injections. Ten rats given 3ugm/gram body weight developed similar signs after 2 - 4 days. Early signs of illness included unsteadyness of gait and splaying of their limbs (Figure 55). The body temperature was well maintained and no rigidity was evident. Estimation of blood sugar levels at this stage using an ames strip revealed hypoglycaemia (< 1.8mMol). Intraperitoneal injection of 10% dextrose led to a normalisation of the blood sugar level without improvement in strength. Generalised muscle rigidity and hypothermia developed if hypoglycaemia was not corrected. Weakness resolved over 24 hours in 2 animals in which the blood sugar level was restored. In these, injections (1.5ug/gm) were recommenced 3 - 5 days after the first episode of weakness and a second episode induced 8 & 14 days later.

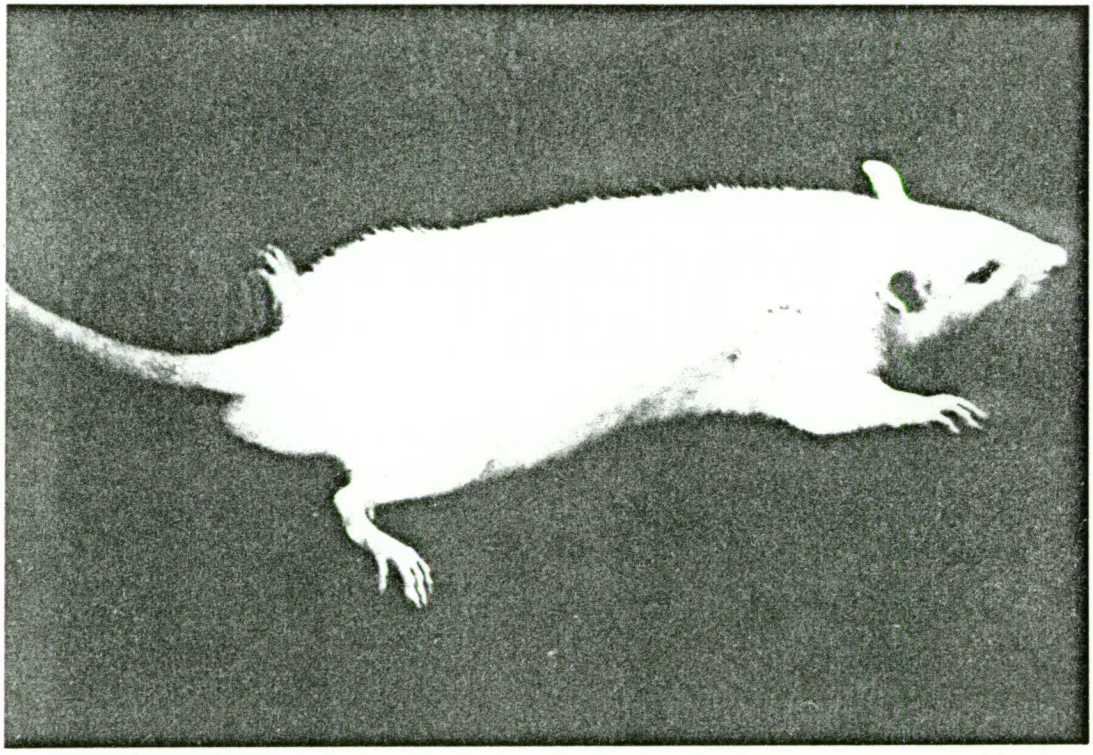
Isometric twitch tensions were recorded in several animals after the onset of weakness. The blood sugar level was restored to 5 - 7mM with intraperitoneal glucose and the rats anaesthetised with urethane. The animals general state remained good throughout recording. Twitch tensions were recorded in 1 animal which developed weakness with 1.5ug/g daily injections (Figure 56) and in 5 animals given 3ug/g daily doses which became weak. A severe force failure with 1c/sec stimulation was seen in the later (Figure 57). The mean initial twitch tension in these animals was 488g (SD 36) and after 15 minutes stimulation at 1c/sec, this had fallen to 45g (SD 40). This fall was associated with a prolongation in ½ relaxation times from an initial mean value of 34 $\pm$ SD 6.5 msec to 96 $\pm$ SD 19 msec, and a prolongation in peak time from 33.6 $\pm$ SD 5.8 msec to 41 $\pm$ SD 2.9 msec (Figure 58). Muscle surface temperature was maintained between 26^{o} and 28^{o} throughout recording. A fall in action potential amplitude closely paralleled twitch failure and a similar pattern of force failure was seen with muscle and with nerve stimulation (Figure 56). Two further animals were studied (3ug/g daily for 2 days) at a stage when walking was unsteady but there was no overt weakness. In these rats, the isometric twitch tension was fairly well preserved after 15 minutes stimulation at 1c/sec (mean initial level 490g, mean 82% of initial tension after 15 minutes stimulation). An abnormal prolongation in the half relaxation time was seen over this period however (mean initial value 34 msec, mean value after 15 minutes stimulation 61 msec). In uncannulated control preparations, isometric twitch tension was 90% or more of the initial value

after 15 minutes stimulation and no prolongation of relaxation times were seen at this stimulus frequency. Studies carried out after 35 days in an animal (with no clinical weakness) given 1.5ugm/gram daily revealed normal isometric twitch tension responses with 1 and 5c sec stimulation.

Figure 55

Title

Myopathic rat after repeated subcutaneous injections of diphenyleneiodonium.



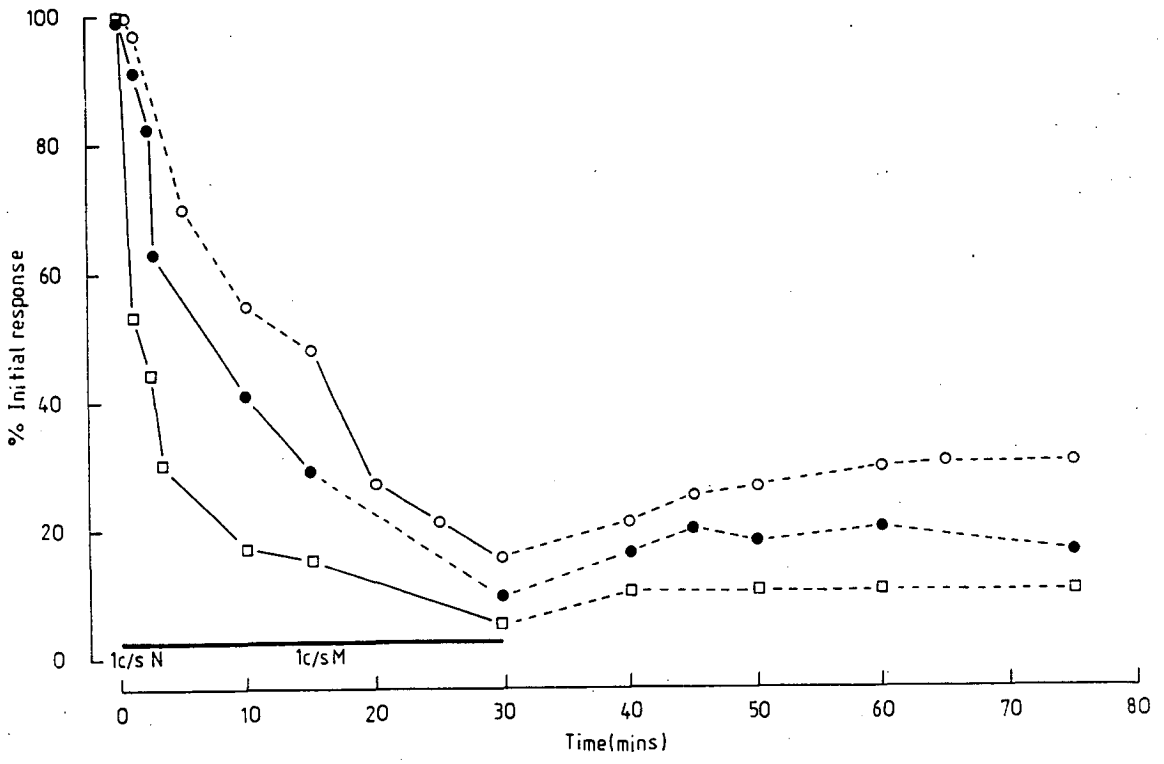
Legend

Animal photographed 4 days after commencement of injections (3ug/g) hypoglycaemia corrected prior to photograph.

Figure 56

Title

Investigation of weak animals after repeated subcutaneous injections of diphenyleneiodonium: typical experiment.



