THE TOXIC EFFECTS OF ANTICHOLINESTERASES ON MUSCLE

by

Helen Elizabeth Townsend

A thesis submitted for the

degree of

Doctor of Philosophy

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SUMMARY

It has been shown that acute administration of ecothiopate iodide *in vivo* caused an approximate 80% depression of acetylcholinesterase activity in the diaphragms of mice. Inhibition of acetylcholinesterase was accompanied by an influx of calcium at the junctional region of the diaphragm, which continued during subsequent progressive development of a severe myopathy located in the same region. Myopathy was accompanied by loss of creatine kinase from the muscle and was represented, at the light microscope level, by hypercontraction, Procion Yellow staining and loss of cross striations within the muscle fibres. It appeared to reach a point of maximum severity approximately 3-6 hours after ecothiopate administration and then, by means of some repair/regeneration process, regained an apparently normal morphology within 72 hours of the intoxication.

At the ultrastructural level, ecothiopate-induced myopathy was recognised by loss of Z-lines, swelling and vacuolation of mitochondria and sarcoplasmic reticulum, dissarray of myofilaments, crystal formation, and sometimes, by the complete obliteration of sarcomeric structure. The development of myopathy *in vitro* was shown to be nerve-mediated and to require a functional acetylcholine receptor for its development. It was successfully treated therapeutically *in vivo* by pyridine-2-aldoxime methiodide and prophylactically by pyridostigmine bromide. However, the use of a range of membrane-ion channel blockers, and of leupeptin, an inhibitor of calcium-activated-neutral-protease, have been unsuccessful in the prevention of ecothiopate-induced myopathy.

Key words: Ecothiopate iodide. Myopathy. Creatine Kinase. Acetylcholinesterase. Intracellular calcium.

With thanks and in loving memory to Mum and Dad

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ABBREVIATIONS

The following non-standard abbreviations were used throughout this work.

ACh	acetylcholine
AChE	acetylcholinesterase
AChR	acetylcholine receptor
anti-ChE	anticholinesterase
ASCh	acetyl-thiocholine
BuChE	butyrylcholinesterase
CANP	calcium-activated-neutral-protease
ChE	cholinesterase
CK	creatine kinase
CROC	calcium-induced release of calcium
DFP	diisopropylfluorophosphate
DTNB	dinitrothiobisnitrobenzoate
ECO	ecothiopate iodide
EDL	extensor digitorum longus
HCL	hydrochloric acid
iCa^{2+}	intracellular calcium
G-6-P	glucose-6 phosphate
MWt	molecular weight
NL	normal Liley
OMPA	tetramono-isopropyl-pyrophosphortetramide
OP	organophosphorus
2PAM	pyridine-2-aldoxime-methiodide
SR	sarcoplasmic reticulum
TCF	temperature constant factor

CHAPTER 1

INTRODUCTION.

The present study is of the development of myopathy induced in the diaphragms of mice exposed to the organophosphorus anticholinesterase ecothiopate iodide (ECO).

1.1. THE TOXICITY OF ORGANOPHOSPHORUS ANTICHOLINESTERASES

Organophosphorus (OP) compounds are used widely in industry, agriculture, vetenary medicine, and clinically for treatment of glaucoma. However, the compounds have been shown to produce serious toxic effects in man and other animals. OP anticholinesterases act by inhibiting the cholinesterase (ChE) systems, most notably acetylcholinesterase (AChE), found throughout the body. As a result of this inhibition the duration of action of ACh, released from presynaptic nerve terminals, is prolonged, its effect on the postsynaptic receptors is enhanced, and this in turn leads to the signs and symptoms of anticholinesterase poisoning.

The effects of anticholinesterase poisoning may be categorised as muscarinic, nicotinic and central. However, since this study is concerned primarily with the OP compound ECO, which does not penetrate the blood brain barrier (Koelle & Steiner, 1956; Schauman & Job, 1958), only the muscarinic and nicotinic effects of poisoning are considered relevant to the present study. Muscarinic effects of OP poisoning include nausea, vomitting abdominal cramps, diarrhoea, urination, increased bronchial constriction, sweating, excessive secretion of tears, bradycardia and hypotension, all signs which may be relieved by prompt

administration of atropine (Grob, 1956; Grob & Johns, 1958; Durham & Hayes, 1962). The nicotinic effects, which usually occur after the muscarinic effects have reached moderate severity (Durham & Hayes, 1962), are the consequence of inhibition of AChE at skeletal muscle neuromuscular junctions, and the corresponding reduced rate of hydrolysis of ACh. The nicotinic effects include muscle fasciculations and cramps, increased fatiguability and mild, generalised muscle weakness which is increased by exertion (Durham & Hayes, 1962). Accompanying the altered neuromuscular activity, induced by OP poisoning, skeletal muscle damage has been shown to develop, initially at the junctional region of the muscle concerned (Ariens et al., 1969; Wecker et al., 1978b; Toth et al., 1983). The myopathy has been shown to develop in fatal exposures to man (Wecker et al., 1986), and death may result from repiratory paralysis (Durham & Hayes, 1962).

The term myopathy can be used to describe any abnormal state which may reside within a muscle fibre. It may be very mild, consisting of slight abnormality only, or gross, possibly resulting in total cell necrosis. There are many different types of myopathy which result from an interference of the normal biochemical and/or physiological parameters that govern neuromuscular transmission and/or muscle function. This particular study is concerned primarily with the myopathy induced by the irreversible inhibition of AChE by the OP compound EOO.

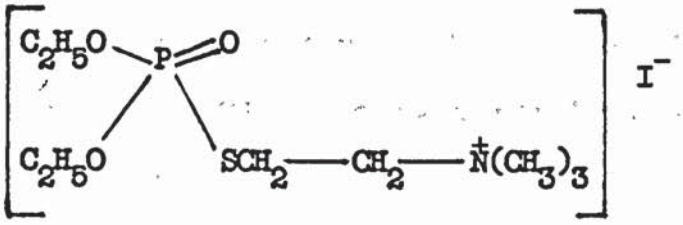
OP-induced myopathy has been most consistently represented by dilatation of sarcoplasmic reticulum (SR) and mitochondria plus dissolution of the Z-line (Laskowski et al., 1975, 1977; Leonard

& Salpeter, 1979, 1980) and loss of sarcoplasmic enzymes such as lactate dehydrogenase (Leonard & Salpeter, 1980), with a consequent increase in serum enzyme activity. However, the mechanism by which OP agents induce myopathy is not yet fully understood. The aim of the present study is to investigate the sequence of events involved in the development of OP-induced myopathy, by using the quaternary OP compound, ECO.

1.2. ECOTHIOPATE IODIDE

Ecothiopate iodide (ECO), also known as Phospholine iodide, is an irreversible inhibitor of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Silver, 1974) and was first synthesised by Tammelin in 1957. ECO is not lipid soluble and

Ecothiopate iodide (ECO)



therefore does not penetrate the central nervous system following injection (Koelle & Steiner, 1956). On entering the general circulation however, it may reach a concentration which will affect the peripheral synapses formed by cholinergic neurones and it will then induce the muscarinic and nicotinic cholinergic symptoms of OP toxicity described above (1.1) (Osserman et al., 1961). ECO has also been shown to induce myopathy in the diaphragms of mice (Ferry & Cullen :- personal communication), but

neither the nature of this myopathy nor its development have been comprehensively studied to the authors knowledge.

1.3 MYOPATHY AND INTRACELLULAR CALCIUM

Myopathies in general have often been associated with elevation of intracellular calcium (Wrogeman & Pena, 1976; Obero & Engel, 1977; Bodensteiner & Engel, 1978; Schanne et al, 1979; Leonard & Salpeter, 1979; Ross-Canada et al., 1983; Toth et al, 1983) although, whether calcium is the cause of, or a result of myopathy is uncertain. There is evidence (Leonard and Salpeter, 1979) to suggest that in anticholinesterase induced myopathies, at least, free intracellular calcium (iCa^{2+}) may be causative, and infact Toth et al (1983) demonstrated cytochemically, the uptake of Ca^{2+} prior to the development of myopathy at the junctional region of diaphragms exposed to the OP cholinesterase inhibitors diisopropylfluorophosphate (DFP) and methylparathion (Wolfatox). However, the role of Ca^{2+} in the development of myopathy is not clear. One possibility is that a calcium-activated-neutral-protease (CANP) may act by attacking the Z-disks (Busch et al., 1972; Dayton et al., 1975; Reddy et al., 1975, 1983; Reville et al., 1976; Inomata, 1983) causing dissassembly of myofilaments whose further degradation may then be lysosomal (Cullen et al., 1978). Alternatively, it has been suggested that muscle degradation may be the result of the activity of catheptic enzymes, released from lysosomes in the presence of high levels of

iCa^{2+} (Iodice et al., 1966; M^{c}Gowan et al., 1976; Libby & Goldberg, 1978). Whatever the mechanism, it seems certain that the development of myopathy is dependent on the elevation of the iCa^{2+} concentration above normal levels (approximately 10^{-7}M; Irvine, 1986). Thus the failure of regulation of iCa^{2+} is vitally important to the myopathic process.

The regulation of the Ca^{2+} level in the cytoplasm is a vast topic and a comprehensive overview is beyond the scope of this thesis. However, certain aspects of Ca^{2+} regulation are considered to be particularly relevant to the development of myopathy and are introduced correspondingly.

1.3.1. Calcium regulation in the cell.

Free Ca^{2+} has been shown to be a vital intracellular second messenger, responsible for the modulation of a vast range of physiological processes, most notably, secretion and contraction. The role of Ca^{2+} as a second messenger necessitates high control of the Ca^{2+} concentration in the cell:-

Intracellular calcium is maintained at about 10^{-7}M, compared with a concentration in the extracellular fluid of about 10^{-3}M (Irvine, 1986). However, this low figure of 10^{-7}M applies only to the free Ca^{2+} in the cytosol, the total calcium inside the cell being much higher, and the bulk of this Ca^{2+} being bound to

proteins, membranes or other cellular constituents, or sequestered inside intracellular organelles, most notably the endoplasmic reticulum (sarcoplasmic reticulum (SR) in muscle) and mitochondria (Irvine, 1986). The level of Ca^{2+} in the cytoplasm is therefore controlled largely by the uptake and release of the cation across the plasma membrane, the inner mitochondrial membrane (the outer membrane being fully permeable to Ca^{2+}), and the SR. Each of these membrane systems possesses distinctive transport mechanisms which act in concert to regulate iCa^{2+}.

1.3.1.1 Calcium regulation by the plasma membrane

Influx of Ca^{2+} across the plasma membranes of excitable cells, such as muscle, occurs via voltage-gated Ca^{2+}-channels (Reuter, 1986), large membrane-spanning glycoproteins in the membrane that allow large numbers of ions to move down the concentration gradient into the cell (Stanfield, 1986) in response to an action potential. The Ca^{2+}-channels were first demonstrated in skeletal muscles of crustacea (Fatt & Ginsborg., 1958) and have been shown to open in response to depolarisation induced by the opening of voltage-dependent Na^{+} channels (Stanfield, 1986). Ca^{2+}-channel opening will itself produce an element of self-reinforcing depolarisation since the Ca^{2+} entering the muscle will further depolarise, opening more Ca^{2+} channels, and allowing more

Ca^{2+} entry. Following the peak of the action potential ionic events return the membrane potential to its resting level and the influx of Ca^{2+} ends (Stanfield, 1986). The % open Ca^{2+}-channels can increase significantly under the influence of cAMP, a second intracellular messenger which activates enzymes that phosphorylate the Ca^{2+}-channels; phosphorylation greatly increasing the probability that a channel will open.

In addition to the voltage-operated channels described above, there exists nonspecific cation channels, located mainly at the endplate region of skeletal muscle. These endplate channels have been shown to open in the presence of ACh (Csillik & Savay, 1963; Miledi, 1973; Evans, 1974; Miledi et al., 1977) and allow the exchange of Na^+, K^+, and Ca^{2+} ions (Takeuchi, 1963) into the soleplate region of the muscle concerned. It is possible that the prolongation of the half-life of ACh, induced by an anticholinesterase, may enhance the entry of Na^+ and Ca^{2+} ions into the muscle fibre.

The plasma membrane of the cell contains two transporters responsible for expelling Ca^{2+} from the cell. The high affinity system is an ATPase (Brandt et al., 1980; Carafoli & Zurini, 1982; Carafoli et al., 1983; Nicholls, 1986): an enzyme that splits ATP to derive energy. The energy enables it to move Ca^{2+} out of the cell against the steep concentration gradient that prevails across the plasma membrane. The pump ejects Ca^{2+} from

the cell, importing protons (H^+) probably in the ratio of 2 H^+ per Ca^{2+} (Carafoli et al., 1983).

The plasma membrane ATPase responds to minute increases in iCa^{2+}. More drastic swings in concentration activate the other Ca^{2+} removal system of the plasma membrane, the Na^+/Ca^{2+} exchanger (Nicholls, 1986). The exchanger was first identified in squid heart muscle and neurones and has since been shown to operate in most mammalian tissues although there is conflicting evidence concerning its existence in skeletal muscle (Yamamoto & Greeff, 1981; Gilbert et al., 1982). The Na^+/Ca^{2+} exchange system has been extensively researched in both cardiac and smooth muscles and is thought to derive some of the energy required to drive Ca^{2+} out of the cell from the concentration gradient of Na^+ across the plasma membrane (Baker, 1986). However, the system has also been shown to be electrogenic, importing 3 singly charged Na^+ for every doubly charged Ca^{2+} exported (Reeves & Hale, 1984) and thus allowing the transport process to tap the resultant energy embodied in the membrane potential. Thus, both chemical and electrical gradients drive the Na^+/Ca^{2+} exchanger of the excitable cell membrane.

1.3.1.2. Calcium regulation by intracellular organelles.

Intracellular processes of reducing sarcoplasmic Ca^{2+}

include the intracellular Ca^{2+}-buffering organelles, most notably the SR and mitochondria (Obero & Engel, 1977).

Under physiological conditions the SR is the major intracellular store for Ca^{2+} (Irvine, 1986) and it functions to govern the short-term fluctuations in free Ca^{2+} that cause the contraction of muscle fibres (Luttgau & Moisescu, 1978). The persistence of the contraction of fast skeletal muscle in the absence of Ca^{2+} in the extracellular fluid indicates that the Ca^{2+} trigger for contraction does not come from the extracellular space but from an intracellular source (Fabiato, 1982). However, the mechanism of release of this Ca^{2+} is still uncertain. It has been suggested that an initial "trigger" pool of Ca^{2+} may be mobilised from the SR in response to inositol (145) triphosphate (IP_3), a second messenger and breakdown product of phosphatidylinositol bisphosphate which is split by certain hormones that bind to the cell surface (Irvine, 1986). It is thought that the IP_3-induced release of Ca^{2+} from the SR may occur via a pore in exchange for K^+ (Muallem et al., 1985). Also, Fabiato has shown (1982), in both heart and skeletal muscle, that a rise in intracellular Ca^{2+} above the resting physiological level can cause a further Ca^{2+}-induced release of Ca^{2+} (CROC) from the SR, supporting similar findings of other authors (Ford & Podolski, 1970; Endo, 1977; Martinosi, 1984). Fabiato suggests (1982) that it is the CROC which is ultimately responsible for muscle contraction.

The complexity of the Ca^{2+}-release mechanisms from SR offers a range of possible sources for an excessive elevation of iCa^{2+} which has been observed in many pathological conditions.

The Ca^{2+} that triggers muscle contraction is pumped back into the SR by a Ca^{2+}-pumping ATPase which comprises an intrinsic component of the SR, representing 50-80% of the total protein embodied in the membrane (Martinosi, 1984). The activity of the ATPase depends on membrane phospholipids and, unlike the plasma membrane Ca^{2+}-ATPase, is relatively insensitive to calmodulin (Martinosi, 1984). The SR Ca^{2+} pump is capable of lowering the cytoplasmic free Ca^{2+} concentration to or below 10^{-8}M (Martinosi, 1984), and is thought to be the main regulator of sarcoplasmic Ca^{2+} under physiological conditions (Irvine, 1986). However, it will not buffer large amounts of Ca^{2+} and it is the mitochondria that are crucial in buffering dangerous increases in cytoplasmic Ca^{2+} after the other Ca^{2+}-regulatory systems become overloaded (Martinosi, 1984). The free Ca^{2+} within the mitochondrial matrix of physiologically normal muscle has been shown to be low (10^{-5}M), supporting the notion that mitochondria do not play a major role in Ca^{2+} regulation under physiological conditions (Martinosi, 1984). It is thought that the Ca^{2+} which does enter the mitochondria forms a complex of hydroxyapatite with inorganic phosphate ions, thus allowing large amounts of Ca^{2+} to be

accumulated by mitochondria with no deterioration of its bioenergetic properties (Nicholls, 1986). The influx of Ca^{2+} into mitochondria is governed by the inner mitochondrial membrane and occurs via a uniporter system powered by the mitochondrial membrane potential of about 150-180mV (matrix negative), with compensatory extrusion of $2H^{+}$ from the matrix (Fiskum & Lehninger, 1982). In pathological circumstances, when Ca^{2+} loading becomes really excessive and especially in the absence of certain components, most notably magnesium ions and adenine nucleotides, the buffering capacity of the mitochondria becomes finite, Ca^{2+} leaks into the sarcoplasm and becomes an ionic assassin of the cell. The reactions that Ca^{2+} normally modulates proceed continuously and uncontrollably and the excess ions activate other reactions that do not occur in a physiologically normal cell. In liver mitochondria, at least, Ca^{2+} uptake has been shown to take primacy over oxidative phosphorylation in utilising energy (Lehninger et al., 1967). In such cases, the lack of ATP production will mean that less energy is available to the cell for the expulsion of excess Ca^{2+}. The result would again be a massive increase in the ionised iCa^{2+} concentration and the resultant initiation of cellular destruction. In less cataclysmic situations the mitochondria buy time for the cell by absorbing the excess Ca^{2+} then, after the Ca^{2+} storm has abated they release the Ca^{2+} at a rate that does not disturb the cell metabolism. Ca^{2+} release takes place through a transporter that, like the exchanger

of the plasma membrane, catalyses the exchange of Na^+ and Ca^{2+} (Nichols, 1986). In contrast to the plasma membrane exchanger, however, which creates a charge imbalance by bringing in 3 Na^+ ions for every Ca^{2+} it exports from the cell, the mitochondrial exchanger is electically neutral: it transports Na^+ and Ca^{2+} ions in the ratio of 2:1 (Fiskum & Lehninger, 1982). Infact Crompton et al., (1976) suggested that the release reaction of Ca^{2+} from mitochondria was mediated by a Na^+/Ca^{2+} exchange carrier which operated in parallel with a Na^+/H^+ antiporter. The Na^+ that had entered mitochondria in exchange for Ca^{2+} would return to the extramitochondrial space in exchange for H^+ producing, ultimately, Ca^{2+}/H^+exchange (Carafoli & Zurini, 1982). It seems that the exchanger system may vary according to the tissue from which the mitochondria originates (Nicholls, 1986) and Fiskum & Lehninger (1982) suggested that in nonexcitable tissue Ca^{2+} efflux is thought to be mediated by a $Ca^{2+}/2H^+$ antiport carrier. However, the establishment of a direct Ca^{2+}/H^+ exchange antiport system is not clear (Carafoli & Zurini, 1982).

1.3.1.3. Ca^{2+} regulation by soluble proteins.

Apart from the membrane systems described above, Ca^{2+} regulation within the cell is also governed by soluble proteins

in the cytoplasm and within the organelles themselves. These proteins mediate the intracellular effects of calcium by controlling the level of free Ca^{2+} in the cell, but the amount of Ca^{2+} a soluble protein can bind is limited by the number of protein molecules. Two examples of soluble Ca^{2+}-binding proteins vital to skeletal muscle physiology are troponin and calmodulin. A rise in iCa^{2+} triggers the contraction of the muscle, an event mediated by troponin (Huxley, 1971; Ebashi et al., 1975; Endo, 1977; Herzberg et al., 1986). It also results in a longer term metabolic change mediated by calmodulin: Ca^{2+}-activated calmodulin mobilises a protein kinase that phosphorylates a second enzyme. Thus activated, the second enzyme catalyses the breakdown of glycogen to glucose which is then metabolised to provide energy for muscle contraction. A third example of sarcoplasmic Ca^{2+}-binding proteins are the parvalbumins whose actual function is unclear although they may play a protective role in prolonged contraction by slowly reducing the intracellular Ca^{2+} concentration (England, 1986).

In the present study attempts were made to discover something of the nature of the myopathy induced by ECO in the diaphragms of mice. Particular attention was paid to the following questions:-

a) Does acute administration of ECO induce myopathy at the junctional region of the diaphragms of mice? If so, what is the

nature of this myopathy (using light and electron microscopy), and is it accompanied by loss of sarcoplasmic enzymes and/or elevation of iCa^{2+}? How can myopathy be quantified?

b) How is the development of ECO-induced myopathy related to inhibition of acetylcholinesterase?

c) Is ECO-induced myopathy mediated by Ca^{2+} ie. can it be prevented by reducing Ca^{2+} entry into the sarcoplasm?

d) Is ECO-induced myopathy nerve mediated and does it require the integral involvement of the acetylcholine receptor (AChR)?

e) How can ECO-induced myopoathy be prevented or treated in the <u>in vivo</u> situation?

CHAPTER 2

MATERIALS AND METHODS.

2.1 ANIMALS.

Male white mice weighing between 40 and 50g were supplied by Bantin and Kingman Ltd. The mice were all exbreeders and, at about 6 months of age, were no longer undergoing any developmental physiological changes (Banker et al, 1982).

2.2 ADMINISTRATION OF ECOTHIOPATE IODIDE (ECO) IN VIVO.

ECO was used as Phospholine iodide (supplied as eyedrops by Ayerst Laboratories). A stock solution of 10^{-2}M-ECO was made by adding 3.62ml of the diluent (containing 0.5% chlorbutanol, mannitol, boric acid and sodium phosphate) to 12.5mg of phospholine (containing 40mg potassium acetate ie. a negligible increase in the potassium concentration, of both the in vivo injection solution and the incubation medium in vitro). The resulting 10^{-2}M-ECO solution was diluted 100-fold with distilled water to give a final 10^{-4}M stock ECO solution. This stock solution was stored at 5^{0}C between experiments thus ensuring minimal degradation.

The injection solution consisted of the 10^{-4}M-ECO stock solution plus 1.43×10^{-4}M-atropine sulphate (in 0.9% sodium chloride) and is henceforth referred to as ECO plus atropine. Atropine was included in the ECO injection solution in order to

minimise the muscarinic effects of ECO (Grob, 1956; Grob & Johns, 1958).

Mice were injected subcutaneously, between the shoulder blades, with 0.1ml of ECO plus atropine per 20g body weight, giving a final dose of ECO of $500nMKg^{-1}$. However, mice used as controls were injected with 0.1ml of atropine saline ($1.43x10^{-4}M$ atropine sulphate in 0.9% sodium chloride solution) per 20g body weight.

2.3 PREPARATION OF HEMIDIAPHRAGMS FROM MICE GIVEN ECO IN VIVO.

Following injection of ECO, mice were killed by cervical dislocation and were exanguinated. The rib cage was exposed and whole diaphragms were removed and rinsed at room temperature in physiological saline similar to that described by Liley (1956) but with an increased dextrose content of 25mM (recommended by Krnjević and Miledi, 1970). The modified Lileys saline (A2) is referred to as normal Lileys saline throughout this study. Hemidiaphragm preparations were then stained with Procion (2.6) and for cholinesterase (ChE) (2.6) in order to assess the extent of myopathy induced by ECO, using the Procion technique (2.7).

2.4. ADMINISTRATION OF ECO IN VITRO: THE IN VITRO TECHNIQUE.

The technique was used to investigate the initiation and early development of ECO-induced myopathy.

Phrenic nerve-diaphragm preparations were dissected according to the technique of Bulbring (1946), in Ca-reduced saline (A2), and were bisected into left and right hemidiaphragms by cutting through the central tendon. The preparations were rinsed at room temperature in Ca-reduced saline and were then stimulated with single pulses, in organ baths containing normal Lileys saline at 37°C. In this way, tissue viability was confirmed and the resting tension of the preparation was adjusted such that maximal contractions were elicited for each nerve impulse. Having established the optimum resting tension for each preparation, the stimulation pattern was fixed at 5 pulses at 50Hz every 5 seconds, and 500nM ECO was added to one bath; the preparation within this bath represented the ECO-treated control and was stimulated thus for 3 hours, the saline (containing 500nM ECO) being renewed approximately half way through the incubation period. In the contralateral organ bath 500nM ECO was added 10 minutes after a modification appropriate to the experiment concerned had been made eg. replacement of normal Lileys saline by Ca-reduced saline, cessation of nerve stimulation etc. In this way, it was hoped that the muscle would have adjusted to its modified conditions prior to exposure to ECO. The "experimental" preparation was incubated thus for 3 hours, the relevant saline being renewed approximately half way through the incubation period.

At the end of the incubation period allpreparations were washed for 15 minutes in Ca-reduced saline in order to minimise

muscle contraction which might otherwise complicate further tissue processing for histology.

Finally, all preparations were stained with Procion (2.6) and for ChE (2.6) in order to assess the extent of myopathy induced by ECO, using the Procion technique (2.7).

2.5. THE IN VIVO/IN VITRO TECHNIQUE.

This technique was devised to maintain as much consistency as possible with the in vivo experiments, by minimising the variation induced by different methods of administration of ECO. Hence a more viable comparison could be made with the results of in vivo experiments. Consistency was maintained in:-

a) the magnitude of the dose of ECO administered.

b) the route of administration of ECO.

c) the time course of acetylcholinesterase (AChE) inhibition.

However the importance of performing internal controls was still recognised and enacted for all in vivo/in vitro experiments.

2.5.1. The procedure.

All mice were injected subcutaneously with ECO plus atropine for a given period, usually 30 minutes, before being killed. Phrenic nerve hemidiaphragm preparations were dissected (Bulbring, 1946) in Ca-reduced saline (A2) and were then stimulated with

single pulses, in organ baths containing normal Lileys saline at 37°C. Tissue viability was thus confirmed and the resting tension of each preparation adjusted such that maximum contractions were elicited. Once the optimum resting tension had been established, the stimulation pattern was fixed at 5 impulses at 50 Hz every 5 seconds, and modifications pertinent to the experiment concerned were made eg. normal Lileys saline was replaced by Ca-reduced saline (chapter 8) or nerve stimulation was stopped (chapter 9) etc.

In most experiments one hemidiaphragm acted as an ECO-treated control, stimulated in normal Lileys saline (A2) throughout the incubation period which ended 3 hours after the time of the injection and was therefore of 2 hours 30 minutes duration.

The respective salines were renewed approximately half way through the incubation period and at the end of the incubation all preparations were washed in Ca-reduced saline to minimise muscle contraction that might otherwise complicate further tissue processing for histology.

Finally, all preparations were stained with Procion and for ChE (2.6) in order to assess the extent of myopathy induced by ECO, using the Procion technique (2.7).

N.B. In both the <u>in vivo/in vitro</u> and the <u>in vitro</u> technques an exposure period to ECO of 3 hours was purposely chosen since it is known to yield a severe myopathy uncomplicated by regeneration or phagocytosis (6.3.2.7 & 6.3.2.8) and is there-

fore more accurately assessed using the Procion technique (2.7). In both techniques all salines were maintained at 37°C throughout the incubation period.

2.6 HISTOLOGICAL METHODS.

2.6.1. Preparation of hemidiaphragms for histological examination.

Fresh hemidiaphragms were pinned, via the costal margin of the ribs and the central tendon, onto sheets of dental wax, with slight stretching, keeping the fibres straight. They were then incubated at room temperature for 60 minutes in a 0.1% solution of Procion Yellow MX4R (ICI) in normal Lileys saline. The staining solution shall be referred to simply as Procion throughout this study. The stained hemidiaphragms were then washed for a total of 30 minutes with three changes of normal Lileys saline and were then fixed for approximately 60 minutes in 4% formaldehyde in 0.1M-acetate buffer at pH 4.0.

2.6.1.1. Cholinesterase staining:- the localisation of endplates.

Procion-stained, fixed hemidiaphragms were trimmed from the ribs and were then incubated, at room temperature, in a solution shown by Karnovsy and Roots (1964) to stain ChE (A3). Staining took approximately 60-90 minutes for untreated preparations, the

endplates finally appearing as patches of brown stain. The preparations were then washed in distilled water, trimmed and mounted in glycerol jelly onto microscope slides. They were viewed by incident ultraviolet light using a Zeiss fluorescence microscope.

Photographs were taken of the preparations using an Olympus PM-6 camera and either colour reversal film, corrected for tungsten illumination for slides, or colour negative film with a blue filter for prints.

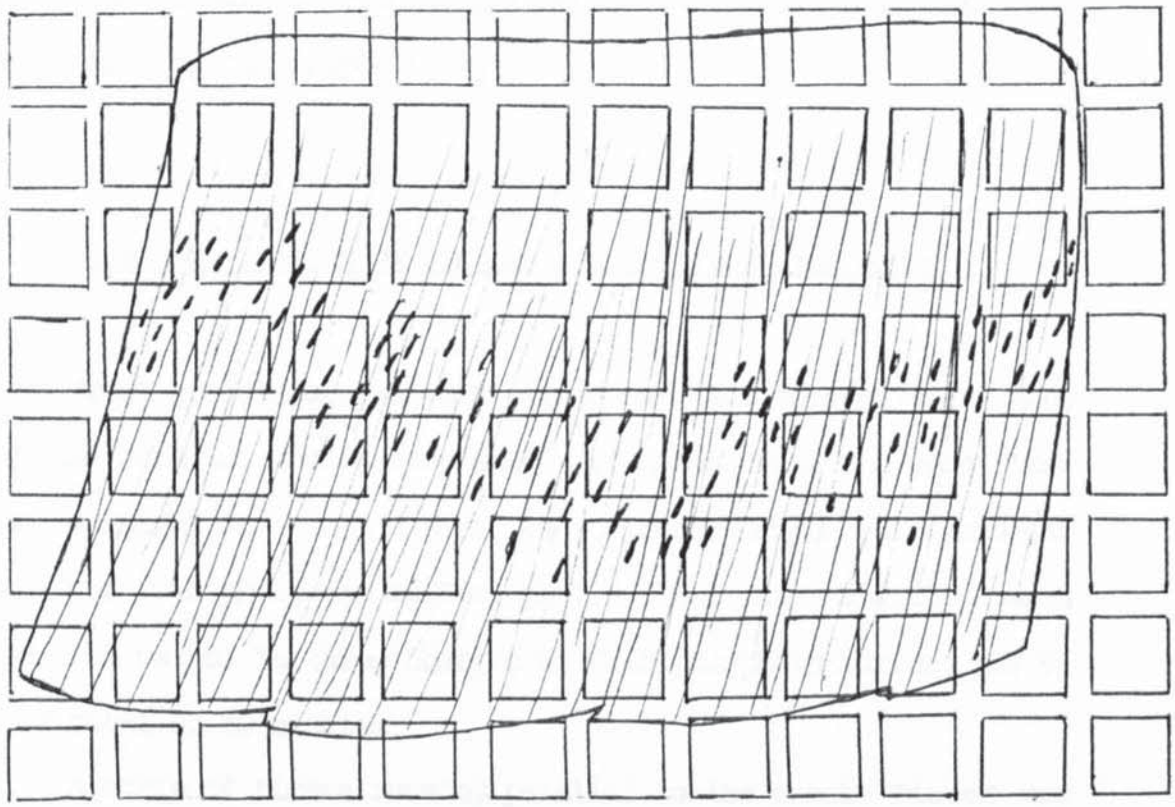
2.7 ESTIMATION OF THE EXTENT OF MYOPATHY IN HEMIDIAPHRAGMS STAINED WITH PROCION :- The Procion technique.

A morphometric point counting technique was used to quantify the extent of Procion staining in hemidiaphragm preparations:- an eyepiece graticule ($0.62MM^2$) of 121 points (the intersections of an 11x11 grid) was placed at regular fields of view over each hemidiaphragm preparation. Fields were selected using a stage micrometer to advance 1mm across the tissue such that the space between consecutive fields measured 0.38mm (figure 2.1). For each field a count was made of the number of points overlying Procion-stained regions of the preparation. This number, x, was expressed as a percentage of the total number of points on the grid.

A total of approximately 12000 points were counted for each preparation (about 100 fields). The value for the overall %

Figure 2.1 The positioning of the eyepiece graticule over hemi-diaphragm preparations in order for an estimate to be made of the % Procion staining at the junctional and nonjunctional regions.

The crus of the diaphragm



The costal margin of the diaphragm

The distance between grids= 0.38mm.

Any grid-square overlying a field containing at least one endplate is counted as part of the junctional region of the diaphragm.

Procion staining of a particular preparation was the arithmetic mean of the % Procion staining of all the fields counted.

A comparison was made between the % Procion staining in the junctional and nonjunctional regions by estimating separately the % Procion points for each region, any field containing one or more endplates being assigned to the junctional region. Results were expressed as the % Procion points at the junctional and non-junctional regions respectively.

2.8 PREPARATION OF TISSUES FOR ELECTRON MICROSCOPY

Fresh hemidiaphragms were pinned out taut through their ribs and the central tendon onto sheets of dental wax, and were fixed thus overnight in a solution of 5% glutaraldehyde (BDH-Aristar grade) in 0.1M phosphate buffer, pH 7.4. The fixed hemidiaphragms were then washed in three changes of 0.1M-phosphate buffer, pH7.4, for a total of 30 minutes.

A strip of tissue running parallel to the muscle fibres was removed such that muscle fibres were kept intact. A second strip of tissue encompassing the intramuscular branch of the phrenic nerve was also removed. It was assumed that most of the endplates within the hemidiaphragm were contained in this strip. Each strip was then post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer for 1-2 hours and was then dehydrated through graded concentrations of alcohol (50%, 70%, 90% and absolute). At each stage two changes of alcohol were used and the tissues were

immersed in each for 15 minutes. Following dehydration the tissues were rinsed in two 5 minute changes of propylene oxide and were then steeped for 30 minutes in a 1:1 mixture of propylene oxide and araldite (A4). Finally, the tissues were left overnight in fresh araldite which was polymerised by heating to 60°C for 24 hours (A4).

2.8.1. Preparation of sections.

Tissue sections were cut from the araldite blocks using glass knives on a Reichert OM U4 ultramicrotome. Survey semithin sections of 1µ thickness were cut first and were floated on a drop of distilled water on a clean glass microscope slide. The slide was then placed onto a hotplate at 60°C and the sections were allowed to dry onto the slide. Sections were stained for approximately 10 seconds with a solution of 1% Toluidine Blue:1% borax in distilled water. The stain was rinsed from the slide with distilled water and the sections were dried on the hotplate before inspection with the light microscope. If these sections appeared suitable, ultrathin sections were cut and prepared for electron microscopy. Ultrathin sections were cut at approximately 60-70 nm thickness and were mounted on Athene new copper grids.

2.8.2. Staining of ultrathin sections.

The ultrathin sections were stained, on the copper grids, in two stages:-

2.8.2.1. Staining with uranyl acetate.

1) A sheet of Parafilm was placed in a petridish.

2) For every grid to be stained, one drop of 30% uranyl acetate in methanol (A3) was placed on the parafilm.

3) Grids were immersed in the stain for 5 minutes.

4) Grids were briefly rinsed in each of 100%, 50% and 25% solutions of methanol.

5) The grids were dried carefully on filter paper.

2.8.2.2. Staining with lead citrate.

1) A sheet of parafilm was placed in a petridish.

2) For each grid to be stained, one drop of lead citrate solution (A3) was placed on the parafilm.

3) Grids were immersed in the stain for 5 minutes.

4) Grids were briefly rinsed in four changes of distilled water.

5) The grids were dried carefully and stored.

The stained sections were inspected on a Jeol 1200 EX electron microscope using an accelaration voltage of 80kV.

Photographs were taken on film which was developed in D19 (Kodak developer) for 4 minutes at 20 °C before being washed in tap water for 20 minutes at 20°C and subsequently dried.

2.9. MEASUREMENT OF THE CALCIUM ACCUMULATION AT THE JUNCTIONAL REGION OF THE DIAPHRAGM.

Calcium accumulation at the junctional region of the diaphragm was assumed to be represented by the difference in the calcium contents of the junctional and nonjunctional regions (adjusted for weight), the difference being negligible in control preparations (5.3).

2.9.1. Assay of the calcium content of the junctional and nonjunctional regions of hemidiaphragm preparations.

Hemidiaphragms, still attatched to their ribs were washed for 15 minutes in Ca-reduced saline to reduce extracellular Ca^{2+}. A 15 minute wash was considered sufficient to reduce extracellular Ca^{2+} since indirect muscle twitch stops within 5-10 minutes of exposure to Ca-reduced saline (8.2.2.1). The washed hemidiaphragms were pinned onto sheets of dental wax via the costal margin and the central tendon such that their fibres were straight and not pierced by the pins. They were then dehydrated in three changes of acetone (BDH AristaR) for a minimum total period of 60

minutes. The ribs, tendons and cut edges were removed from the hemidiaphragms, viewed beneath a dissection microscope. With appropriate illumination the intramuscular nerve could be seen traversing the hemidiaphragm approximately perpendicular to the axis of the muscle fibres. Associated with this nerve is the junctional region of the muscle, which, in damaged preparations was clearly visible as a shadow surrounding the nerve. By incising as close as possible either side of this area the junctional region of each hemidiaphragm was removed as a strip, approximately 2mm wide, leaving the two nonjunctional strips.

Acetone was allowed to evaporate from the junctional and nonjunctional strips which were then weighed separately and digested overnight in 0.1ml of 10M-HCl (BDH-AristaR- grade) in conical bottomed polystyrene vials. The resulting solutions were evaporated in a fume cupboard using a laboratory hotplate and the residues were then redissolved in 0.8ml of a 0.1% solution of lanthanum chloride (BDH AristaR- grade) in 0.1M HCl.

The samples were assayed using a Perkin Elmer atomic absorption spectrophotometer against a blank of 0.1% lanthanum chloride in 0.1M-HCl. The spectrophotometer was previously calibrated using Ca^{2+} standards of 1 and 4 parts per million (ppm) (table 2.1).

N.B. Whilst the technique measures ionised calcium (Ca^{2+}), much of this calcium may have been bound prior to digestion of the tissue in HCl (plus lanthanum chloride). The results therefore reflect the total calcium content of the tissues concerned.

Table 2.1 Composition of the calcium standards used to calibrate the atomic absorption spectrophotometer.

	Standards	
	4ppm	1ppm
Blank	99ml	75ml
10^{-2}M calcium chloride	1ml	-
4ppm standard	-	25ml

Readings of the Ca^{2+} concentration of the samples were in parts per million (ppm) and were converted into a measure of the total calcium content of the tissues ($nmol\,mg^{-1}$) using the following equation:-

$$\text{calcium content } (nmol\,mg^{-1} \text{ tissue}) = \frac{25 \times V \times X}{\text{dry weight of tissue}}$$

25 = $nmol\,ml^{-1}$ equivalent of 1ppm $CaCl_2$.

V = Total sample volume (0.8ml).

X = Ca^{2+} concentration (ppm) of the sample as read from the spectrophotometer.

Hence the calcium contents of both the junctional and nonjunctional regions were found.

2.9.2. Conversion of calcium content results to a value of calcium accumulation at the junctional region of the hemidiaphragm.

The calcium accumulation at the junctional region of the diaphragm was calculated using the following formula:-

$$\text{calcium accumulation at the junctional region} = \frac{\text{Jwt (J-NJ)}}{\text{Total weight}}$$

Jwt = dry weight of the junctional strip.

J = calcium content of 1mg of junctional region (nmols mg^{-1})

NJ = calcium content of 1mg of nonjunctional region (nmols mg^{-1})

Total weight = combined dry weight of the junctional and non-junctional regions (mg).

Hence the final results were expressed as the accumulation of calcium at the junctional region of the diaphragm ($nmolmg^{-1}$).