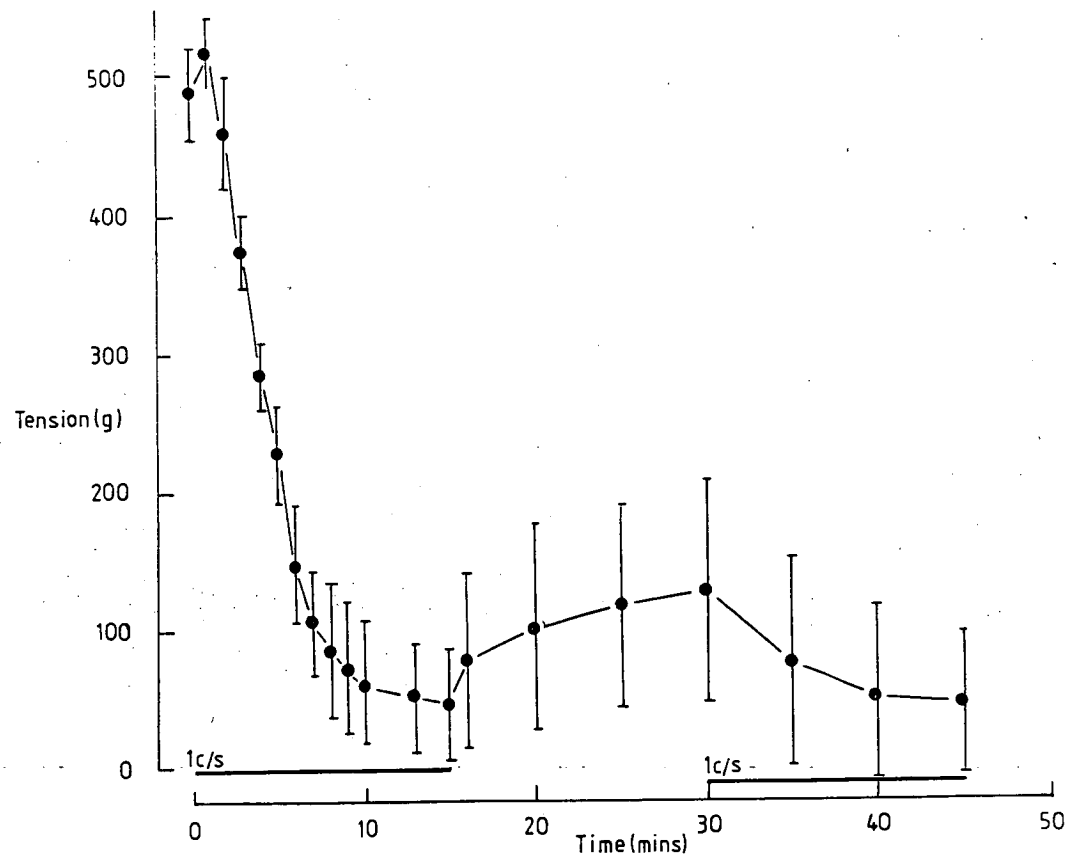
Legend

Mechanical and electrical responses from the right gastrocnemius muscle recorded in an animal which became weak after 10 days of injection of 1.5uq/g body weight daily subcutaneously of diphenyleneiodonium. Hypoglycaemia was corrected with intraperitoneal glucose prior to recording. The animals general state was stable for 4 hours after commencement of recording. 1c/sec nerve stimulation was given from 0 to 15 minutes and 1c/sec direct muscle stimulation from 0 to 30 minutes. Ordinate - % of initial response isometric twitch tension (nerve stimulation) ● , (muscle stimulation) ○ , and muscle action potential (nerve stimulation) □ .

Figure 57

Title

Investigation of weak animals after repeated subcutaneous injections of diphenyleneiodonium: isometric twitch tension with 1c/sec stimulation. Group data.



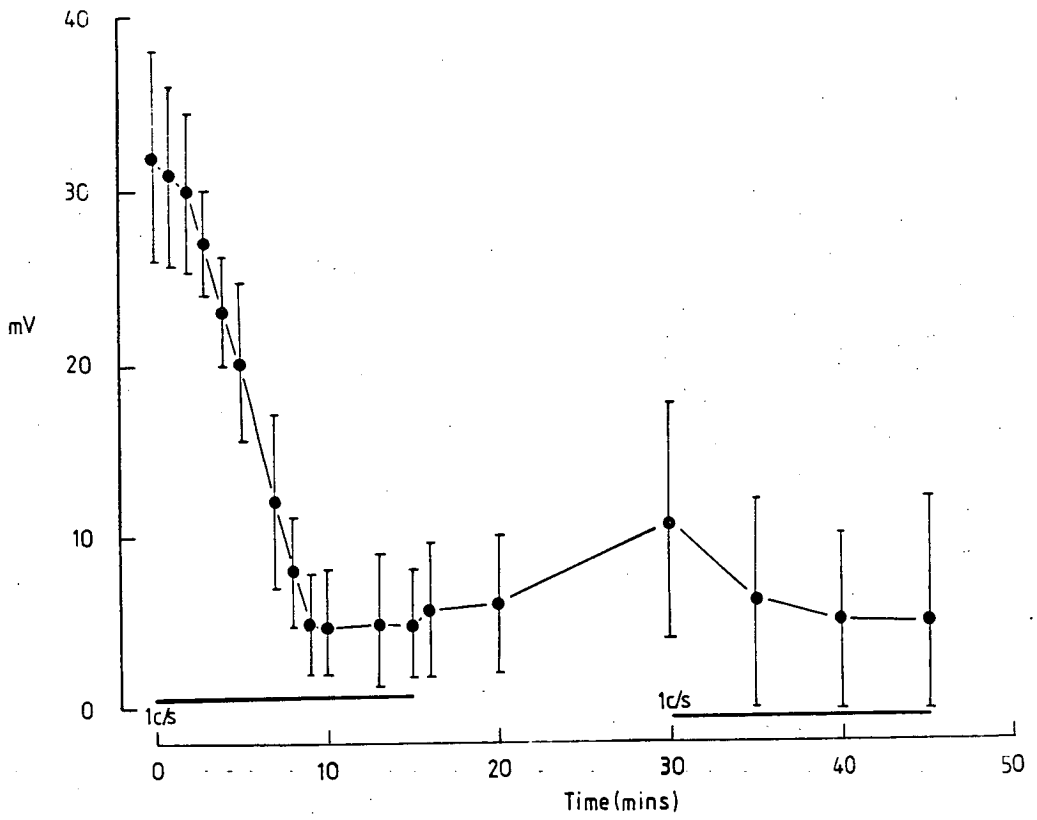
Legend

Ordinate - twitch tension (g). Abscissa - time in minutes. The graph shows the mean and standard deviation for 5 experiments in which weakness developed on the third day of injection of diphenyleneiodonium (3ug/g body weight per day). Hypoglycaemia was corrected prior to stimulation. 1c/sec nerve stimulation was carried out from 0 to 15 and from 30 to 45 minutes. All experiments were terminated by freeze clamping the gastrocnemius muscle for biochemical analysis at 45 minutes.

Figure 58

Title

Investigation of weak animals after repeated subcutaneous injections of diphenyleneiodonium: compound muscle potential amplitude during 1c/sec stimulation. Group data.



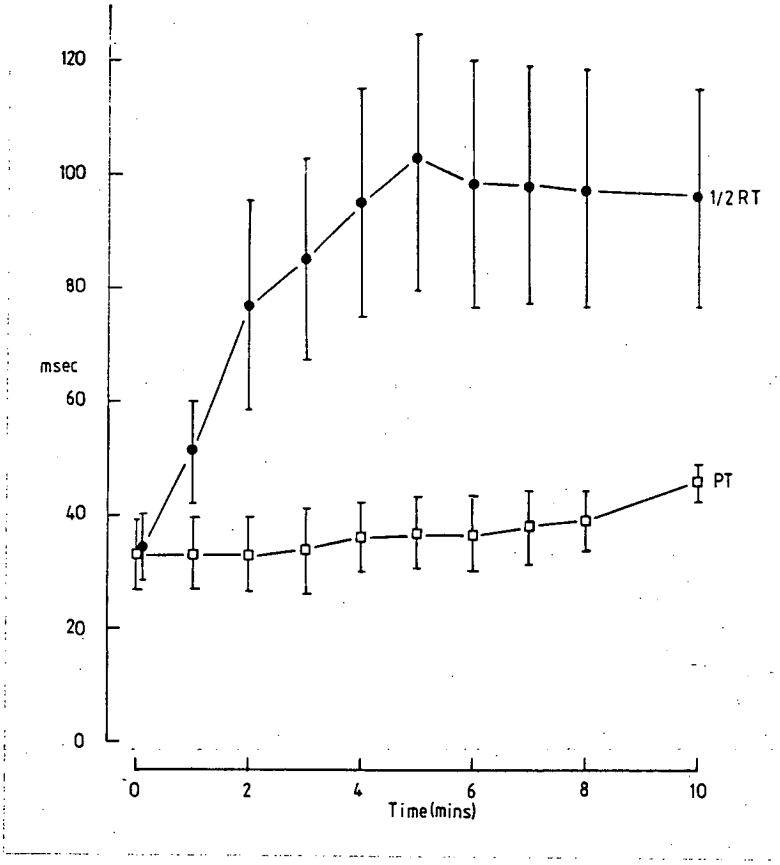
Legend

Ordinate - compound muscle action potential in mV. Data for the same experiments shown in Figure 62. Bars indicate standard deviation.

Figure 59

Title

Investigation of weak animals after repeated subcutaneous injections of diphenyleneiodonium: isometric twitch characteristics during 1c/sec stimulation. Group data.



Legend

Ordinate - ½ relaxation time ● and peak time □ in msec. Abscissa - time in minutes. Bars indicate standard deviation. The data is for the first 10 minutes of stimulation in the experiments shown in Figure 62.

2.1.2 Histology and Electron Microscopy. (2 Experiments)

The gastrocnemius was fixed for histology and electron microscopy in 2 animals after the onset of weakness. Occasional ragged red fibres ($\simeq$ 5% total) were seen with the Gomori trichrome stain. Increased activity with the succinic dehydrogenase stain was seen in the same fibres. These changes were not so marked as after intra-arterial DNP.

With electron microscopy, large numbers of abnormal mitochondria were visible. These were most prominent in subsarcolemmal aggregates but intermyofibrillar mitochondria were also involved. Degenerating mitochondria, with lysed crista, were frequent. Many mitochondria had changed into myelin figures. Occasional linear intracristal inclusions were identified. The changes seen resembled the more acute findings after intraarterial dinitrophenol (Figure 60).

Figure 60

Title

Investigation of weak animals after repeated subcutaneous injections of diphenyleneiodonium: electron microscopy.

Legend

60a. Section from the medial head of the gastrocnemius muscle in an animal which developed weakness 3 days after commencement of subcutaneous injections of 3ug/g body weight diphenyleneiodonium per day. Note degenerating mitochondria with concentric crista and myelin figures.

60b. Section from medial gastrocnemius from an animal which developed two episodes of weakness. The first episode developed 4 days after commencement of injections (3ug/g) day and the rat recovered over 24 hours with intraperitoneal glucose. Injections (1.5ug/g) were recommenced after an interval of 3 days and the gastrocnemius muscle fixed 8 days later during a second episode of weakness. Note swollen degenerating mitochondria with lysed crista and myelin figures.

Figure 60a

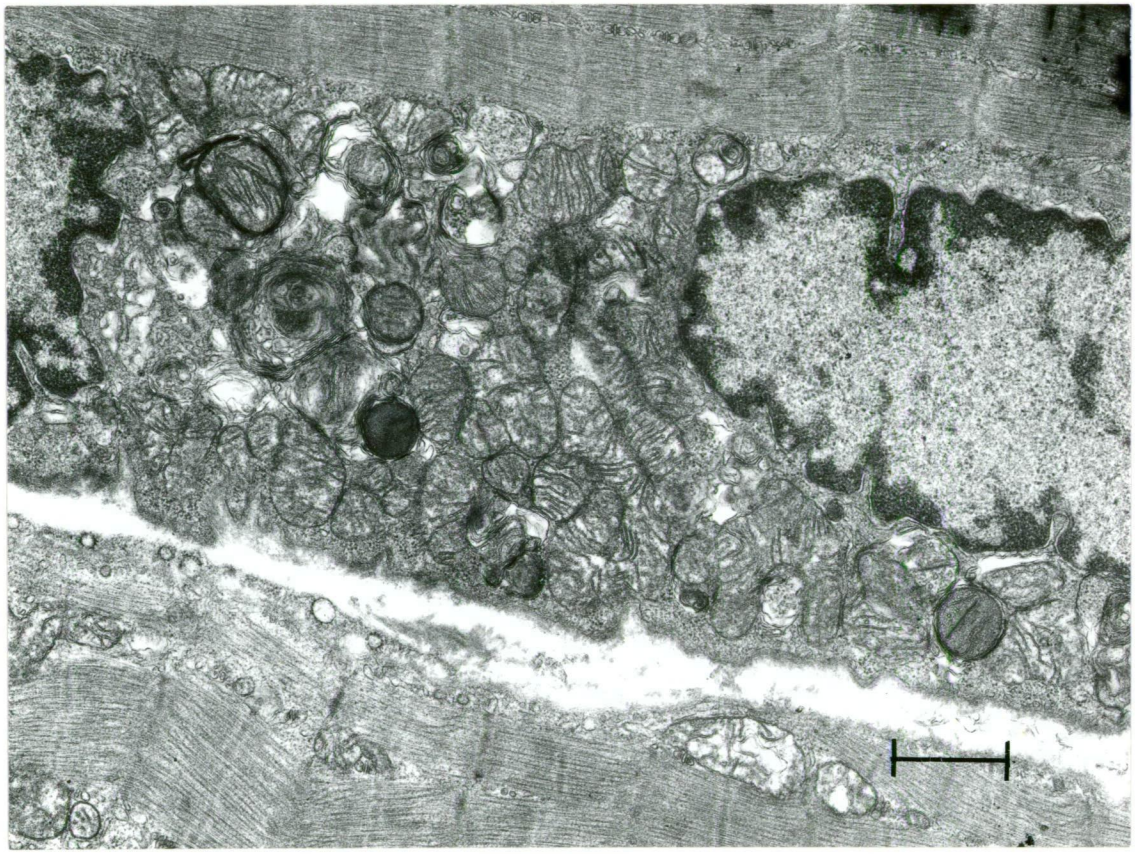
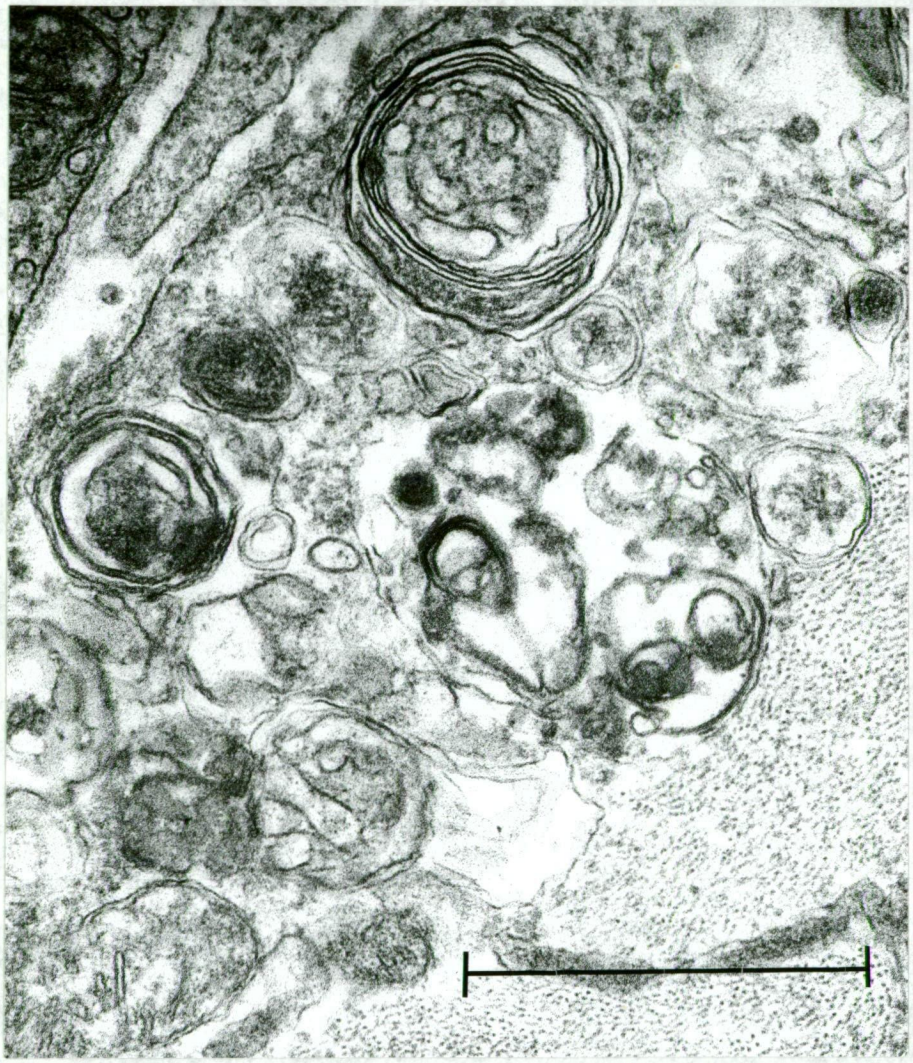


Figure 60b



2.1.3 Biochemistry.

The stimulated gastrocnemius muscle was freeze clamped in 5 experiments after a severe twitch failure had developed. The unstimulated left gastrocnemius was then freeze clamped immediately afterwards. The results of biochemical analysis are compared with those obtained from control experiments in which the same protocol was used in Table 9. PCr, glycogen and ATP levels in unstimulated muscle in the weak rats were normal. Following stimulation a marked rise in muscle lactate levels was seen. A significant accumulation of IMP and AMP accompanied force failure. ATP levels, however, were not significantly lower than in the control experiments after an equivalent amount of work. PCr levels by contrast fell approximately 25% of the control value.

TABLE 10

Title

Subcutaneous Injection of Diphenyleneiodonium: Metabolite and nucleotide levels in stimulated and unstimulated muscle from weak rats and a control group.