2.10 ASSAY OF SERUM CREATINE KINASE (CK)

Mice were anaesthetised with 2% Halothane in a 50:50 mixture of nitrous oxide and oxygen. The femoral artery was exposed and severed taking particular care not to damage the surrounding muscle. Approximately 1ml of blood was collected using a polystyrene syringe, without anticoagulant. The blood was placed into an Eppendorf tube and the mouse was then killed by cervical dislocation whilst anaesthetised. Within 4 hours of collection the blood was spun at 12,000g for 2 minutes. The serum was removed and stored at 4°C in the dark (Paterson & Lawrence, 1972). The assay itself was usually performed on the day of blood collection.

CK was assayed in the serum using a modification (Rosalki, 1967) of the technique devised by Oliver (1955) and sold as a Sigma 46 UV CK assay kit.

The kit contains:-

Reagent A:- ADP, NADP, creatine phosphate, AMP, N-acetyl cysteine, hexokinase ›24U (yeast), glucose-6-phosphate dehydrogenase ›14U (yeast), ethylenediaminetetraacetic acid, diadenosine pentaphosphate, stabilisers.

Reagent B:- D-glucose in imidazole buffer, magnesium ions and sodium azide.

The principal of the assay is the phosphorylation of adenosine diphosphate (ADP) by CK to produce creatine and

adenosine triphosphate (ATP), creatine phosphate serving as the phosphate donor.

$$\text{ADP + creatine phosphate} \xrightarrow{\text{CK}} \text{creatine + ATP}$$

The auxiliary enzyme, hexokinase (HK) catalyses glucose phosphorylation to produce glucose-6-phosphate (G-6-P) and ADP, ATP serving as the phosphate donor.

$$\text{ATP + glucose} \xrightarrow{\text{HK}} \text{ADP + G-6-P}$$

Glucose-6-phosphate is then oxidised to 6-phosphogluconate with the concomittant production of nicotinamide adenine dinucleotide phosphate (reduced form:- NADPH).

$$\text{G-6-P + NADP} \xrightarrow{\text{G-6-P dehydrogenase}} \text{6 phosphogluconate + NADPH + } H^+$$

Thus the rate of NADPH formation, measured at 340nm is directly proportional to the CK activity of the serum sample.

The Beckman Acta V spectrophotometer with attached chart recorder was used for all CK assays and the change in absorbance observed at 340nm.

Preliminary experiments showed that ECO itself, when added to the reaction mixture, did not affect NADPH formation in any way

2.10.1 The procedure.

A manual procedure for measuring the rate of NADPH formation was used:-

Using the reagents supplied in the Sigma 46-UV CK assay kit, 6.5ml of Reagent B was added to Reagent A and the resulting solution was mixed until Reagent A had dissolved. 3ml of this reaction mixture was pipetted into two glass cuvettes of 1cm light path and warmed to 30°C in a preheated cuvette stand. Two separate cuvettes, containing distilled water, were placed in the water-jacketed cuvette holders of the spectrophotometer, one in the path of the reference beam and the other (the blank), in the path of the sample beam .

Once the reaction mixtures had reached 30°C the assays were begun:-

At the begining of each assay the spectrophotometer was zeroed using the distilled water blank.

A cuvette containing prewarmed reaction mixture was then placed in the constant temperature compartment of the spectrophotometer in the path of the sample beam. A steady reading of the absorbance of this solution was obtained before 50µl of a sample (serum) was added. The reaction mixture, plus sample were mixed using a plastic rod. The rate of NADPH production was measured as the increase in absorbance (ΔA) of the reaction mixture with time and was recorded on the chart recorder using a chart speed of 1.5 inches/5 minutes.

Following each assay the temperature of the reaction mixture was checked using a mercury thermometer.

Having obtained a mean value for the rate of increase in absorbtion over a 5 minute period (ΔA/5min) the following equation was used to calculate the CK activity (UL^{-1}) of the serum samples, where one international unit, U, of enzyme is capable of converting one μmol of substrate per minute:

$$CK\ (UL^{-1}) = \frac{\Delta A/5min \times V \times 1000 \times TCF}{6.22 \times v \times 5}$$

V = total reaction volume (3.05ml)

1000 = conversion of activity ml^{-1} to activity L^{-1}

TCF = temperature correction factor (TCF at 30°C = 1)

v = volume of sample (50μl)

5 = conversion of ΔA/5min to ΔA/min.

6.22 = absorption coefficient of CK

Providing that the recommended volumes are used, the equation may be abbreviated to:

$$CK\ (UL^{-1}) = \Delta A/5min \times 2000 \times TCF$$

The final results of serum CK levels were expressed in UL^{-1}.

2.11. MEASUREMENT OF THE CK CONTENT OF THE JUNCTIONAL AND NONJUNCTIONAL REGIONS OF THE DIAPHRAGM.

Diaphragms were removed from mice, and were separated into hemidiaphragms (2.3). Each hemidiaphragm was trimmed parallel to the alignment of fibres, minimising the number of cells damaged and therefore leakage of intracellular enzymes. Both hemidiaphragms were blotted dry and the fat plus connective tissue were removed. Under appropriate illumination the intramuscular nerve could be seen traversing each hemidiaphragm approximately perpendicular to the axis of the muscle fibres. Working quickly to minimise enzyme leakage, a strip of tissue, approximately 2mm either side of the nerve, was removed from each hemidiaphragm. These "junctional" strips were weighed wet then homogenised in 5ml of ice cold 0.1M-KCl (BDH AnalaR), using a Tri-R Stir-R homogeniser at position 8.

For each hemidiaphragm one nonjunctional strip had to be removed from the ribs before all four nonjunctional regions of the whole diaphragm could be combined, weighed wet and homogenised in 5ml of 0.1M-KCl.

The homogenates of the junctional and nonjunctional regions were spun separately at 20,000 rpm for 30 minutes in a Beckman J2-21 centrifuge. The supernatants were removed and kept on ice whilst the pellets were washed in 5ml 0.1M-KCl. Supernatants from the second spin were combined with those from the first, and the resulting solutions were diluted 10 fold with 0.1M-KCl. Aliquots of 50ul were assayed for CK using the Sigma 46-UV CK assay kit.

Having obtained the values for the rate of change in absorption ($\Delta A/5min$) for each sample, the CK activities of the whole supernatant fractions were calculated using the following equation:

$$CK\ (U\ supernatant^{-1}) = \frac{\Delta A/5min \times V \times 100 \times TCF}{6.22 \times v \times 5}$$

V = total reaction volume (3.05ml)

100 = conversion of activity ml^{-1} to activity of the total supernatant (100ml) ie. the activity of the tissue.

TCF = temperature correction factor

v = volume of sample (50µl)

5 = conversion of $\Delta A/5$ min to $\Delta A/min$.

6.22 = absorption coefficient of CK

The equation gives a value of the total CK activity existing in each tissue preparation. In order to calculate the enzyme activity per mg tissue this value was divided by the total wet weight of the tissue concerned. The overall equation becomes:-

$$CK\ (Umg^{-1}tissue) = \left(\frac{\Delta A/5min \times V \times 100 \times TCF}{6.22 \times v \times 5}\right) \div \text{wet wt.of tissue(mg)}$$

Hence the final results were expressed in Umg^{-1} tissue for both the junctional and nonjunctional regions of the diaphragm.

2.12. ASSAY OF ACETYLCHOLINESTERASE (AChE) IN BLOOD AND IN THE DIAPHRAGM

The technique used is based on that of Ellman et al (1961) and is quick, simple and economical to use. ChE activity is measured by following the increase in absorbtion which occurs when thiocholine reacts with the dithiobisnitrobenzoate ion (DTNB)

$$\text{acetylthiocholine} \xrightarrow{\text{cholinesterase}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{DTNB} \longrightarrow \text{yellow colour}$$

The change in absorbtion is measured at 412nm and the reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting (Ellman et al., 1961). The main disadvantage of the technique of Ellman et al, is that it lacks specificity where both AChE and pseudocholinesterase (BuChE) are present. In the present study this has been overcome by the use of ethopropazine, a specific inhibitor of BuChE. Ethopropazine had previously been shown to inhibit diaphragm BuChE by 100% and diaphragm AChE by only 20% (S.Das personal communication).

2.12.1. Preparation of tissues for the cholinesterase assay.

2.12.1.1. Blood.

Mice were anaesthetised and 0.1ml of blood was obtained from the femoral artery (2.10). The blood was mixed with 18ml of phosphate buffer, pH 8.0 (A5), plus 2ml of 1M-ethopropazine (ie. to give a final ethopropazine concentration of $1x10^{-4}$M as recommended by Silver (1976) for the complete inhibition of BuChE). The blood solution was kept on ice until the assay was performed.

2.12.1.2. Diaphragm.

Mice were killed following the removal of blood and whilst still anaesthetised. The diaphragm was removed and cut into hemidiaphragms. One hemidiaphragm was prepared for assay of AChE as follows:-

Fat and connective tissue were removed from the hemi-diaphragm which was then trimmed parallel to the axis of the muscle fibres. The muscle was blotted dry, weighed then homogenised in 2ml of ice cold 0.1M phosphate buffer, pH 8.0 (A5) for 1 minute using a TRI-R STIR-R K41 homogeniser set at position 8. Phosphate buffer pH 8.0 (A5) (2ml) was then added to the homogenate in a specimen tube, and kept on ice. The homogenate was sonicated on ice for 30 seconds, using a Soniprep 150

sonicator at medium frequency and position 5, and was then poured into a centrifuge tube. The specimen tube was rinsed out with 1ml of a 50% mixture of phosphate buffer, pH 8.0 (A5), and 1.0M-ethopropazine, which was then added to the centrifuge tube. The 0.5ml of ethopropazine gives a final concentration of $1x10^{-4}$M ethopropazine (ie that recommended by Silver (1974) for the complete inhibition of BuChE). The sonicate (now of total volume 5ml) was spun at 3000rpm for 15 minutes at 4°C using a Beckman J2-21 centrifuge in order to remove the connective tissue fragments from the solution. The resulting supernatant was kept on ice until the assay was performed.

2.12.2. The cholinesterase assay:- The procedure.

Using the method of Ellman et al (1961) two blank reactions must be subtracted from the test reaction. The first blank, the substrate blank, corrects for the spontaneous hydrolysis of the substrate in the absence of the enzyme: The blank value for each new substrate solution must be determined before it is used. The second blank, the tissue blank corrects for any reaction of the thiol groups in the tissue with DTNB, in the absence of the substrate: This must be determined for every tissue sample assayed and it is run alongside the sample assay.

In the present study, a Pye Unicam spectrophotometer, with attatched chart recorder was used. It was set up to assay in duplicate, at a fixed wavelength of 412nm and at 30°C. In this

way readings of absorbance were recorded for each sample against its blank, at one minute intervals, and the rate of the absorbance change (ΔA) with time was followed.

2.12.2.1. The substrate blank.

Four 10ml test tubes were placed in a waterbath at 30°C. To each tube 1ml of phosphate buffer, pH 8.0 (A5) was added plus 1ml DTNB (A5). 1ml of substrate (acetylthiocholine: A5) was then added to two of the tubes (the test samples) and 1ml of distilled water was added to the two remaining (reference) tubes. The contents of each tube were mixed rapidly and poured into separate transparent, plastic cuvettes. The two test cuvettes and the two reference cuvettes were placed in the spectrophotometer sample and reference beams respectively. The change in absorbtion of the substrate blanks was monitored for 10 minutes at 412nm.

2.12.2.2. Test sample and tissue blank.

Each sample was assayed at 30°C, in duplicate, with corresponding duplicate tissue blanks. Each of 4 test tubes contained a sample volume of 1ml, plus 1ml DTNB solution (A5). Into two of the tubes (the tissue blanks) 1ml of distilled water was added, and into each of the two remaining tubes (the test samples) 1ml of acetylthiocholine (A5) was added. The contents of each tube were mixed for a few seconds and poured into separate

cuvettes. Each test sample was read against its corresponding tissue blank for approximately 10 minutes, or until a suitable number of absorbance readings had been recorded.

2.12.3. Calculations.

2.12.3.1. Substrate blank.

The rate of increase in absorbtion was obtained from the slope of the record produced. First and last readings were ommitted from the measurements, minimising distortion of the results by equilibration of the reaction temperature after substrate addition, or by slowing of the reaction due to rapid hydrolysis of the substrate.

In the present study the average change in absorbance for the substrate blank was approximately 0.001 absorbance units per minute.

2.12.3.2. Test sample and tissue blank.

For each sample, the rate of increase in absorbance was obtained from the slope of the record produced. Again the first and last readings were ignored.

2.12.3.3. Conversion of absorbance units to enzyme units.

The calculation was performed using the following expression:-

$$\text{Total N}^{o}\ \mu\text{m ASCh hydrolysed} = \frac{\Delta A/\text{min}}{\mu\text{mol}} \times 1 \times \frac{V}{v}$$

ASCh = Acetyl thiocholine (substrate).

$\Delta A/\text{min}$ = change in absorbance per minute.

μmol = μmolar extinction coefficient of DTNB at 412nm ($13.6 \mu\text{mol ml}^{-1}$).

l = path length of cell, 1cm.

V = volume of cuvette.

v = sample volume.

2.12.3.4. Calculation for whole blood determination.

$$\text{ASCh hydrolysed} = \frac{\Delta A/\text{min}}{13.6} \times 1 \times 3 \times 200\ \mu\text{mol min}^{-1}\text{ml}^{-1}\ \text{blood.}$$

3 = volume of cuvette, V.

$$200 = \frac{1}{\text{volume of blood used in assay (5ul or 0.005ml)}}$$

The equation may be abreviated to:-

$$\Delta A/min \times 44.18\ \mu molmin^{-1}ml^{-1}\ blood.$$

2.12.3.5. Calculation for the hemidiaphragm determination.

$$\text{ASCh hydrolysed} = \frac{\Delta A/min}{13.6} \times 1 \times \frac{3 \times 1000}{\text{conc. of homogenate}} \quad \mu molmin^{-1}mg^{-1}$$

3 = volume of cuvette.

$$\text{conc. of homogenate} = \frac{\text{wet weight of tissue}}{5} \quad mgml^{-1}$$

1000 = conversion of µl to ml.

The equation may be abbreviated to:-

$$\Delta A/min \times \frac{220.59}{\text{concentration of homogenate}} \quad \mu molmin^{-1}mg^{-1}$$

CHAPTER 3

THE PROGRESS OF THE DEVELOPMENT OF MYOPATHY FOLLOWING ACUTE SUBCUTANEOUS ADMINISTRATION OF ECQ.

3.1 INTRODUCTION.

It has been reported (Ferry, personal communication) that an acute injection of ECO induces myopathy in skeletal muscle of mice. An experiment was designed to answer the following questions:-

a) Does ECO induce myopathy in the diaphragm of mice, and if so, is it located largely at the junctional region of the muscle, as would have been expected from its anticholinesterase reaction?

b) Can the extent of ECO-induced myopathy be adequately measured using the Procion technique (2.7)?

c) Is the myopathy associated with elevation of iCa^{2+} and serum CK? Can these parameters be used as adequate measures of the extent of myopathy induced in the diaphragm?

d) How does the myopathy progress with time following ECO administration; assessed using the Procion technique, measurement of serum CK activity and of calcium accumulation at the junctional region of the diaphragm?

e) What is the sequence of events associated with the myopathy induced in the diaphragms of mice following an injection of ECO?

3.2 EXPERIMENTAL DESIGN.

The effect of ECO on mice was observed at various times following its administration in vivo (20mins, 30mins, 1hr, 2hr, 3hr, 6hr, 12hr, 24hr, 48hr, 72hr).

Each group contained at least five mice and was injected with ECO plus atropine (2.2). Another group was administered atropine only (2.2) and acted as a control. At the appropriate times mice were anaesthetised and blood was obtained from the femoral artery for analysis of serum CK (2.10). Mice were killed whilst anaesthetised, their diaphragms were removed and cut into left and right hemidiaphragms (2.3). For each mouse, one hemidiaphragm was assayed for calcium accumulation at its junctional region (2.9), whilst the other was stained with Procion and assessed for myopathy using the Procion technique (2.7). Selection of the left and right hemidiaphragms for the two procedures was alternate.

The histology of the stained hemidiaphragms was studied, then photographed in both ultraviolet and tungsten lights using a fluoresence microscope: Procion-stained myopathic regions were easily recognised as bright yellow fluorescing areas.

3.3 RESULTS AND DISCUSSION.

The acute administration of ECO caused severe cholinergic symptoms in mice, most noticeably, ataxia and overall body tremor, the result of spontaneous fasiculations within individual muscles. These peripheral motor effects began within 15 minutes of the intoxication, were maximal for approximately 2 hours and then declined gradually, dissappearing within 6 hours of ECO administration.

3.3.1. Histological examination of hemidiaphragms.

Hemidiaphragms from atropine-treated control mice consisted of muscle fibres aligned in parallel with one another along their longitudinal axes (plate 3.1). The fibres displayed the normal transverse striations representative of the regular sarcomere structure pertaining to skeletal muscle (Huxley & Hanson, 1960), with endplates being recognised as patches of brown stain, usually approximately half way along the length of the fibres (plate 3.1). When viewed with ultraviolet light some fibres possessed a green autofluorescence (plate 3.1), which, as yet, remains unexplained. None of the fibres fluoresced yellow (plate 3.1), implying lack of Procion penetration, although Procion did stain the central tendon of the diaphragm plus the connective tissue between and enveloping the muscle fibres.

Following administration of ECO, the earliest sign of abnormality was the appearance of a swollen endplate region in

Plate 3.1. Control hemidiaphragm preparation (x125): viewed with ultraviolet light. The muscle fibres run parallel to each other and may fluoresce green. Endplates are represented by patches of brown stain. Procion has stained connective tissue but has not penetrated the fibres themselves.

Plate 3.2. Hemidiaphragm preparation 20 minutes after ECO administration in vivo (x125): viewed with tungsten light. Procion has stained the connective tissue but has not penetrated the muscle fibres themselves.

Plate 3.3. Hemidiaphragm preparation 20 minutes after ECO administration *in vivo* (x125): viewed with incident ultraviolet light. Procion has stained the connective tissue but has not penetrated the muscle fibres themselves.

some muscle fibres within 20 minutes (plate 3.2) although most fibres remained unaffected. Only the connective tissue components of the muscles stained with Procion, the fibres remaining impenetrable, and often fluorescing green (plate 3.3).

As the duration of exposure to ECO increased, hypercontracted regions appeared in muscle fibres, at and close to their endplate regions (plate 3.4). The hypercontractions, usually stained with Procion, appeared as fairly symmetrical bulging of the fibre often encroaching onto neighbouring fibres (plate 3.4). Within hypercontracted regions the spacing between cross-striations was reduced.

Following longer exposure to ECO the frequency and severity of the hypercontractions increased, sometimes detatching completely from the rest of the muscle fibre (as contraction clumps) (plate 3.6). Often hypercontractions occured in chains along the length of an individual fibre (plate 3.7) and, within an individual contraction clump cross-striations sometimes were not recognisable due to the intensity of the contraction. Hypercontractions were usually stained with Procion but a small number, particularly at the earlier times after ECO administration were not (plate 3.5). The spread of hypercontractions and contraction clumps, even in the absence of Procion-staining, is one indicator of the spread of ECO-induced myopathy.

Procion staining of muscle fibres was first noticed in hemidiaphragms of mice 30 minutes after ECO administration and was restricted to the small number of hypercontractions present at

Plate 3.4. Hemidiaphragm preparation 30 minutes after ECO administration *in vivo* (x125): viewed with incident ultraviolet light. Notice the appearance of hypercontractions within some muscle fibres. Most of the hypercontractions are stained with Procion and fluoresce yellow (Y).

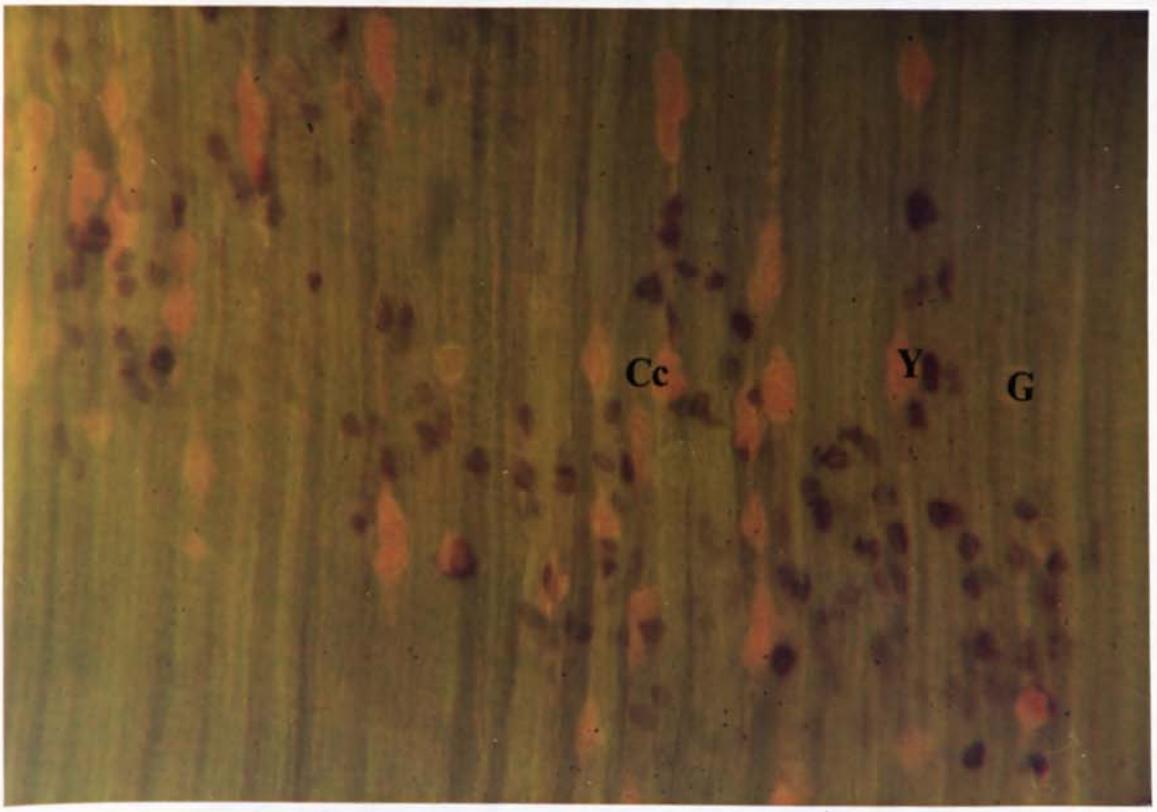


Plate 3.5. Hemidiaphragm preparation 1 hour after ECO administration in vivo (x125): viewed with incident ultraviolet light. Most hypercontractions fluoresce yellow (Y), but some are unstained by Procion and fluoresce green (G). Some contractions are so intense that they have broken away from the rest of the fibre, forming contraction clumps (Cc's) Hypercontractions and Procion staining are more extensive than at 30 minutes after ECO (plate 3.4).

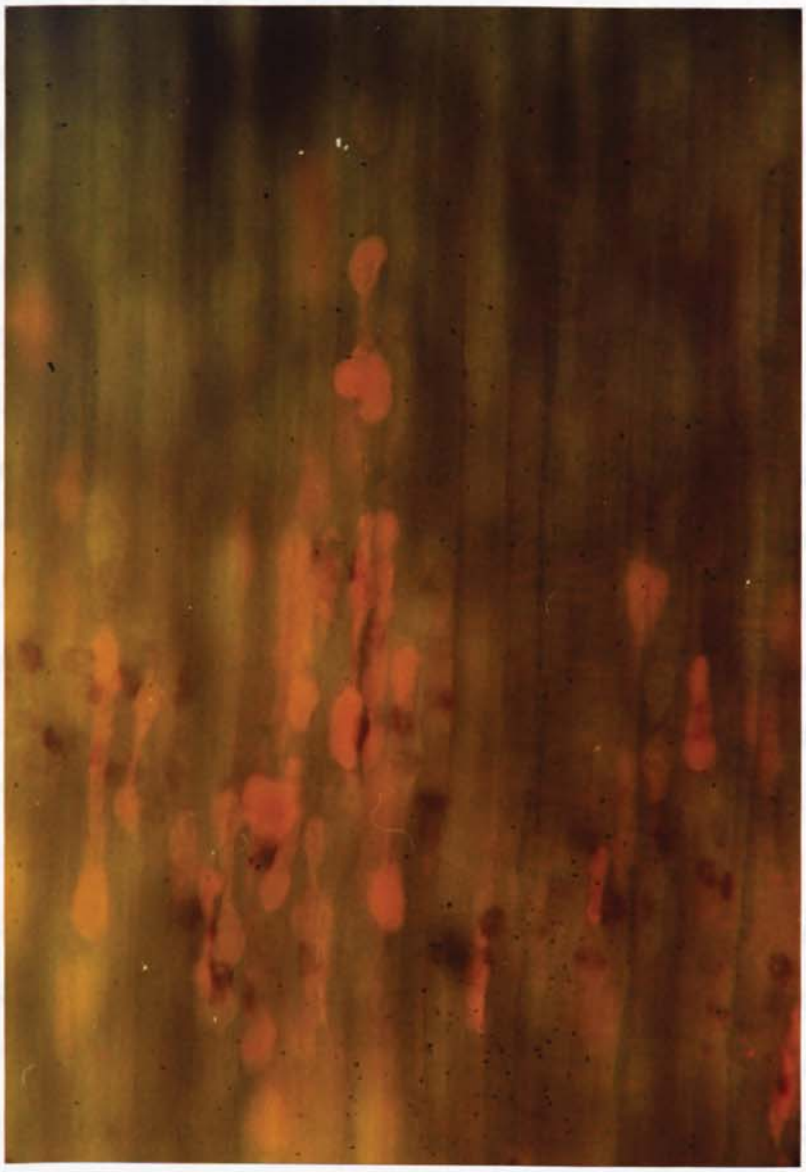


Plate 3.6. Hemidiaphragm preparation 3 hours after ECO administration in vivo (x125): viewed with incident ultraviolet light. Hypercontractions and Procion staining are more extensive than at 1 hour after ECO (plate 3.5).

this time (plate 3.4). At longer times after ECO administration Procion penetrated the muscle fibres between hypercontractions, and the overall degree of Procion staining increased. Some fibres lost their cross-striations and appeared granular.

The extent of Procion staining and the frequency of hypercontractions both appeared to be maximal approximately 6-12 hours after ECO administration, at which time, several endplate regions had lost their striations, appearing granular : plates 3.3, 3.4 3.5, 3.6, 3.7 and 3.8 represent hemidiaphragm preparations at times 20 minutes, 30 minutes, 1, 3, 6, and 12 hours respectively, after ECO. At no time were the extrajunctional regions of the diaphragm severely affected by ECO, and although the number of fibres exhibiting damage increased with time, there was always a large proportion of unaffected fibres.

Following the occurence of maximal myopathy at 6-12 hour after ECO, a decrease in both Procion staining and the frequency of hypercontractions was observed. Plate 3.9 represents a Procion-stained hemidiaphragm 24 hours after ECO administration. Minimal amounts of Procion staining and hypercontraction were regained within 48 hours of the ECO injection (plate 3.10) despite the persistant loss of striations at the endplate regions of the fibres concerned. Apparent morphological normality was not acheived until 72 hours after the intoxication (plate 3.11).

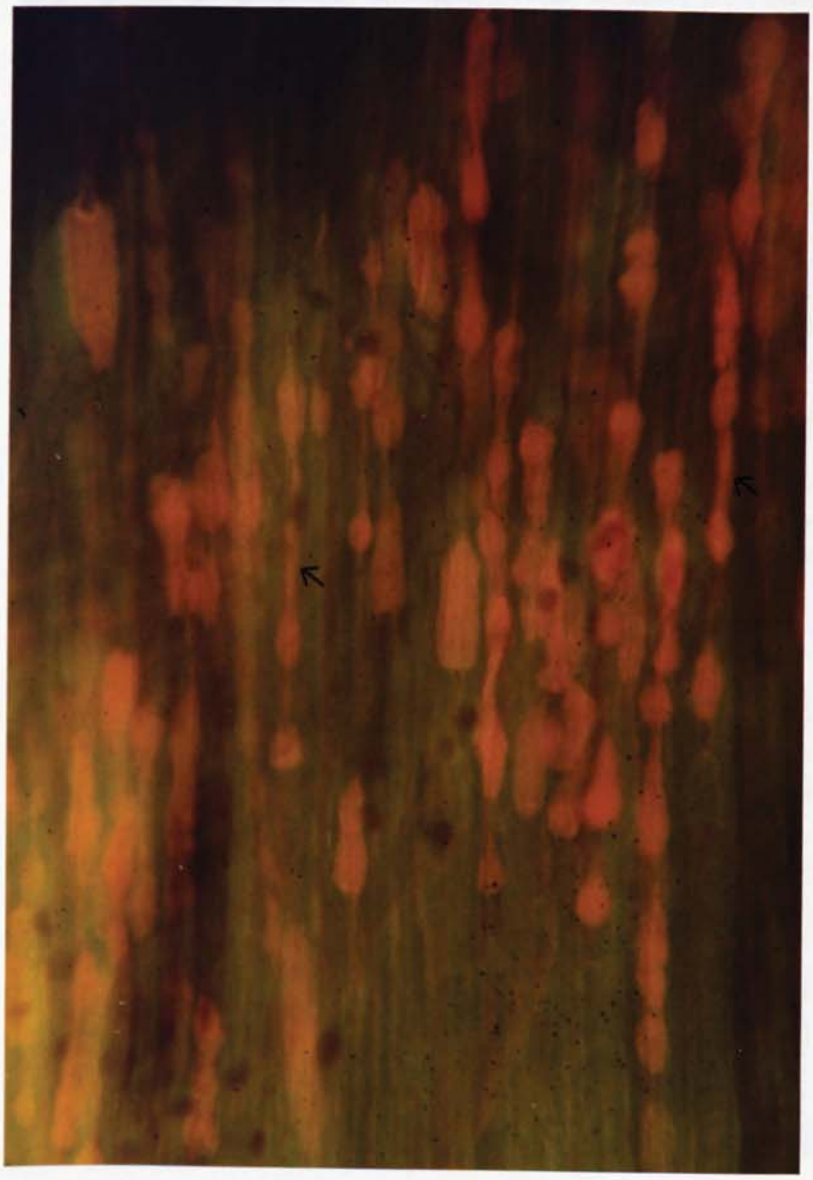


Plate 3.7. Hemidiaphragm preparation 6 hours after ECO administration in vivo (x125): viewed with incident ultraviolet light. Hypercontractions and Procion staining are more extensive than at 3 hours after ECO (plate 3.6), the contractions often occurring in chains within the length of individual muscle fibres (↑).

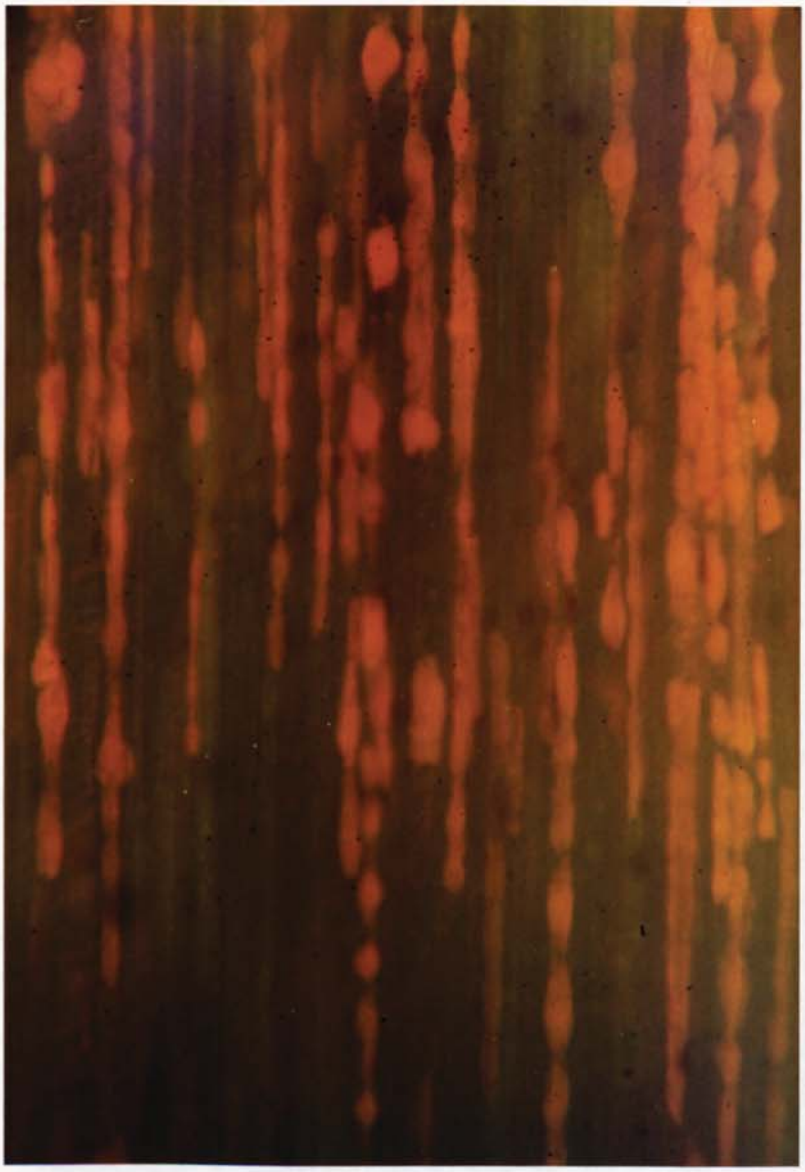


Plate 3.8. Hemidiaphragm preparation 12 hours after ECO administration in vivo (x125): viewed with incident ultraviolet light. Procion staining and hypercontractions are more extensive than at 6 hours after ECO administration (plate 3.7). Hypercontractions tend to exist in chains within individual muscle fibres.

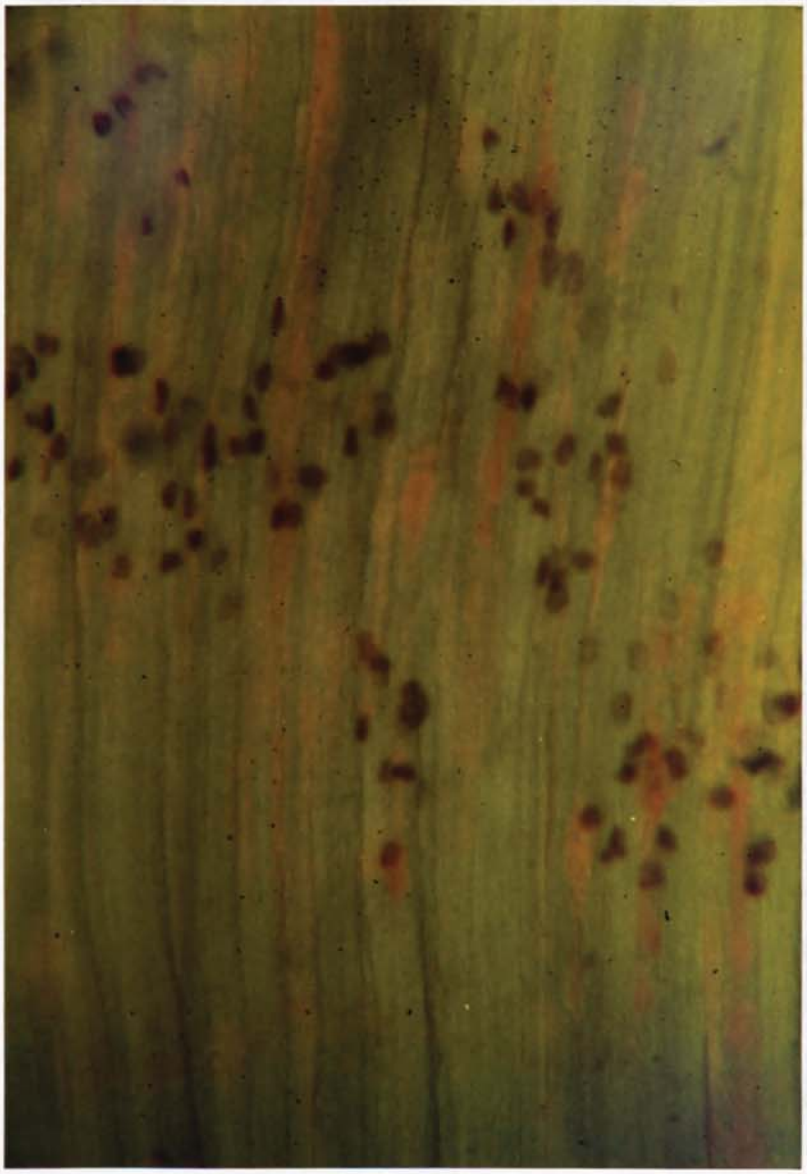


Plate 3.9. Hemidiaphragm preparation 24 hours after ECO administration *in vivo* (x125): viewed with incident ultraviolet light. The extent of Procion staining and the frequency of hypercontractions have decreased with respect to that observed in preparations 12 hours after ECO administration.

Plate 3.10. Hemidiaphragm preparation 48 hours after ECO administration in vivo (x125): viewed with incident ultraviolet light. Procion staining is minimal and hypercontractions are infrequent. Procion staining is largely of the connective tissue within the preparation.

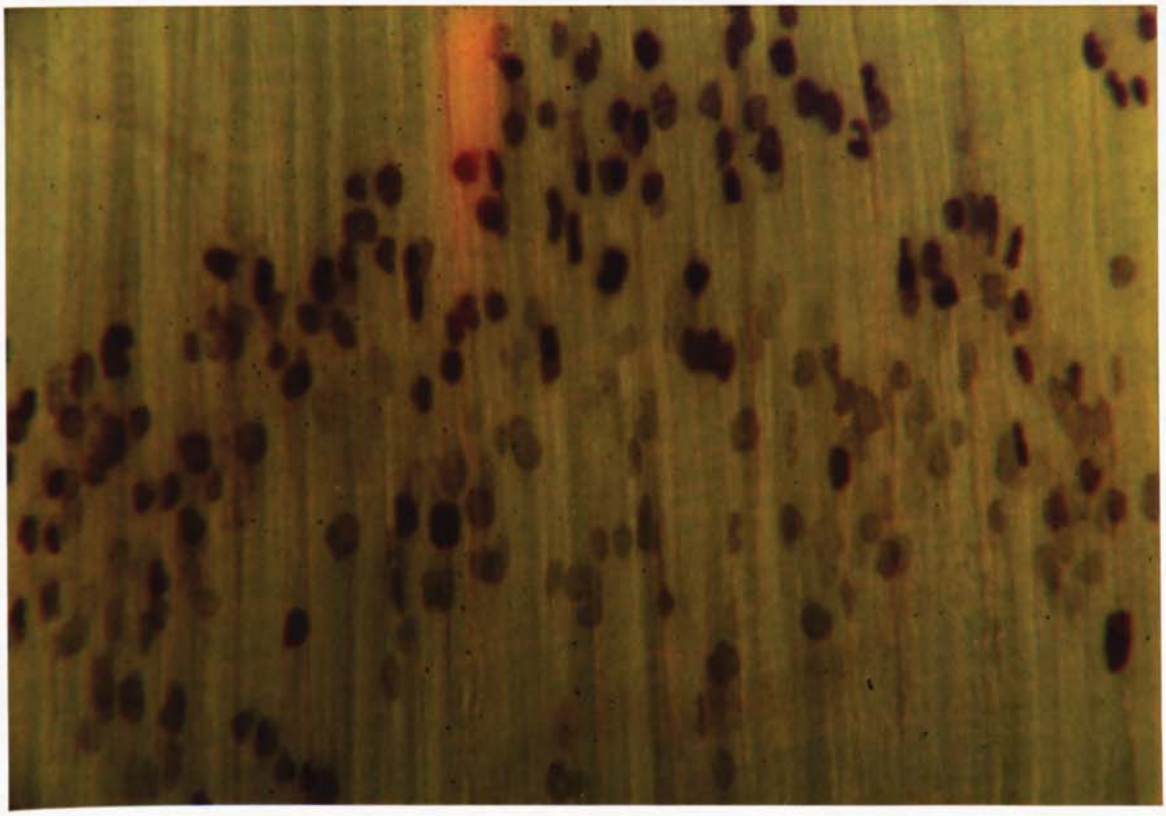


Plate 3.11. Hemidiaphragm preparation 72 hours after ECO administration in vivo (x125): viewed with incident ultraviolet light. There is no Procion staining and no hypercontractions within muscle fibres.

3.3.2. The % Procion staining using the Procion technique.

Table 3.1 shows that Procion staining at the junctional region of the diaphragm did not increase significantly until 30 minutes after ECO administration ($P<0.01$), implying that the sarcolemmal membranes of the muscle fibres remained intact until this time. However % Procion staining at the nonjunctional region did not increase significantly until 6 hours after ECO ($P<0.001$: table 3.1), and was never extensive throughout the development of myopathy (figure 3.1). This suggests either that the sarcolemma in the nonjunctional region did not become permeable to Procion until 6 hours after ECO administration, or that, within this 6 hour period, the Procion that had entered at the junctional region had diffused from its site of entry into the nonjunctional region of the diaphragm. The former of these suggestions is thought to be more likely since Procion stains by binding to intracellular proteins (Bradley & Fulthorpe, 1978) and is therefore unlikely to diffuse far from its point of entry. Whichever hypothesis is correct however, the % Procion results of the present study confirm the histological observations that ECO-induced myopathy was initiated at the junctional region of the diaphragm.

Once Procion staining had begun at the junctional region of the diaphragm it increased quite rapidly with increasing time after ECO administration (figure 3.1). This progressive increase in % Procion staining was shown to be highly significant using the analysis of variance ($P<0.001$). The more gradual increase in % Procion at the nonjunctional region (figure 3.1) was also

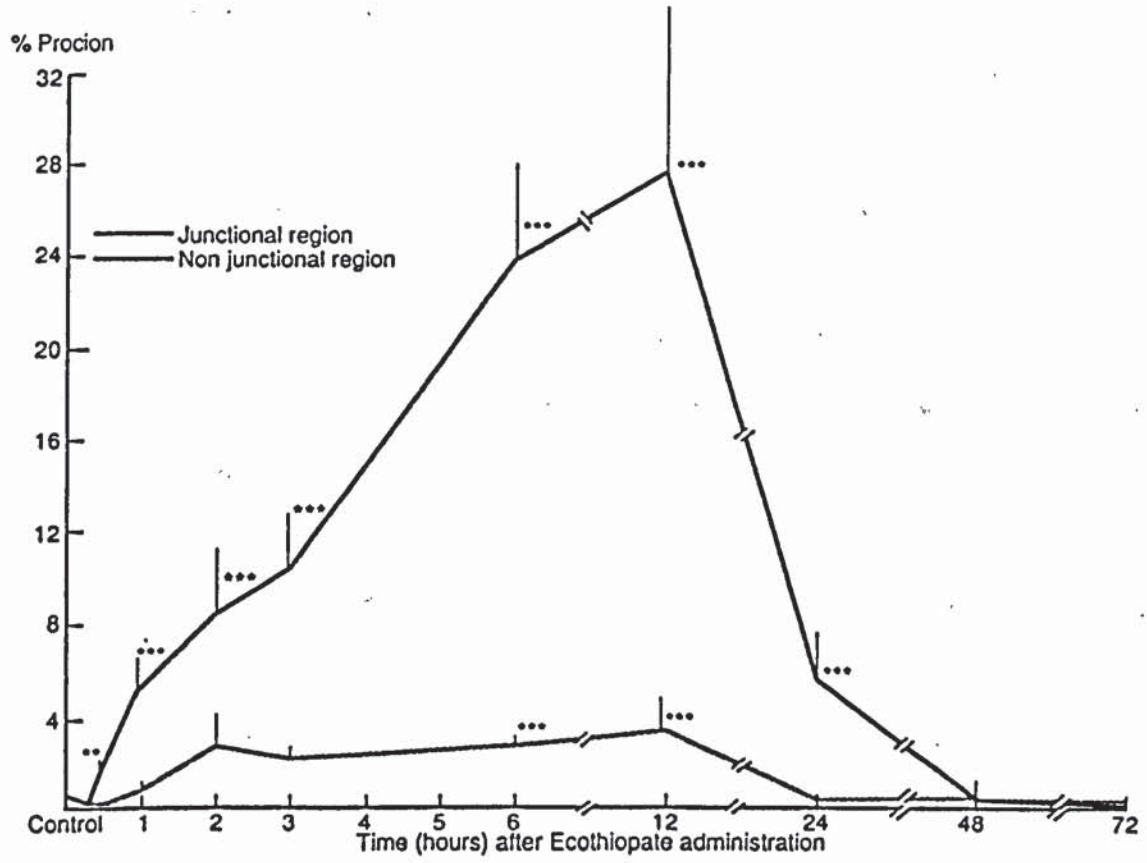
Table 3.1.- % Procion staining of the diaphragm (junctional and nonjunctional regions) at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 values.

	% Procion staining	
	junctional	nonjunctional
Control	0.29 ± 0.21	0.67 ± 0.42
20 mins.	0.04 ± 0.04	0.03 ± 0.02
30 mins.	** 1.63 ± 0.62	0.08 ± 0.03
1 hr.	*** 5.37 ± 1.69	0.79 ± 0.47
2 hr.	*** 8.58 ± 2.50	2.85 ± 1.30
3 hr.	*** 10.10 ± 3.22	2.08 ± 1.01
6 hr.	*** 23.70 ± 4.33	2.70 ± 0.51 ***
12 hr.	*** 27.80 ± 7.05	3.43 ± 1.38 ***
24 hr.	*** 5.67 ± 2.10	0.36 ± 0.20
48 hr.	0.64 ± 0.50	0.44 ± 0.04
72 hr.	0.05 ± 0.05	0.44 ± 0.04

*** and ** indicate results that are significantly different from those of the control group at the 0.1% and 1% levels respectively, using Students-unpaired-"t"-test.

Further statistical analysis of the data was performed using the analysis of variance.

Figure 3.1. % Procion staining at the junctional and nonjunctional regions of the diaphragm at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 experiments.



***, ** indicate results which are significantly different from the control group, at the 0.1% and 1% levels respectively.

significant ($P<0.05$) using analysis of variance. Finally, following the maximum % Procion staining at approximately 6-12 hours after ECO administration (for both the junctional and the nonjunctional regions: table 3.1) a rapid decrease was observed, regaining control levels within 48 hours (figure 3.1). Possibly, regenerative/repair processes had started during the plateau phase of myopathy, apparent normal muscle morphometry, at the light microscope level, being regained within 72 hours (plate 3.11).

3.3.3. The Procion technique: Is it an accurate measure of myopathy?

Procion is a water soluble fluorescent dye known to penetrate and stain necrotic muscle fibres which might exist in an otherwise healthy tissue. It is a small molecule (MWt 500 daltons: Bennet et al., 1969) which will not penetrate intact, live myocytes (Bradley and Fulthorpe, 1978). Procion stains by binding to intracellular proteins and may be used on living or fixed tissues, *in vivo* or *in vitro* (Bradley and Fulthorpe, 1978): It has frequently been used in the vital tracing of neurons (Stretton and Kravitz, 1968; Payton, 1969) after intracellular injection.

The Procion technique (2.7) was used to quantify ECO-induced myopathy, which progressed with time reaching a maximum at approximately 6-12 hours following intoxication (figure 3.1). However, the fact that % Procion staining had decreased substantially by 24 hours, when structural abnormality (ie. loss

of cross-striations) was still prevalent in the diaphragm, does cast some doubt on the validity of the technique.

It is possible that by 24 hours, some fibres had reached such a gross stage of damage that very little protein structure remained onto which the Procion could bind. Hence the stain would have entered the fibres but would have subsequently been washed out. Certainly the ultrastructural analysis performed, at a later stage in the present study (6.3.1), did confirm complete loss of sarcomeric structure in several fibres at the later stages of myopathy development. Alternatively, it has been suggested that most cell membranes have turnover rates of 24-48 hours (Finean et al., 1978; Siekevitz, 1972): if sarcolemmal repair occurred in the ECO-treated diaphragm within 24-48 hours, Procion may thereafter have been denied entry into necrotic fibres. Again, ultrastructural evidence, in the present study, confirms fibre regeneration within 48 hours of ECO administration (6.3.2).

Hence, at the later stages of myopathy, the Procion technique is a useful measure only of the permeability of the sarcolemmal membranes of muscle fibres comprising the diaphragm. At the earlier stages of myopathy (0-6 hours), it is presumed that the Procion technique provides a better estimate of the degree of myopathy.

Another drawback of the Procion technique is its tendency to underestimate extensive myopathy. Underestimation may result from the overlap of two or more damaged, Procion-stained fibres such that only one would be recorded by the technique. This probably occurs less in slightly damaged muscles, but more frequently in

extensively myopathic tissues. Therefore, the more extensive the myopathy, the greater the likelihood that it will be underestimated by the Procion technique. However, the problem of underestimation is not considered to be detrimental to the present study, and if corrected for, would infact enhance the significant differences in the myopathy index between the various times after ECO. It is not considered likely that Procion itself induces myopathy in the diaphragm, during the 1 hour incubation period, since the muscle fibres of control hemidiaphragm preparations, untreated with ECO, were unstained by Procion and were apparently undamaged.

3.3.4. Serum creatine kinase (CK) activity

Table 3.2 shows that, accompanying the development of myopathy in the diaphragm, after ECO administration, there was a progressive, but slightly delayed elevation of serum CK activity which reached a maximum approximately 6 hours after the intoxication. The progression was shown to be highly significant ($P<0.001$) by the analysis of variance although serum CK activity was not significantly greater than that in control mice until 1 hour after ECO administration ($P<0.001$:table 3.2) ie. later than the first significant uptake of Procion at the junctional region of the diaphragm at 30 minutes (table 3.2). The observed delay in elevation of serum CK activity with respect to Procion staining of the diaphragm may be due to masking of a small increase in serum CK activity, at the earlier times after ECO administration, by

Table 3.2. Serum CK (UL^{-1}) and % Procion staining of the diaphragm at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 values.

	% Procion staining		Serum CK
	junctional	nonjunctional	(UL^{-1})
Control	0.29±0.21	0.67±0.42	137±34
20min	0.04±0.04	0.03±0.02	77±20
30min	**1.63±0.62	0.08±0.03	248±63
1hr	***5.37±1.69	0.79±0.47	495±54***
2hr	***8.58±2.50	2.85±1.30	1640±580 ***
3hr	***10.10±3.20	2.08±0.76	2100±534 ***
6hr	***23.70±4.30	2.70±0.50***	4640±831***
12hr	***27.70±7.10	3.43±1.40***	971±490
24hr	***5.67±2.10	0.36±0.20	238±59
48hr	0.64±0.50	0.44±0.19	204±98
72hr	0.05±0.05	0.44±0.37	77±10

*** and ** indicate results that are significantly different from those of the control group at the 0.1% and 1% levels respectively using Students-unpaired-"t"-test.

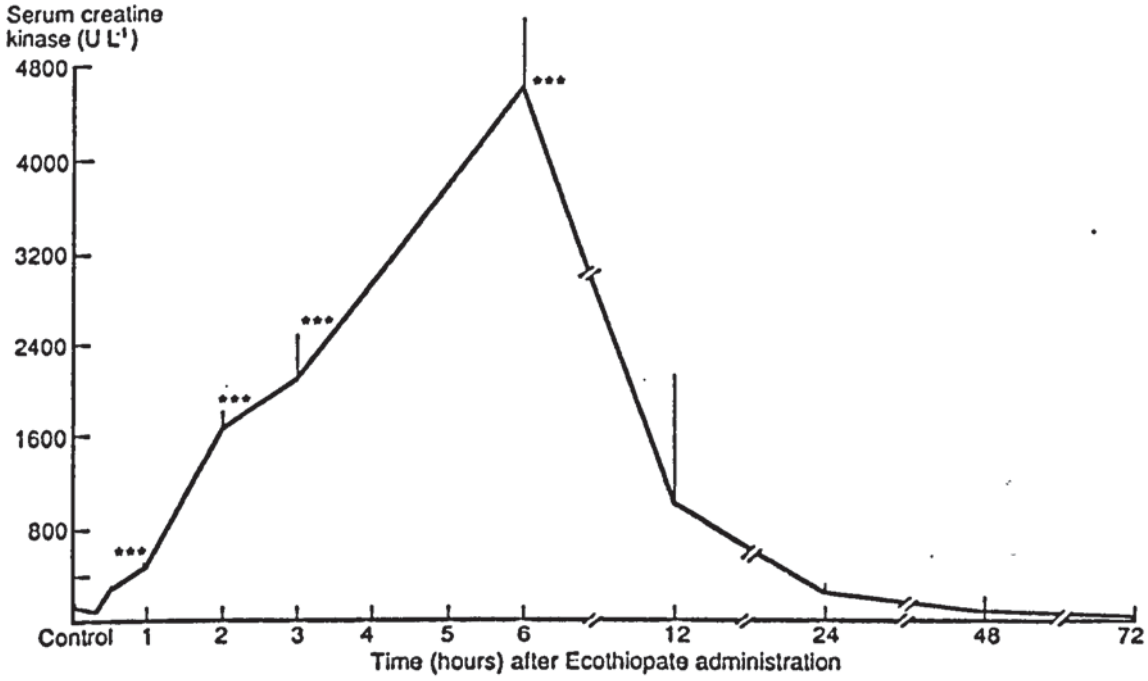
Further statistical analysis of the data was performed using the analysis of variance.

slight effluxes of CK from muscle incurred during the dissection and severing of the femoral artery. Alternatively it may be a consequence of the lower molecular weight of the Procion molecule (Procion= 500da: Bennet et al., 1969. CK=80,000da: Dawson et al., 1967) such that Procion entry into muscle fibres occurred prior to CK efflux. Another explanation for the delay in serum CK elevation may be that, in ECO-poisoned muscles, breakdown of the sarcolemma may occur prior to sarcomere destruction

The maximum level of serum CK activity was recorded 6 hours after ECO administration (figure 3.2) ie. during the plateau phase of myopathy development (figure 3.4). Following this peak, serum CK declined rapidly, returing to control values within 24 hours of the intoxication (table 3.2). Presumably therefore, after 6 hours very little CK was released from the muscles, and its clearance from the blood promoted an overall drop in its activity in the serum.

Serum CK activity seems to reflect the extent of Procion staining in the diaphragm throughout the development and repair of myopathy. However, since the muscle fibres of the diaphragm were histologically abnormal at 24 hours and 48 hours after ECO administration (3.3.1), serum CK activity may not accurately reflect the degree of myopathy existing in the diaphragm at times greater than 6 hours after ECO administration. Therefore, serum CK activity may be considered as a reasonable measure of the rate of development of ECO-induced myopathy only.

Figure 3.2. Serum creatine kinase (CK) activity in mice at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 values.



*** indicates that a result is significantly different from that of the control group, at the 0.1% level, using Students-unpaired-'t'-test.

Since CK is specific to the brain, skeletal and cardiac muscle (Hess, 1969), and knowing that ECO cannot penetrate the blood-brain barrier (Koelle and Steiner, 1956; Schauman and Job, 1958), the enzyme is presumed to have been released from skeletal muscle, cardiac muscle, or from both. ECO must have injured myocytes, including those of the diaphragm, causing release of CK into the blood stream. Serum CK activity is therefore indicative of the extent of myopathy induced in the whole animal. For confirmation of enzyme release from a particular muscle (eg.the diaphragm) it would be necessary to assay CK activity of the muscle itself (chapter 4).

3.3.5. Calcium accumulation at the junctional region of the diaphragm.

Table 3.3 indicates that calcium accumulation accompanied increased Procion staining at the myopathic junctional region of diaphragms exposed to ECO. The calcium accumulation at the junctional region was significant as early as 20 minutes after ECO administration ($P<0.001$: table 3.3) ie. preceding Procion staining at the junctional region. The calcium accumulation is thought to be responsible for the hypercontractions observed throughout the development of myopathy (Podolski and Costantin, 1964; Huxley, 1972), hinting that much of it may be ionised and that the cytosolic Ca^{2+} concentration must be greater than 10^{-7}M. Some of the hypercontractions, particularly at early stages of the

Table 3.3. % Procion staining of the diaphragm, serum CK and calcium accumulation at the junctional region of the diaphragm at various times after ECO administration in vivo: the results represent the mean ± standard error of at least 5 values.

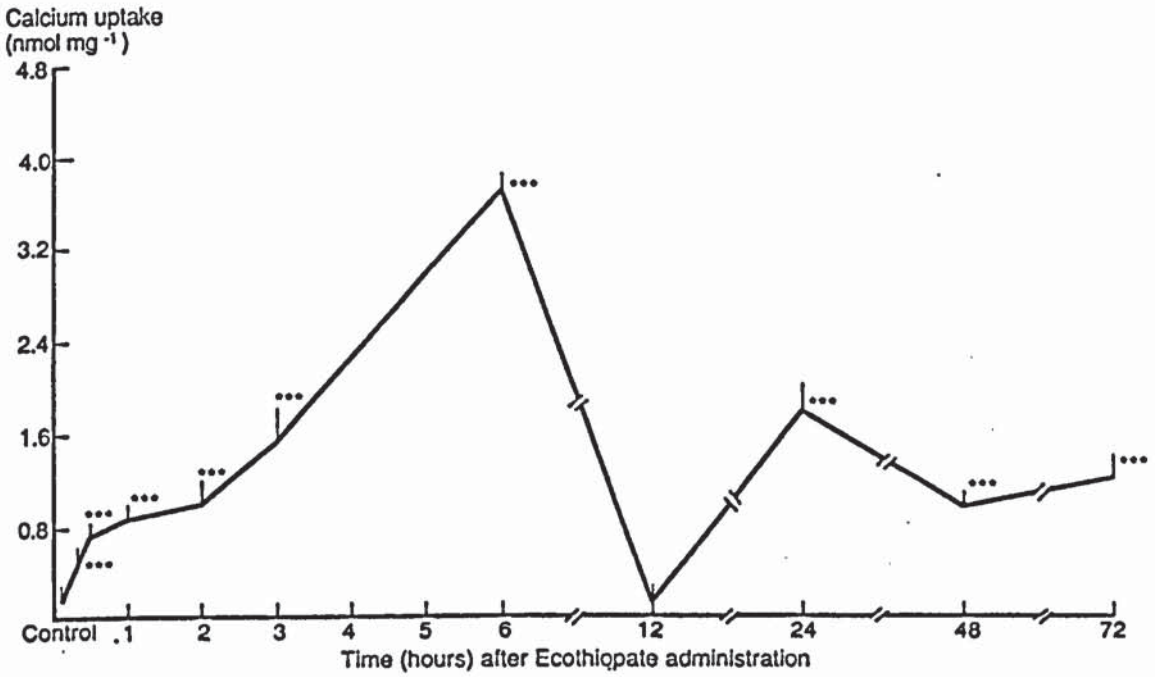
	% Procion staining		Serum CK	Calcium accum.
	J	NJ	(UL^{-1})	at J ($nmol mg^{-1}$)
Control	0.29±0.21	0.67±0.42	137±34	0.128±0.17
20min	0.04±0.04	0.03±0.02	77±20	0.536±0.13***
30min	** 1.63±0.62	0.08±0.03	248±63	0.762±0.07***
1hr	*** 5.37±1.69	0.79±0.47	495±54***	0.876±0.11***
2hr	*** 8.58±2.50	2.85±1.30	1640±580***	1.07 ±0.15***
3hr	***10.10±3.20	2.08±0.76	2100±534***	1.53 ±0.30***
6hr	***23.70±4.33	2.70±0.51***	4640±831***	3.70 ±1.52***
12hr	***27.80±7.05	3.43±1.38***	971±490	0.131±0.13
24hr	*** 5.67±2.10	0.36±0.20	238±59	1.77 ±0.27***
48hr	0.64±0.50	0.44±0.19	204±98	0.952±0.14***
72hr	0.05±0.03	0.44±0.04	77±10	1.20 ±0.26***

J, NJ indicates the junctional and nonjunctional regions respectively.

***, ** indicates results which are significantly different from those of the control group at the 0.1% and 1% levels respectively, using Students-unpaired-"t"-test.

Further statistical analysis of the data was performed using the analysis of variance.

Figure 3.3. Calcium accumulation at the junctional region of the diaphragm at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 values.

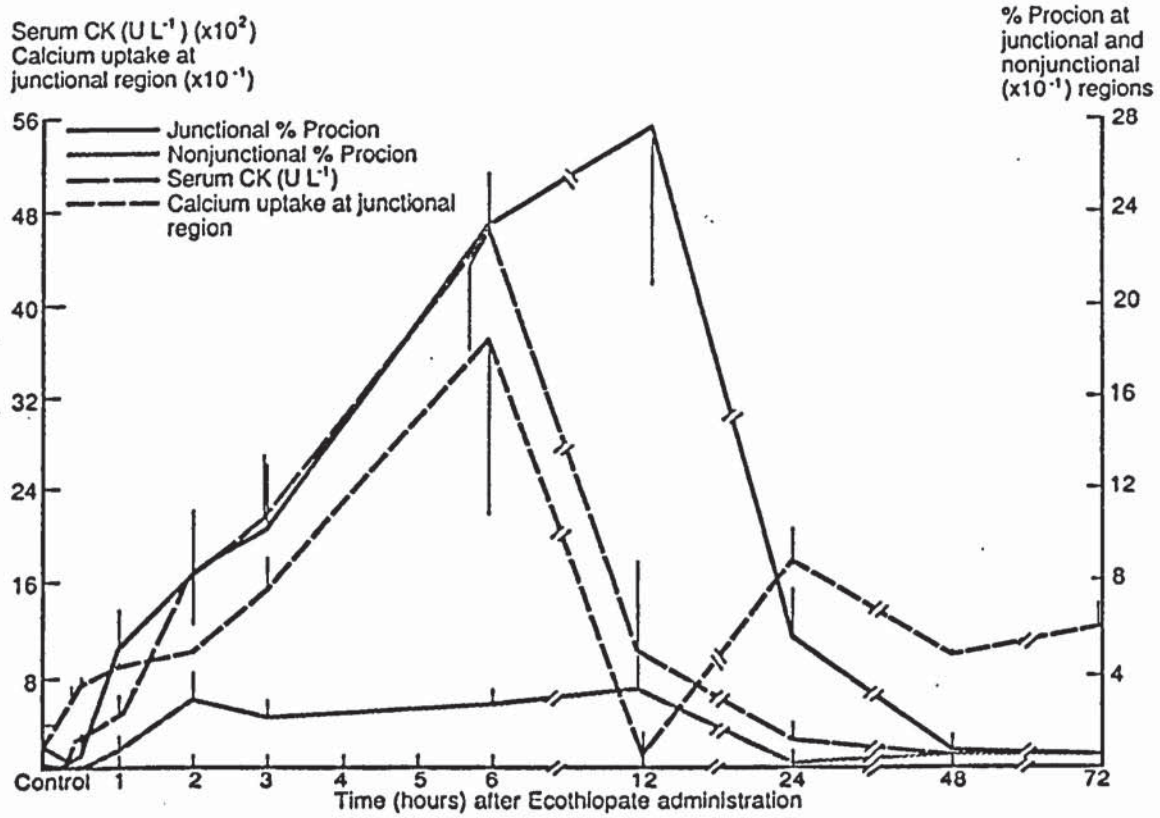


*** indicates that a result is significantly different from the control group, at the 0.1% level, using Students-'t'-test.

myopathy, were not stained with Procion, suggesting that the intracellular free Ca^{2+} was increased in fibres prior to damage to the sarcolemma.

Calcium accumulation continued with increasing time after ECO administration, reaching a maximum value at approximately 3-6 hours (figure 3.3) ie. prior to the maximum % Procion staining at about 6-12 hours (figure 3.4) supporting the suggestion that intracellular calcium may be elevated in fibres prior to damage to the sarcolemma. The gradual increase in calcium accumulation was found to be significant ($P<0.01$) using the analysis of variance. However, it is not yet known whether the high intracellular calcium exists purely in fibres prior to necrosis, in myopathic fibres or in fibres undamaged by ECO. The observation that at 12 hours after ECO administration, when Procion staining was maximal, the junctional intracellular calcium had regained control levels (figure 3.4), suggests that calcium was not retained in the damaged fibres. Perhaps the elevated calcium exists in fibres at an early stage of myopathy but is lost once damage reaches a certain degree of severity. This suggestion seems more feasible in the light of ultrastructural results (6.3.3) demonstrating gross destruction of sarcomeric structure at later stages of myopathy development; if intracellular calcium is normally bound to intracellular proteins, then the destruction of such proteins may cause the release of free calcium into the sarcoplasm. Calcium may then be lost from the myopathic fibres via holes in the sarcolemmal membrane. The location of the accumulated calcium

Figure 3.4. % Procion staining of the diaphragm (at the junctional and nonjunctional regions), serum CK and calcium accumulation at the junctional region of the diaphragm at various times after ECO administration in vivo:- the results represent the mean ± standard error of at least 5 experiments.



is discussed at a later stage (5.3) in this study.

Since intracellular calcium was lost within 12 hours of ECO administration, when myopathy was maximal, the calcium content of the diaphragm can only be considered a reasonable measure of myopathy for times upto and including 6 hours following the intoxication.

The reason for the secondary elevation of junctional intracellular calcium in the diaphragm, 24 hours after ECO (figure 3.3) is uncertain. Possibly a second population of fibres underwent a delayed onset myopathy whose maximum calcium accumulation occurred at 24 hours and certainly, an ultrastuctural study (6.3.2) confirmed fibres in an early stage of myopathy 24 hours after ECO administration (table 6.2). Alternatively, it is possible that phagocytes, which have been shown to invade necrotic muscle fibres following acute cholinesterase inhibition (Wecker et al., 1978), may raise the calcium level of the diaphragm at a time of severe necrosis. There is infact ultrastuctural evidence (6.3.2.7) of the presence of phagocytes within grossly necrotic muscle fibres 24 hours after ECO administration in vivo, and in the light of reports made by Westwick & Poll (1986), of elevated intracellular calcium within activated phagocytes, this suggestion seems viable.

3.3.6. The sequence of events associated with the development of myopathy following administration of ECO to mice

It seems that the sequence of events associated with the development of ECO-induced myopathy began with calcium accumulation plus the formation of hypercontractions at the junctional region of the diaphragm, was rapidly followed by slight Procion staining of the myopathic regions, and then by increased serum CK activity (figure 3.4). All four parameters increased gradually with time, until a plateau of % Procion staining and hypercontraction frequency was reached at 6-12 hours after the intoxication. During this plateau calcium accumulation and serum CK activity both decreased rapidly, regaining control values within 12 hours of ECO administration. However, calcium accumulation increased a second time, at 24 hours after ECO, when % Procion staining had begun to decrease. This secondary calcium accumulation remained elevated throughout the period under investigation whilst % Procion staining, serum CK activity and hypercontraction frequency remained at control levels.

CHAPTER 4

DOES ECO CAUSE LOSS OF CREATINE KINASE (CK) FROM THE MYOPATHIC REGIONS OF THE DIAPHRAGM?

4.1 INTRODUCTION

The contraction of muscle is an energy dependent process and ATP must be rapidly replenished following muscle contraction (Squire, 1981). The energy reserve closest to ATP is creatine phosphate (PCr). In the presence of this molecule and the enzyme creatine kinase (CK) the regeneration of ATP occurs.

$$ADP + PCr \xrightarrow{CK} ATP + Cr$$

The CK used in the resynthesis of ATP exists largely in the sarcoplasm of the muscle fibres (Sherwin et al., 1969) but a small percentage has been shown to be a major component of the M-line of the sarcomere (Walliman et al., 1977; Eppenberger & Strehler, 1982; Walliman & Eppenberger, 1983) where it is thought to form the transverse M-bridges linking myosin filaments together (Walliman et al., 1975; Walliman & Eppenberger, 1983; Woodhead & Lowey, 1983).

It has been established that there is an elevation of serum CK activity in mice exposed to ECO (3.3.4) and it is speculated that this elevation might be due, at least in part, to loss of the enzyme from myopathic tissues including the junctional region of the diaphragm. An experiment was designed to determine whether some of the elevated serum CK could be attributed to loss of the enzyme from the myopathic region of the diaphragm.

4.2 EXPERIMENTAL DESIGN

Eight mice were injected with ECO plus atropine and a control group was injected with atropine only (2.2). Six hours after injection a blood sample was obtained from the tail for analysis of serum CK (2.10) in order to confirm that enzyme release had infact occurred in the treated mice. 24 hours after the injection a second blood sample was taken, this time from the femoral artery, for analysis of serum CK (2.10), in order to confirm that enzyme release was complete in the treated mice. The mice were killed whilst anaesthetised and diaphragms were assayed for junctional and nonjunctional CK using a modified version of the technique recommended by Oliver (1953) (2.11). Values of tissue CK were expressed as Umg^{-1} wet weight of muscle.

Statistical comparisons were made between the junctional and nonjunctional regions of the same diaphragms and between 6 hour and 24 hour serum samples within each group of mice, using the Students paired-"t"-test. Statistical comparisons were made between the two groups using the unpaired-"t"-test.

4.3 RESULTS AND DISCUSSION

Table 4.1 shows that a highly significant ($P<0.001$) depletion of CK occurred in the junctional regions of diaphragms exposed to ECO. There was no such depletion from the non-junctional regions of the same diaphragms nor from either region of the diaphragms of control mice (table 4.1). The CK content of the nonjunctional regions of diaphragms treated with ECO were not significantly different from those of either region in the control diaphragms (table 4.1).

Since the depletion of CK occurred only from the junctional regions of diaphragms exposed to ECO, it is concluded that enzyme depletion occurs from the myopathic regions of the diaphragm only and not from the preserved regions.

The experiment has revealed that the elevation of serum CK following ECO administration can be partially attributed to loss of the enzyme from myopathic regions of the diaphragm. The result corresponds to observations made by Leonard & Salpeter, in 1979, of an efflux of the cytoplasmic marker enzyme lactate dehydrogenase from whole muscles exposed to carbamylcholine *in vitro*.

Table 4.1 The effect of ECO on the CK content of mouse diaphragms:- the results represent the mean ± standard error of at least 8 experiments.

	Atropine only (control) mice	ECO + atropine
Serum CK at 6 hr (UL^{-1})	84.4±16.3	$5690\pm1160^{***}_{+}$
Serum CK at 24 hr (UL^{-1})	72.6±17.3	$379\pm58.3^{***}$
Junctional CK at 24 hr (U_{mg}^{-1})	4.36±0.10	$3.59\pm0.22^{**}_{+++}$
Nonjunctional CK at 24 hr (U_{mg}^{-1})	4.41±0.18	4.39±0.10

***, ** indicate a significant difference exists, at the 0.1% and 1% levels respectively, between the results of the ECO-treated mice and control mice, using Students-unpaired-'t'-test.

+ indicates a significant difference, at the 5% level, between the serum CK activities of a group of mice at 6 hrs and 24 hrs after ECO administration, using Students-paired-'t'-test.

+++ indicates a significant difference, at the 0.1% level, between junctional and nonjunctional CK activity 24 hrs after ECO administration, using Students-paired-'t'-test.

CHAPTER 5

THE SOURCE AND STATE OF THE ECO-INDUCED ELEVATION OF CALCIUM AT THE JUNCTIONAL REGION OF THE DIAPHRAGM

5.1. INTRODUCTION.

Acute subcutaneous administration of ECO induces an early accumulation of calcium at the junctional region of the diaphragm, prior to any evidence of myopathy (3.3). The location of this elevated calcium within the muscle structure is likely to be intracellular since free extracellular calcium was washed from the tissue using Ca-reduced saline at the begining of each assay. However, the source of the accumulated calcium is not clear. There may have been a simple redistribution of the ion from the nonjunctional region to the junctional region within the muscle although this is unlikely since free Ca^{2+} ions are rapidly buffered intracellularly, inhibiting their diffusion within the cell. It is more probable therefore, that there may have been an influx of Ca^{2+} from the extracellular fluid. It is not yet clear however, whether the accumulated intracellular calcium in diaphragms exposed to ECO, is bound or free. If it is free in the myopathic fibres which had allowed Procion entry, one might have expected it to have been washed out of the tissue during the 15 minute wash in Ca-reduced saline.

An experiment was designed to determine the source of the accumulated calcium at the junctional region of the diaphragm and also to determine whether intracellular calcium was infact lost from myopathic fibres during the 15 minute wash in Ca-reduced saline.

5.2 EXPERIMENTAL DESIGN.

The effect of a 15 minute wash in Ca-reduced saline, on the calcium content of the diaphragm, was observed at various times following an injection of ECO plus atropine (20min, 30min, 1hr, 3hr, 6hr, 12hr, 24hr) in order to establish whether the intracellular state of calcium changes with the development of myopathy. For each time investigated 6 mice were used and control mice were administered atropine only (2.2).

The mice were killed and their diaphragms, still attatched to the ribs were removed and divided into hemidiaphragms (2.3). One hemidiaphragm was immediately rinsed quickly in Ca-reduced saline and was then prepared for assay (2.9.1). The second hemidiaphragm was washed for 15 minutes in Ca-reduced saline before being prepared for the assay (2.9.1). The selection of left and right hemidiaphragms as "washed" and "unwashed" preparations was alternated.

The Ca^{2+} contents of the junctional and nonjunctional regions were calculated (2.9.1) at each time for both the "washed" and "unwashed" preparations and statistical comparisons were made using Students-paired-'t'-test.

The calcium accumulation at the junctional region was then calculated at each time (2.9.2) and the results of the washed and unwashed preparations were compared using Students paired-"t"-test.

5.3. RESULTS AND DISCUSSION.

The calcium content of both the junctional and nonjunctional regions increased significantly with time after ECO administration (table 5.1) indicating that there must have been an influx of Ca^{2+} from the extracellular fluid (ie. not simply a redistribution of Ca^{2+} causing its accumulation at the junctional region of the muscle). It is concluded that ECO must cause an influx of Ca^{2+} into muscle fibres of the diaphragm, particularly at the junctional region.

There was no significant difference in either the calcium content or the degree of Ca^{2+} influx between the "washed" and "unwashed" preparations (table 5.2), implying that the elevated intracellular calcium may be bound within the damaged fibres and therefore not lost during the wash in Ca-reduced saline. Since intracellular calcium has been shown to accumulate in the SR and mitochondria of damaged fibres (Obero & Engel, 1975, 1977), it seems likely that these organelles may buffer the Ca^{2+} entering the muscle fibres of the diaphragm following exposure to ECO. Alternatively, since the Ca-reduced saline was shown to contain trace amounts of Ca^{2+} ($5x10^{-6}$M: S. Das, personal communication), it is unlikely that Ca^{2+} would have leaked out of the diaphragm against the prevailing, still strong electrochemical gradient. The experiment would have been more conclusive had EGTA been used in the Ca-reduced saline. However, the experiment did provide

reassurence that iCa^{2+} is not lost from hemidiaphragms during their preparation for the Ca-assay.

Table 5.1: The effect of a 15 minute wash in Ca-reduced saline on the calcium content of hemidiaphragm preparations: the results represent the mean ± standard error of 6 experiments each.

	Calcium content of J and NJ regions ($nmol\,mg^{-1}$)			
	Washed		Unwashed	
	J	NJ	J	NJ
Control	3.39±0.22	0.79±0.07	2.40±0.39	1.00±0.15
20mins	2.61±0.41	1.07±0.15	3.14±0.61	0.88±0.05
30mins	4.25±0.92	0.61±0.10	2.68±0.64	0.81±0.10
1hr	5.53±0.88	1.29±0.21	3.88±1.14	1.31±0.18
3hrs	9.89±1.75	2.67±0.28	10.01±1.41	3.73±0.79
6hrs	4.94±0.19	0.65±0.03	5.81±0.70	1.34±0.42
12hrs	4.18±1.01	0.93±0.09	3.85±1.10	0.88±0.09
24hrs	8.55±0.90	2.86±0.56	10.40±1.08	3.27±0.53

J, NJ indicate the junctional, nonjunctional regions respectively.

No significant difference was found to exist in the Ca^{2+} contents of the "washed" and "unwashed" EGO-treated preparations at either the junctional or the nonjunctional regions.

Table 5.2. The effect of a 15 minute wash in Ca-reduced saline on the influx of calcium at the junctional region of the diaphragm: the results represent the mean ± standard error of 6 experiments each.

	Calcium influx ($nmol\,mg^{-1}$)	
	Washed	Unwashed
Control	0.876±0.064	0.646±0.137
20mins.	0.615±0.110	0.554±0.253
30mins.	0.990±0.129	0.748±0.214
1hr.	1.554±0.390	1.185±0.406
3hr.	2.482±0.228	2.850±0.628
6hr.	1.661±0.217	1.595±0.130
12hr.	1.115±0.378	1.163±0.288
24hr.	2.100±0.355	2.825±0.387

No significant difference was found to exist in the Ca^{2+} influx of the "washed" and "unwashed" preparations.

CHAPTER 6

THE FINESTRUCTURE OF THE DIAPHRAGM AT VARIOUS TIMES FOLLOWING ECO ADMINISTRATION IN VIVO.

6.1. INTRODUCTION

An earlier experiment (3.3.1) revealed loss of cross-striations at the junctional regions of some fibres within diaphragms exposed to ECO for three hours or more, implying that degradation of myofilaments was taking place. A more detailed study was undertaken, at the ultrastructural level, to investigate further details of the ECO-induced myopathy. The general fine-structure of the diaphragm was observed but the study was particularly aimed at:

1) Does ECO cause disruption of the Z-disks and is an involvement of calcium-activated-neutral-proteases (CANP) therefore implicated?

2) Observation of the changes in the appearance of the SR and/or mitochondria, which may be due to sequestration of the elevated iCa^{2+} (Oberc & Engel, 1975, 1977). Both these organelles have frequently been reported to be dilated following exposure to anticholinesterase compounds (Laskowski et al., 1975, 1977; Laskowski & Dettbarn, 1977).

3) Assessment of whether the subcellular changes in the muscle fibres of the diaphragm could be attributed to an action of ECO at the endplate.

6.2. EXPERIMENTAL DESIGN.

The effect of ECO on the ultrastructure of the diaphragm was observed at various times (20mins, 30mins, 1hr, 3hr, 6hr, 12hr, 24hr, 48hr, and 72hr) following administration *in vivo*. Only one mouse was used at each time point due to limitations of time and equipment availability, hence the results have been reported without statistical comparison.

Mice were injected with ECO plus atropine and a control mouse was injected with atropine only (2.2). At each given time a mouse was anaesthetised and a blood sample was obtained from its femoral artery for analysis of serum CK (2.10). The control mouse was anaesthetised 3 hours after an injection of atropine (ie. at a time when myopathy is known to be extensive in ECO-treated mice). Mice were killed whilst anaesthetised and the diaphragm was removed and cut into hemidiaphragms (2.3). One hemidiaphragm was prepared for electron microscopy (2.8) and the other was stained with Procion (2.6).

Serum CK analysis and the Procion-technique were performed simply to confirm that myopathy had been induced by ECO and that the specimens for ultrastructural study were typical of damage at that time. Having confirmed this, the ultrastructural study was performed.

6.2.1. Estimation of the % abnormal fibres in semithin toluidine blue-stained sections of the diaphragm, at various times after ECO administration in vivo.

Before electron microscopic observation of ultrathin sections of the diaphragm was performed, semithin toluidine blue-stained sections were viewed in the light microscope at a magnification of x400 which was sufficient for recognition of grossly damaged muscle fibres (6.3.1).

Using both longitudinal and transverse sections, a count was made of the number of normal and abnormal fibres in each section of the junctional region, at each time after ECO (sections from the nonjunctional region were excluded from the count since myopathy had already been shown to be localised to the junctional region: 3.3.2, 6.3.1). Over 100 fibres were counted per mouse, the results were summed and the % abnormal fibres was calculated (table 6.1).

$$\% \text{ Abnormal fibres} = \frac{N^{o} \text{ abnormal fibres in both T.S and L.S}}{\text{Total } N^{o} \text{ of fibres counted}}$$

The results of such an analysis can only be considered as a preliminary assessment of the % fibres affected by ECO since at such a low magnification the true state of the muscle fibres cannot accurately be assessed: an apparent abnormality in a fibre may be artifactual, or a fibre may be damaged to a degree not detectable by light microscopy.