	Control Rats (N3)		DPI Rats (N5)	
	Unstimultd. muscle	Stimultd. muscle	Unstimultd. muscle	Stimultd. muscle
Glycogen	3.50±0.33	1.66±0.23	3.88±2.00	1.33±0.36
Lactate	1.79±0.64	3.86±0.68	3.27±0.57	18.09±2.76
PCr	8.91±0.80	4.72±0.72	9.56±2.11	1.73±0.49
ATP	7,60±0.04	5.88±0.79	8.47±1.36	5.54±0.99
ADP	0.78±0.083	0.74±0.06	0.99±0.19	1.27±0.266
AMP	0.004±0.001	0.003±0.001	0.004±0.013	0.111±0.028
IMP	0.524±0.08	0.185±0.06	0.132±0.08	1.769±0.36
ATP/ADP Ratio	10.391±0.128	6.62±0.85	9.50±1.57	6.93±1.28

Legend

Data from 3 control experiments and 5 experiments in which animals with overt weakness after subcutaneous DPI were investigated. (The physiological data from the later experiments is shown in Figures 57-59). An identical protocol (15 minutes stimulation at 1c/sec, 15 minutes rest, 15 minutes stimulation at 1c/sec) was followed in all experiments and freeze clamping was carried out at the end of the second stimulus run. Mean twitch tension in DPI injected animals

was 8.9% of the initial level at the time of freeze clamping, whereas in all 3 control experim nts, the twitch tension was over 90% of the initial level at that time. Note that (a) the metabolic status of unstimulated muscle in weak rats is "normal" (b) in stimulated muscle, a similar fall in ATP levels is seen in control and DPI injected animals inspite of marked difference in force failure (c) the mean PCr in stimulated control muscle was 52.8 ($\pm$ SD 4.3) of the unstimulated level and the mean PCr in DPI treated stimulated muscle was 18.24 ($\pm$ 3.26) % of the level in unstimulated muscle (d) a marked rise in intramuscular lactate levels accompanied force failure in DPI injected animals.

DISCUSSION

Repeated subcutaneous injection of diphenyleneiodonium produced an acute illness with severe generalised muscle weakness. This was associated with hypoglycaemia confirming that the mitochondrial block is not confined to skeletal muscle. Weakness persisted, however, after correction of hypoglycaemia and a severe force failure associated with a marked prolongation of the duration of relaxation was documented during 1c/sec stimulus patterns. The muscle action potential also failed in parallel with twitch tension failure. These results resemble those seen after intra-arterial injection of DPI.

Biochemical analysis of fatigued muscle revealed a massive rise in lactate concentrations. The energy status of the fatigued muscles was impaired with abnormal accumulation of IMP and a small fall in the adenylate energy change. ATP levels, however as in experiments with arterial infusion were surprisingly high. These observations again contrast markedly with those seen in DNP poisoned muscle and suggest that the energy deficit is less severe. The findings support the suggestion that ATP depletion per se is not responsible for contractile failure after iodonium. Depletion of PCr itself (Saks et al 1976) or the marked intracellular lactate accumulation (Hermansen 1981) might underlie twitch failure and this possiblility will be discussed further in the general discussion.

Ultrastructural examination in animals rendered weak by repeated subcutaneous injection of DPI revealed much more severe morphological changes in muscle mitochondria than after intraarterial injections, probably reflecting the longer period of exposure. The similarity of these changes to those seen more acutely after DNP, suggest that they represent a non specific morphological response to any agent which leads to a low energy state in the inner mitochondrial membrane.

2.2.1 Dinitrophenol.

Twelve rats were injected with dinitrophenol subcutaneously in a dose ranging between .02 and.04mg/Kg on 4 or 5 days a week for upto 5 weeks. Several rats died overnight but no animals with weakness were identified. Normal weight gain occurred over the period. The subcutaneous route of administration is ineffective with this agent. Physiological studies on one animal after 5 weeks of injections showed normal isometric twitch tension responses with 1 and 5c/sec stimulation.

2.2.2 Antimycin A.

Antimycin a was injected subcutaneously in 8 animals in a dose between .15 and .4mg a day. Three animals died overnight, but no ill effects were seen in the remainder and the experiments were discontinued after 2 weeks. Injection of a large single dose of antimycin a (9ugm/gram) resulted in severe weakness with splaying of the limbs and death within 30 minutes.

DISCUSSION

Attempts to produce a myopathic illness with subcutaneous DNP and Antimycin a were unsuccessful.

3. In Vitro Experiments. Twitch tensions and single fibre electrical recordings in soleus.

3.1 Control Observations

The soleus was mounted in a perspex chamber containing oxygenated Krebs ringer solution as described in the methods. Five stimuli at 1c/sec by nerve stimulation and 5 stimuli at 1c/sec by direct muscle stimulation were administered every 5 minutes. Isometric twitch tension amplitudes (measureing the last twitch response in each stimulus train) were stable for 90 minutes (Figure 61,63).

In another series of experiments, intracellular recordings were made with a glass filament electrode. Only superficial fibres or those one deep were examined. Fibres were sampled continuously and the results grouped in 5 minute periods. The resting membrane potential, muscle action potential amplitude, action potential rise time and % of excitable fibres entered remained stable for 60 minutes. The mean initial resting membrane potential was - 84mV (SD 10.9), the mean action potential amplitude was 90mV (SD 15.6) and the mean rise time was 1.8msec (SD 0.2). After 60 minutes recording, the mean resting membrane potential was - 89.9mV (SD 9.2), action potential amplitude 81.5mV (SD 15.4) and rise time 1.8msec (SD 0.4). Approximately 10 fibres were sampled in each 5 minute period.

3.2 Dinitrophenol.

i. Twitch Tension Measurements

Dinitrophenol was added to the bath to produce a concentration between 0.1 and 1mM. A rapid and identical fall in isometric twitch tension developed, with nerve and with direct muscle stimulation. The rate of force failure was strikingly dose dependant (Figure 61&63). Contracture developed in all experiments. This followed the onset of twitch failure in a similar time sequence to that established in vivo in experiments with gastrocnemius (Figure 62, 63).

ii. Action Potential Measurements

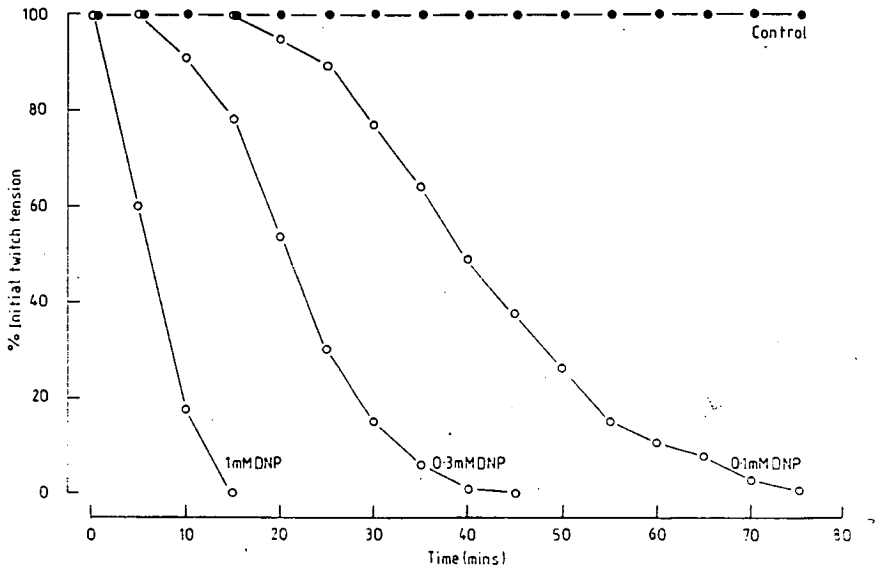
Single fibre electrical recordings were made with a 0.3 mM concentration of dinitrophenol in the bath. A typical experiment is illustrated in Figure 45. The mean resting membrane potential fell slightly over 60 minutes (initial value mean 86.9mV ($\pm$ SD 6.8 N10), values at 60 minutes mean value -72.6mV ($\pm$ SD 4.6 N10). An increasing number of fibres became inexcitable, the action potential amplitude in excitable fibres fell with time and the action potential rise time prolonged. The variability in the rate of fibre failure was wide however, some being unresponsive, at times when others were capable of conducting a low amplitude, or a normal amplitude propogated action potential (Figure 64). No excitable fibres were identified after 60 minutes.

A similar pattern of failure was seen in 2 other experiments. The changes in action potential amplitude, rise time and in the number of inexcitible fibres after addition of DNP are shown in Figure 65a-c.

Figure 61

Title

In vitro experiments: isometric twitch tension responses in soleus after exposure to dinitrophenol.



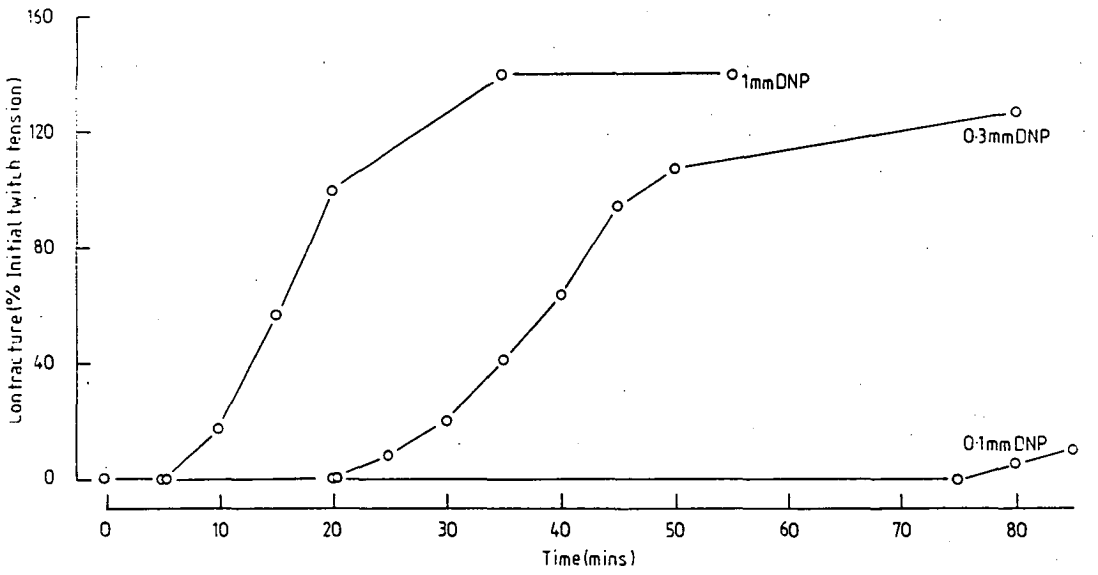
Legend

Ordinate - isometric twitch tension nerve stimulation. Abscissa - time in minutes. A control experiment and 3 experiments with concentrations of dinitrophenol between 0.1 and 1mM are illustrated. 5 stimuli at 1c/sec were given every 5 minutes. The amplitude of the last response in each train was measured.