6.2.2. Estimation of the % abnormal fibres in ultrathin sections of the diaphragm at various times after ECO administration in vivo

Having observed the ultrathin sections of the junctional regions of diaphragms at the various times after ECO administration (6.3.2), a categorisation of fibres with respect to their fine-structural integrity was performed. Six categories of muscle morphology were used:-

1) Normal fibres:- (plates 6.5 and 6.6)

2) Mild damage:- ie. slight dilatation of SR and/or occassional swelling of mitochondria (plates 6.7 and 6.9).

3) Moderate damage:- ie. dilatation of SR with swelling and/or vacuolation of mitochondria; Z-line loss and crystal formation within the mitochondria; Stretching of myofilaments adjacent to hypercontracted regions (plates 6.8 and 6.10).

4) Severe damage:- ie. dilatation of SR so gross that the profiles may be confused with vacuoles. Vacuolation and crystal formation within mitochondria is common. Myofilaments may be stretched or disintegrated and Z-line loss is apparent. Sarcomeres have often broken down completely, and hypercontracted regions (contraction clumps) may have torn away from the rest of the over-stretched muscle fibre (plates 6.13 and 6.15).

5) Gross damage:- ie. complete loss of structure within the sarcoplasm which may therefore appear as an amorphous mass containing no recognisable organelles. Empty sarcolemmal tubes may lie adjacent to contraction clumps, and crystals may occupy large volumes of the cells. Macrophages are common features at such a gross stage of necrosis (plates 6.16 and 6.20).

6) Regeneration:- represented by the presence of satellite cells and myoblasts possibly in a state of fusion, or by fusion of a regenerating area with a 'spared', non-necrotic region of a fibre (plates 6.21 and 6.22).

A count was made of the number of muscle fibres existing in each of groups 1-6. Similarly, a count was made of the total number of fibres in the sections pertaining to each time. The % fibres at each stage of myopathy was calculated for each time observed after ECO:-

$$\% \text{ fibres in stage X at time Y} = \frac{N^{o} \text{ fibres in stage X at time Y} \times 100}{\text{Total } N^{o} \text{ fibres counted at time Y}}$$

The % fibres at each stage of myopathy was thus calculated for each time investigated after ECO (table 6.2). Having classified the muscle fibres of the diaphragm into groups corresponding to the degree of damage induced by ECO (6.3.3), a morphometric study was made of particular subcellular structures

6.2.3. Morphometric analysis of subcellular changes observed at the junctional region of the diaphragm at various times after ECO.

The analysis was performed on electron micrographs (x7500 magnification), of transverse sections cut from the junctional regions of diaphragms, from control mice and from mice exposed to ECO for periods upto and including 12 hours following intoxication. At times greater than 12 hours after ECO administration muscle fibre morphology became complicated by phagocytosis (6.3.2.7) and regeneration (6.3.2.8) such that a morphometric study would have been difficult. Thus photomicrographs of muscle fibres exposed to ECO for 24 to 72 hours after ECO were analysed purely qualitatively. Morphometric analysis was not performed on diaphragms exposed to ECO for 30 minutes because the transverse sections cut from the junctional region showed no signs of myopathy (6.4). Hence the analysis was performed at times 20 minutes, 1 hour, 3 hour, 6 hour and 12 hour after ECO and on control preparations, administered atropine only. At each time, micrographs were made of damaged muscle fibres present in each section, the criterion for damage being the dilatation or complete loss of SR.

In control preparations a random sample of 6 fibres was photographed. At 20 minutes and 1 hour after ECO administration all damaged fibres were photographed, but at later times, as more fibres became involved in the myopathy, a random sample of 6 damaged fibres per section was selected for photography. At each time after ECO administration one or two micrographs were taken of

apparently 'normal' fibres in the sections for comparison with control fibres.

6.2.3.1. Morphometric analysis of electron-micrographs.

Small points were drawn on to an acetate sheet such that each point represented the intersection of a regular two dimensional lattice or grid. The grid measured 20cm by 30cm, and was large enough to cover the whole of one micrograph. The grid was placed over each micrograph in turn, and a record was made for each, of the number of points overlying particular features:-

a) normal myofilaments

b) normal mitochondria

c) sarcoplasmic reticulum (SR)

d) sarcoplasm

e) lipid

f) Z-lines

g) nucleus

h) stretched myofilaments (Stretched myofils)

i) disintegrated myofilaments (Disintegrated myofils)

j) damaged mitochondria

k) vacuoles

l) crystals

m) amorphous mass

n) invaginations in the sarcolemma (Invaginations)

o) cellular inclusions (Inclusions)

The data obtained from all the micrographs made at one time after ECO were combined. For each time, the number of points overlying a particular feature was expressed as a fraction of the total number of points within the cell profile, providing an estimate of the fraction of the total area of the cell occupied by that particular feature (Aherne & Dunhill, 1982). Using the principle of Delesse (1847), the fractional area (Aa) of a feature can be used as an estimate of the volume fraction (Vv) of the same feature in the cell. For example:- if in a particular group of micrographs, taken 60 minutes after ECO, the total number of points overlying damaged mitochondria was 8 and the total number of points counted in the cell profiles was 100 then the fractional volume (Vv) of damaged mitochondria would be 8% of the cell volume.

The volume fractions (Vv) for each feature were calculated for each time after ECO such that the progression of myopathy could be studied at the subcellular level.

6.3. RESULTS

6.3.1. Analysis of semithin toluidine blue-stained sections at various times following ECO administration in vivo.

In sections from the control mouse, the muscle fibres were seen as intact blue profiles which appeared elongated and striated in longitudinal section (plate 6.1), or irregularly shaped in transverse section (plate 6.2). Following administration of ECO, large vacuoles appeared in some muscle fibres (plate 6.3) at the junctional regions only. Longitudinal sections at the endplate demonstrated contraction clumps, stretching, and loss of striations within the affected fibres (plate 6.4). However, at no time were such abnormalities seen in sections cut from the nonjunctional regions.

A count was made of the % abnormal fibres at each time after ECO administration (6.2.1).

Table 6.1 shows that abnormal fibres appeared at the junctional region of diaphragms within 20 minutes of ECO administration *in vivo*. The % abnormal fibres generally increased with time after ECO administration, reaching a plateau between 3 hours and 24 hours and decreasing thereafter (table 6.1). This reflects the progressive myopathy observed in an earlier experiment (3.3.2) in which muscles were stained vitally with Procion Yellow. However, in this earlier experiment recovery of muscle morphology appeared to be complete within 72 hours after ECO. This clearly was not the case in the present experiment,

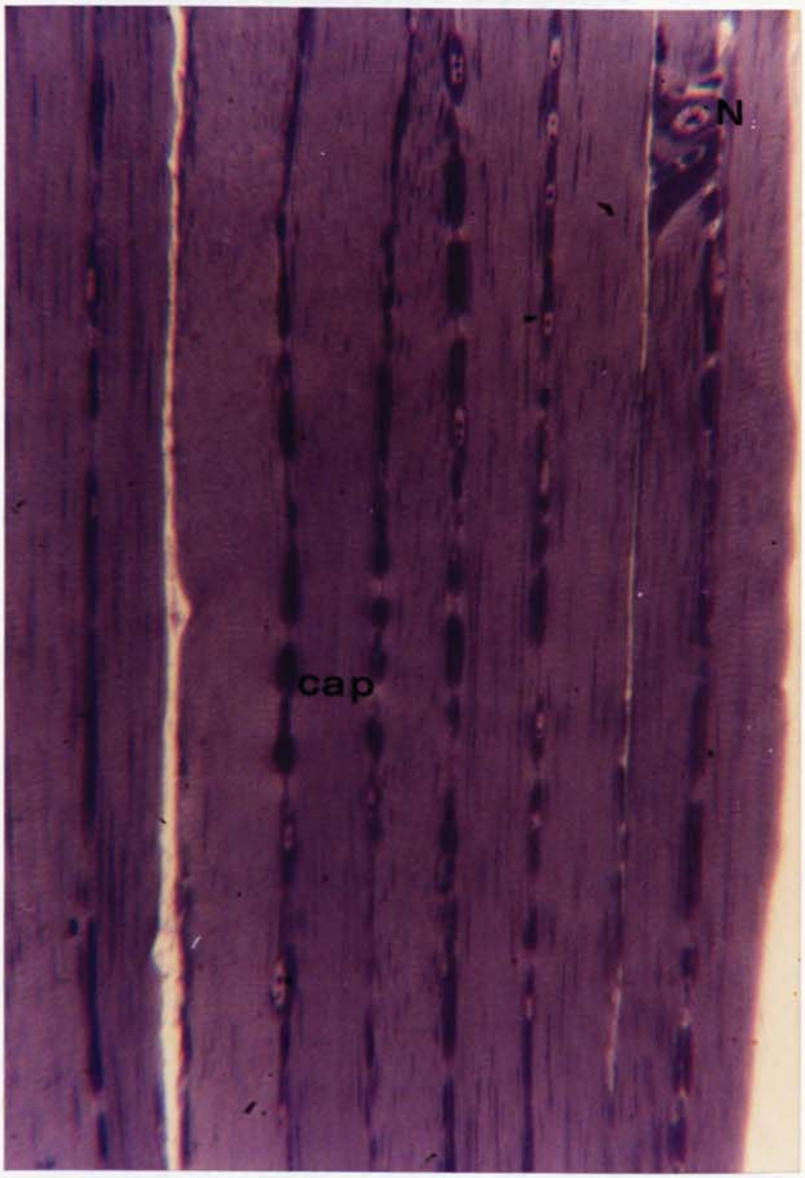


Plate 6.1. Toluidine-blue-stained longitudinal section from a control hemidiaphragm preparation (x400). The muscle fibres are represented by intact elongated profiles, between which run blood capillaries (cap) and nerves (N). Striations are clearly visible running perpendicular to the axis of the fibres.

Plate 6.2. Toluidine blue-stained transverse section of a control hemidiaphrgm preparation (x400). The muscle fibres are represented by intact, irregularly shaped profiles surrounded by blood capillaries and nerves. Within the fibre profiles mitochondria are recognised as small dense spots, more common in some fibres than in others.

Plate 6.3. Toluidine-blue-stained transverse section from a hemidiaphragm preparation 6 hours after ECO administration in vivo (x400). The muscle fibre profiles are represented by irregularly shaped profiles containing vacuoles.

Plate 6.4. Toluidine-blue-stained longitudinal section from a hemidiaphragm preparation 6 hours after ECO administration in vivo (x400). The muscle fibres are represented by elongated profiles containing vacuoles. Many of the profiles are hypercontracted or torn and striations are not visible in all the fibres.

Table 6.1. % Abnormal fibres in toluidine blue-stained sections cut from the junctional region of diaphragms at various times after ECO administration.

	% Abnormal Fibres
Atropine only (controls)	0.0
20mins. after ECO.	20.1
30mins. after ECO.	25.6
1hr. after ECO.	18.8
3hr. after ECO	31.5
6hr. after ECO.	24.2
12hr. after ECO.	30.4
24hr. after ECO.	31.6
48hr. after ECO.	10.4
72hr. after ECO.	16.0

since at 72 hours, although the number of abnormal fibres had decreased, 16% of the muscle fibres at the junctional region were still abnormal (table 6.1). Perhaps, as was suggested in chapter 3 (3.3.3), the myopathy still existing in some fibres at times greater than 24 hours after ECO is so severe that Procion can no longer bind intracellularly.

However, it seems that repair/regeneration may have occurred in at least some of the fibres within 72 hours of ECO administration. The true condition of the muscle fibres may only be satisfactorily answered at the ultrastructural level.

6.3.2. The ultrastructure of the muscle fibres of the diaphragm at various times after ECO administration in vivo: a descriptive morphological study.

The ultrastructural analysis of ECO-induced damage to muscle fibres was restricted to sections cut from the junctional region of the diaphragm only.

6.3.2.1. Control sections.

Sections of control hemidiaphragm showed no signs of myopathy, the bulk of each fibre being occupied by the contractile system. At magnification x7500 the myofibrils were represented, in transverse section, by closely packed, irregularly shaped profiles surrounded by sarcoplasm in which were situated the fibre nuclei, various granular inclusions, mitochondria and SR (plate

Plate 6.5. Transverse section of a control hemidiaphragm preparation (x7500). Muscle fibrils are represented by closely packed, irregularly shaped profiles surrounded by sarcoplasm within which lie mitochondria (mit) and sarcoplasmic reticulum (SR). Z-lines (Z) can be recognised as electron-dense regions traversing the myofibrils.

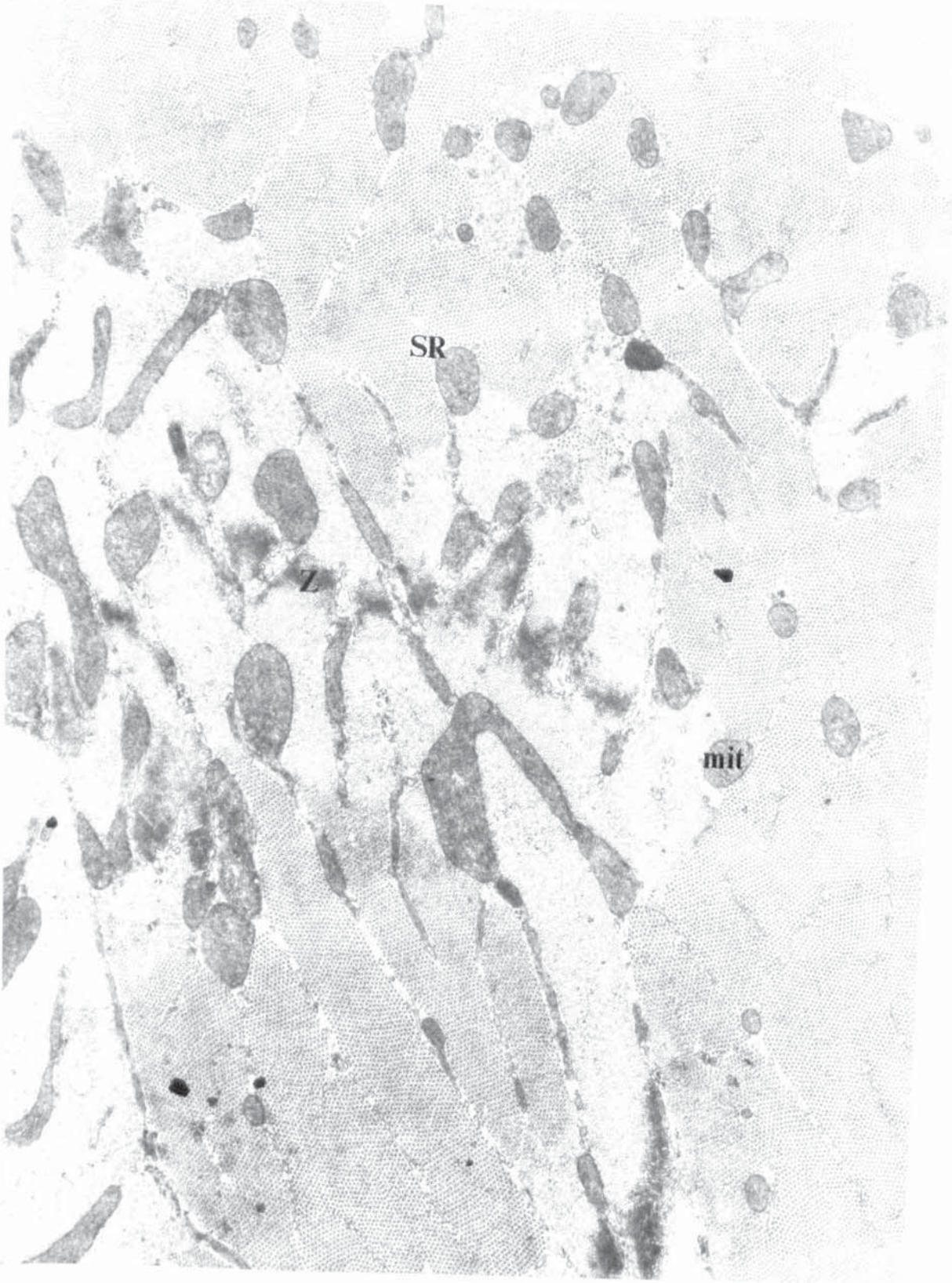
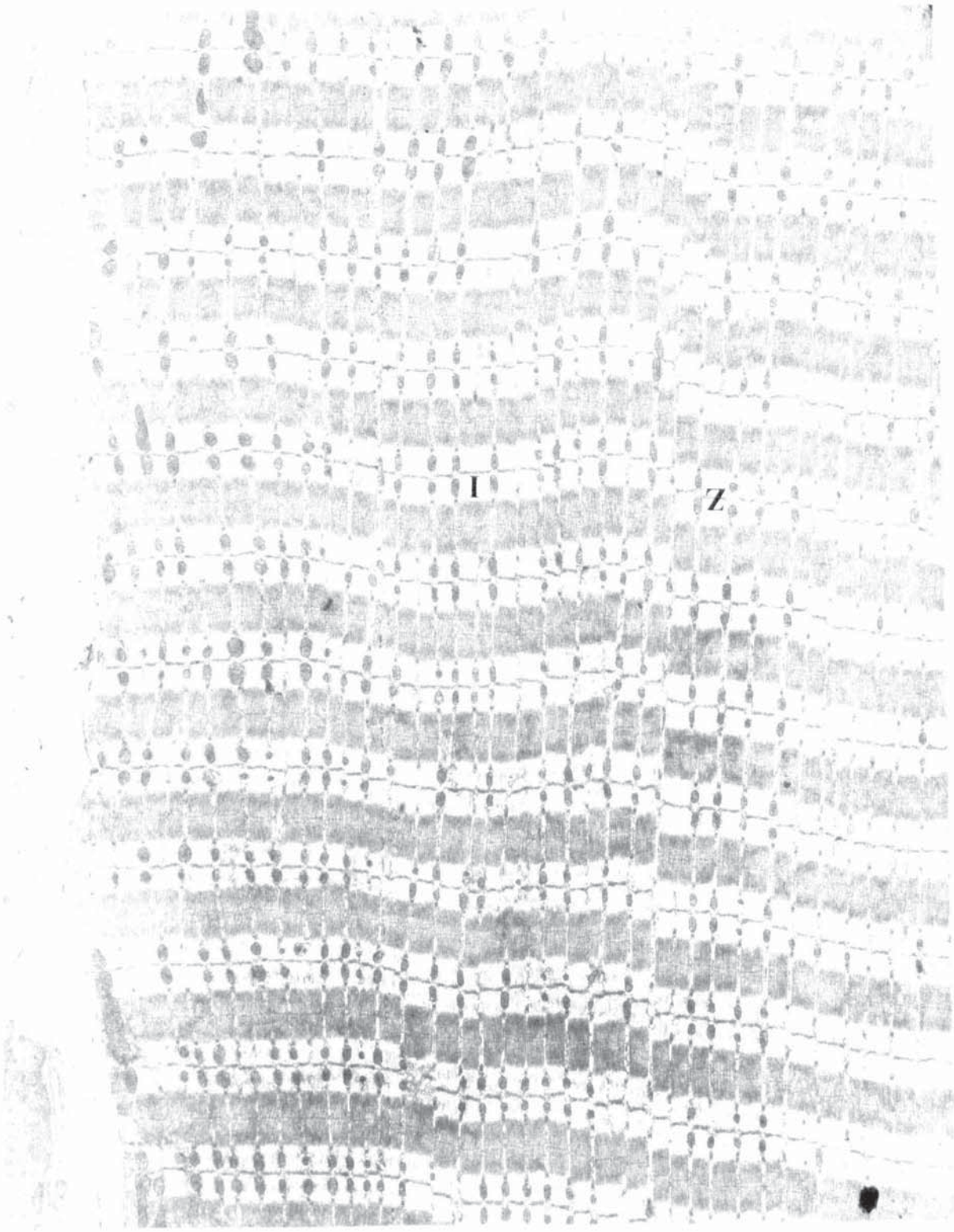

SR
Z
mit

Plate 6.6. Longitudinal section of a control hemidiaphragm preparation (x4000). The photograph demonstrates the repeating system of striations within individual muscle fibrils. The bands of successive fibrils are generally aligned in phase across the fibre and the basic repeating unit of staiations, the sarcomeres, are demarcated by electron-dense Z-bands (Z) running through the pale I-bands (I). Mitochondria can be seen lining the Z-lines within the I-bands.


I
Z

6.5). The mitochondria were irregularly shaped and electron-dense whilst the SR appeared as groups of small circular profiles: Z-lines were recognised as more dense bands traversing the myofibrils (plate 6.5).

Longitudinal sections of normal muscle demonstrated the repeating system of bands or striations (plate 6.6). The bands of successive fibrils were generally aligned in phase across the fibre and the basic repeating unit of striations, the sarcomeres, were demarcated by the electron dense Z-bands (plate 6.6). In plate 6.6 mitochondria can be seen lining the Z-bands in the I-bands, and SR can be seen running between the myofibrils.

6.3.2.2. 20 minutes after ECO administration.

Abnormalities were seen in the diaphragm as early as 20 minutes following ECO administration: a minority of muscle fibres were affected (7.7%: table 6.2) and generally they showed only slight abnormalities. Damage was largely represented by dilatation of SR, the transverse profiles of which were up to 15 times greater in diameter than those measured in control sections (0.75μm:0.05μm), (plate 6.7). In some fibres mitochondria were less electron-dense due to apparent swelling, causing separation of their cristae, whilst in a small number of mitochondria crystal formation had occurred (plate 6.8). Myofilaments appeared to be stretched or disintegrated and the sarcoplasm was swollen; Z-lines were observed less frequently than in control sections.

Plate 6.7. Transverse section of a hemidiaphragm preparation 20 minutes after ECO administration in vivo (x7500). Damage is primarily represented by dilatation of the sarcoplasmic reticulum (SR) and slight swelling of the mitochondria (mit). Myofibrils are generally intact and Z-lines (Z) are clearly visible.

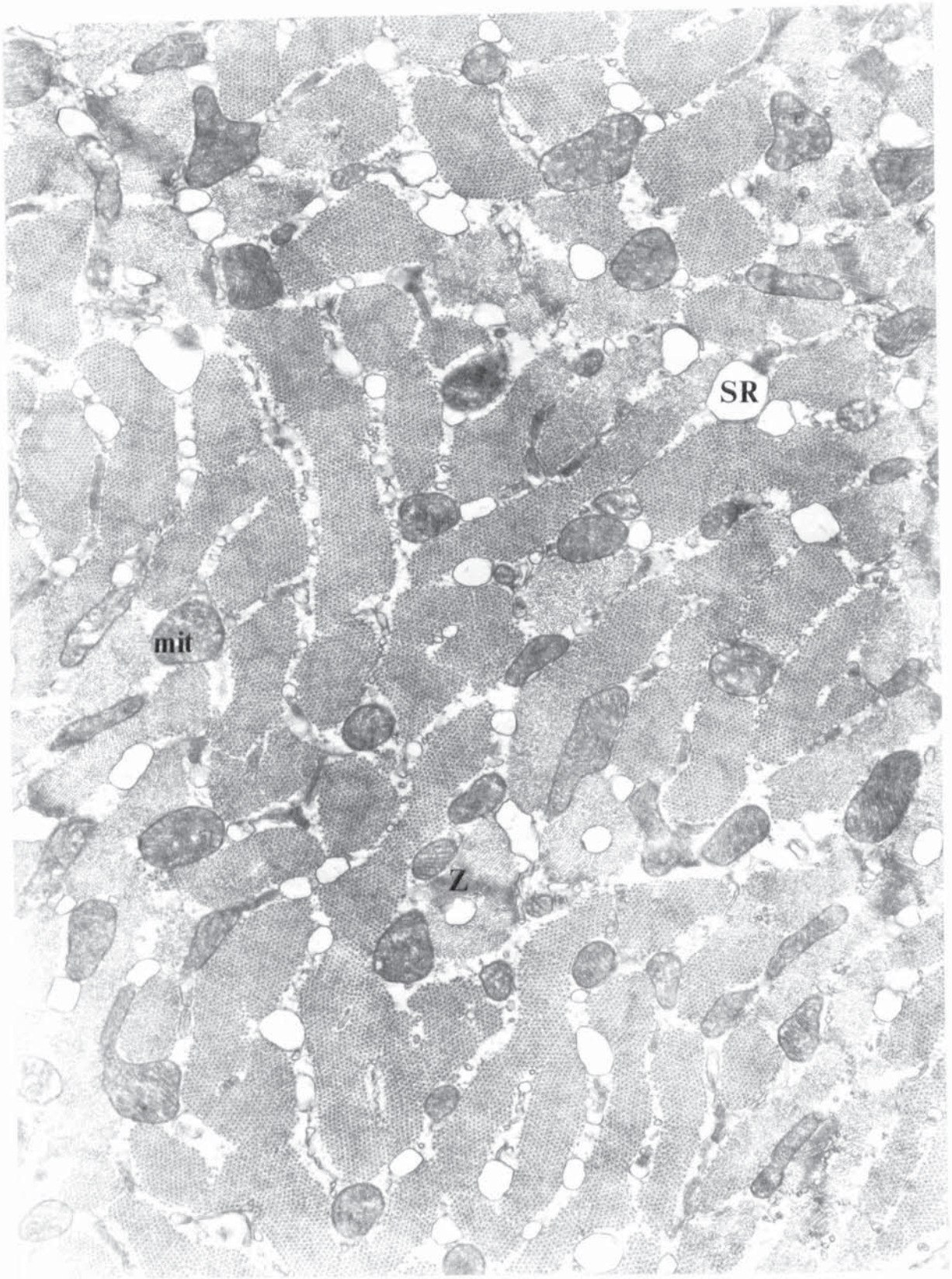
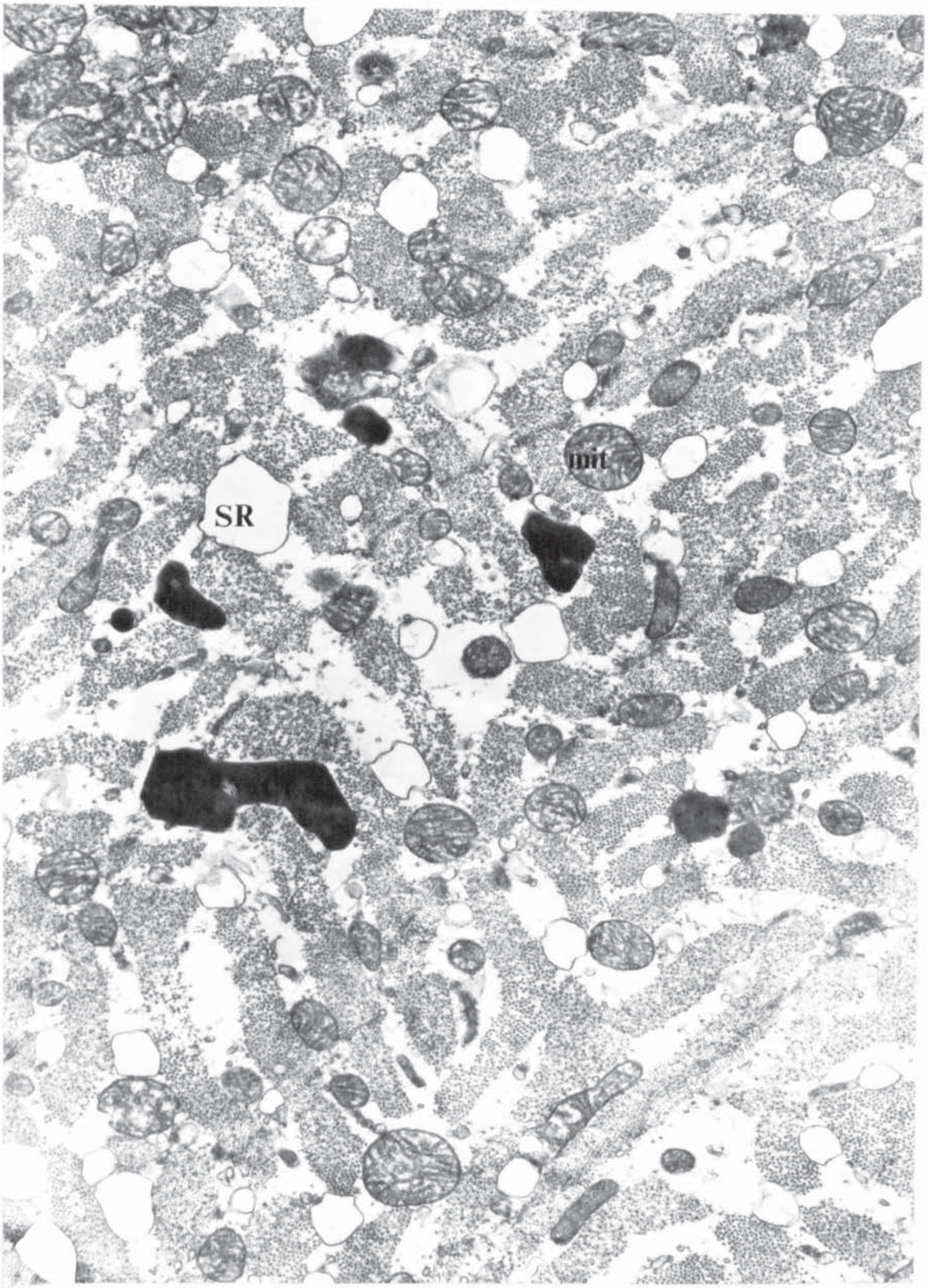

SR
mit
Z

Plate 6.8. Transverse section of a hemidiaphragm preparation 20 minutes after ECO administration in vivo (x7500). Damage is represented by dilatation of sarcoplasmic reticulum (SR) and mitochondria (mit), but also by disintegration of myofibrils and the appearance of large electron-dense crystals (Cr).


SR
mit

6.3.2.3. 30 minutes after ECO administration.

At 30 minutes after ECO no abnormalities were detected in the transverse sections of the diaphragm although, in longitudinal sections, some fibres contained swollen SR (plate 6.9). In one fibre a typical example of Z-line loss accompanied by myofilament disintegration was observed in a stretched length of fibre adjacent to a hypercontracted region (plate 6.10): again the SR was dilated but the mitochondria appeared to be normal. The number of muscle fibres affected by ECO at 30 minutes was small (3.7%: table 6.2) and it is thought that the transverse sectioning of the diaphragm may not have sampled the damaged regions at all. For this reason no morphological analysis was performed on the ultrastructure of muscle fibre profiles at 30 minutes after ECO (6.3.3).

6.3.2.4. 1 hour after ECO administration.

At 60 minutes after ECO the % damaged fibres had increased (4.9%: table 6.2), abnormalities being observed in both transverse and longitudinal sections. This does not conform with the analysis of toluidine blue-stained sections (6.4), where a slight drop in the % abnormal fibres was observed 60 minutes after ECO administration (table 6.1). Finestructural damage to muscle fibres at this time was manifested consistently by dilatation of SR, and often by severely swollen mitochondria (plate 6.11). In one fibre these features were accompanied by the appearance of

Plate 6.9. Longitudinal section from a hemidiaphragm 30 minutes after ECO administration in vivo (x7500). Damage is represented by dilatation of sarcoplasmic reticulum (SR), streaming of Z-lines (Z) plus misalignment of myofibrils.

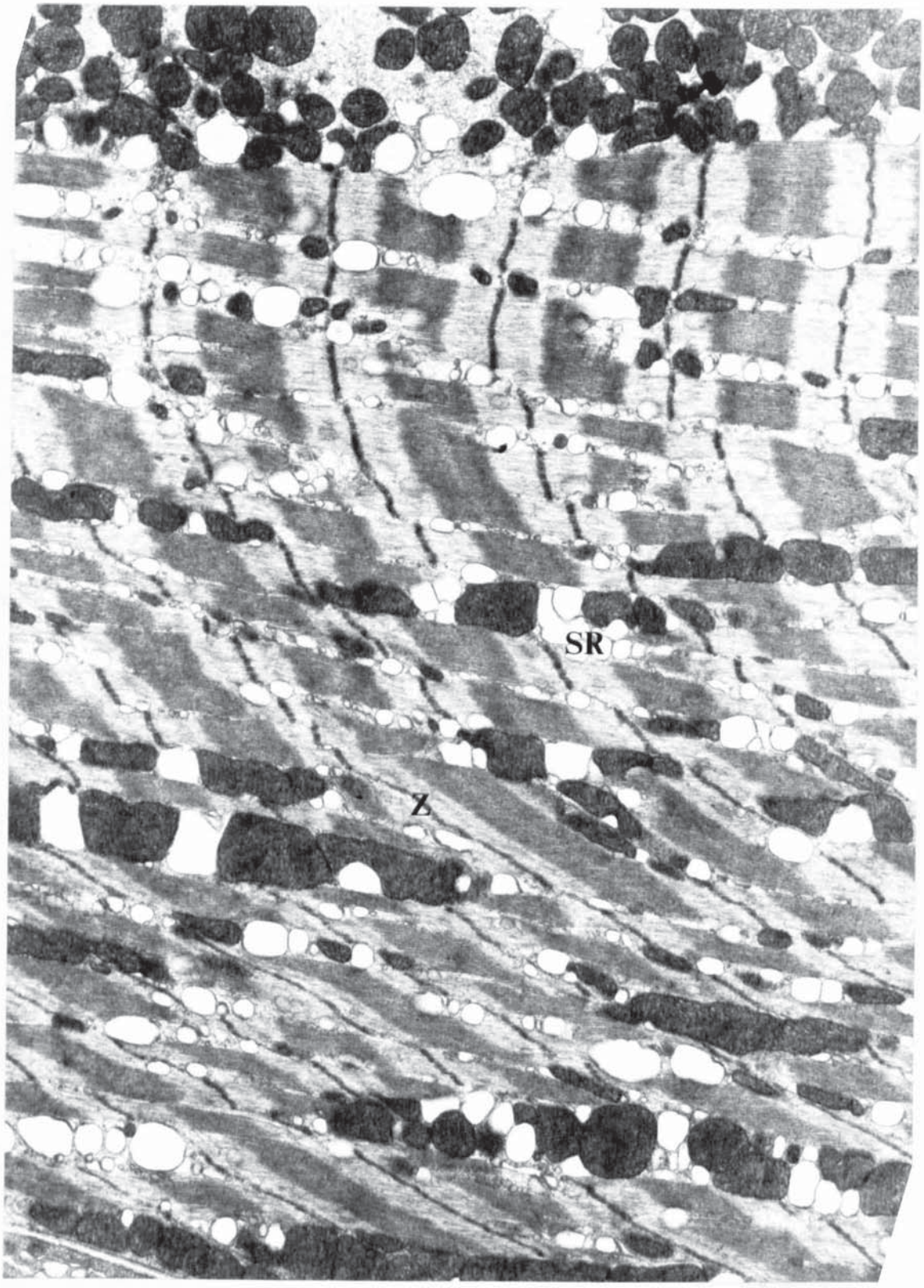

SR
Z

Plate 6.10. Longitudinal section from a hemidiaphragm preparation 30 minutes after ECO administration *in vivo* (x4000). Damage is represented by dilatation of sarcoplasmic reticulum (SR), Z-line loss, myofilament degradation and misalignment in a stretched length of fibre (str) adjacent to a hypercontracted region (hyp). Within the hypercontraction, little sarcomeric structure can be recognised due to the intensity of the contraction.

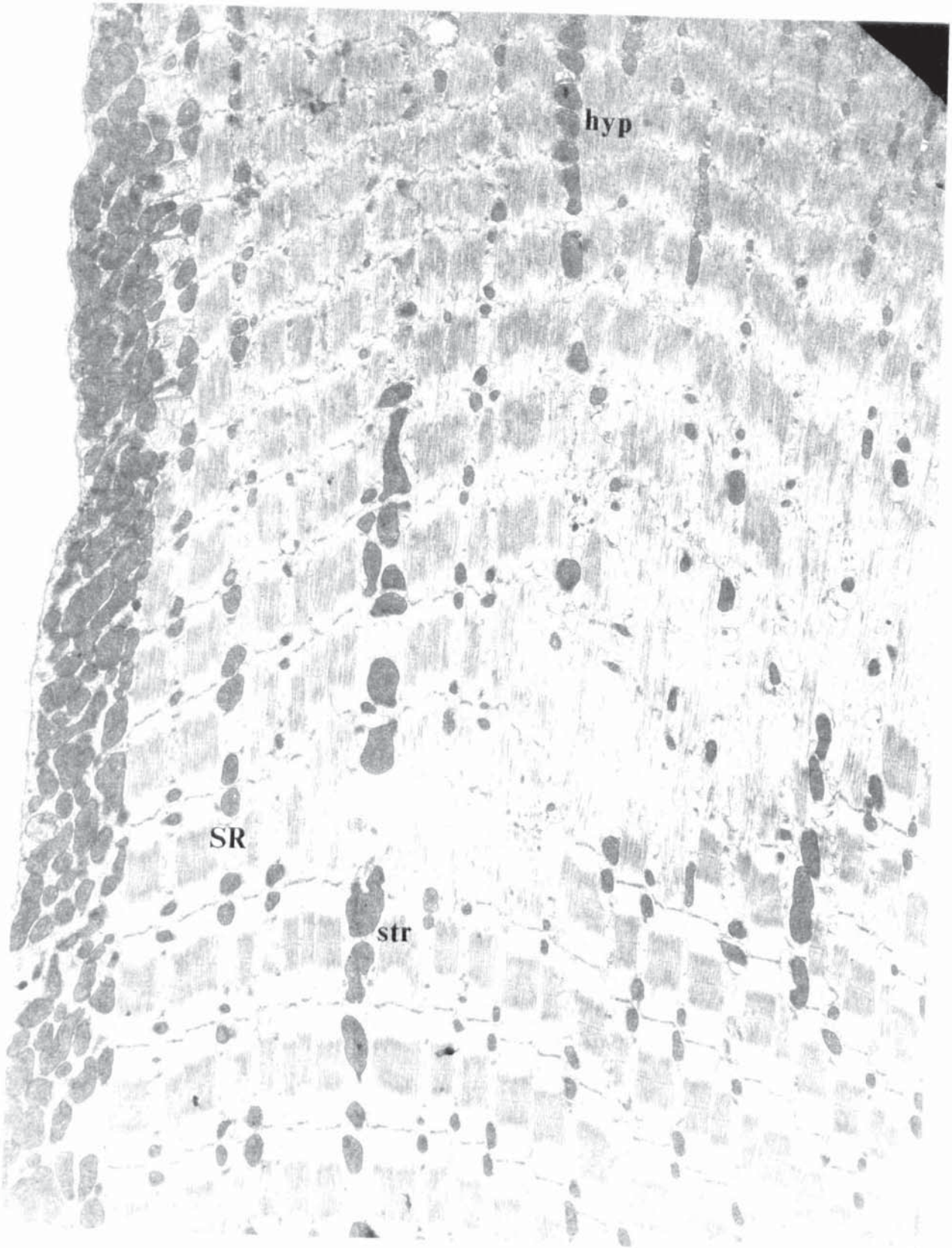
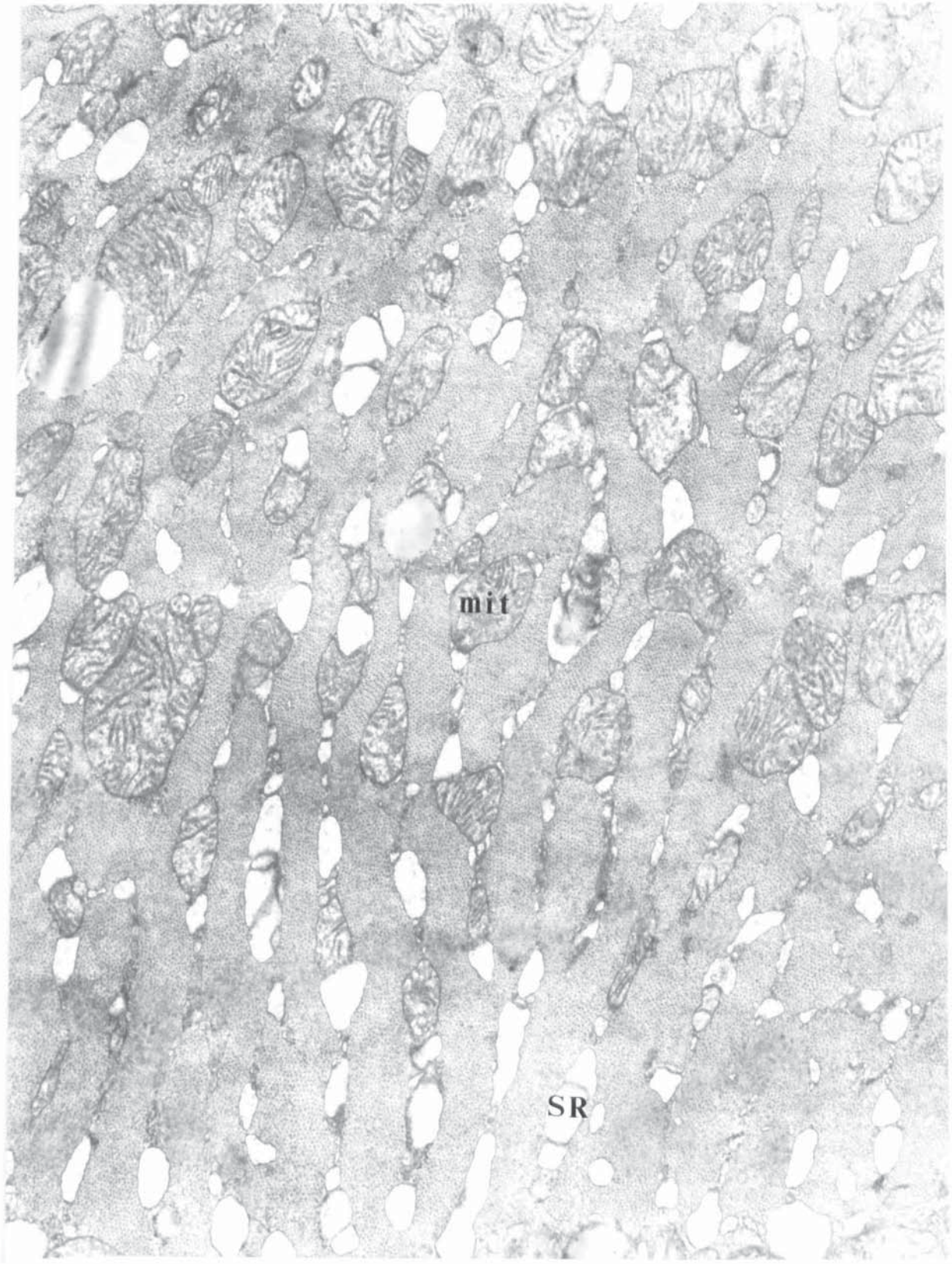

hyp
SR
str

Plate 6.11. Transverse section from a hemidiaphragm preparation 1 hour after ECO administration in vivo (x7500). Damage is represented by dilatation of sarcoplasmic reticulum (SR) and mitochondria (mit) only.


mit
SR

crystals developing within vacuolated mitochondria (plate 6.12). In another cell (plate 6.13), severe necrosis was observed in an area adjacent to the nerve terminal; invaginations of the sarcolemma were seen opposite the nerve terminal and complete loss of sarcoplasmic structure had occurred. In the same cell, the few mitochondria that were recognisable were swollen and 'patchy' and the SR was dilated or vacuolated. In places the organelles of the sarcoplasm were unrecognisable, but away from the nerve terminal the myopathy was less severe.

6.3.2.5. 3 hours after ECO administration.

At three hours after ECO 30.2% of the fibres were damaged (table 6.2). Several of the fibres were so grossly damaged that the SR and/or mitochondria were no longer recognisable or had vacuolated. Myofilaments were sometimes completely broken down and crystal formation was common within vacuoles which appeared to have originated from damaged mitochondria (plate 6.14). Such gross necrosis was usually seen in close association with a nerve terminal (plate 6.15), the areas of the fibre away from the terminal being much less severely affected.

6.3.2.6. 6 hours after ECO administration.

At 6 hours after ECO the number of fibres affected by the intoxication had increased, 39.7% of the fibres being damaged (table 6.2). All damaged fibres exhibited loss of Z-lines whilst

Plate 6.12. Transverse section from a hemidiaphragm 1 hour after ECO administration *in vivo* (x7500). Damage is represented by dilatation and vacuolation of sarcoplasmic reticulum (SR) and mitochondria (mit), misalignment of myofilaments and the appearance of small crystals (Cr) developing within vacuolated mitochondria.

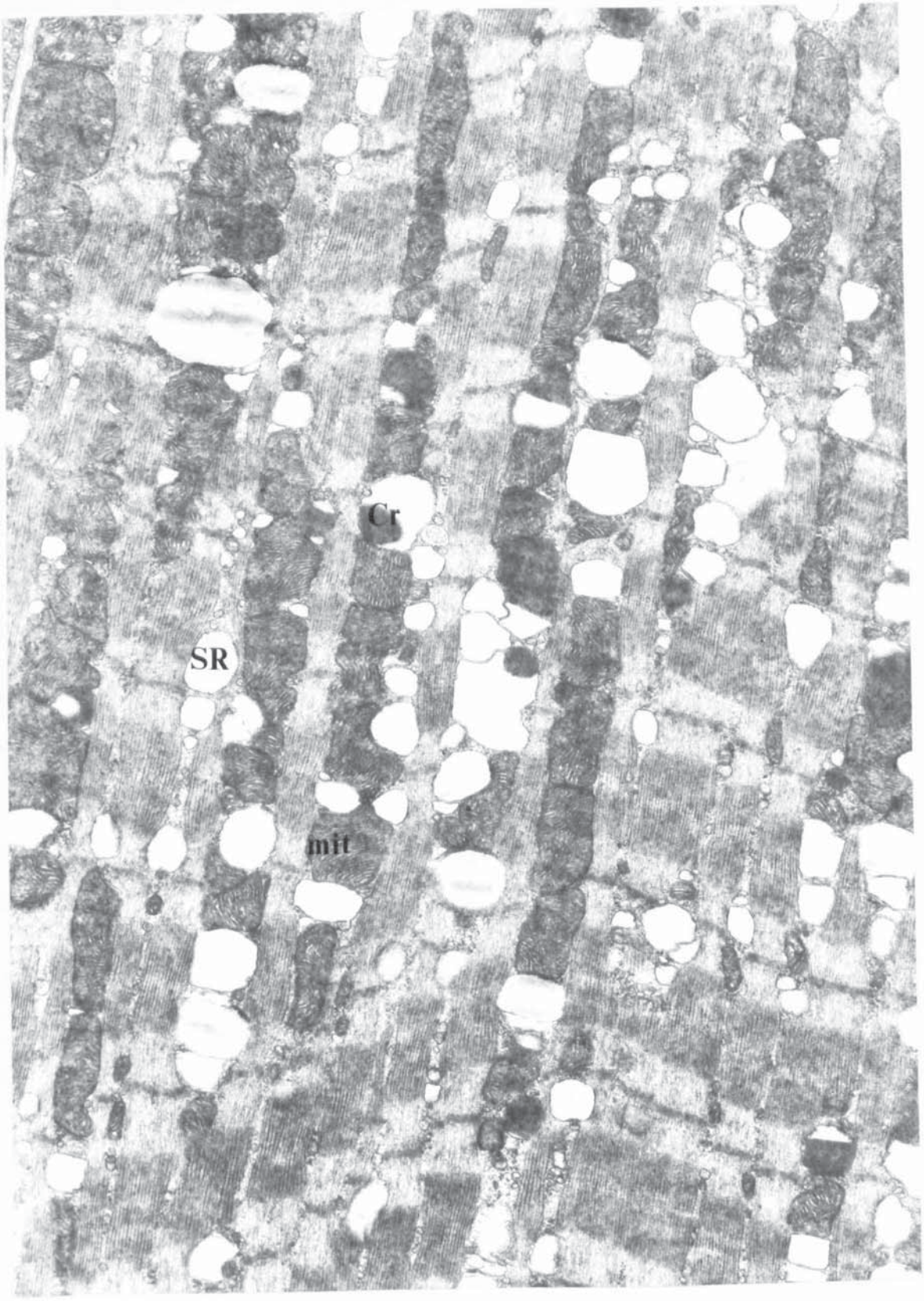

Cr
SR
mit

Plate 6.13. Transverse section from a hemidiaphragm preparation 1 hour after EDO administration in vivo (x7500). Damage is represented by gross dilatation and vacuolation of sarcoplasmic reticulum (SR) and mitochondria (mit), complete loss of sarcomeric structure plus crystal formation (Cr) within vacuolated mitochondria. This gross necrosis was located adjacent to the nerve terminal (NT) pertaining to the fibre: invaginations (inv) of the sarcolemma were seen opposite the nerve terminal. In this particular section the transverse profile of a fibre nucleus (nuc) is clearly seen.

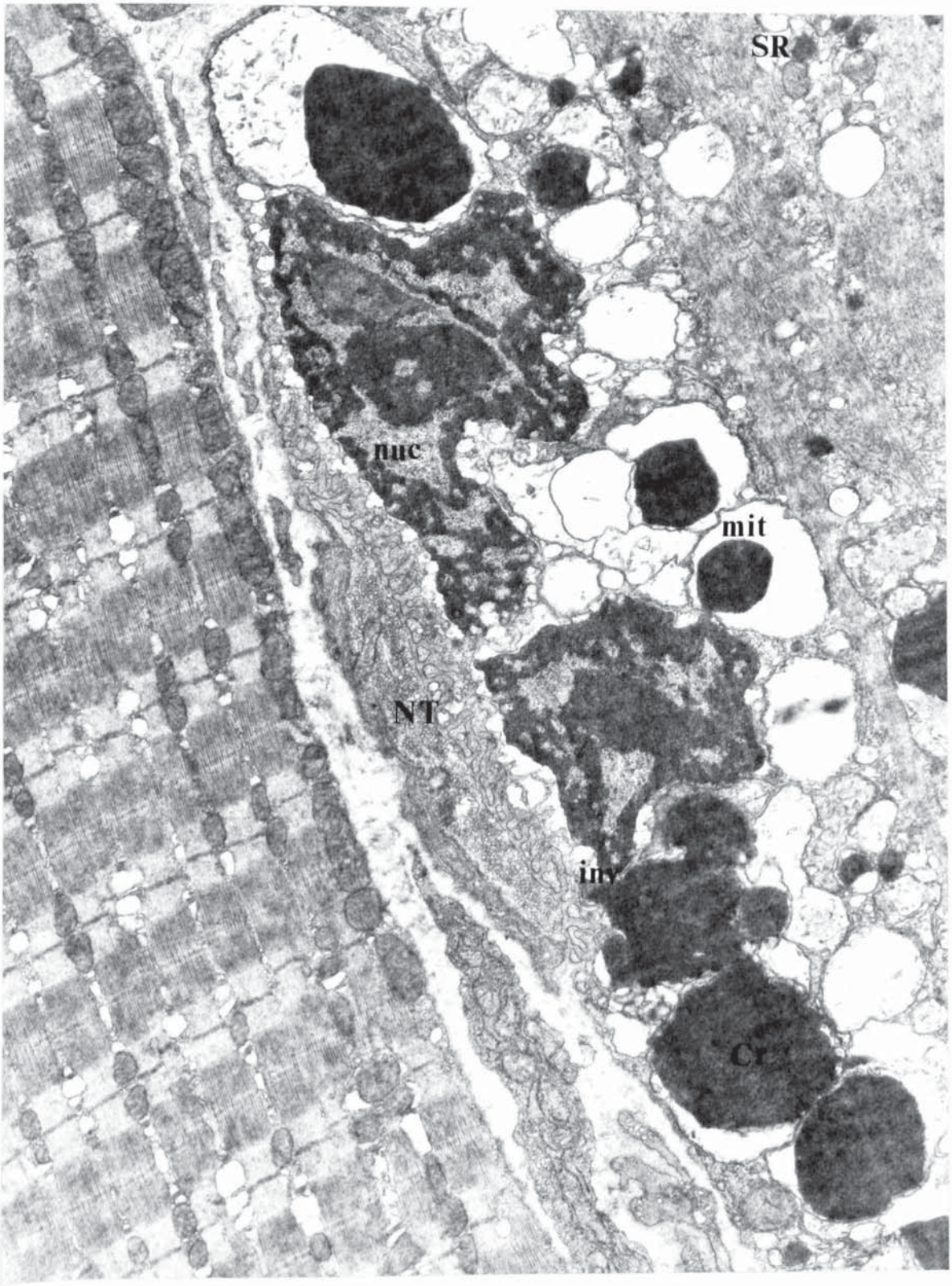

SR
nuc
mit
NT
inv
Cr

Table 6.2:- The % of muscle fibres at the various stages in the development of ECO-induced myopathy.

	Normal %	Mild %	Moderate %	Severe %	Gross %	Regen. %
Control	100	0.0	0.0	0.0	0.0	0.0
20 mins.	92.3	1.5	1.5	4.7	0.0	0.0
30 mins.	96.3	1.85	1.85	0.0	0.0	0.0
1 hr.	95.1	2.9	1.0	0.0	1.0	0.0
3 hr.	69.8	7.6	7.6	5.7	9.3	0.0
6 hr.	60.3	13.5	4.5	16.1	5.4	0.0
12 hr.	66.4	14.6	9.1	5.2	4.7	0.0
24 hr.	76.8	3.6	0.0	3.6	16.1	0.0
48 hr.	78.4	0.0	1.8	3.4	6.2	11.0
72 hr.	89.4	0.0	0.0	3.0	1.5	6.1

N.B. Approximately 100 fibres were counted at each time point.

Plate 6.14. Transverse section from a hemidiaphragm preparation 3 hours after EDO administration in vivo (x10K). The photograph represents a nerve terminal (NT) lying adjacent to a severely myopathic muscle fiber (MF). Vesicles (v) and mitochondria (mit) can be easily identified within the nerve terminal, the processes (P) of which can be identified lying close to the muscle fiber. Myopathy within the myofiber is very severe and is represented by gross sarcoplasmic disruption plus vacuolation and crystal formation within mitochondria.

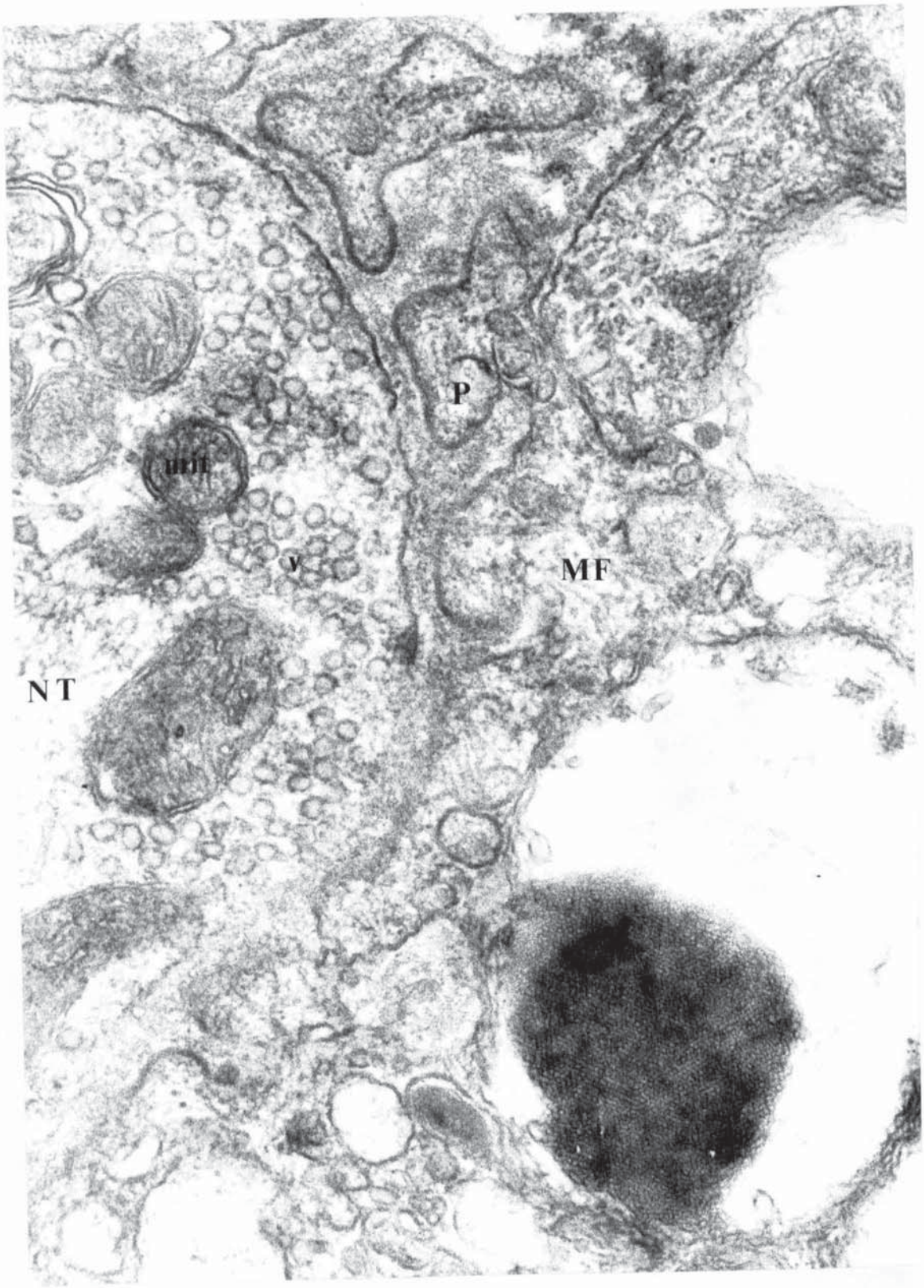
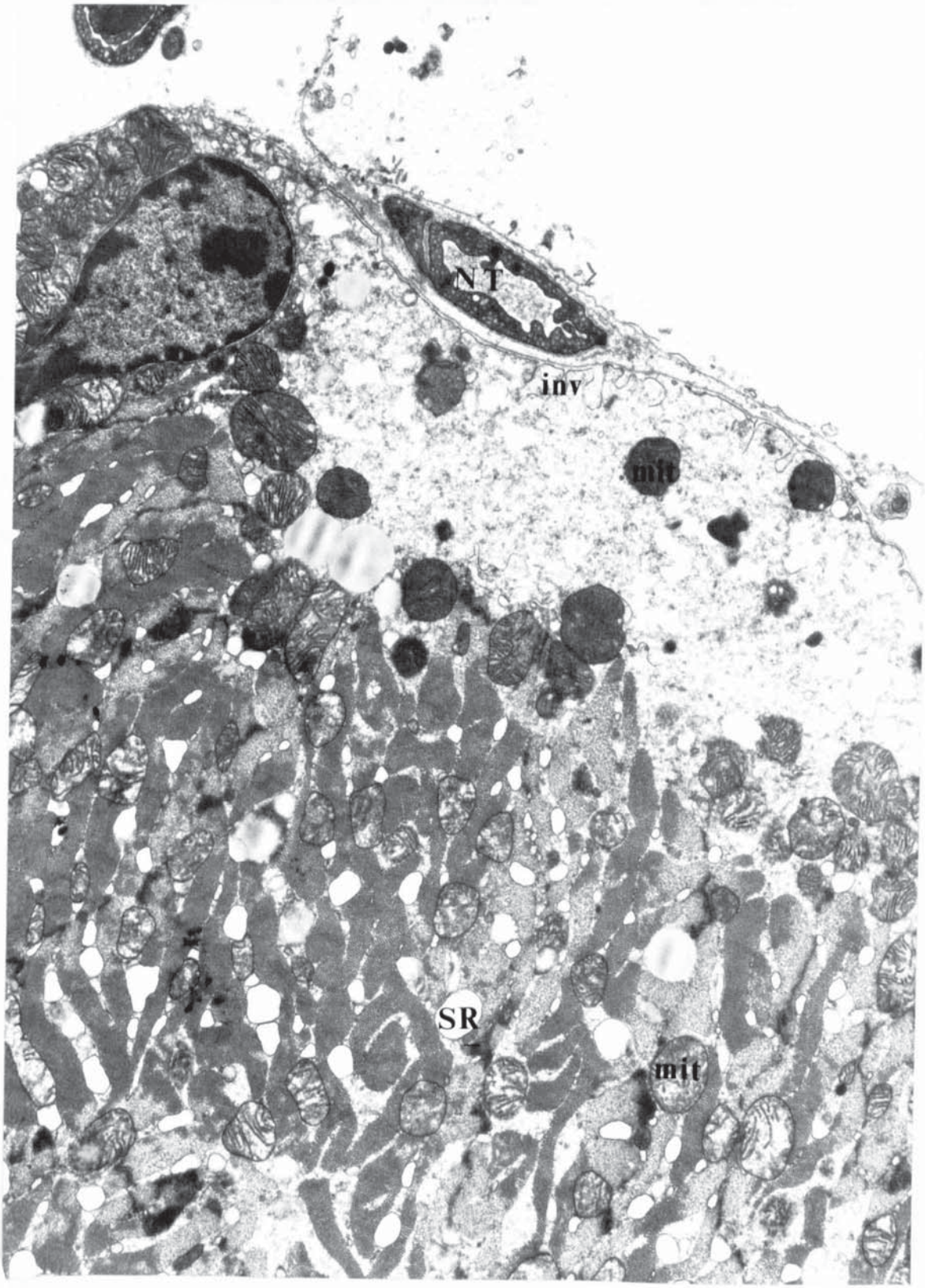

P
MF
NT

Plate 6.15. Transverse section from a hemidiaphragm preparation 3 hours after ECO administration *in vivo* (x7500). Damage is most severe close to the nerve terminal (NT) where invaginations (inv) can be seen in the sarcolemmal membrane. The sarcoplasm adjacent to the nerve terminal is completely disrupted, containing only one or two identifiable mitochondria (mit). Further away from the nerve terminal myopathy is less severe: mitochondria and sarcoplasmic reticulum (SR) are dilated and the sarcoplasm appears to be swollen but the basic myofibrillar structure is still recognisable.


NT
inv
mit
SR
mit

most showed breakdown of myofilaments and hence loss of sarcomeric structure. Mitochondria were swollen, SR dilated and crystal formation was common, the crystals occasionally occupying large areas of the fibre profile (plate 6.16).

Again the cells were more severely affected close to the nerve terminals. In some fibres all subcellular organelles were lost completely, leaving an amorphous mass of sarcoplasm and cell debris (plate 6.17), whilst in others, sarcoplasmic organelles had been 'squeezed' out of a hypercontracted region into adjacent stretched lengths of the same fibre (plate 6.18).

Multilayered membraneous structures of undetermined origin were observed in some muscle fibres at 3 hours, 6 hours and 12 hours after ECO, and appeared to be developing from damaged mitochondria (plate 6.19).

6.3.2.7. 12 hours after ECO administration.

At 12 hours after ECO 34% of the muscle fibres were damaged (table 6.2): many fibres had dilated SR, mitochondria were swollen and occasionally vesiculated but crystal formation was not observed (plate 6.19). Misalignment and disintegration of myofilaments was common but Z-lines were recognised despite their complete dissappearence at both 6 and 24 hours after ECO (plate 6.19). Some fibres were grossly affected at 12 hours but myopathy in general appeared to be less severe than that observed at both 6 hours and 24 hours after intoxication. No nerve terminals were

Plate 6.16. Transverse section of a hemidiaphragm preparation 6 hours after ECO administration in vivo (x7500). Damage is gross on one side of the cell where sarcoplasmic reticulum (SR) and mitochondria (mit) are vacuolated and crystal (Cr) formation vast. Away from this grossly necrotic region the cell is less severely affected, myopathy being represented by dilatation of sarcoplasmic reticulum and some mitochondria. Z-lines were not visible throughout the fibre profile.

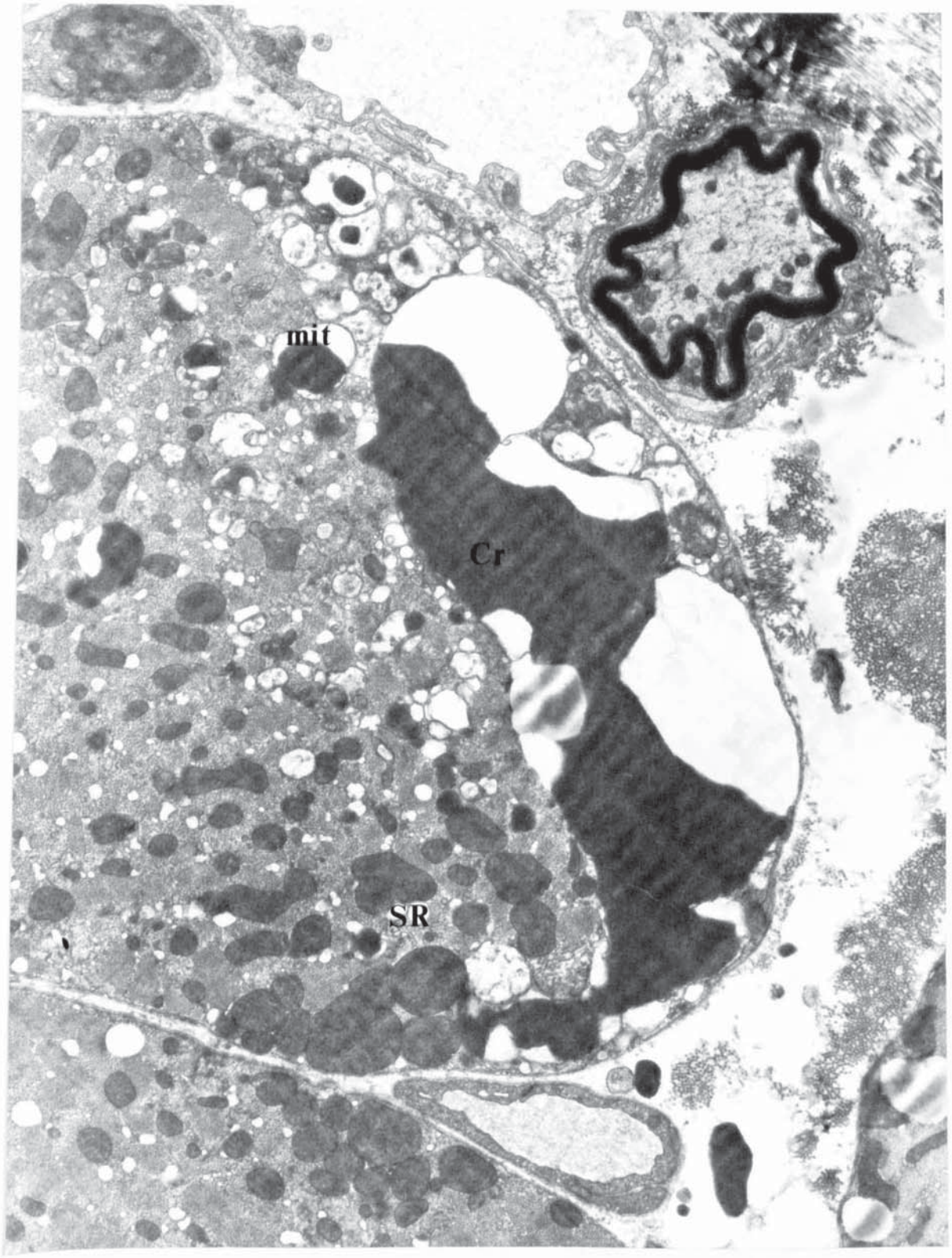

mit
Cr
SR

Plate 6.17. Transverse section of a hemidiaphragm preparation 6 hours after ECO administration *in vivo* (x7500). The photograph represents a muscle fiber (MF) which has been completely destroyed. Most of the sarcoplasm is amorphous, containing only fragmented myofilaments plus one or two recogniseable mitochondria (mit).

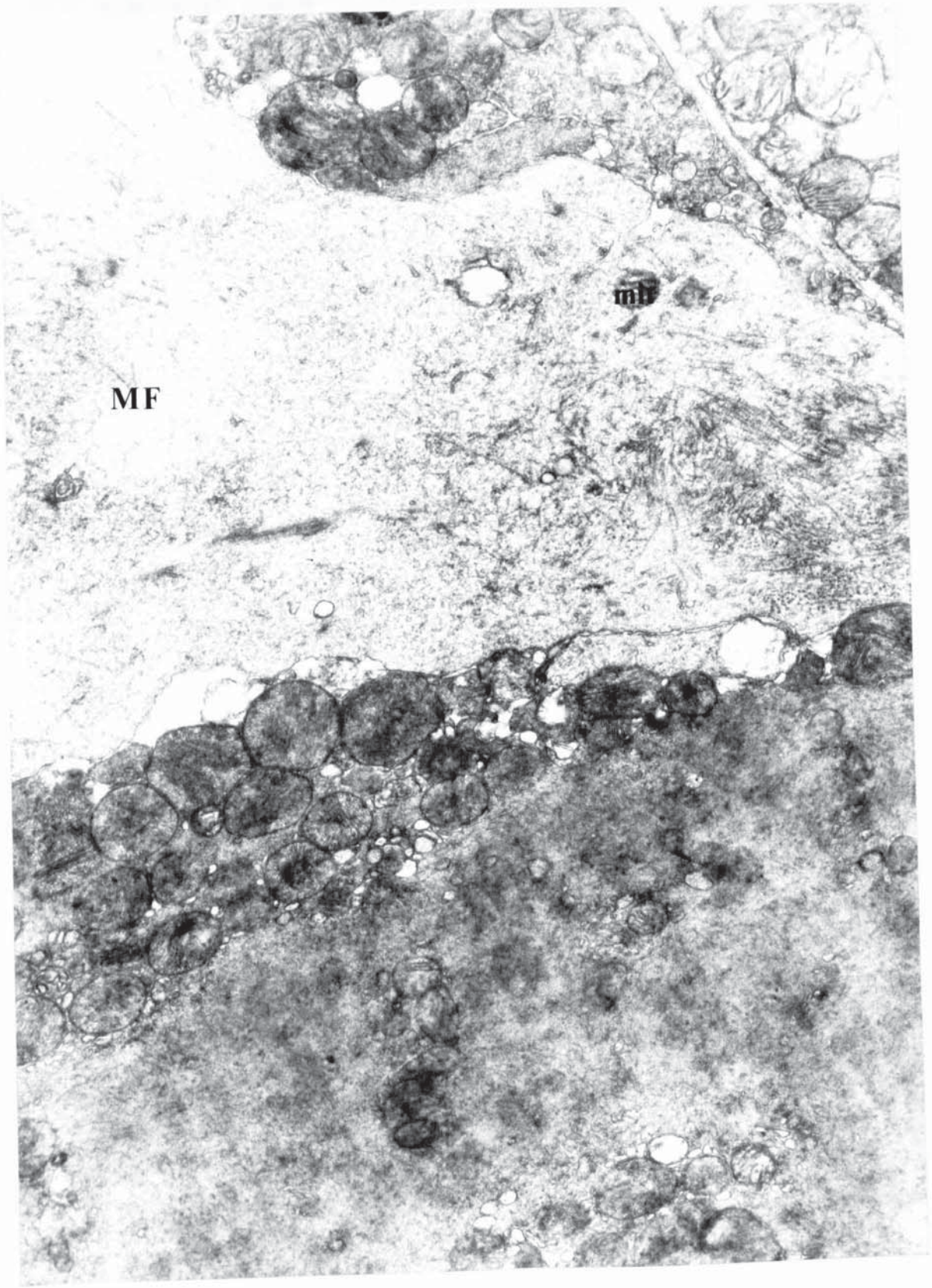

MF
mit

Plate 6.18. Longitudinal section of a hemidiaphragm preparation 6 hours after ECO administration in vivo (x4000). The photograph represents a contracted region of a muscle fibre damaged by ECO. Within the contraction (C) itself most organelles have been squeezed out of the sarcoplasm and sarcomeric structure is not recognisable due to the intensity of the contraction. The adjacent, stretched lengths of fibre contain dilated sarcoplasmic reticulum (SR) plus misaligned and disintegrated myofilaments.

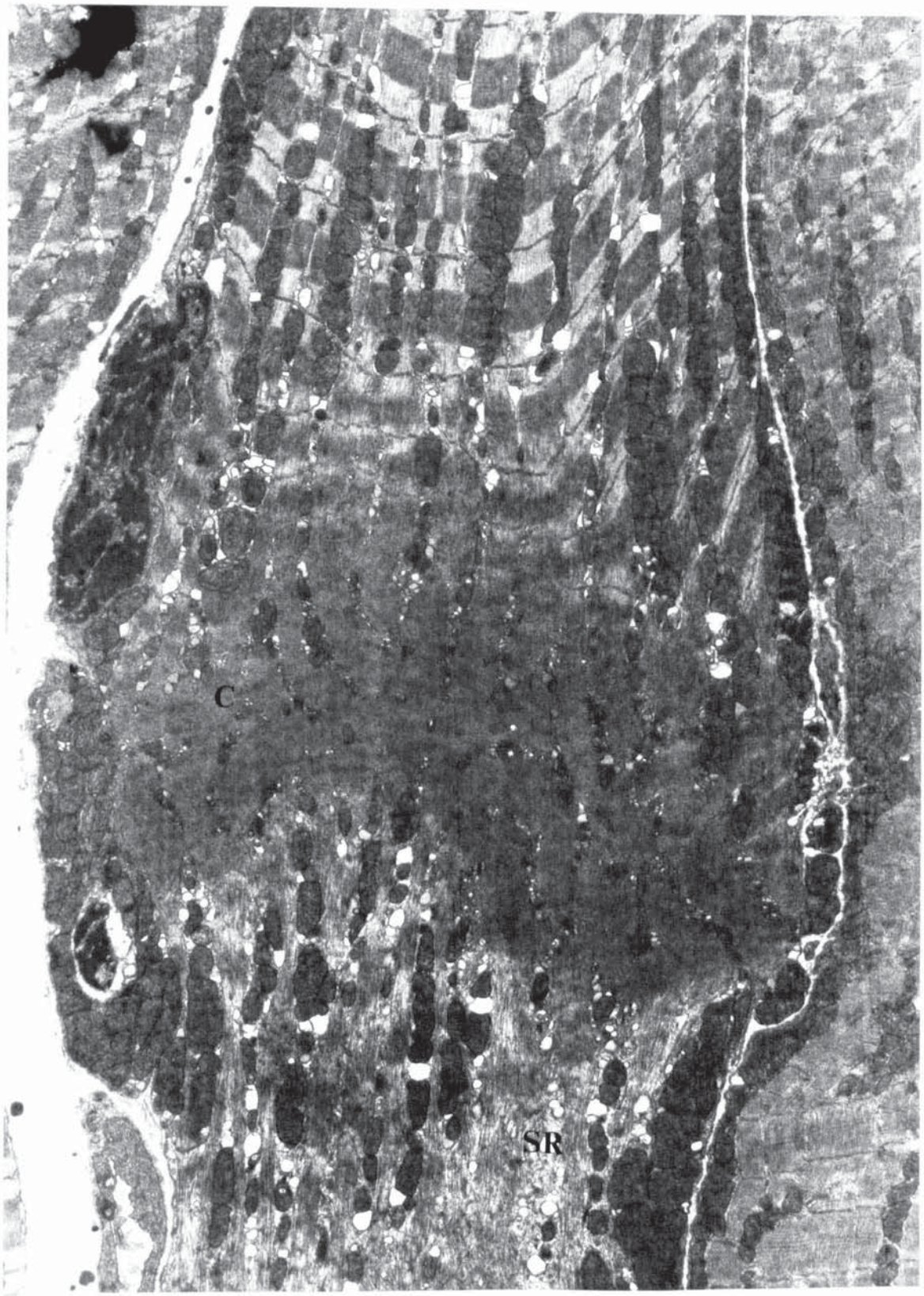
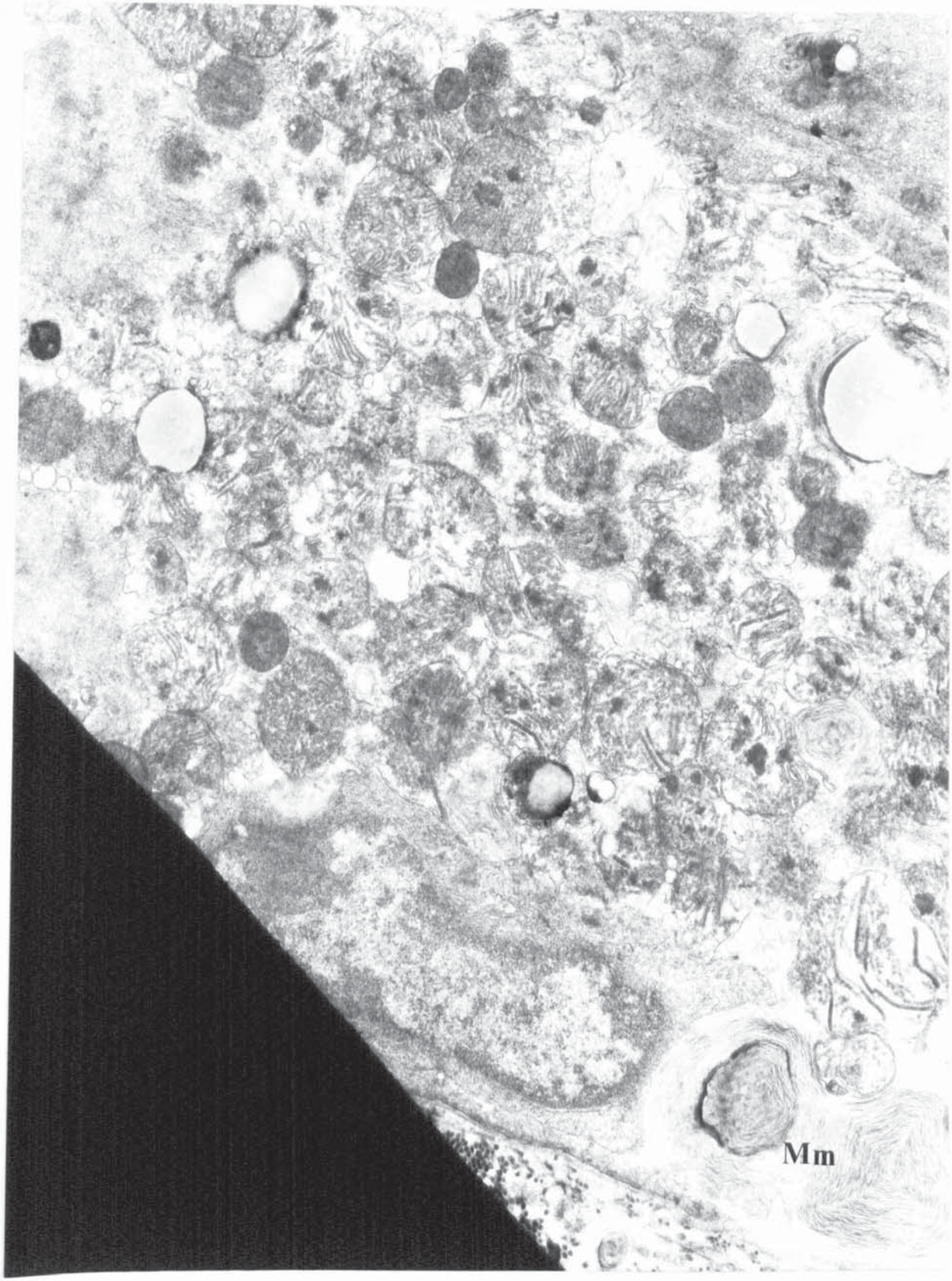

C
SR

Plate 6.19. Transverse section of a hemidiaphragm preparation 12 hours after ECO administration *in vivo* (x7500). Multilayered membraneous structures (Mm) were observed in some muscle fibres and were usually associated with gross sarcoplasmic destruction: They appeared to be originating from damaged mitochondria.


Mm

recognised in the sections cut from diaphragms 12 hours after ECO administration.

6.3.2.8. 24 hours after ECO administration.

By 24 hours after ECO most of the affected fibres were grossly damaged and many were seen to be invaded by phagocytes and macrophages (plate 6.20), not observed at the earlier stages of myopathy development. It is thought that the active phagocytes may be responsible for the secondary elevation of iCa^{2+} at 24 hours, observed at an earlier stage in this study (3.3.5). Approximately 23% of the fibres were affected by ECO (table 6.2).

6.3.2.9. 48 hours after ECO administration.