Figure 62

Title

In vitro experiments: contracture evolution after exposure to dinitrophenol in soleus.



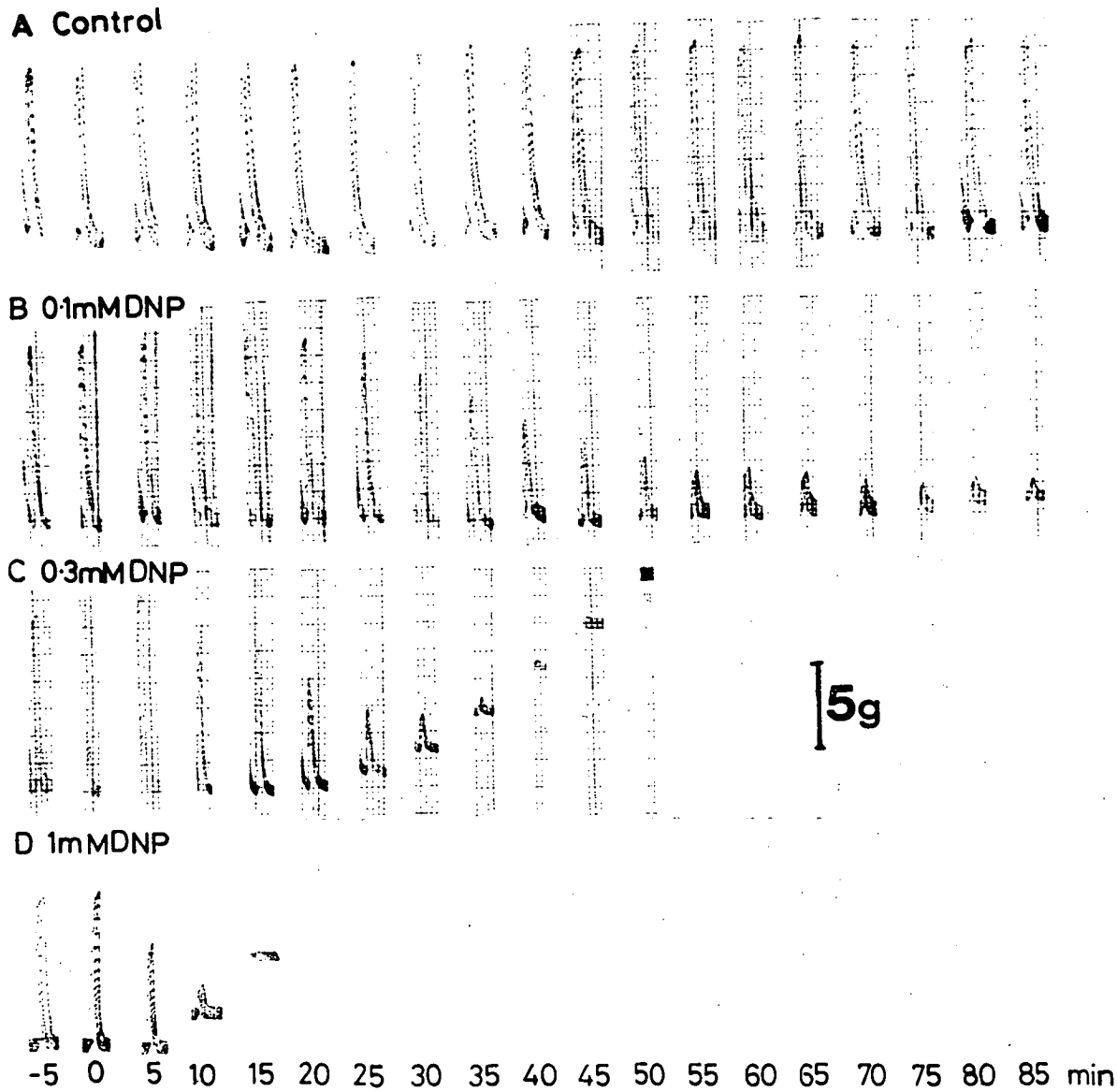
Legend

Ordinate - contracture expresssed as a % of the initial twitch tension in 3 experiments in which concentrations of dinitrophenol between 0.1 and 1mM were added to the bath. No contracture was seen in controls.

Figure 63

Title

In vitro experiments: isometric twitch responses.



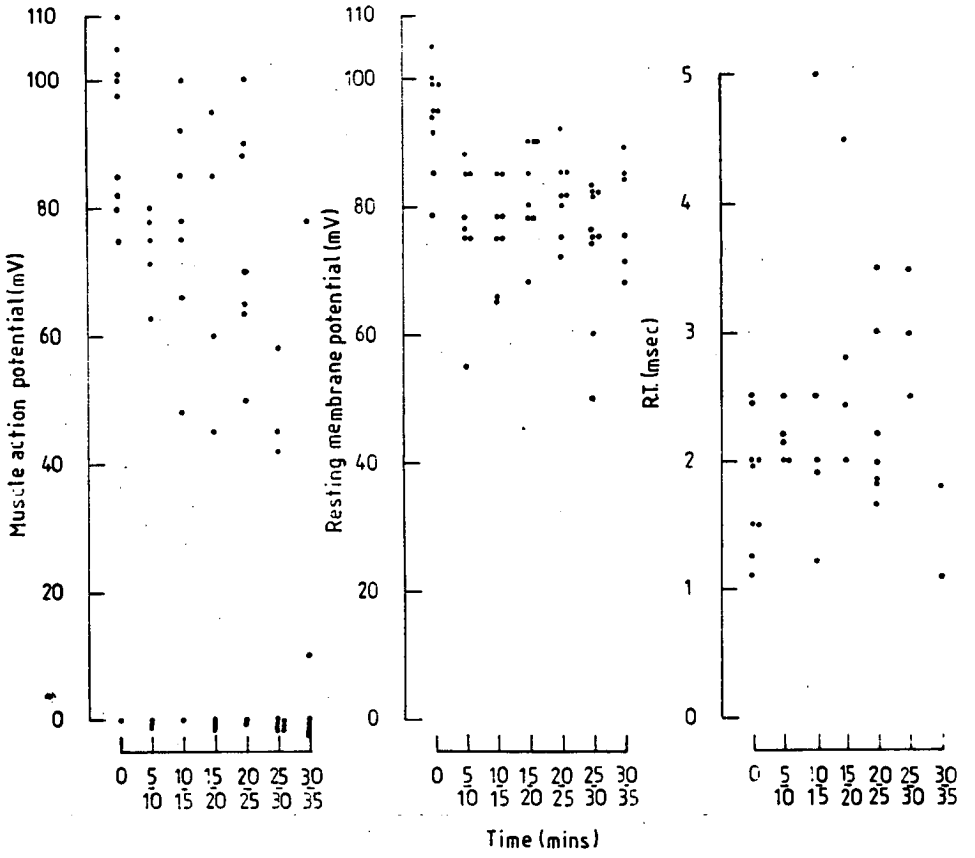
Legend

Isometric twitch tension (nerve stimulation) in 4 experiments. (A control, B-D 0.1-1mM DNP added to chamber) 5 stimuli at 1c/sec were given every 5 minutes and the fifth response in each train is illustrated.

Figure 64

Title

In vitro experiments: single fibre electrical recordings in soleus exposed to dinitrophenol. Typical experiment.