At 48 hours few fibres were still in an early stage of myopathy. Regeneration was common, being manifested by satellite cells and myoblasts. In one section, the beginings of new myofibrils was noticed in the sarcoplasm of a myoblast (plate 6.21) whilst in other sections myoblasts were seen to be fusing with each other (plate 6.22). In addition, regenerating areas were seen to be fusing with 'spared' areas which had presumably never been necrotic: a montage of two micrographs has been constructed to illustrate this phenomenon (plate 6.23).

Plate 6.20. Transverse section of a hemidiaphragm preparation 24 hours after BOO administration *in vivo* (x7500). Damage is represented by gross disruption and crystal-formation (Cr) within mitochondria (mit) plus loss of sarcomeric structure. A macrophage (mac) has invaded the necrotic cell and seems to be ingesting the damaged mitochondria.

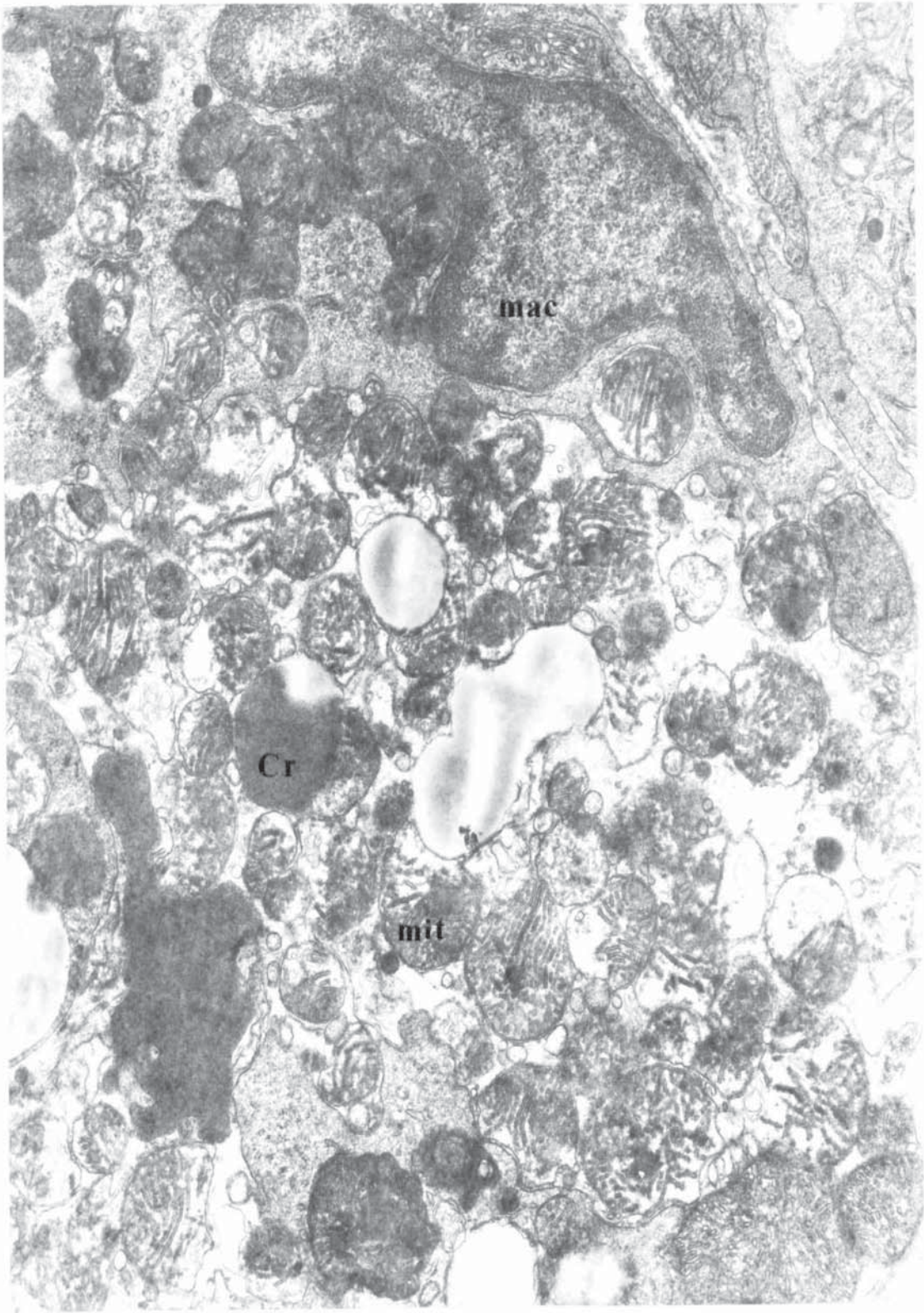

mac
Cr
mit

Plate 6.21. Transverse section of a hemidiaphragm preparation 48 hours after ECO administration in vivo (x7500). New myofilaments (myo) can be seen in the sarcoplasm of a destroyed cell suggesting that regeneration is under way.

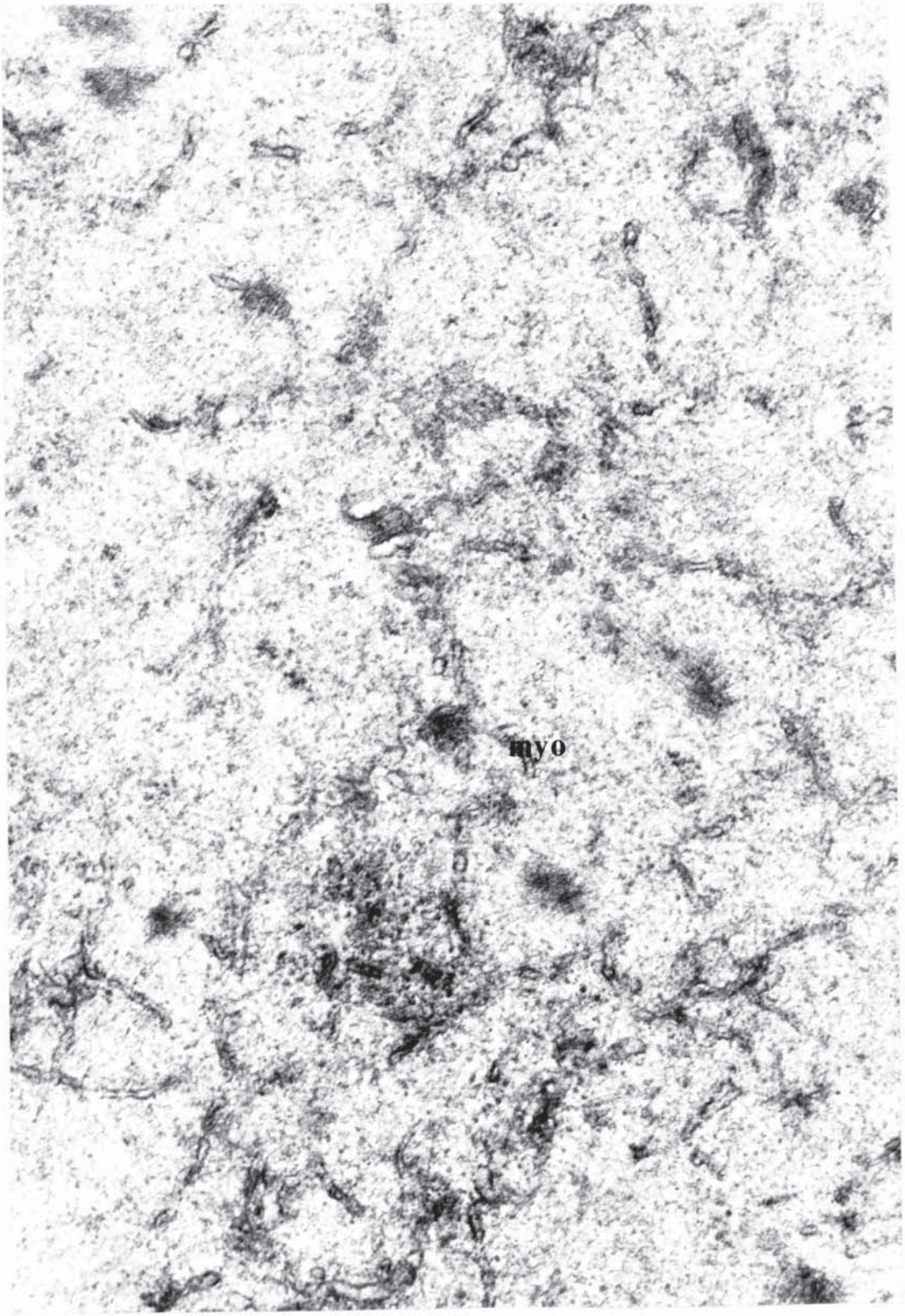

myo

Plate 6.22. Longitudinal section of a hemidiaphragm preparation 48 hours after BCO administration *in vivo* (x7500). The photograph represents two myoblasts fusing together presumably in the early stages of muscle regeneration.

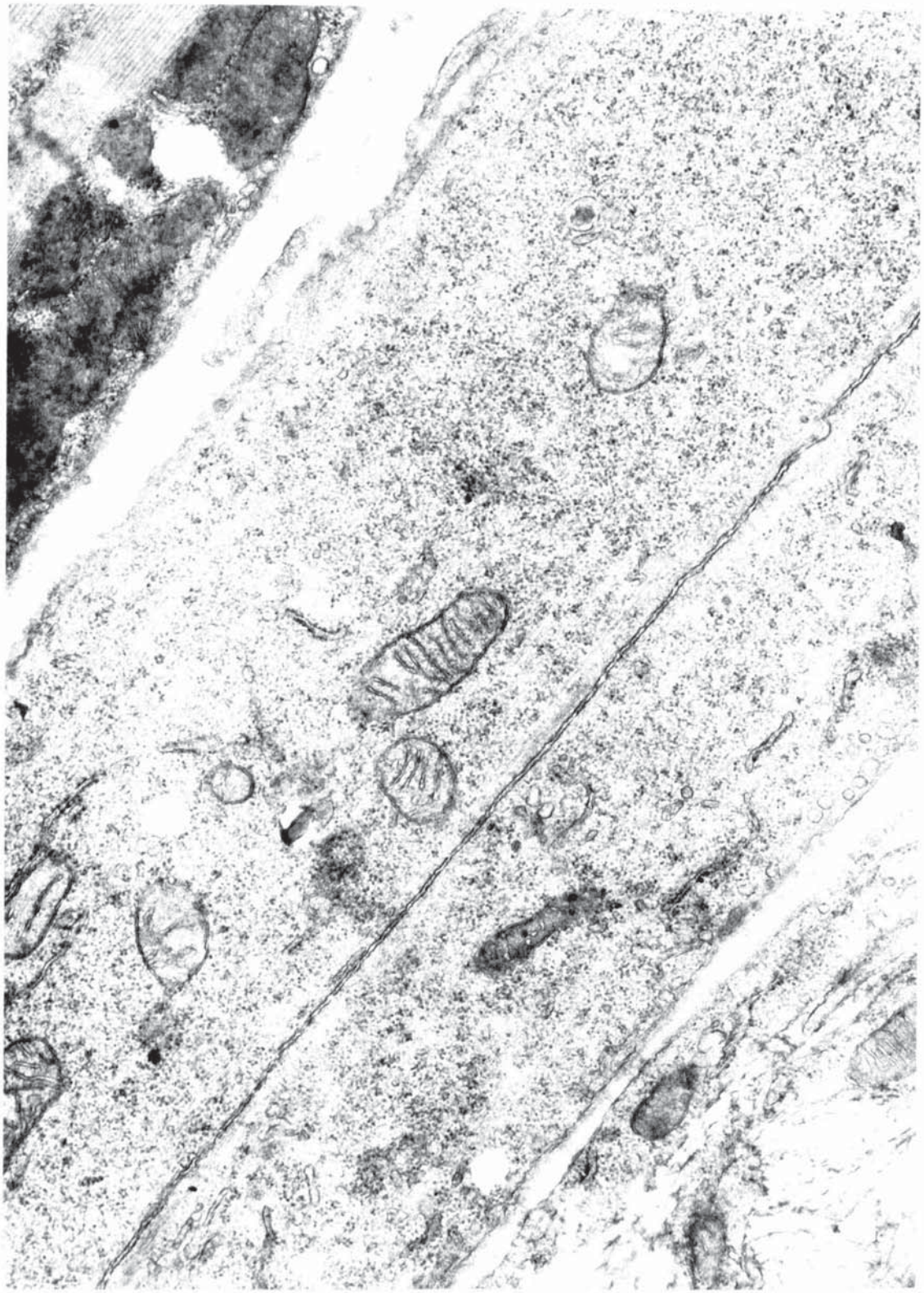
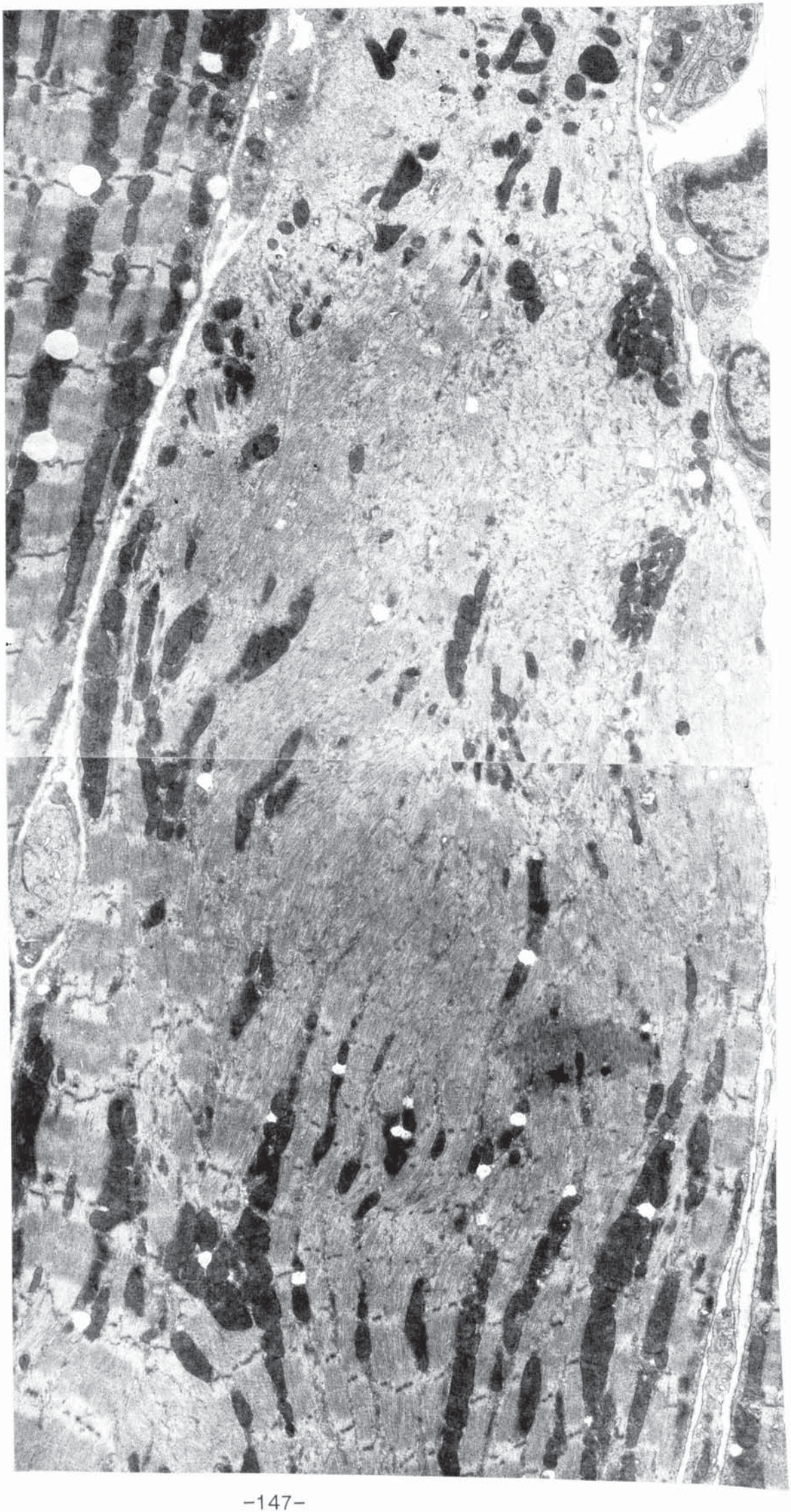


Plate 6.23. Longitudinal section of a hemidiaphragm preparation 48 hours after ECO administration in vivo (x4000). The montage represent the fusion of a regenerating myofiber with a "spared" area which presumably had never been necrotic.



6.3.2.10. 72 hours after ECO administration.

Regeneration was still observed in the diaphragm 72 hours after ECO administration. Only one fibre was necrotic and was still undergoing phagocytosis but most fibres were regenerative or had presumably already regenerated and appeared normal.

An interesting feature of ECO-induced myopathy is that, at all stages of its development, the majority of fibres were ultrastructurally normal. Plate 6.24 shows an apparently normal fibre 6 hours after ECO, a time when many fibres were grossly necrotic. Apparently normal fibres often lie adjacent to severely myopathic fibres (plate 6.25) and it is not known why some fibres become myopathic whilst others, in the same muscle are resistant to ECO treatment. The discrete nature of the myopathy supports the idea that it is due to a specific biological mechanism and not an artifact of tissue processing.

Contraction clumps were observed at all stages of ECO-induced myopathy, the contractions appearing as electron-dense areas, often containing only a few organelles and no recogniseable sarcomeric structure (plate 6.18). Within a contraction the sarcoplasm may have been completely broken down and the fibre expanded transversely (plate 6.18). Lengths of fibre adjacent to the contraction clump often contained the sarcoplasm and organelles which had been 'squeezed' from the contracted region, and Z-lines were misaligned due to stretching of the myofilaments towards the contraction (plate 6.18). Myofilaments may have been

Plate 6.24. Transverse section of a hemidiaphragm preparation 6 hours after ECO administration in vivo (x7500). A "normal-looking" fibre, unaffected by ECO, in a muscle known to contain many grossly necrotic fibres.

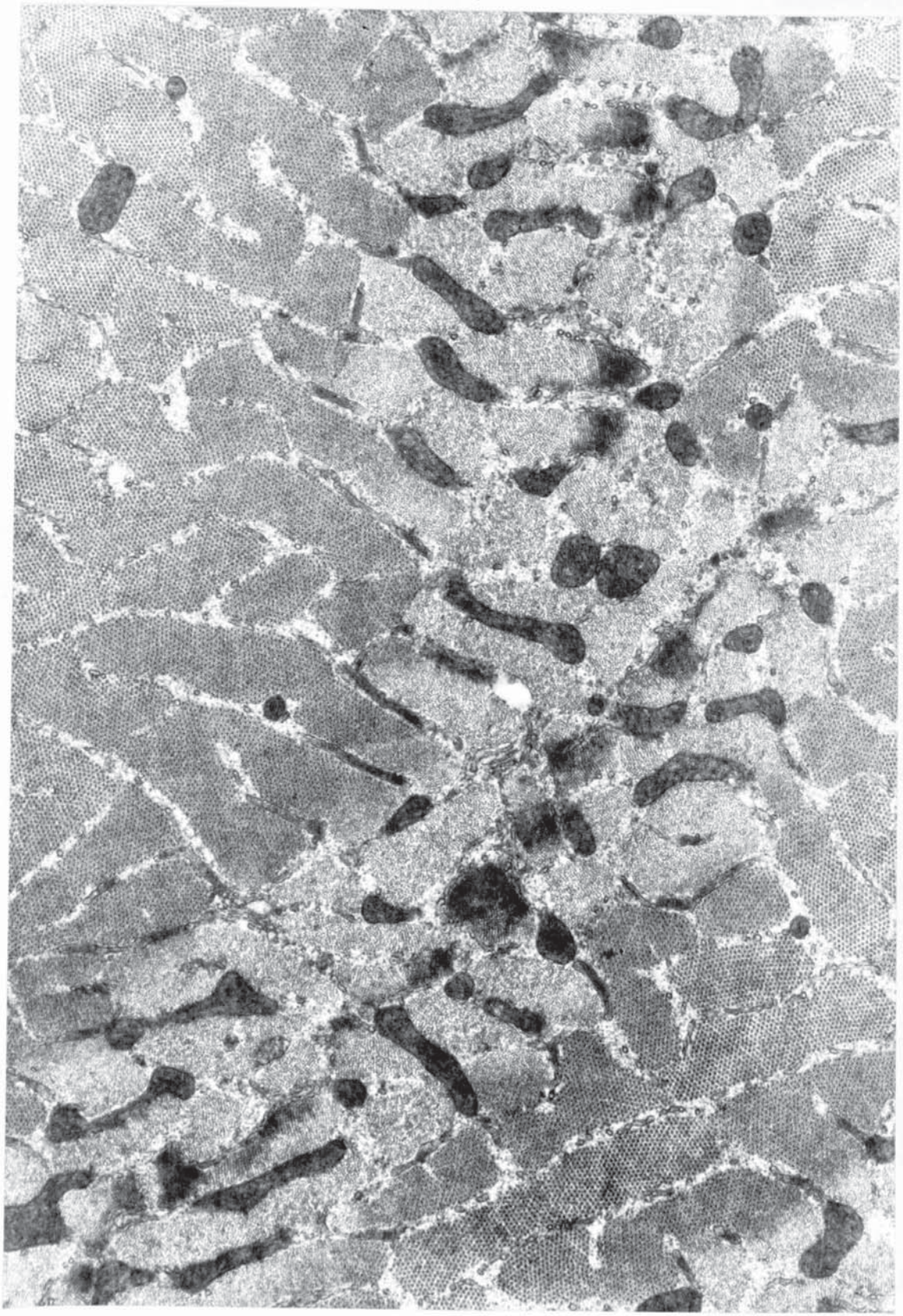
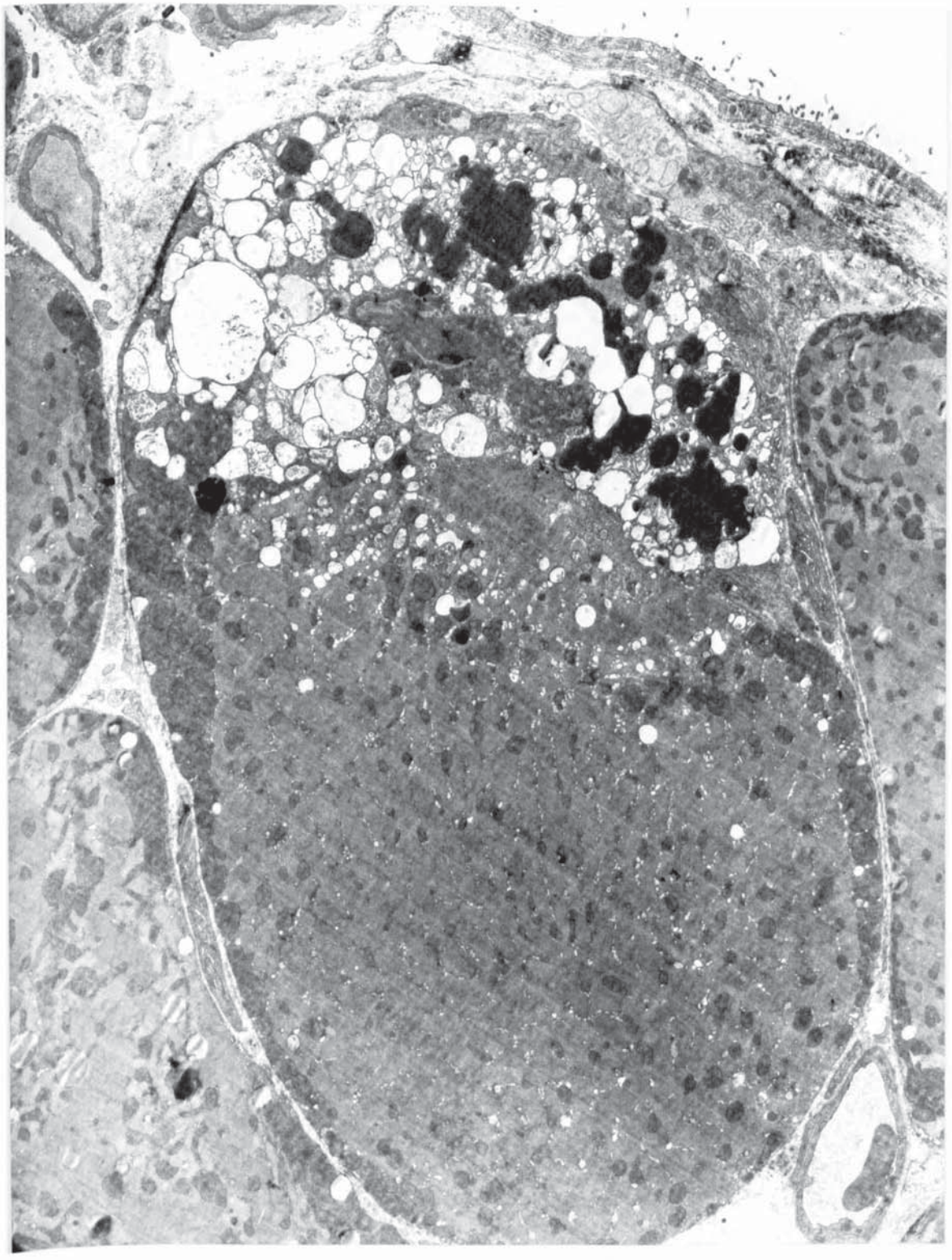


Plate 6.25. Transverse section of a hemidiaphragm preparation 6 hours after ECO administration in vivo (x4000). A severely necrotic fibre may lie adjacent to fibres apparently unaffected by ECO.



torn apart and, in very severe cases, the contraction may have detatched from the rest of the fibre.

In summary, observation of the fine structure of the diaphragm revealed that the number of fibres involved in ECO-induced myopathy increased with time after ECO administration. The % normal fibres decreased with increasing time after ECO, reaching a minimum value at about 6 hours (table 6.2) and the % abnormal fibres increased correspondingly (table 6.2). As the myopathy progressed there was a general increase in the numbers of mild, moderate and severely damaged fibres until approximately 6-12 hours after the intoxication. Grossly damaged fibres appeared at 1 hour after ECO administration and increased in number thereafter, being most frequent at 24 hours (table 6.2) by which time phagocytosis had begun and few fibres were at an early stage of myopathy development. Regenerating fibres were first observed at 48 hours, indicating that recovery of the muscle was underway; The % normal fibres increased accordingly although complete recovery was not achieved within the 72 hour period studied (table 6.2).

At all times during the development and recovery from myopathy the fine-structure of the majority of muscle fibres appeared to be normal.

6.3.3. Morphometric analysis of the subcellular changes observed at the junctional region of the diaphragm at various times after ECO administration in vivo.

Table 6.3 shows that in control tissues the majority of the muscle fibre volume (72.2%) was occupied by normal myofilaments but that following ECO administration *in vivo* the volume fraction (Vv) of normal myofilaments decreased progressively with the development of myopathy. Accompanying the decreased Vv normal myofilaments was a decrease in the Vv normal mitochondria and a corresponding increased index of damaged mitochondria (table 6.3). The Vv damaged mitochondria reached a maximum value (17.1%) one hour after ECO, after which it decreased slightly (table 6.3). Having already ascertained that mitochondria became vacuolated at the later stages of myopathy (6.3.2), this reduction in total Vv mitochondria (both normal and damaged) was not unexpected and table 6.3 indicates that it was infact accompanied by an increased index of vacuoles in the sarcoplasm. The Vv vacuoles was maximal at 3 hours but decreased by 6 hours presumably due to breakdown of mitochondria as the myopathy progressed further (6.3.2). Vacuolation and subsequent obliteration of sarcoplasmic organelles may also account for the drop in the Vv SR at 6 hours, following its dilatation soon after ECO.

The Vv SR, Vv normal mitochondria and the Vv Z-lines were all increased at 12 hours after ECO with respect to their values at 6 hours after ECO, suggesting perhaps that damage at 12 hours was less severe than that at 6 hours after ECO (table 6.3).

Table 6.3:- The volume fractions (Vv) of various organelles in muscle fibres at different times after ECO (6.2.3).

	C.F	N.F	20min	1hr	3hr	6hr	12hr
Normal myofilaments	72.2%	66.0%	50.3%	48.7%	45.0%	32.5%	33.1%
Normal mitochondria	13.0%	18.6%	2.6%	7.3%	2.7%	7.7%	9.5%
SR	10.8%	10.2%	14.0%	13.2%	15.1%	9.8%	16.6%
Sarcoplasm	1.4%	11.9%	15.5%	2.5%	0.32%	2.2%	0.08%
Lipid	0.2%	0.0%	0.0%	1.1%	0.0%	1.6%	0.5%
Z-lines	2.5%	3.3%	1.4%	2.0%	1.4%	0.0%	0.84%
Nucleus	0.0%	0.0%	0.0%	0.0%	1.1%	0.0%	1.6%
Stretched myofils.	0.0%	0.0%	1.6%	0.0%	0.0%	10.6%	2.6%
Disintegrated myofils.	0.0%	0.0%	0.0%	0.0%	0.12%	1.3%	1.6%
Damaged mitochondria	0.0%	0.0%	13.3%	17.1%	14.3%	15.5%	9.1%
Vacuoles	0.0%	0.0%	0.0%	0.36%	10.1%	3.4%	7.7%
Crstals	0.0%	0.0%	1.2%	0.29%	2.8%	3.9%	0.0%
Amorphous mass	0.0%	0.0%	0.0%	0.72%	7.1%	9.8%	14.6%
Invaginations	0.0%	0.0%	0.0%	0.21%	0.04%	0.0%	0.0%
Inclusions	0.0%	0.0%	0.0%	0.14%	0.4%	1.7%	2.4%

C.F- fibres from control diaphragms.

N.F- "normal" fibres in damaged diaphragms.

myofils - myofilaments.

Similarly, crystal formation and membraneous inclusions in muscle fibres became more common as the myopathy progressed but did not feature at all 12 hours after ECO. However the Vv vacuoles and Vv amorphous masses did increase at 12 hours indicating that in some fibres damage was severe (table 6.3), and the % abnormal fibres was still high.

One very interesting feature shown in table 6.3 is the drop in Z-line index within 20 minutes, and its complete obliteration within 6 hours of ECO administration, suggesting that CANP may be involved in the necrotic process.

Generally the volume fractions of normal features decreased whilst those of abnormal features increased progressively after ECO. However, by 6 hours, many areas of muscle fibres had become so badly affected that subcellular organelles, normal or otherwise were completely lost. At this time, such areas often appeared as an amorphous mass of sarcoplasm perhaps containing damaged mitochondria and/or SR.

A comparison of the apparently "normal" fibres in damaged muscles with those of control muscles revealed that the Vv indices of most 'normal' features were similar (table 6.3). The slight drop in Vv normal myofilaments seen in the 'normal' fibres appears to have been compensated by an increased Vv normal mitochondria. Whether this observation was due to non-uniform representation of fibre-type profiles (red fibres containing a higher proportion of mitochondria than white fibres: Gauthier & Padykula, 1966) or to a genuine effect of ECO is not known. It is possible that fibres

containing more mitochondria may be more resistant to attack by ECO.

6.4. DISCUSSION.

In the present study, estimation of the proportions of fibres affected by ECO, by light microscopy (table 6.1), often differed from those made at the ultrastructural level (table 6.2), particularly at the earlier times after ECO administration. The ultrastructural results are considered to be more trustworthy since the higher magnification enabled a more accurate assessment of the morphological state of individual muscle fibres to be made. The low magnification used in the examination of toluidine blue-stained sections could not accurately distinguish genuine myopathy from processing artefacts. In addition, lipid droplets within fibres might have been mistaken for vacuolation resulting from damage due to ECO and slight myopathy may have been missed altogether. However, the general trend of the development of ECO-induced myopathy (ie. increasing with time after ECO administration then recovering gradually), was reflected in the toluidine blue-stained sections which were useful also for the location of necrotic areas within a given section.

At all times following intoxication the majority of fibres remained unaffected by ECO (table 6.2) and there was great variation between fibres with regard to the extent of myopathy induced. Hence some fibres of the diaphragm, a muscle thought to

be composed of a mixture of fibre types (Padykula & Gauthier, 1963) must have been more resistant to ECO than were others. This variability between fibres with regard to the extent of antiChE-induced myopathies was previously noticed by Hudson and Foster (1984) in an investigation of the effect of pyridostigmine on the neuromuscular junction of the rat diaphragm. Subsequently, Hudson et al., (1985) demonstrated that there did not appear to be any selectivity for myopathy associated with a specific fibre type. The reason for the great variability in the extent of the myopathy between fibres, is still unclear: Possibly anticholinesterases act preferentialy at particular motor units, perhaps those which are more active.

Despite the substantial variability in the severity of myopathy between individual muscle fibres of the diaphragm, there was a general, gradual incease in severity with increasing time, reaching a maximum level between approximately 3 hours and 24 hours after ECO administration. However, the observation that myopathy appeared to be less severe in the 12 hour sections than that in both the 6 hour and 24 hour sections was surprising since it seems improbable that myopathy would reach a peak at 6 hours, decrease by 12 hours and then peak again at 24 hours after ECO administration. On observation of the micrographs of the 12 hour sections, no nerve terminals were observed, unlike their regular appearance in sections cut at other times after ECO. It is suggested that a sampling error may have occurred such that the 12 hour sections had not been cut from the 'true' junctional region of the diaphragm at all. The 'true' junctional region could have

been missed at one of two stages during the preparation of the diaphragm for electron microscopy:-

a) the removal of the junctional strip from the glutaraldehyde-fixed hemidiaphragms may have been inaccurate.

b) the trimming and sectioning of the tissue block during the preparation of semithin sections may have passed through the junctional region.

Since myopathy has been shown to be maximal at the junctional region of the diaphragm it will inevitably be less severe in sections cut away from this region although the number of fibres seen to be involved in the myopathy would not be greatly misrepresented. Such a sampling error would also explain the absence of myopathy in transverse sections, 30 minutes after ECO, despite the appearance of necrotic fibres in longitudinal sections of the same muscle. The problem of sampling highlights the necessity for a larger sample size in this study and an obvious progression to this work, given more time, would be to repeat the experiment several times in order to attain an acceptable sample size.

Aswell as the large degree of variability of the severity of ECO-induced myopathy between individual muscle fibres, there was also a huge degree of variability within the affected fibres themselves, not only along their lengths (myopathy being less severe further from the junctional region) but across their diameters, at the junctional region. Severe necrosis consistently appeared to be associated with the nerve terminals, adjacent areas demonstrating a graded degree of damage becoming less severe

further away from the subsynaptic region. Infact, across the diameter of a single fibre, damage was often seen to range from the complete loss of muscle structure and organelles on one side, to simple dilatation of SR at the other (plate 6.15). The results suggest a close involvement of the synapse in ECO-induced myopathy, a feature which has been implicated in other OP-induced myopathies (Fenichel et al., 1972; Kawabuchi et al., 1975; Laskowski et al., 1975; Wecker et al., 1978b).

The severity of myopathy in individual fibres was maximal approximately 24 hours after ECO (table 6.2), when phagocytosis was first observed and the majority of affected fibres were grossly damaged. Regeneration was first observed 48 hours after intoxication when almost 50% of the affected fibres were regenerating (table 6.2). By 72 hours most muscle fibres had regenerated and appeared to be morphologically normal (table 6.2).

Dilatation of SR remained a salient feature throughout ECO-induced myopathy development until profiles became so expanded that they appeared as large vacuoles. This presented itself as an initial increase in the Vv SR followed by an increase in the Vv vacuoles (table 6.3). Finally the vacuoles disappeared and there was an overall drop in both SR and vacuolar indices at 6 hours after ECO (table 6.3). Similarly, an initial increase in the Vv mitochondria, alongside a decrease in the Vv normal mitochondria, was followed by an increase in the Vv vacuoles. The vacuoles eventually disrupted and there was an overall drop in the mitochondrial index with increasing time after intoxication.

Swelling of mitochondria is a recognised early feature of OP poisoning (Laskowski et al, 1975,1977; Leonard & Salpeter, 1982) and it has been suggested (Hudson et al., 1985) that the mitochondrial changes might reflect altered ionic concentrations within the fibres concerned. Since both SR and mitochondria are believed to form an integral part of the iCa^{2+} buffering system of skeletal muscle (Oberc & Engel, 1975) this suggestion seems plausible and, in 1979, Leonard and Salpeter proposed that the extreme disruption of SR and mitochondria beneath the endplate may reflect overloading of the muscle's Ca^{2+} binding capacity. In the present study, SR and mitochondria were seen to swell and vacuolate, presumably, releasing their Ca^{2+} load into the sarcoplasm. It has been suggested that such a massive release of ionised Ca^{2+} may be responsible for the gross cellular destruction associated with many myopathies (Wrogeman & Pena, 1976). In the present study, crystal formation was frequently observed in many mitochondria, and is thought to be due to precipitation of excess intramitochondrial Ca^{2+} with inorganic phosphate ions which accumulate in mitochondria alongside Ca^{2+} (Carafoli & Crompton, 1976). The precipitate is recognised as an electron dense deposit in electron micrographs, and has been seen to occupy the majority of the matrix space when very high levels of Ca^{2+} loading are reached (Brierly & Slauterback, 1964; Greenawalt et al, 1964; Peachy, 1964; Greenawalt & Carafoli, 1966). The calcium phosphate precipitate has been shown to be initially amorphous but

to take on a crystalline form of hydroxyapatite $Ca_3(PO_4)_3Ca(OH)_2$ under certain conditions (Weinbach & Von Brand, 1965). The deposition of calcium phosphate crystals within mitochondria is beleived to reduce the ionised Ca^{2+} in the sarcoplasm but it is a reversible process since much of the Ca^{2+} can still leave the mitochondria under appropriate conditions (Greenawalt et al, 1964). However, such a release of the accumulated Ca^{2+} is very slow and it is unlikely that the deposits can participate in any rapid Ca^{2+} fluxes between the mitochondria and the sarcoplasm (Carafoli & Crompton, 1976). It is possible that, in the current study, deposition of crystals was exacerbated by the use of phosphate buffer during processing of the diaphragms. However, since crystals were not observed in control sections from untreated diaphragms, their deposition must also be a consequence of ECO.

The results of this ultrastructural study implicate an early involvment, possibly by the sequestration of iCa^{2+}, of mitochondria and SR in the development of myopathy in the diaphragm following ECO administration in vivo. It is not yet clear how these early abnormalities are related to the gross destruction of the sarcoplasm but the accompanying loss of Z-lines may implicate involvement of CANP (Busch et al., 1972).

CHAPTER 7

INHIBITION OF ACETYLCHOLINESTERASE (AChE) IN THE BLOOD AND THE DIAPHRAGM DURING THE DEVELOPMENT OF ECO-INDUCED MYOPATHY

7.1 INTRODUCTION

The mechanism of action of OP anti-ChE in the development of myopathy in skeletal muscle is still uncertain. It is possible that the anticholinesterases exert a direct effect on muscle, not involving cholinesterase inhibition at all (Karczmar, 1984), although it is considered most likely that the development of myopathy is due to the resultant accumulation of acetylcholine (ACh) in the synapse following irreversible inhibition of acetylcholinesterase (AChE) (Katz et al., 1973; Fenichel et al., 1974).

Although OP compounds inhibit both AChE and butyrylcholinesterase (BuChE), it is the inactivation of the former that is considered to be crucial to the development of myopathy in skeletal muscle. Wecker et al., (1978a) showed that 90% inhibition of BuChE with OMPA (tetramono-isopropyl-pyrophosphortetramide) did not affect the diaphragm, whilst 85% inhibition of AChE with Paraoxon was accompanied by gross morphological destruction of the muscle. Other workers have also associated OP-induced myopathy development with severe AChE inhibition (Toth et al., 1983; Wecker et al., 1986).

The present study has shown that ECO induced severe necrosis in the junctional region of the diaphragm of mice. Since ECO is known to be a potent inhibitor of AChE (Koelle and Steiner, 1956), it is believed that myopathy is a result of the consequential accumulation of ACh at the neuromuscular junction.

An experiment was designed to assess the extent of inhibition of blood and diaphragm AChE at various times throughout the development and regeneration of ECO-induced myopathy, and to see if whole blood AChE activity reflects the degree of inhibition of tissue AChE as has previously been suggested (Durham & Hayes, 1962; Namba et al., 1971).

7.2 EXPERIMENTAL DESIGN.

Whole blood and diaphragm AChE was measured in groups of mice at various times after ECO administration (20min, 30min, 1hr 3hr, 6hr, 12hr, 24hr, 48hr, 72hr), and in control mice given atropine only (2.2). For each experimental group 6 mice were injected with ECO plus atropine. The mice were anaesthetised at the appropriate times and blood was collected from the femoral artery for analysis of serum CK (2.10) and AChE (2.12). The mice were killed whilst anaesthetised and their diaphragms were removed and cut into hemidiaphragms. One hemidiaphragm was assayed for AChE (2.12), and the other was assessed for myopathy using the Procion technique (2.7).

Statistical analysis of the data was carried out using Students-unpaired-"t"-test.

7.3. RESULTS.

7.3.1 Inhibition of blood and diaphragm AChE at various times after ECO administration.

Table 7.1 shows that ECO caused a highly significant reduction of AChE activity, both in the blood and the diaphragm, within 20 minutes of its administration *in vivo*. However, a more extensive inhibition of diaphragm AChE than blood AChE occurred (table 7.2): In the diaphragm, the mean % inhibition prior to the onset of recovery, 12 hours after ECO, was 83±1% whilst in the blood it was 53±6%.

In blood the early inhibition of AChE was maintained for approximately 6 hours after ECO administration (figure 7.1), enzyme activity begining to recover at 12 hours (figure 7.1), when its activity was shown to be significantly greater than that at 6 hours, ($P<0.02$: unpaired-"t"-test), and not significantly different from that of control blood. Enzyme recovery continued gradually until, by 72 hours blood AChE activity was significantly greater than that of control mice ($P<0.001$: table 7.1).

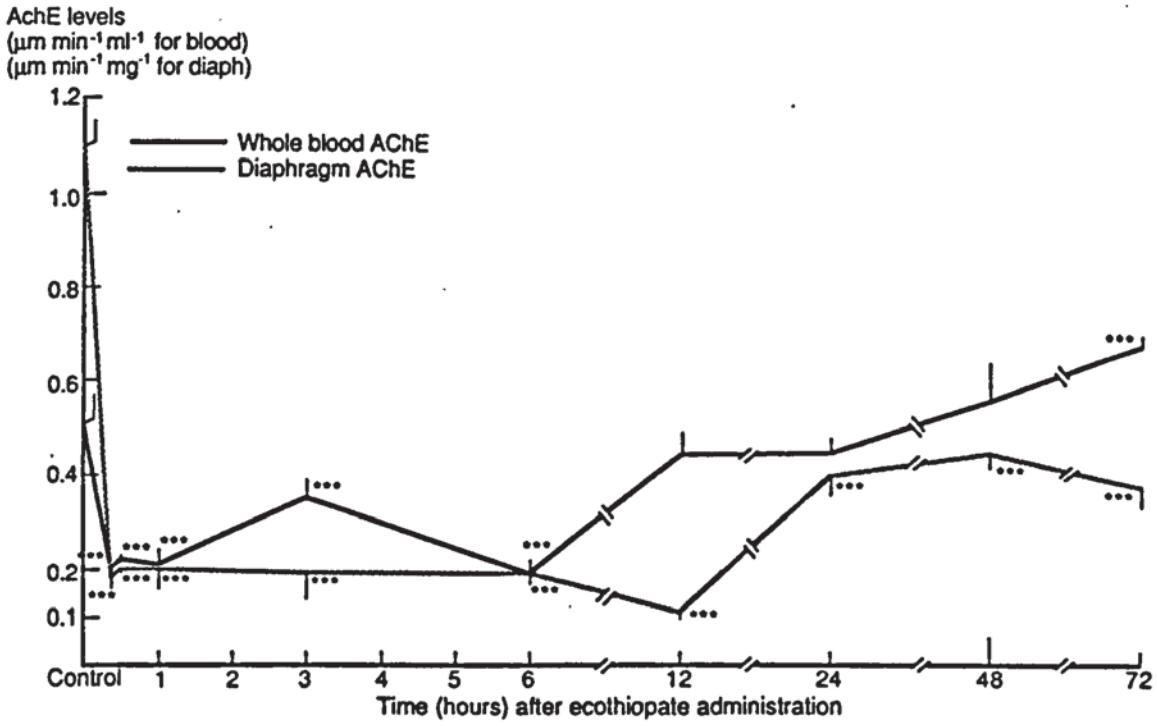
In the diaphragm the extent of inhibition remained at approximately 83% for about 6 hours, after which it increased further still, ($P<0.02$), reaching a maximum level approximately 12 hours after ECO administration (figure 7.1). There was some recovery of diaphragm AChE at 24 hours after ECO (table 7.2), but

Table 7.1 Blood and diaphragm AChE activity at various times after ECO administration in vivo: the results represent the mean±standard error from 6 experiments.

	Whole Blood AChE ($\mu mmin^{-1}ml^{-1}$)	Diaphragm AChE ($\mu mmin^{-1}mg^{-1}$)
Control	0.52 ± 0.05	1.11 ± 0.05
20min	0.20 ± 0.03 ***	0.19 ± 0.02 ***
30min	0.23 ± 0.01 ***	0.20 ± 0.01 ***
1hr	0.22 ± 0.03 ***	0.20 ± 0.04 ***
3hr	0.36 ± 0.04 ***	0.19 ± 0.05 ***
6hr	0.19 ± 0.03 ***	0.19 ± 0.02 ***
12hr	0.44 ± 0.08	0.14 ± 0.01 ***
24hr	0.44 ± 0.05	0.40 ± 0.04 ***
48hr	0.55 ± 0.07	0.45 ± 0.02 ***
72hr	0.68 ± 0.02 ***	0.38 ± 0.03

*** indicates results which are significantly different from those of the control group at the 0.1% level, using Students-unpaired-'t'-test.

Figure 7.1. Blood and diaphragm AChE activity at various times after ECO administration in vivo:- the results represent the mean ± standard error of 6 experiments.



*** indicates that a result is significantly different from the control group, at the 0.1% level, using Students-unpaired-'t'-test.

Table 7.2 The % inhibition of AChE in the blood and the diaphragm at various times after ECO administration in vivo.

	% Inhibition of AChE	
	Blood	Diaphragm
Control	0	0
20min	60	83
30min	55	82
1hr	57	79
3hr	31	83
6hr	63	83
12hr	27	88
24hr	15	64
48hr	-9	59
72hr	-32	66

The % inhibition of AChE was calculated at each time for both blood and diaphragm using the formula:-

$$\% \text{ inhibition at time X} = 100-\left(\frac{\text{AChE activity at time X}}{\text{Control AChE activity}}\right)$$

A sample size of 6 mice was used at each time studied.

a high, fairly constant level of inhibition was maintained (averaging 63%) for the remainder of the period under study (72hrs).

7.3.2. Morphology of the diaphragm in relation to % inhibition of blood and diaphragm AChE.

Control hemidiaphragm preparations exhibited the normal skeletal muscle morphology, (3.3.1). Striations were evident within the muscle fibres, no hypercontractions were seen, and Procion staining was restricted to connective tissue only.

Accompanying the highly significant inhibition of blood and diaphragm AChE within 20 minutes of ECO administration, was the appearance of hypercontracted endplates in some fibres, a feature that has already been shown to represent the initial stages of myopathy development induced by ECO *in vivo* (3.3.1). All other aspects of muscle morphology were unaffected and, at 30 minutes, there was no further change in the histological appearance of the tissue.

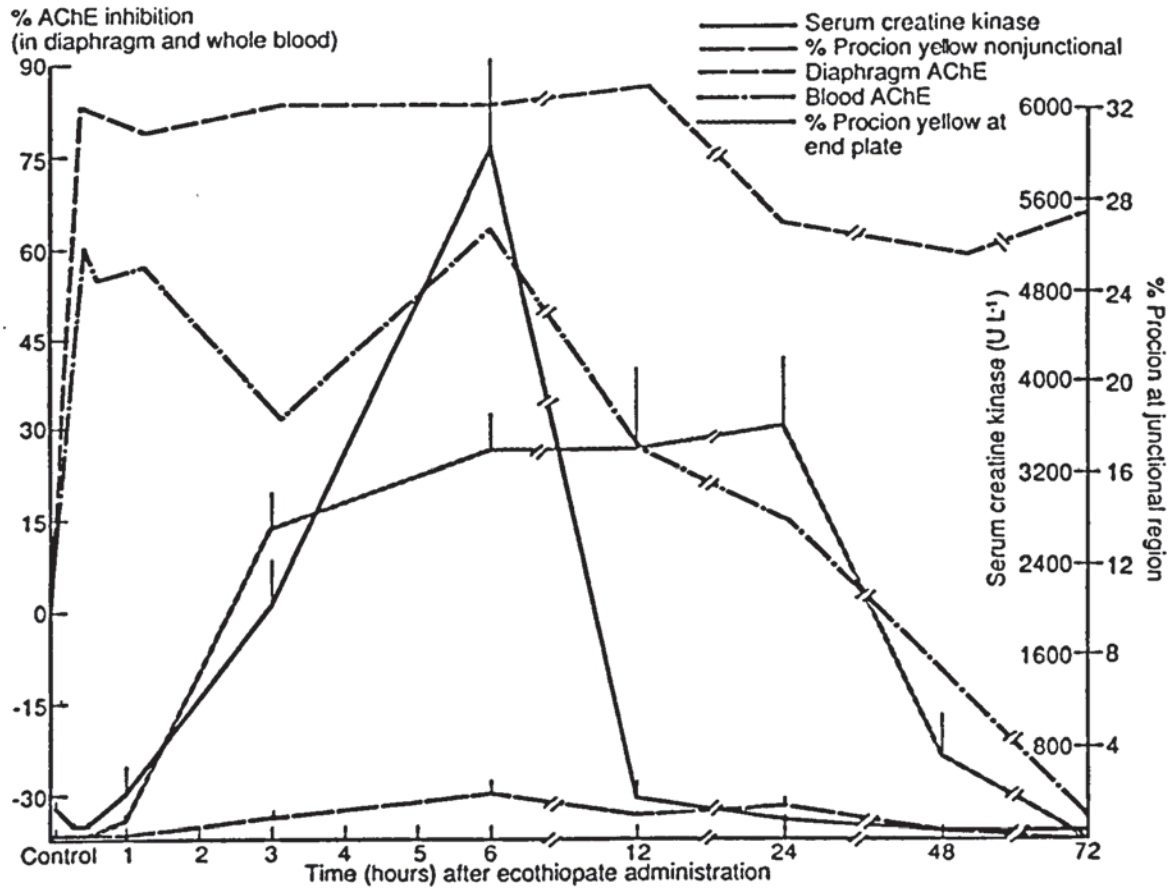
AChE activity remained depressed at a fairly constant level, whilst Procion staining plus hypercontractions appeared, then proliferated within the muscle fibres as the myopathy progressed (table 7.3) ie. increased enzyme inhibition was not required for the further development of myopathy nor for the subsequent progressive elevation of serum CK (figure 7.2). However, it has already been shown (Wecker et al., 1978b) that AChE activity must be inhibited to a critical degree to induce

Table 7.3. The relationship between AChE inhibition, serum CK activity, and % Procion staining in the diaphragm, at various times after ECO: the results represent the mean +/- standard error of 6 experiments.

	% Inhibition AChE		Serum CK	% Procion staining	
	Blood	Diaphragm	(UL^{-1})	junctional	nonjunctional
Control	0%	0%	294±113	0.21±0.19	0.07±0.04
20min	60%***	83%***	126±60***	0.07±0.06	0.01±0.01*
30min	55%***	82%***	125±55	0.01±0.01	0.02±0.03
1hr	57%***	79%***	440±181	0.78±0.36	0.03±0.02
3hr	31%***	83%***	2057±420***	13.50±1.70***	0.56±0.14***
6hr	63%***	83%***	6120±757***	16.90±1.65***	1.87±0.60***
12hr	27%	88%***	391±122	17.10±3.50***	0.93±0.32***
24hr	15%	64%***	153±48	18.10±2.70***	1.46±0.14***
48hr	-9%***	59%	114±24	3.70±1.77*	0.57±0.34
72hr	-32%***	66%***	101±28	0.30±0.29	0.19±0.14

*** and * indicate results which are significantly different from those of control mice, at the 0.1% and 5% levels respectively, using Students-unpaired-"t"-test.

Figure 7.2. The relationship between AChE inhibition, serum CK and % Procion staining in the diaphragm at various times after ECO administration in vivo:- the results represent the mean ± standard error of 6 experiments.



severe skeletal muscle fibre necrosis in rat skeletal muscle. In her experiments, Wecker found the critical level of AChE inhibition to be approximately 85%, a value similar to the 83% observed in the current study.

In the current study, within 12 hours of ECO administration, when blood AChE had recovered significantly, the % Procion staining of the diaphragm plateaued and serum CK activity had decreased dramatically (figure 7.2) probably due to the reduced myopathy development plus elimination of the enzyme from the blood stream. Following this plateau of maximum myopathy the muscle seemed to recover its normal morphology, within the 72 hour period studied, despite persistently low AChE activity in the diaphragm itself (figure 7.2). It seems that further AChE recovery in the diaphragm was not required for the complete repair of ECO-induced myopathy.

7.4 DISCUSSION.

It has been reported that the *in vivo* administration of an acute dose of OP causes a high degree of AChE inhibition in mammals (Koelle & Steiner, 1956; Wecker et al.,1978 a&b; French et al., 1983; Toth et al., 1983), and it is generally agreed that enzyme inhibition occurs within 30 minutes of the exposure (Wecker & Dettbarn, 1976; Wecker et al., 1978a&b; Goudou & Reiger, 1983). In the present study, 83% of diaphragm AChE was inhibited within 20 minutes of ECO administration (table 7.2), inhibition remaining at this level for 12 hours. The % inhibition of blood AChE (63%) was considerably less than that of diaphragm AChE (table 7.2), implying that, contrary to reports by Durham & Hayes, (1962), and Namba et al., (1971) inhibition of the blood enzyme is not always a good indicator of synaptic inhibition.

In both blood and the diaphragm there was prolonged AChE inhibition between 20 minutes and 6 hours (table 7.2), after which blood AChE rapidly recovered reaching a value, at 72 hours, which was 32% greater than the control activity. However, diaphragm AChE recovered by only about 25% at 24 hours after the intoxication, there being no further recovery of AChE activity within the duration of the experiment. It is possible that maximal inhibition of blood AChE may be indicative of maximal inhibition in the diaphragm for the period between 20 minutes and 6 hours after ECO, but neither the extent of inhibition nor recovery of blood AChE reflects that of the diaphragm in any way.

In the present study, the recovery halflife of blood AChE was approximately 12 hours, whilst that of diaphragm AChE was considerably longer and was even in excess of the 72 hour period under study. It is possible that such a discrepancy may have been a real affect of ECO, or simply a result of the experimental technique, possibly due to a more rapid hydrolysis of the ECO-AChE complex in blood once in vitro circumstances prevailed.

During the recovery of OP-inhibited AChE, two reactions occur simultaneously in vivo. The first is regeneration of inhibited enzyme by spontaneous hydrolysis of the enzyme-inhibitor complex, and the second is synthesis of new enzyme. In 1984 Newman et al., reported that whilst non-endplate AChE (inhibited by ECO) had a turnover (regeneration) rate with a halflife of 13 hours, endplate AChE turnover was significantly slower. It is possible therefore that the higher proportion of endplate AChE existing within the diaphragm (approximately 65%:35%; Newman et al., 1984) may lower the overall AChE turnover rate within the muscle. However, in the same study (Newman et al., 1984), a spontaneous reactivation of ECO-inhibited AChE with a halflife of 27 hours was observed. Therefore, even if the turnover of AChE in the diaphragm was completely ignored, the halflife of AChE recovery should still be approximately 27 hours. This was obviously not so in the current study.

The fact that blood AChE recovered so rapidly after only 12 hours of intoxication by ECO suggests that either spontaneous hydrolysis of the ECO-AChE complex, or synthesis of new AChE occurs more rapidly in the blood than in the diaphragm. It also

conflicts with reports (Durham & Hayes, 1962; Wulfson et al., 1966; Namba et al., 1971) of a very slow enzyme recovery in the blood, taking 3-4 weeks to regain control levels, the authors concluding that since blood AChE remained inhibited after the dissappearance of cholinergic symptoms, the recovery of tissue AChE must be more rapid than that of blood AChE. It was even suggested (Grob & Harvey, 1953; Durham & Hayes, 1962) that blood AChE is not regenerated at all and that the rate of recovery reflects replacement of new erythrocytes, therefore requiring 90-100 days to regain control values. This clearly was not the case with ECO, and blood AChE appears to have been both less susceptable to inhibition than diaphragm AChE and more efficient at recovering from the inhibition (table 7.2). The fact that by 72 hours blood AChE activity actually exceeded that of control mice may be due partly to the production of new erythrocytes by the spleen, but it is obvious from the rate of recovery that some enzyme regeneration must have occurred.

By 72 hours after ECO administration, when recovery of muscle morphology was complete, diaphragm AChE was still inhibited by about 66% (table 7.2), indicating a slow rate of spontaneous reactivation and de novo synthesis of enzyme, but supporting the observation that very little functional AChE is required for the normal operation of the muscle cell physiology (Hobbiger, 1976).

CHAPTER 8

THE ROLE OF CALCIUM IN THE DEVELOPMENT OF ECO-INDUCED MYOPATHY.

8.1. INTRODUCTION.

The present study has established that myopathy, induced by ECO at the junctional region of the diaphragm, is preceeded and accompanied by an influx of Ca^{2+} from the extracellular fluid (5.3). Since elevated intracellular calcium has previously been implicated as a causative agent in many myopathies (Wrogeman & Pena, 1976; Bodensteiner & Engel, 1978; Cullen et al, 1978; Ross-Canada et al., 1983), including those induced by OP agents such as diisopropylfluorophoshate (DFP: Leonard & Salpeter, 1979; Toth et al., 1983) it was suspected that the elevated iCa^{+}, induced by ECO, might be responsible for the subsequent development of myopathy. However, the role of iCa^{2+} in promoting muscle damage is still undetermined. One theory is that a calcium activated neutral protease (CANP) may cause dissassembly of intact myofibrils (Busch el al, 1972; Dayton, 1975; Reville, 1976; Inomata et al, 1983; Reddy et al, 1975, 1983). CANP is known to specifically destroy Z disks when applied to isolated myofilaments (Reddy et al, 1975; Dayton et al., 1975; Reville et al, 1976; Dayton & Schollmeyer, 1980; Ishiura et al, 1980; Sugita, 1980) and since, in the present study, Z-line loss was shown to be a prominent feature of ECO-induced myopathy (6.3.2), the possibility that CANP might be involved in the early stages of myopathy development was considered.

Several substances have been found capable of inactivating CANP (M^{c}Gowan et al, 1976; Libby & Goldberg, 1978; Toy-Oka et

al, 1978; Sugita et al, 1980) but, of these, leupeptin has been found to be the most consistently effective and the most potent inhibitor of CANP (Suzuki et al, 1981). Moreover, leupeptin has been shown to delay the degeneration of skeletal and cardiac muscles both *in vitro* and *in vivo* (McGowan et al, 1976; Stracher & McGowan, 1978; Libby & Goldberg, 1978).

The aims of the experiments described in this chapter were:

1. To investigate the requirement for extracellular Ca^{2+} at various stages during the development of ECO-induced myopathy. This was achieved by exposing ECO-treated phrenic nerve-diaphragm preparations to Ca-reduced saline (A2) using the *in vivo/in vitro* technique (2.5) since reduction of extracellular Ca^{2+} is not possible *in vivo*

2. To investigate the effect on the development of myopathy, of preventing the entry of Ca^{2+} into muscle fibres, by using the Ca-antagonist diltiazem *in vitro*. Both the *in vivo/in vitro* plus the *in vitro* techniques were used (sections 2.5 & 2.4 respectively).

3. To investigate the effect *in vivo*, of a range of membrane ion channel blockers on the development of ECO-induced myopathy, when administered prior to ECO *in vivo*. It was hoped that, by preventing the entry of certain ions (eg. Na^+, Ca^{2+}), elevation of iCa^{2+} would be prevented and myopathy therefore not produced.

4. To investigate a possible role for CANP in the development of ECO-induced myopathy, using leupeptin. The *in vivo/in vitro* plus the *in vitro* techniques were used (sections 2.5 & 2.4 respectively) since leupeptin is reported to be rapidly metabolised and excreted when administered *in vivo* (Libby & Goldberg, 1978).

8.2. EXPERIMENTS INVESTIGATING THE REQUIREMENT FOR EXTRACELLULAR CALCIUM FOR THE DEVELOPMENT OF ECO-INDUCED MYOPATHY.

8.2.1. Experimental Design.

8.2.1.1. The requirement for extracellular Ca^{2+} in the progression of myopathy *in vitro* in the diaphragms of mice exposed to ECO for 30 minutes *in vivo*.

Six mice were injected with ECO plus atropine (2.2) and were killed 30 minutes later. Their phrenic nerve-diaphragm preparations were prepared according to the *in vivo/in vitro* technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in Ca-reduced saline (A2) throughout the incubation period *in vitro*, and was then stained with Procion: The contralateral preparation was stained immediately on its removal from the animal (ie. 30 minutes after ECO administration). Myopathy was assessed using the Procion technique and Students-paired-'t'-test was used to compare the two treatments. It was thus established whether myopathy had progressed *in vitro* in preparations exposed to reduced extracellular Ca^{2+}.

Further statistical comparison was made, using Students-unpaired-'t'-test, between the myopathy developed in preparations stimulated in Ca-reduced saline (2hours 30 minutes) and that

developed in preparations stimulated in normal Lileys saline (A2) for the duration of the incubation period (2 hours 30 minutes). The latter preparations (n=7) were prepared for an investigation of the requirement for nerve stimulation during the development of ECO-induced myopathy (9.2).

8.2.1.2. The requirement for extracellular Ca^{2+} in the progression of myopathy *in vitro* in diaphragms of mice exposed to ECO for 60 minutes *in vivo*.

Six mice were injected with ECO plus atropine (2.2) and were killed 60 minutes later. The phrenic nerve-diaphragm preparations were then prepared according to the *in vivo/in vitro* technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in Ca-reduced saline (A2) and the other in normal Lileys saline (A2) for the duration of the incubation period (2 hours) *in vitro*. Both sets of preparations were finally stained with Procion and assessed for myopathy. Students-paired-'t'-test was used to compare the two treatments and therefore to establish whether the reduction of extracellular Ca^{2+}, 60 minutes after ECO administration *in vivo*, had any effect on the subsequent progression of myopathy *in vitro*.

8.2.1.3. The effects on the in vitro progression of myopathy, of delaying a 30 minute exposure to extracellular Ca^{2+}, for increasing periods following ECO administration in vivo.

Thirteen mice were injected with ECO plus atropine and were killed 30 minutes later. Their phrenic nerve-diaphragm preparations were prepared according to the in vivo/in vitro technique described in section 2.5. and the preparations were divided into 4 groups:-

Group 1 (n=7): Phrenic nerve-diaphragm preparations were stimulated in normal Lileys saline (A2) throughout the incubation period (2 hours 30 minutes).

Group 2 (n=6): Phrenic nerve-diaphragm preparations were stimulated in normal Lileys saline (A2) for 30 minutes and then in Ca-reduced saline (A2) for the remainder of the incubation period (2 hours).

Group 3 (n=6): Phrenic nerve-diaphragm preparations were stimulated in Ca-reduced saline (A2) for 30 minutes, then in normal Lileys saline (A2) for 30 minutes and finally in Ca-reduced saline (A2) for the remainder of the incubation period (1 hour 30 minutes).

Group 4 (n-6): Phrenic nerve-diaphragm preparations were stimulated in Ca-reduced saline (A2) for 60 minutes, then in normal Lileys saline (A2) for 30 minutes and finally in Ca-reduced saline for the remainder of the incubation period (1 hour).

All preparations were then assessed for the extent of myopathy, using the Procion technique. Statistical comparisons were drawn between the groups using Students-unpaired-'t'-test.

8.2.2. Results and Discussion.

Preliminary experiments using the *in vivo/in vitro* and *in vitro* techniques confirmed that:-

a) stimulation of untreated phrenic nerve-diaphragm preparations *in vitro* in normal Lileys saline, for up to 3 hours, does not induce muscle necrosis and the tissues were not stained by Procion.

b) following an *in vivo* exposure to ECO of greater than 30 minutes, subsequent development of myopathy could be supported *in vitro* by stimulation of the preparation in normal Lileys saline.
N.B. A 15 minute exposure to ECO *in vivo* was found to be insufficient to initiate a myopathy which could be developed *in vitro*.

8.2.2.1. The requirement for extracellular Ca^{2+} for the progression of myopathy *in vitro* in the diaphragms of mice exposed to ECO for 30 minutes *in vivo*.

Contraction records.

Control preparations, stimulated in normal Lileys saline following a 30 minute exposure to ECO *in vivo* (9.2), elicited strong contractions throughout the incubation period (figure 8.1): on exposure to Ca-reduced saline, at the end of the 3hr incubation period, the contraction magnitude decreased rapidly dissappearing entirely within 5-10 minutes.

Experimental preparations, stimulated in Ca-reduced saline following a 30 minute exposure to ECO *in vivo*, exhibited a decline in contraction magnitude begining immediately on exposure to Ca-reduced saline. After 5-10 minutes the contractions stopped altogether (figure 8.2).

Since muscle contraction is known to depend on the release of Ca^{2+} from intracellular SR (Sandow, 1965; Ebashi & Endo, 1968; Costantin, 1975) and not on the influx of Ca^{2+} from the extracellular fluid, the decline in contraction magnitude, observed in preparations following the removal of extracellular Ca^{2+}, is not thought to be due to a reduced influx of Ca^{2+} at the post-synaptic membrane. It is more likely to be the result of an inhibition of ACh release from the nerve terminal on exposure to a reduced extracellular Ca^{2+}.

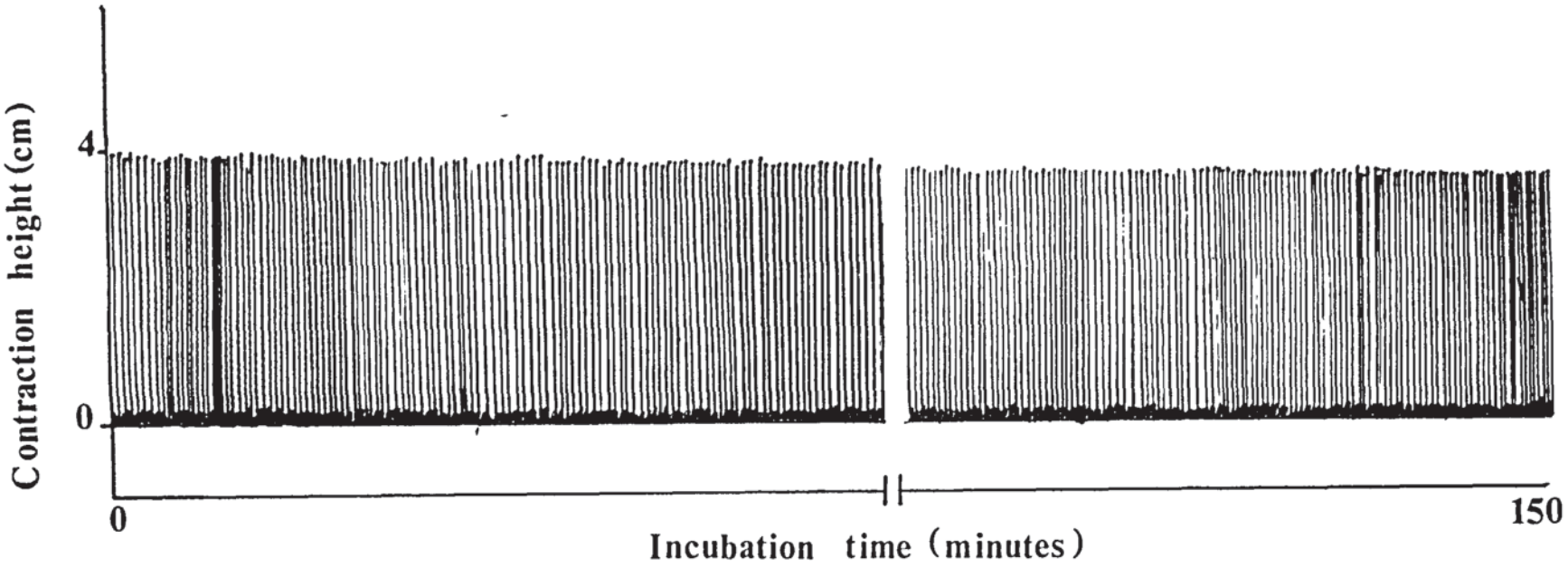


Figure 8.1. Control preparation stimulated in normal Lileys saline for 2 hours 30 minutes, following a 30 minute exposure to ECO in vivo (9.2), elicited strong contractions throughout the incubation period.

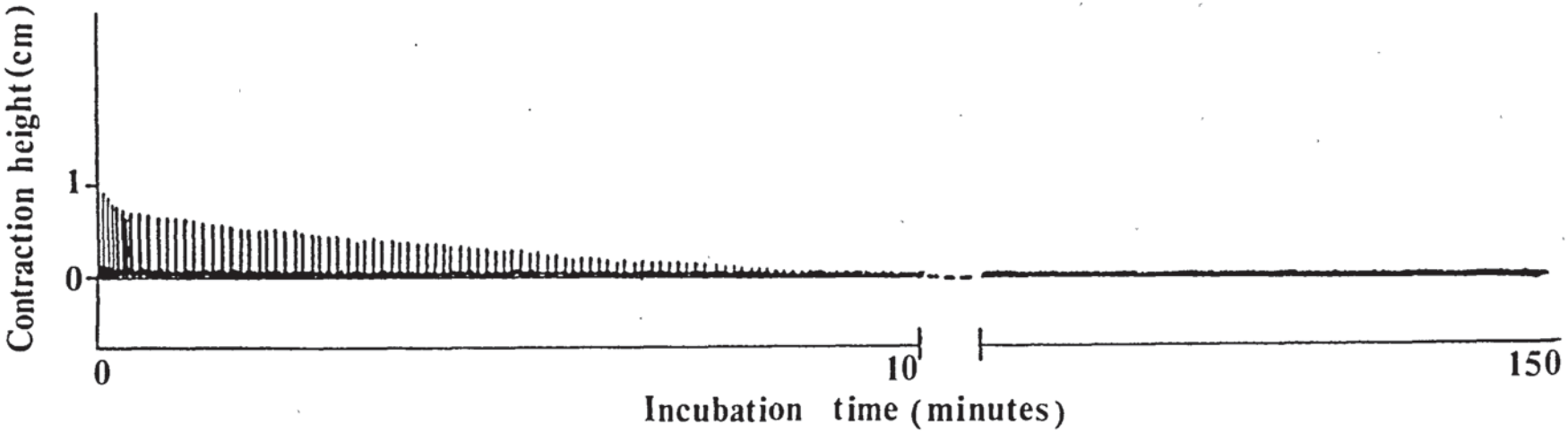


Figure 8.2. Preparations stimulated in Ca-reduced saline for 2 hours 30 minutes, following a 30 minute exposure to EDO in vivo, elicited a decline in contraction magnitude begining immediately on exposure to Ca-reduced saline. After approximately 10 minutes the contractions stopped altogether.

Histological examiniation of preparations.

Those preparations that were stained with Procion 30 minutes following ECO administration *in vivo*, showed signs of only slight myopathy manifested by slight Procion staining and hyper-contraction at the junctional region of the diaphragm, the non-junctional region being unaffected. This was consistent with the 30 minute hemidiaphragm preparations observed in earlier experiments (3.3.2 & 7.3.2).

Preparations that were stimulated in normal Lileys saline (for 2hrs 30mins), following a 30 minute exposure to ECO *in vivo*, had developed severe myopathy, represented by extensive Procion staining (table 8.1) and hypercontraction at the junctional region of the diaphragm ie. myopathy development was supported *in vitro* by stimulation of the preparation in normal Lileys saline, and the % Procion staining was not significantly different from that induced by a 3 hour exposure to ECO *in vivo* (3.3.2: %Procion staining = 10.10±3.2 (2.08±1.01) at the junctional (non-junctional) regions respectively).

However, myopathy did not progress much *in vitro* in the preparations stimulated in Ca-reduced saline, following a 30 minute exposure to ECO *in vivo* (table 8.1). Myopathy existing at the junctional region of these preparations was greater than that induced in hemidiaphragms 30 minutes after ECO administration ($P<0.1$: table 8.1), but less than that developed in normal Lileys saline 30 minutes after ECO administration *in vivo* ($P<0.1$: table 8.1).

Table 8.1. % Procion staining of phrenic nerve-diaphragm preparations exposed to ECO for 30 minutes *in vivo* and of those stimulated in normal Lileys saline (NL)/Ca-reduced saline following a 30 minute exposure to ECO *in vivo*:- the results represent the mean± standard error of 7 experiments.

	% Procion staining		
	ECO 30min	ECO 30min + stim 2hr. 30min in NL	ECO 30min + stim 2hr. 30min in Ca-reduced saline.
Junctional	2.49±0.27	19.0±5.70***	5.17±1.00$^{**}_{++}$
Nonjunctional	0.05±0.02	2.5±0.62***	0.59±0.18$^{***}_{++}$

stim indicates stimulation.

***, ** indicates results which are significantly different, at the 0.1% and 1% levels respectively, from the preparations of mice injected with ECO for 30 minutes *in vivo*, with no further treatment *in vitro*, using Students-'t'-tests.

++ indicates results which are significantly different, at the 1% level, from the preparations of mice injected with ECO for 30 minutes then stimulated in normal Lileys saline for 2 hours 30 minutes, using Students-unpaired-'t'-tests.

It is concluded that the reduction in the extracellular Ca^{2+} concentration significantly reduced the development of myopathy normally induced by ECO in the diaphragms of mice (figure 8.3).

Since the Ca-reduced saline contained trace elements of Ca^{2+} ($5x10^{-6}$M: S.Das, personal communication) it is unlikely that Ca^{2+} would have leaked out of the diaphragm against the, still strong inward electrochemical gradient. However, it is possible that Ca^{2+} entry into diaphragms exposed to Ca-reduced saline was minimised such that elevation of iCa^{2+} might have been reduced and myopathy development thus regulated. Certainly, it has been suggested that when the extracellular Ca^{2+} concentration is less than 10^{-5}M, Na^{+} and K^{+} influx may occur via voltage operated calcium channels, in preference to Ca^{2+} influx (Almers & M^{c}Clesky, 1984). It would be an interesting progression to the current experiment to actually assay the calcium contents of the preparations concerned, in order to determine whether Ca^{2+} influx had infact been reduced. A further progression would be to repeat the experiment using EGTA in the Ca-reduced saline, as was done by Leonard and Salpeter (1979): perhaps then, by preventing Ca^{2+}-influx completely, ECO-induced myopathy would be completely prevented?

It has already been suggested (8.2.2.1), that the reduction of extracellular Ca^{2+} is likely to have had a presynaptic effect on the phrenic nerve preparation, possibly reducing the release of

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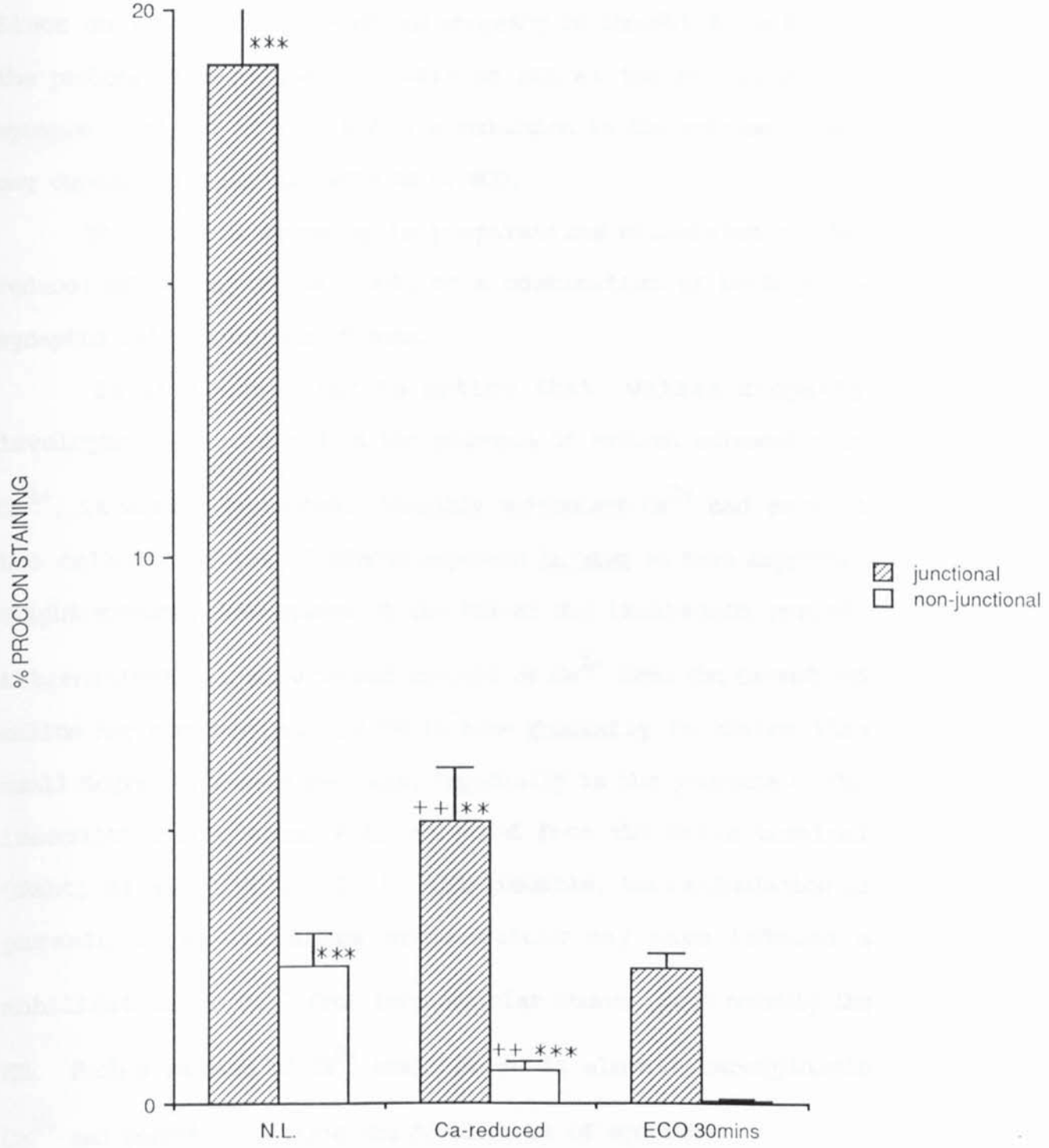
Figure 8.3. % Procion staining of phrenic nerve-diaphragm preparations exposed to ECO for 30 minutes in vivo and of those stimulated in normal Lileys saline (NL)/Ca-reduced saline following a 30 minute exposure to ECO in vivo:- the results represent the mean ± standard error of 7 experiments.



***, ** represent results which are significantly different, at the 0.1% and 1% levels respectively, from preparations pertaining to mice injected with ECO for 30 minutes with no further treatment in vitro, using Students-'t'-tests.

++ indicates results which are significantly different, at the 1% level, from preparations pertaining to mice injected with ECO for 30 minutes then stimulated in normal Lileys saline (NL) for 2 hours 30 minutes in vitro, using Students-unpaired-'t'-test.

ACh from the nerve terminal: the contraction records (figure 8.2) indicate that the stimulated release of ACh from nerve terminals may have stopped in preparations exposed to Ca-reduced saline. Since anticholinesterase-induced myopathy is thought to be due to the prolongation of the half-life of ACh at the neuromuscular synapse (Fenichel et al., 1974), a reduction in the release of ACh may oppose the myopathic effects of ECO.