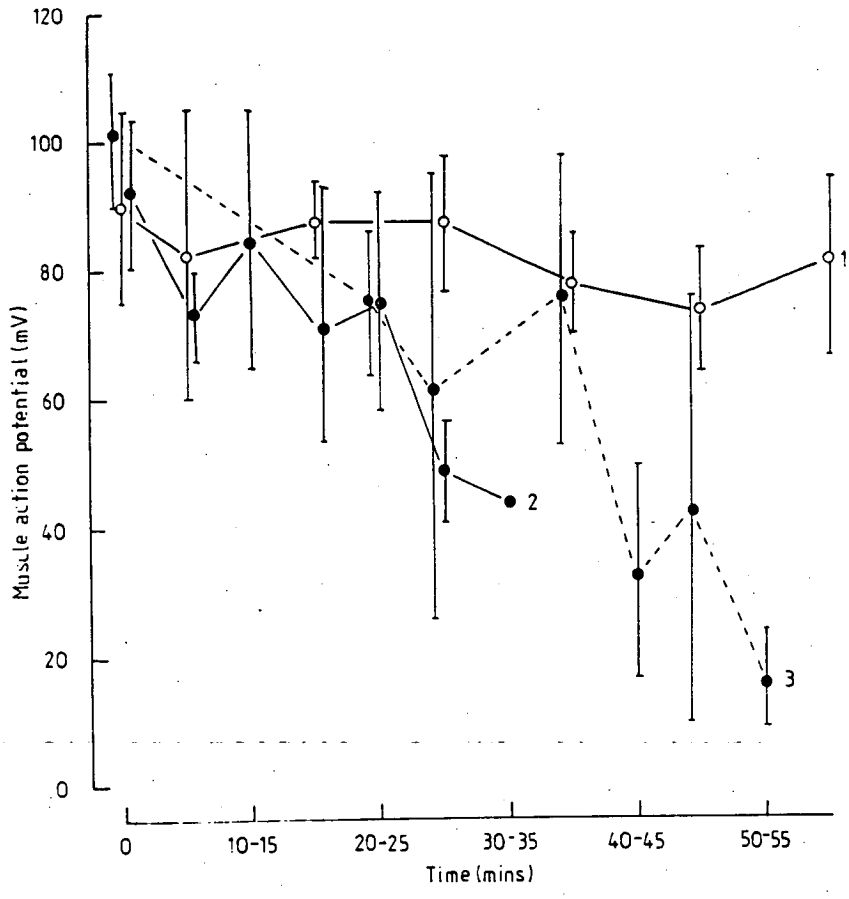
Legend

0.3mM DNP was added to the chamber after the first set of observations. Muscle fibres were sampled continuously and the results are grouped in 5 minute periods. Changes in muscle action potential amplitude are shown on the left, resting membrane potential recordings are shown in the centre graph and the rise time of the action potential is shown on the right. Each point represents a single fibre sampling.

Figure 65a

Title

In vitro experiments: excitability changes in soleus induced by dinitrophenol.



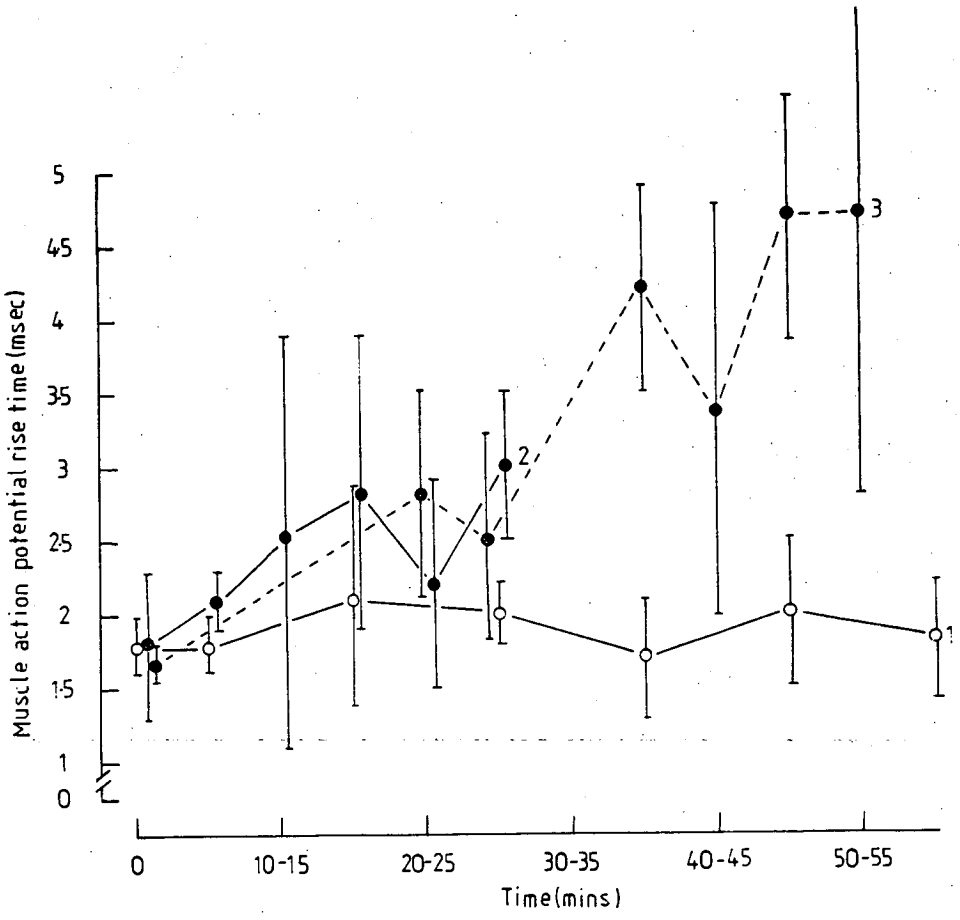
Legend

Data from a control ○ and from 2 experiments with 0.3mM DNP added to the chamber ● . Approximately 10 fibres were sampled in each 5 minute period. The bars indicate standard deviation. Ordinate - muscle action potential amplitude in mV. Note the wide variation in the rate of action potential failure between individual fibres. Selected single fibre action potential responses in a control and in 2 experiments after addition of DNP are shown in Figure 65d.

Figure 65b

Title

In vitro experiments: excitability changes in soleus induced by dinitrophenol.



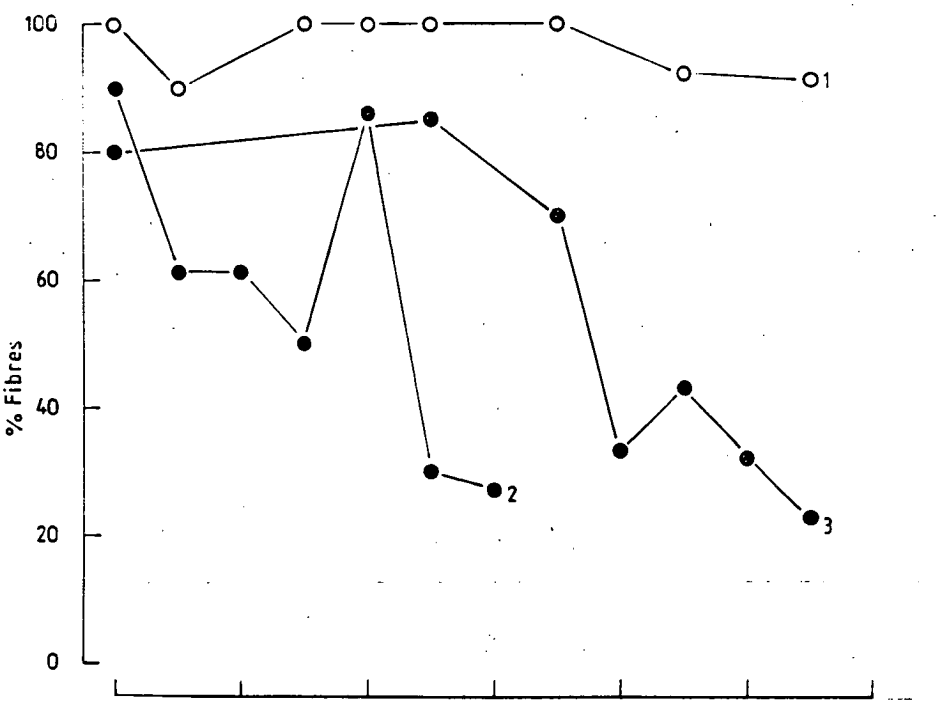
Legend

Muscle action potential rise times in the same experiments. The bars indicate standard deviation. Note the prolongation in mean rise time after exposure to dinitrophenol and the wide spread of results at any one time.

Figure 65c

Title

In vitro experiments: excitability changes in soleus induced by dinitrophenol.



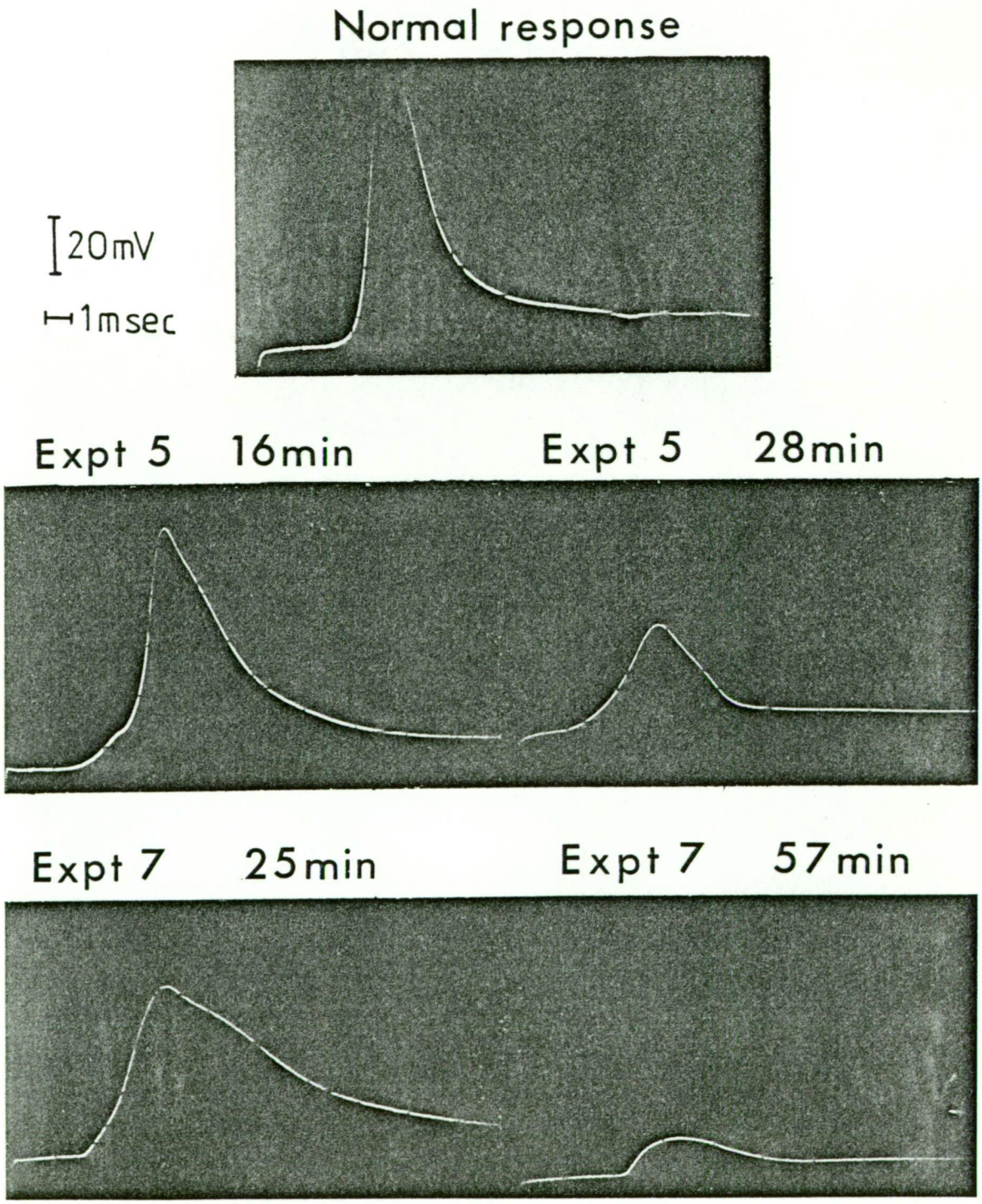
Legend

% of excitable fibres in a control ○ and in 2 experiments with 0.3mM DNP ● . Each point is an average of 7-12 observations in different fibres. Inexcitable fibres were not included in the data shown in Figures 48a and 48b. The points illustrated represent samplings over 5 minute periods.

Figure 65d

Title

In vitro experiments: excitability changes in soleus induced by dinitrophenol.



DISCUSSION

Insertion of an intracellular electrode may block action potential propogation along muscle fibres (Krnjevic and Miledi 1962) and in control experiments occasional fibres sampled were inexcitable but the proportion remained stable at less than 10% of all fibres entered over 60 minutes of recording. After the addition of 2 - 4 dinitrophenol to the bath, a fall in action potential amplitude was seen and this was associated with a marked increase in the rise time. Eventually no excitable fibres could be identified. These changes in membrane properties are paralleled by some earlier observations in vitro referred to in the introduction. Abood et al (1961) found that concentrations of less than 0.5mM DNP had little effect on the electrical properties of frog striated muscle in vitro but 1 - 2mM concentrations resulted in a progressive fall in action potential amplitude and prolongation in duration, with eventual inexcitability. These changes were associated with partial depolarisation (to 55mV) and excitability was restored by anodal hyperpolarisation, paralleling earlier observations of Lorente de No in frog nerve depolarised by metabolic inhibitors. Goldsmith et al (1975) in studies using frog sartorius muscle in vitro and De Mello (1969) in studies on cardiac muscle also found that DNP induced a partial membrane depolarisation. The fall in resting membrane potential seen in this study was not nearly so great as that reported by Abood et al. This is not surprising however as Fink and Luttgau in studies on metabolically exhausted muscle and Krnjevic et al in studies on neurones exposed to DNP, have shown that membrane inexcita-

bility is not always associated with depolarisation. The crucial underlying mechanism in this situation is a massive increase in membrane k^t conductance and this can lead to hyperpolarisation as well as to depolarisation. Increased cytosolic calcium levels may account in part for the increase in potassium conductance induced by dinitrophenol (Krnjevic et al 1978).

GENERAL DISCUSSION AND CONCLUSIONS

The main findings and the extent to which the original objectives of the project were fulfilled will be discussed in this final chapter. The chief aim was to induce selective defects in the mitochondrial respiratory chain or in respiration/phosphorylation coupling in muscle mitochondria in an experimental animal, and to categorise the properties of working muscle in this model from several viewpoints. This approach was designed to further knowledge of the pathophysiology in human mitochondrial myopathies. A focal respiratory chain block involving NADH CoQ reductase (with DPI) and uncoupling of respiration from phosphorylation (with DNP) were successively produced and studied in vivo. An attempt to produce a viable animal model with a respiratory chain block between cytochromes b and c_1 was less successful because of the high systemic toxicity of Antimycin a, the inhibitor used.

Dinitrophenol was selected for investigation because earlier studies with DNP have demonstrated structural changes in rat skeletal muscle mitochondria and it has been suggested that acute DNP myopathy is a useful model for some human mitochondrial myopathies (Melmed et al 1976; Sahgal et al 1979). Several key differences emerged, however, between the physiological findings with DNP in this study and observations in patients with defective mitochondrial coupling. Isometric twitch tension failure in DNP treated animals was invariably associated with electrically silent contrac-

ture, a finding never reported in patients. Failure of the mechanical evoked response in dinitrophenol treated animals occurred in resting muscle activated only infrequently to monitor tension changes, indicating a progressive metabolic deterioration not linked to external work. This finding was confirmed with 31P NMR studies and is in contrast to the findings in human disease where a recovery of muscle function, albeit delayed, occurs with rest (see Morgan-Hughes 1981; Radda et al 1982). No recovery in muscle function was seen in acute DNP myopathy in this study whereas in the earlier studies where clinical and histological observations were made over 24 hours, rigidity eventually disappeared (Melmed et al 1976; Sahgal et al 1979). The marked delay in recovery in these studies strongly suggests that this followed either a disassociation of DNP from the inner mitochondrial membrane or a generation of new mitochondria rather than recovery in the presence of severe uncoupling.

The electrical properties of skeletal muscle poisoned with DNP and in human mitochondrial myopathies are also different. Sarcolemmal inexcitability was found both in vivo and in vitro in muscle poisoned with DNP in this study. These observations confirm and extend earlier observations in frog muscle in vitro (Abood et al 1961). The limited studies which have been carried out in patients with proven mitochondrial blocks have shown dissimilar electrical changes in that muscle action potential amplitude is well preserved in the presence of force failure (De Jesus et al 1974; Edwards and Wiles 1981; Morgan-Hughes et al 1977, 78 & 79).

The results are not directly comparable however as the experimental protocols used in patients either employed a less protracted work load (De Jesus 1974) or muscle was activated voluntarily rather than by direct stimulation during fatigue prior to recording a single twitch response (Morgan-Hughes et al 1979). The rapid failure of tetanic tension in DNP poisoned animals with nerve stimulation suggests that a defect in neuromuscular transmission accelerates tension failure at tetanic stimulus frequencies. DNP is known to enhance neuromuscular junction transmitter release at rest (Alnaes and Rahaminoff 1975; Sandoval 1981). Mitochondrial calcium release and reuptake may, by determining calcium levels in the terminal presynaptic expansions, play an important modulatory role in normal quantal release. Dinitrophenol blocks mitochondrial calcium uptake and releases mitochondrial calcium stores (Carafoli et al) and the resultant rise in cytosolic calcium levels probably underlies increased acetylcholine release at rest. It is possible that this process may deplete transmitter stores and underlie premature neuromuscular failure at rapid stimulus frequencies. Neuromuscular failure was not evident with 1 and 5c/sec stimulus patterns in this study and is unlikely to be important in human mitochondrial myopathies where there is no evidence that intraneural mitochondria are involved in the disease process.

Biochemical analysis of DNP poisoned muscle with the techniques of phosphorus NMR and analysis of freeze clamped muscle showed a severe depletion of phosphocreatine and ATP.

Rapid PCr depletion in resting muscle confirms that DNP accelerated ATP hydrolysis as well as blocking oxidative phosphorylation. ATP depletion correlated well with twitch tension failure and in end stage contracture almost all of the ATP stores had been converted to IMP. Severe ATP depletion in frog muscle in vitro (Abood et al 1961) and in rat diaphragm preparations (Barnes et al 1955) are known to follow DNP poisoning and Sahgal et al (1979) demonstrated PCr and ATP depletion in rat hind limb muscles in vivo. Biochemical and mechanical events in vivo following DNP poisoning have not been correlated however in the past. Twitch failure is probably not related exclusively to changes in any one nucleotide or metabolite and rise in ADP levels (Maruyama et al 1967) fall in PCr (Saks et al 1978), intracellular acidosis due to lactate accumulation and proton release during ATP hydrolysis (Hermansen et al 1981) as well as ATP depletion may all contribute to pathological muscle fatigue. Analysis of contractured muscle exposed to DNP revealed low ATP levels and similar findings were found in ischaemic contracture and in iodoacetate contracture in vivo in this study, paralleling observations in in vitro studies with metabolic inhibitors in the past (Murphy et al 1966). As discussed earlier, ATP depletion may not be the sole immediate cause of contracture evolution and the marked increase in cytosolic calcium levels following failure of energy linked sarcoplasmic reticulum calcium reuptake probably has a synergistic effect.

This postulated difference in the biochemical basis of twitch failure and contracture if correct would explain the observation in this study that twitch failure is always well advanced before contracture begins in DNP poisoned muscle.

Ultrastructural studies showed a picture of severe mitochondrial damage 60 minutes after DNP infusion, similar to that seen in other studies (Melmed et al 1976; Walter et al 1981). Swollen mitochondria with fractured or lysed crista, linear intracristal inclusions and myelin figures were abundant. These changes reflect a more severe mitochondrial damage than that seen in patients (see Busch et al 1981).

Dinitrophenol clearly produces a more severe striated muscle failure than that seen in either Lufts syndrome or in patients with loose coupling without hypermetabolism. The reason for this may be intrinsic to its action. DNP uncouples, rather than loosely couples, oxidative phosphorylation and thereby produces a total block to ATP regeneration, whereas in patients with loose coupling, oxidative phosphorylation is still functioning although ADP has no control over electron flow in the transport chain. Furthermore DNP is known to activate mitochondrial and myosin ATPase in vitro (Levy et al 1964) thereby accelerating ATP hydrolysis as well as blocking resynthesis and the current study suggests this action is important in vivo. This results in a more severe energy deficit than could occur naturally and explains why acute DNP myopathy has no human parallel.

The physiological findings in dinitrophenol and iodoacetate poisoned muscle in vivo were very similar. Failure of contractility, sarcolemmal inexcitability and severe rigor was seen with both metabolic inhibitors. Severe ATP and PCr depletion and accumulation of IMP were found in contractured muscle with both agents. These physiological changes are therefore not specific to DNP and follow a severe energy deficit of any cause.

Diphenyleneiodonium, when administered by the subcutaneous or by the intraarterial routes, leads to a rapid failure of isometric twitch tension during 1 and 5c/sec stimulation patterns. In contrast to the findings with dinitrophenol, contracture evolution was not an invariable association of twitch failure but only developed as a late finding in some animals given large doses of DPI. A marked prolongation in half relaxation time accompanied force failure paralleling earlier observations in metabolically blocked muscle in vitro and in human muscle in vivo (Edwards et al 1972 & 75). The compound muscle action potential failed at a similar rate to the twitch response. It is not therefore possible to state from these results whether the force failure seen is due to a true failure of contractility or a failure of excitation. The close association of mechanical and electrical fatigue suggests, however, that muscle membrane inexcitability influences the pattern of force failure observed.

In several experiments, a pattern of rapid twitch fatigue followed by delayed incomplete recovery was established

by interrupted 5c/sec stimulus patterns. Contracture did not develop in these animals. Abnormal fatiguability is a major symptom in patients with a proven site I mitochondrial block as well as in some other patients with less well classified mitochondrial myopathies (Morgan-Hughes et al 1979; Price et al 1967, Munstat et al 1967; Rawles & Weller 1974). The finding of rapid force failure with 5c/sec stimulation followed by a delayed recovery therefore has a close resemblance to the clinical picture in these patients.

Biochemical studies on fatigued muscle in animals given diphenyleneiodonium by the intraarterial or by the subcutaneous routes revealed a different picture to that found in DNP poisoned muscle. The close association between force failure and ATP depletion evident in experiments with the uncoupler was not apparent in DPI experiments. Fall in ATP levels in freeze clamped muscles in which a major fall in twitch tension had developed (during 5c/sec stimulation after intraarterial DPI and 1c/sec stimulation after subcutaneous DPI) was similar to that seen in control experiments in which identical stimulus paradigms were carried out. ATP depletion cannot, therefore, be the immediate basis of contractile failure in muscle with a site I respiratory chain block.

Two alternative possibilities are suggested by the metabolite assays. Firstly, PCr levels were much lower in muscles freeze clamped after DPI injection than in comparable controls. For many years, PCr has been thought to act as an energy store, transferring high energy phosphate bonds

to ADP as needed (Mommaerts 1971). If this is the sole function of PCr, then a contraction of the total PCr pool with preservation of ATP levels could not cause contractile failure. This long held view may be incorrect and Saks et al (1976) have recently postulated that PCr has a more major role. They suggest that ATP is compartmentalised and does not freely diffuse from the mitochondria to the cytosol. Instead mitochondrial ATP generates phosphocreatine locally and this has a crucial transport role in transferring high energy phosphate bonds to ADP in the immediate vicinity of the myofilaments and in the sarcoplasmic reticulum. Sak's et al theory envisages that only catalytic amounts of ADP are present in the mitochondrial compartment. PCr depletion with high concentrations of ATP, could therefore only lead to fatigue if firstly free ATP in the cytosol is unavailable for utilisation by myosin ATPase and secondly cannot directly transfer the terminal phosphate bond to ADP bound on myosin. If PCr has an essential transport role in the delivery of high energy phosphate bonds to ADP bond at sites of peripheral utilisation, then PCr depletion could cause fatigue in the presence of "normal" ATP levels.

Hermansen (1981) has suggested that muscle fatigue in normal subjects follows an increased production of hydrogen ions during vigorous exercise. Such an increase might impair myofibrillar ATPase activity. In experiments with subcutaneous injection of DPI, muscle fatigue was

associated with a 5 times greater increase in muscle lactate levels than was seen in equivalent control animals, and it is possible that a fall in intramuscular pH may contribute to twitch failure.

However lactate accumulation in experiments where a considerable force failure was seen after intraarterial DPI was no greater than in equivalent controls and acidosis cannot be the sole mechanism of fatigue. The biochemical basis of the fatigue seen with DPI has not been definitely established, and both PCr depletion and intracellular acidosis may be important factors.

In muscle poisoned with DPI freeze clamped after the onset of contracture, the ATP concentration was much lower than in non contractured muscles. This observation again suggests that contracture does not begin to evolve until ATP concentrations have fallen to low levels.

The morphological mitochondrial changes seen after intraarterial injection of DPI were minor, with many unusually rounded mitochondria visible but no degenerating mitochondria or inclusions. An acute functional mitochondrial block can therefore lead to contraction failure before morphological changes become marked. In experiments with repeated subcutaneous injection, where the duration of exposure to DPI was longer, the morphological changes were much more severe with many degenerating mitochondria containing myelin figures and enlarged mitochondria with fractured crista.

These structural changes, as after DNP injection were not identical to those seen in human disease. The finding that morphological evidence of mitochondrial disruption is more pronounced in the experimental model than in patients probably reflects both a more severe block to oxidative phosphorylation and a more acute mitochondrial insult.

Acute diphenyleneiodonium myopathy in the rat has considerable potential as a model for human mitochondrial myopathies. The animal model produced may also be useful in the assessment of possible treatments. Agents which bypass proximal blocks in the respiratory chain are recognised and include the artificial electron acceptor menadione. The utilisation of 31P NMR and tension measurements, would enable potential theraputic agents to be rapidly tested.

The series of experiments reported in this thesis represent the first comprehensive attempt to establish and characterise a mitochondrial myopathy in an experimental animal. Considerable success in producing a disease model followed use of the agent diphenyleneiodonium. Differences between the biochemical and physiological behaviour of muscle poisoned with the uncoupler DNP and with the respiratory chain inhibitor diphenyleneiodonium are emphasised.

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APPENDIX HISTOLOGICAL METHODS

1) Modified Gomori trichrome stain
   a) Harris's haematoxylin for 10 minutes
   b) distilled water rinse
   c) trichrome solution for 10 minutes +
   d) wash in 2% acetic acid
   e) dehydrate and mount in DPX

Trichrome Solution Composition:

Chromotrope 2R 1.2g, Fast green FCF 600mg, phosphotunstic acid 1.2g, glacial acetic acid 2 mls, distilled water 198mls. Final pH 3.7

2) Periodic Acid-Schiff-Leuco-Fuschin stain
   a) Carnoys solution + for 5 minutes
   b) distilled water rinse
   c) 0.5% periodic acid for 5 minutes
   d) distilled water rinse
   e) Schiff's solution * for 10 minutes
   f) tap water wash 10 minutes
   g) dehydrate and mount in DPX

Carnoy's Fluid Composition: +
Chloroform 30 mls, absolute alcohol 60 mls, glacial acetic acid 10 mls.
Schiff's Solution Composition: *
Parasanaline 1g, distilled water 200mls. Boiled and filtered at 50°C. 20 mls NHCL, 1g sodium metabibulphite stored in a dark place overnight. Charcoal 1g.

3) Succinic dehydrogenase reaction
   a) 0.2m tris buffer pH 7.4 10mls +
   b) nitroblue tetrazolium 10mg
   c) sodium succinate 100mg
   d) phenazine methosulphate 0.2s mg.

Buffered to pH 7.2 to 7.6. The sections were incubated at 37°C for 1 hour, washed in distilled water and marked in glycerine jelly. (+0.2m Trisma (Hydroxymethyl amino methane) 25mls. 0.1M HCl 42mls, distilled water 33 mls. Buffered to pH 7.4.)