The reduced myopathy in preparations stimulated in Ca-reduced saline may be the result of a combination of both post-synaptic and presynaptic effects.

It is interesting to notice that, whilst myopathy development was reduced in the presence of reduced extracellular Ca^{2+}, it was not prevented. Possibly sufficient Ca^{2+} had entered the cell during the 30 minute exposure in vivo to have supported slight myopathy development by the end of the incubation period. Alternatively, a slow inward current of Ca^{2+} from the Ca-reduced saline may have been sufficient to have gradually initiated this small degree of muscle necrosis, especially in the presence of Ca-insensitive nonquantal ACh, released from the nerve terminal (Tabti et al., 1987). It is also possible, that stimulation of phrenic nerve-diaphragm preparations may have induced a mobilisation of Ca^{2+} from intracellular stores, most notably the SR. Such a release of Ca^{2+} would serve to elevate sarcoplasmic Ca^{2+} and possibly initiate the development of myopathy.

Whatever the mechanism, the reduction of extracellular Ca^{2+}, 30 minutes after ECO did significantly reduce the subsequent

development of myopathy *in vitro* (figure 8.3) and it was decided to investigate whether the reduction of extracellular Ca^{2+} would effectively hinder myopathy at a later stage in its development.

8.2.2.2. The requirement for extracellular Ca^{2+} for the progression of myopathy *in vitro* in diaphragms of mice exposed to ECO for 60 minutes *in vivo*.

Contraction records.

Control preparations, stimulated in normal Lileys saline for 2 hours following a 60 minute exposure to ECO *in vivo*, elicited strong contractions throughout the incubation period.

Preparations stimulated in Ca-reduced saline for 2 hours, following a 60 minute exposure to ECO *in vivo*, showed a gradual decline in the contraction magnitude begining immediately on exposure to Ca-reduced saline and stopping altogether after 5-10 minutes.

Histological examination of preparations.

Both sets of preparations were severely myopathic at their junctional regions, nonjunctional regions being comparatively unaffected (table 8.2) ie. the reduction of extracellular Ca^{2+} 60 minutes after ECO administration *in vivo*, did not affect the

Table 8.2. % Procion staining of phrenic nerve-diaphragm preparations stimulated in normal Lileys saline (NL)/Ca-reduced saline following a 60 minute exposure to ECO *in vivo*:- the results represent the mean ± standard error of 6 experiments.

	% Procion staining	
	NL saline	Ca-reduced saline
junctional	27.6±7.4	20.9±5.9
nonjunctional	2.00±0.65	1.82±0.77

Using Students-paired-"t"-test, no significant difference was found to exist between the two sets of preparations at either the junctional or the nonjunctional regions.

further development of myopathy *in vitro*. The myopathy developed was significantly greater, at the junctional region, than that induced by a 60 minute exposure to ECO *in vivo*, with no further treatment *in vitro* (table 3.1: %Procion=5.37±1.69), suggesting that the development of myopathy may be time-dependent aswell as Ca^{2+}-dependent.

However, when extracellular Ca^{2+} was reduced 30 minutes after ECO administration *in vivo* (8.2.2.1), myopathy was significantly reduced. Possibly an extra 30 minutes exposure to ECO *in vivo* allowed additional entry of Ca^{2+} into muscle fibres such that the potential for the development of full myopathy was triggered and was then irreversible by the removal of extracellular Ca^{2+}. Again, assays of iCa^{2+} would be necessary to confirm this idea.

Two questions were addressed following the result of this experiment:-

a) Does the extra 30 minute exposure to extracellular Ca^{2+} need to occur *in vivo* for myopathy to develop to completion?

b) In order for myopathy to develop to completion, must the extra 30 minute exposure to extracellular Ca^{2+} immediately follow the 30 minute period *in vivo* or would any 30 minute exposure to Ca^{2+} be as effective?

A series of experiments was designed (8.2.1.3) to determine the effect of different 30 minute exposures to extracellular Ca^{2+} on the development of myopathy *in vitro* in preparations already exposed to extracellular Ca^{2+} for 30 minutes *in vivo*.

8.2.2.3. The effects on the *in vitro* progression of myopathy, of delaying a 30 minute exposure to extracellular Ca^{2+}, for increasing periods following ECO administration *in vivo*.

Contraction records.

Control preparations, stimulated for the whole incubation period in normal Lileys saline, contracted maximally throughout the experiment.

Experimental preparations contracted maximally during stimulation in normal Lileys saline, but in Ca-reduced saline the contractions declined and dissappeared entirely within 5-10 minutes, reappearing when normal Lileys saline was reapplied (figure 8.4).

Histological examination of preparations.

All preparations were severely myopathic at their junctional regions, the nonjunctional regions being comparatively unaffected (table 8.3). There was no significant histological differences between the treatments (table 8.3) suggesting that, as long as the preparations exposed to ECO for 30 minutes *in vivo* are then exposed to extracellular Ca^{2+} for 30 minutes at some time during the next 2 hours, they will ultimately develop severe myopathy.

Since myopathy was hindered when extracellular Ca^{2+} was reduced 30 minutes after ECO (8.2.2.1), but was severe following

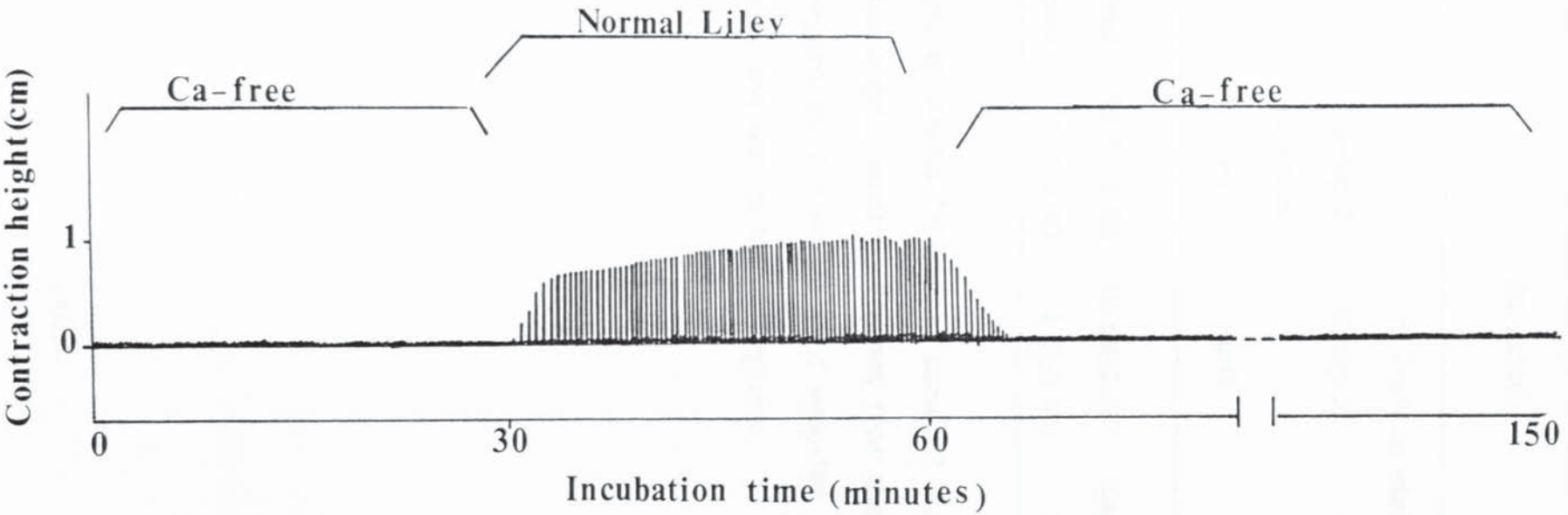


Figure 8.4. Preparations already exposed to ECO *in vivo* for 30 minutes did not contract on exposure to Ca-reduced saline. However, when normal Lileys saline was administered the preparations contracted strongly for the duration of the exposure (30 minutes). Contractions dissappeared once more on reintroduction of Ca-reduced saline.

Table 8.3 % Procion staining in preparations exposed to ECO for 30 minutes *in vivo* and then for various 30 minute periods to extracellular calcium (normal Lileys saline) during stimulation *in vitro*.

	% Procion staining			
	Group 1 (control) n=7	Group 2 n=6	Group 3 n=6	Group 4 n=6
junctional	18.92±3.90	15.65±2.63	24.70±5.40	28.60±7.50
nonjunctional	1.2±0.50	1.1±0.35	2.2±0.53	3.4±1.10

Using Students-"t"-test, none of the experimental groups gave results significantly different from the control group (group 1) with regard to the severity of myopathy induced at either the junctional or the nonjunctional regions.

any subsequent 30 minute exposure to Ca^{2+} *in vitro* (table 8.3) or *in vivo* (table 8.2), there must have been an additive effect on the development of myopathy, due to the continuous or repeated exposure of preparations to extracellular Ca^{2+}: a total 60 minute continuous exposure to extracellular Ca^{2+} was sufficient to promote the subsequent development of full myopathy *in vitro*, longer exposures (ie. stimulation in normal Lileys saline for greater than 2 hours tables 8.1 & 8.2) failing to produce a more severe myopathy.

Knowing that the development of myopathy is significantly hindered on the reduction of extracellular Ca^{2+} 30 minutes after ECO (8.2.2.2) it was surprising that the reintroduction of Ca^{2+} towards the end of the incubation period (ie. 90 minutes after ECO) induced a myopathy equal in severity to that induced by its reintroduction earlier during the incubation (ie. 30 minutes after ECO). One might have expected the myopathy developed in the former instance to have been less than that of the latter since the second Ca^{2+} exposure would have had less time to effect myopathy.

However, there is recent evidence (Ruigrok, 1985) that the reintroduction of Ca^{2+} following a minimum 2 minute perfusion with Ca-free saline, caused rapid, gross morphological damage to the mammalian heart. The phenomenon, termed "The Calcium Paradox", is based on the idea that, following Ca^{2+} depletion a phase of Ca^{2+} repletion causes enhanced entry of Ca^{2+} into the muscle concerned.

It is suggested that the route of Ca^{2+} entry during the repletion period, may include the glycocalyx, the slow channels, the Na^{+}/Ca^{2+} exchange system, passive diffusion and/or abnormal sites of entry (Ruigrok, 1985). Infact, Putney et al., (1978) demonstrated that if the intracellular Ca^{2+} stores are exhausted and then the ACh receptor (AChR) is blocked, the stores can be refilled by a brief application of extracellular Ca^{2+}. Then, after the extracellular Ca^{2+} is removed again, mobilisation of the stored iCa^{2+} may reoccur (Putney et al., 1978). The existence of a calcium paradox in skeletal muscle is controversial (Armani et al., 1984; Zuurveld et al., 1985) but, if it does take place, the consequent elevation of iCa^{2+} might initiate the rapid development of a severe myopathy.

It would be an interesting progression to measure:-

a) the Ca^{2+} content of phrenic nerve-diaphragm preparations following each 30 minute <u>in vitro</u> exposure to extracellular Ca^{2+} ie. is there an enhanced influx of Ca^{2+} on the reintroduction of Ca^{2+} to muscles following exposure to Ca-reduced saline?.

b) the severity of myopathy induced by an early <u>in vitro</u> exposure to extracellular Ca^{2+} (ie the second 30 minutes) during a shorter incubation period (ie. 90 minutes). If the Calcium Paradox is in operation one would expect myopathy to develop to a degree similar to that induced following a 2 hr 30 minute <u>in vitro</u> incubation of such preparations in normal Lileys saline.

8.3 EXPERIMENTS DESIGNED TO BLOCK THE ENTRY OF CALCIUM INTO MUSCLE FIBRES EXPOSED TO ECO.

8.3.1. Experimental Design.

8.3.1.1. The effect of 10^{-5}M diltiazem on the *in vitro* development of ECO-induced myopathy.

8.3.1.1.1. In phrenic nerve-diaphragm preparations exposed to ECO *in vivo* for 30 minutes (the *in vivo/in vitro* technique: 2.5).

Five mice were injected with ECO plus atropine and were killed 30 minutes later. Their phrenic nerve-diaphragm preparations were prepared according to the *in vivo/in vitro* technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in normal Lileys saline (A2) for the duration of the incubation period (2 hours 30 minutes) whilst the contralateral preparation was stimulated in 10^{-5}M diltiazem (in normal Lileys saline). Finally, all preparations were assessed for myopathy using the Procion technique. Statistical comparisons were made between the two sets of preparations using Students-paired-'t'-test.

8.3.1.1.2. In phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro (the in vitro technique;2.4).

Six mice were killed and their phrenic-diaphragm nerve preparations prepared according to the in vitro technique described in section 2.4. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in 500nM ECO (in normal Lileys saline) for the duration of the incubation period (3 hours) whilst the contralateral preparation was stimulated in 10^{-5}M diltiazem (in normal Lileys saline) for 10 minutes prior to addition of 500nM ECO: the preparation was stimulated thus for 3 hours. Finally, all preparations were assessed for myopathy using the Procion technique. Statistical analysis between the two sets of preparations was made using Students-paired-'t'-test.

8.3.1.2. The effect of a range of membrane ion channel blockers on the development of myopathy, when administered prior to ECO in vivo.

36 mice were divided into 6 groups:-

Group 1 (control group):- Mice were injected with ECO plus atropine only (2.2).

Group 2:- Mice were injected with verapamil (1.14-3.42 $mgKg^{-1}$), an antiarrhythmic Ca-antagonist which has also been shown to block endplate channels (Wachtel, 1987) and to reduce cardiac necrosis

in dogs (Reimer et al., 1977). 15 minutes later mice were injected with ECO plus atropine.

Group 3:- Mice were injected with the Ca-antagonist diltiazem (1.71-3.42 $mgKg^{-1}$), which has been shown to block endplate channels (Miledi & Parker, 1981; Wachtel, 1987). 15 minutes later mice were injected with ECO plus atropine.

Group 4:- Mice were injected with mecamylamine (5.5 $mgKg^{-1}$), a ganglionic blocker which acts by preventing the action of ACh at the post-junctional membrane. 15 minutes later mice were injected with ECO plus atropine.

Group 5:- Mice were injected with disopyramide phosphate (2.14-4.28 $mgKg^{-1}$), a membrane stabilising compound which has been shown to block open endplate channels at the neuromuscular junction (Harvey et al., 1984). 15 minutes later mice were injected with ECO plus atropine.

Group 6:- Mice were injected with procainamide (3.0-6.0 $mgKg^{-1}$), a membrane stabilising compound which blocks open endplate channels in the neuromuscular junction (Harvey et al., 1984). 15 minutes later mice were injected with ECO plus atropine.

NB. All injections were administered subcutaneously between the shoulder blades; all drug solutions were made up in normal Lileys

saline and the doses administered were equivalent to or greater than that recommended for use clinically and were corrected for body weight.

Mice were anaesthetised 3 hours after ECO administration and blood was obtained from the femoral artery for analysis of serum CK (2.10). The mice were killed whilst still anaesthetised and their diaphragms were removed. One hemidiaphragm was assayed for Ca^{2+} influx at its junctional region (2.9) and the other was assessed for myopathy using the Procion technique (2.7).

The results of each experimental group were compared with those of the control group (injected with ECO plus atropine only) in order to establish whether the pretreatment had been effective against the development of myopathy. All statistical comparisons were made using Students-unpaired-"t"-test.

8.3.2. Results and Discussion.

8.3.2.1. The effect of 10^{-5}M diltiazem on the *in vitro* development of ECO-induced myopathy (8.2.4)

8.3.2.1.1. In phrenic nerve-diaphragm preparations exposed to ECO for 30 minutes *in vivo*.

Contraction records.

Diltiazem had no apparent effect on the contractions elicited by ECO-treated phrenic nerve-diaphragm preparations in normal Lileys saline (figure 8.5) suggesting that diltiazem did not compromise neuromuscular transmission either pre- or post-synaptically.

Histological examination of preparations.

Severe myopathy was induced at the junctional region of preparations stimulated in normal Lileys saline and also in those stimulated in 10^{-5}M diltiazem (table 8.4), the nonjunctional regions in each case being comparatively unaffected ie. diltiazem had no effect on the *in vitro* development of myopathy when applied 30 minutes after ECO administration *in vivo*.

Possibly diltiazem failed to prevent Ca^{2+} influx into the diaphragm but in order to confirm this it would be necessary to

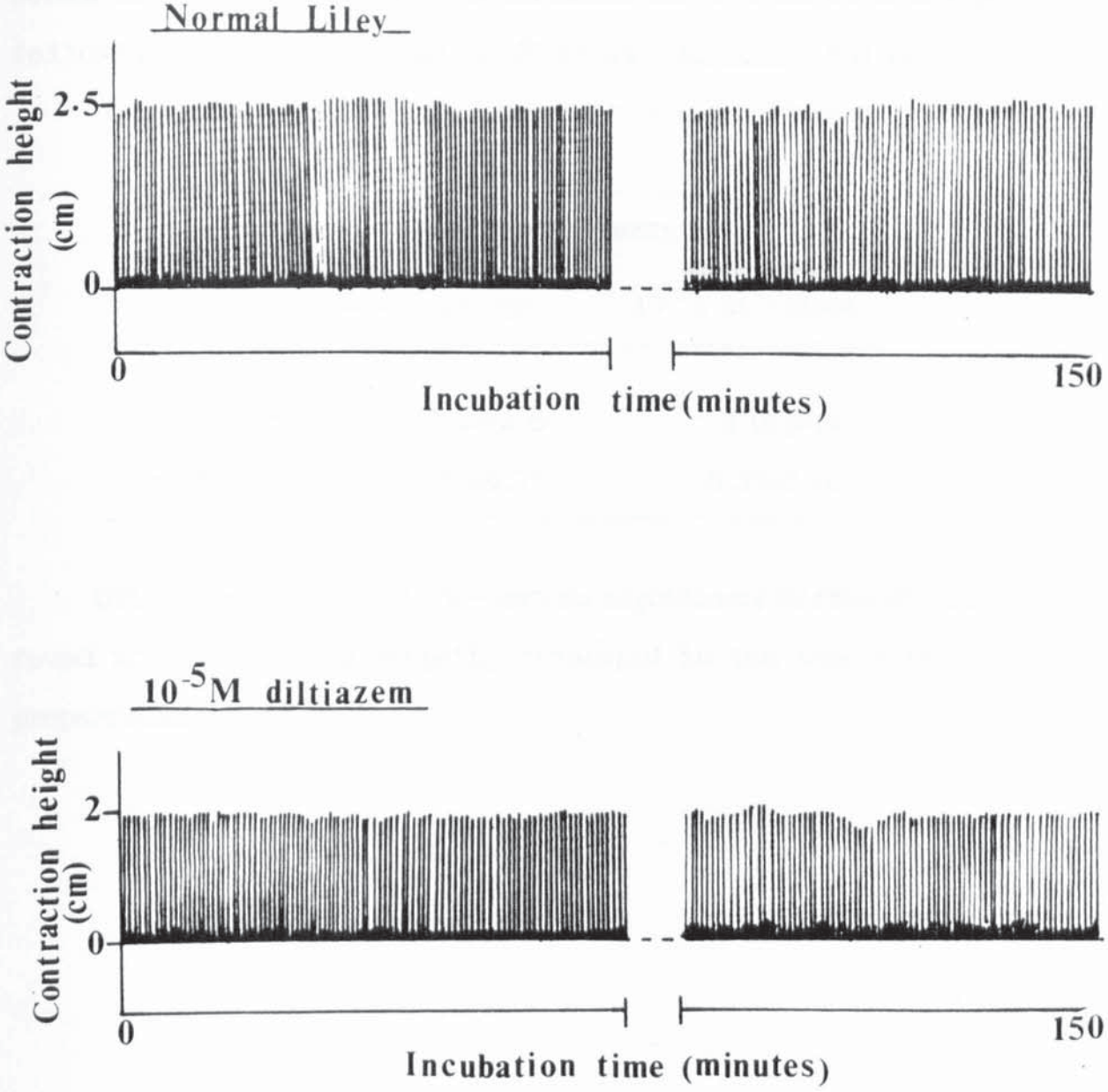


Figure 8.5. Preparations stimulated in normal Lileys saline or diltiazem, 30 minutes following exposure to ECO in vivo, showed no difference in contraction pattern.

Table 8.4 % Procion staining in preparations stimulated in normal Lileys saline or in 10^{-5}M diltiazem for 2 hours 30 minutes following an exposure to ECO of 30 minutes in vivo:- the results represent the mean ± standard error of 5 experiments.

	% Procion staining	
	Normal Lileys	10^{-5}M diltiazem
junctional	10.56±2.6	9.01±3.4
nonjunctional	0.39±0.11	0.37±0.16

Using Students-paired-'t'-test no significant difference was found to exist in the myopathy developed in the two sets of preparations.

perform the appropriate assays of the calcium content of the preparations concerned.

It is possible that diltiazem did prevent Ca^{2+} entry into the diaphragm once it had actually reached the tissue. However, the delay of access of diltiazem, due to its late administration (30 minutes after ECO) and to the time taken for it to reach the diaphragm thereafter, may have allowed sufficient Ca^{2+} influx to have occurred, both pre- and post- synaptically, to trigger the development of myopathy before the Ca^{2+} antagonist had even reached the neuromuscular synapse.

It was decided to investigate the effect of an earlier application of diltiazem ie. prior to ECO administration in vitro, on the development of myopathy.

8.3.2.1.2. In phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro.

Contraction records.

Control (figure 8.6):- When ECO was added to a bath containing normal Lileys saline a short-lived potentiation of muscle contraction was elicited. The contraction magnitude then gradually decreased over an approximate 30 minute period, to a level below that observed prior to ECO administration. Finally,

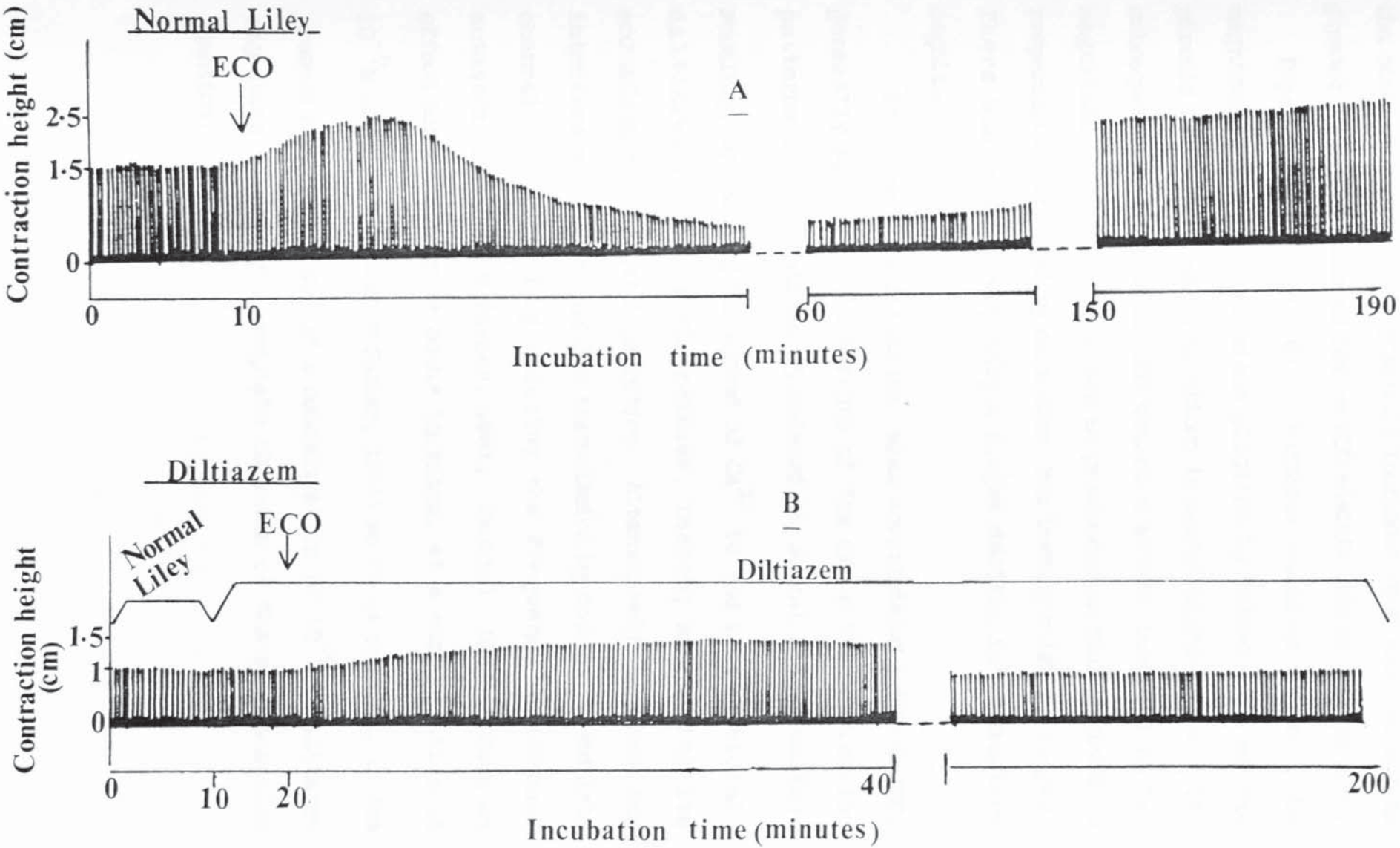


Figure 8.6. A. ECO elicited a short-lived potentiation of muscle contraction when applied to preparations in vivtro. B. Diltiazem produced no change in the contraction magnitude of preparations stimulated in normal Lileys saline, and reduced the potentiation of the contraction normally induced by ECO.

the contraction magnitude gradually increased once more, to a size greater than that observed before ECO administration (figure 8.6).

Experimental (figure 8.6):- Diltiazem caused no change in the magnitude of the contractions elicited by stimulation of the phrenic nerve-diaphragm preparations in normal Lileys saline. The subsequent application of ECO caused a slight increase in the magnitude of contractions, not as pronounced as that observed in preparations untreated by diltiazem, but lasting slightly longer. There was subequently only a slight decline in contraction magnitude.

It seems that diltiazem, when administered prior to ECO, generally minimised the effects of the OP on the contraction patterns of preparations stimulated in normal Lileys saline. Possibly, by reducing the access of Ca^{2+} to the nerve terminal, diltiazem inhibited ACh release, thereby minimising its accumulation during AChE inhibition. Alternatively, diltiazem may interfere with neuromuscular transmission by decreasing endplate channel lifetime and by reducing the frequency of channel activation (Miledi & Parker, 1981; Wachtel, 1987). Such an effect has been shown to occur <u>in vitro</u>, at a concentration of 10^{-7}M diltiazem (Miledi & Parker, 1981) so it is possible, in the present study also, that at a concentration of 10^{-5}M, diltiazem may have blocked the endplate channels of the neuromuscular junction.

Histological examination of preparations.

Control preparations:- stimulation of phrenic nerve-diaphragm preparations for 3 hours in 500nM ECO in normal Lileys saline induced myopathy located largely at the junctional region of the diaphragm (table 8.4). The myopathy was represented by Procion staining and hypercontractions but was less severe than that induced by ECO in the *in vivo/in vitro* experiments (table 8.4:$P<0.05$ at the junctional region and $P<0.005$ at the non-junctional region) suggesting that perhaps the nerve stimulation pattern used *in vitro* did not accurately mimic that occurring *in vivo*. Whatever the reason for this anomaly, the problems of extrapolation of results from *in vitro* experiments to an *in vivo* situation are clear.

Experimental preparations:- pretreatment of phrenic nerve-diaphragm preparations with diltiazem prevented the myopathy normally induced by ECO *in vitro* (table 8.5). Procion staining was minimal and the muscle fibres were not hypercontracted.

Since diltiazem is thought to block both voltage operated channels (Stanfield, 1986) and endplate channels (Miledi & Parker, 1981; Wachtel, 1987), it seems that the ECO-induced influx of Ca^{2+} at either the pre- or post-synaptic sites, or both, must occur through either channel type, or both, since if this were not so diltiazem would not have been effective against the development of myopathy.

Table 8.5. % Procion staining of phrenic nerve-diaphragm preparations stimulated in 500nM ECO and those treated with 10^{-5}M diltiazem before and during stimulation in 500nM ECO:- the results represent the mean ± standard error of 6 experiments.

	% Procion staining	
	500nM ECO.	10^{-5}M diltiazem in 500nM ECO.
junctional	5.36±0.98	0.12±0.08***
nonjunctional	0.18±0.05	0.08±0.03

*** indicates that the result was found to be significantly different, at the 0.01% level, using Students-paired-'t'-test ie. diltiazem significantly prevented the myopathy induced by ECO at the junctional region of the phrenic nerve-diaphragm preparation: no significant difference was found in the extent of myopathy developed at the nonjunctional region.

Contraction records of the control and experimental preparations concerned, indicate that diltiazem reduced the ECO-induced increase in the contractile response of the diaphragm. This effect suggests a postsynaptic effect of diltiazem, reducing the ECO-induced repetitive firing in the diaphragm. Since the preparations did continue to contract strongly, a significant presynaptic effect of diltiazem is considered unlikely. It is thought that, by minimising the postsynaptic response of the diaphragm to ECO, diltiazem may have reduced also the ECO-induced influx of Ca^{2+} into the muscle and thereby prevented subsequent development of myopathy. However, to confirm such an hypothesis, assays of the Ca^{2+} content of the preparations would have to be performed at the appropriate stages of the experiment.

Since diltiazem successfully prevented myopathy, when applied prior to and during administration of ECO (figure 8.7), one might have expected it to have reduced myopathy when it was applied *in vitro*, 30 minutes after ECO administration *in vivo*. Yet diltiazem in this instance was completely ineffective (figure 8.7). Possibly the delay between the application of diltiazem to the preparations and its actual access to its site of action was such that Ca^{2+} influx continued, ultimately causing a more severe myopathy than that observed when extracellular Ca^{2+} was minimised 30 minutes after ECO administration (8.2.2.2). In addition, the slow, continual entry of Ca^{2+} into both the pre- and post synaptic sites via routes other than those blocked by diltiazem, may have

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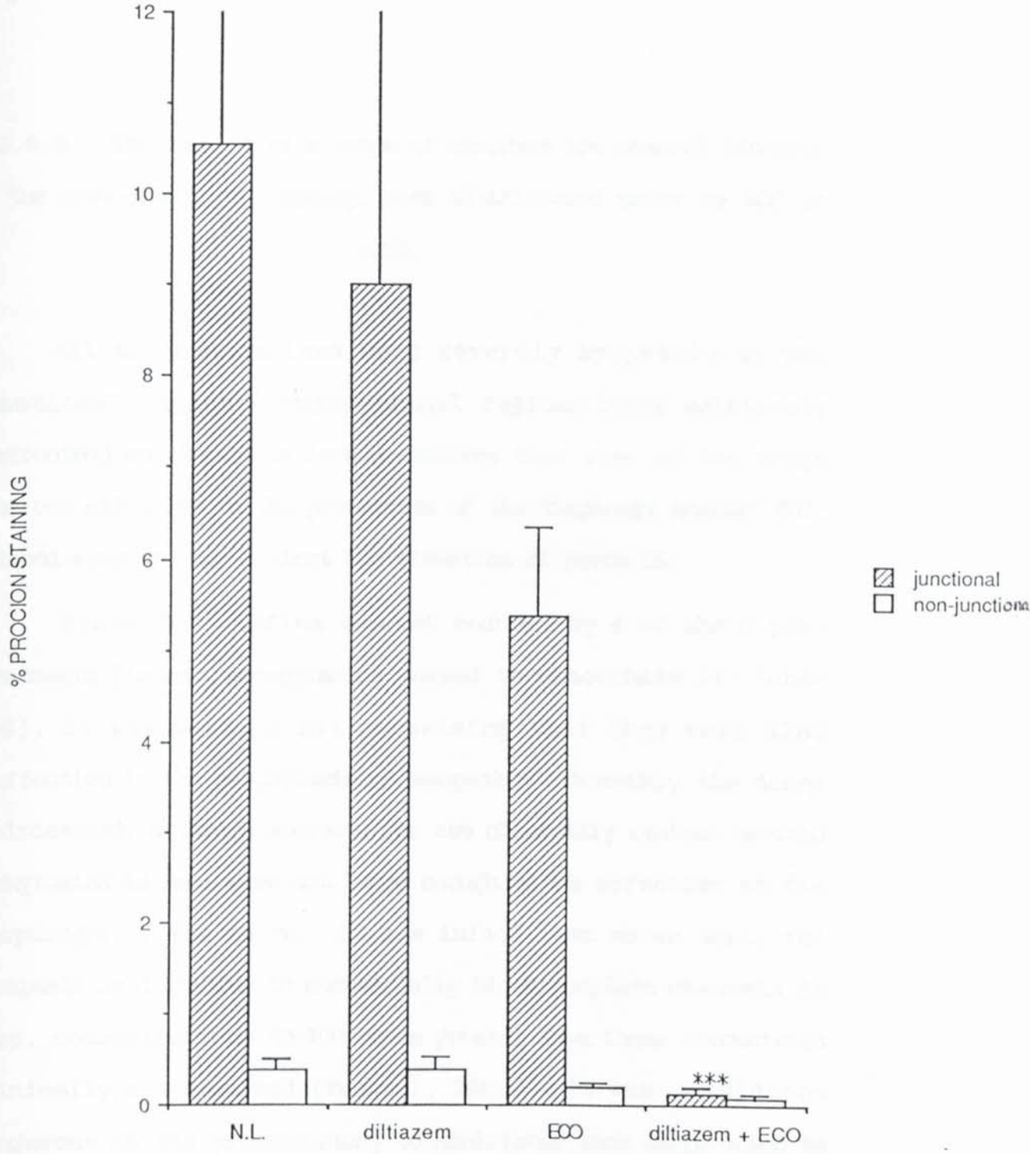
Figure 8.7. The in vitro effect of 10^{-5}M diltiazem on the development of myopathy, when applied 30 minutes after ECO administration in vivo (ie. the in vivo/in vitro experiments), or when applied 10 minutes before ECO administration in vitro (ie. the in vitro experiments):- the results represent the mean ± standard error of at least 5 experiments.



NL indicates preparations stimulated in normal Lileys saline in vitro following exposure to ECO in vivo for 30 minutes.

*** indicates that a significant difference exists, at the 0.1% level, between the preparations treated with diltiazem plus ECO in vitro and those treated purely with ECO in vitro, using Students-paired-'t'-test.

lead to further development of myopathy. Again, in order to substantiate such theories, assays of the Ca^{2+} content of the preparations would have to be performed.

8.3.2.2. The effect of a range of membrane ion channel blockers on the development of myopathy, when administered prior to ECO *in vivo*.

All the preparations were severely myopathic at the junctional region (nonjunctional regions being relatively unaffected) and infact table 8.6 confirms that none of the drugs used was effective in the protection of the diaphragm against ECO-induced myopathy nor against the elevation of serum CK.

Since Ca^{2+} influx was not reduced by 4 of the 5 pre-treatments (infact, mecamylamine seemed to exacerbate it: table 8.6), it was perhaps not surprising that they were also ineffective in the prophylaxis of myopathy. Possibly the doses administered, although adequate for use clinically against cardiac arrhythmias in man, were not large enough to be effective at the diaphragm of the mouse. It has infact been shown that, for verapamil or diltiazem to successfully block endplate channels *in vivo*, concentrations 20-100 times greater than those encountered clinically are required (Wachtel, 1987). It was considered dangerous in the present study to administer such large doses to mice due to the unpleasant side effects of the drugs concerned.

Table 8.6:- The prophylactic effect of various membrane ion channel blockers against ECO-induced myopathy.

	% Procion staining		Serum CK	Ca^{2+} influx at
	J	NJ	(UL^{-1})	J ($nmolmg^{-1}$)
ECO	21.4±2.3	2.29±0.91	4366±719	1.56±0.18
Verapamil + ECO	16.4±4.2	1.80±0.46	4700±1247	0.97±0.83
Diltiazem + ECO	16.4±4.2	1.63±0.56	3759±1144	0.586±0.11**
Mecamylamine + ECO	15.1±2.0	0.86±0.38	1865±239*	2.49±0.55*
Disopyramide + ECO	19.6±4.2	2.59±0.43	3267±977	1.40±0.34
Procainamide + ECO	15.6±3.4	2.11±0.05	5019±1866	1.82±0.36

J, NJ = junctional, nonjunctional regions respectively

**, * = results which are significantly different from those induced by ECO only at the 1% and 5% levels respectively, using Students unpaired-'t'-test.

Diltiazem however, did significantly reduce Ca^{2+} influx at the junctional region of the diaphragm (table 8.6) possibly by Ca^{2+} antagonism rather than by endplate channel block. However, Ca^{2+} influx was not prevented completely and was infact similar to that recorded in an earlier experiment (3.3.5), 20 minutes after ECO administration *in vivo*. Possibly this small elevation of iCa^{2+} is sufficient to initiate the development of a severe myopathy by the end of the 3 hour post-injection period. Alternatively, there may have been a Ca^{2+}-induced release of Ca^{2+} from the SR (Ford & Podolski, 1970; Endo, 1977; Martinosi, 1984), and/or a depolarisation-induced release of Ca^{2+} from SR (Endo, 1977; Nakajima & Engel, 1973) due to the prolongation of ACh half-life at the neuromuscular junction. The prolonged action of ACh at the endplate might also have indirectly caused release of Ca^{2+} from mitochondria, in response to an elevation of intracellular Na^{+}. Whatever the mechanism of Ca^{2+} release from intracellular stores, no elevation of iCa^{2+} would have been recorded by the technique used in the current study since no change in the total cell Ca^{2+} is involved. However, it is probable that an internal redistribution of Ca^{2+} does occur alongside Ca^{2+} influx on exposure of muscles to ECO.

Since there was no block of the entry of Ca^{2+} into muscle fibres exposed to ECO *in vivo*, it was decided to investigate

alternative methods of ameliorating ECO-induced myopathy, by interfering with the role of iCa^{2+} per se.

8.4. The use of 25μM leupeptin in an investigation of the possible role of a Ca^{2+}-activated neutral protease (CANP) in the development of ECO-induced myopathy.

8.4.1. Experimental design.

8.4.1.1. In phrenic nerve-diaphragm preparations exposed to ECO *in vivo* for 30 minutes (the *in vivo/in vitro* technique).

Six mice were injected with ECO plus atropine and were killed 30 minutes later. Their phrenic nerve-diaphragm preparations were prepared according to the *in vivo/in vitro* technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in normal Lileys saline (A2) for the duration of the incubation period (2 hours 30 minutes) whilst the contralateral preparation was stimulated in 25μM leupeptin (in normal Lileys saline). Finally all preparations were assessed for myopathy using the Procion technique. Statistical comparisons were made between the two sets of preparations using Students-paired-'t'-test.

8.4.1.2. In phrenic nerve preparations exposed to 500nM ECO *in vitro* (the *in vitro* technique).

Six mice were killed and their phrenic nerve-diaphragm preparations prepared according to the *in vitro* technique described in section 2.4. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in 500nM ECO (in normal Lileys saline) for the duration of the incubation period (3 hours) whilst the contralateral preparation was stimulated in 25μM leupeptin (in normal Lileys saline) for 10 minutes prior to addition of 500nM ECO: the preparation was stimulated thus for 3 hours. Finally all preparations were assessed for myopathy using the Procion technique. Statistical analysis between the two sets of preparations was made using Students-paired-'t'-test.

8.4.2. Results and Discussion.

8.4.2.1. In phrenic nerve-diaphragm preparations exposed to ECO *in vivo* for 30 minutes.

Contraction records.

Leupeptin had no effect on the contractions elicited in normal Lileys saline by preparations treated with ECO *in vivo*.

Table 8.7. % Procion staining of preparations stimulated in normal Lileys saline/25 μM leupeptin following a 30 minute exposure to ECO *in vivo*:- the results represent the mean ± standard error of 6 experiments.

	% Procion staining	
	Normal Lileys	25μM leupeptin
junctional	10.68±0.70	9.71±0.87
nonjunctional	0.15±0.06	0.29±0.09

Using Students paired-'t'-test no significant difference was found to exist in the extent of myopathy induced in the two sets of preparations at either the junctional or nonjunctional regions.

Histological examination of preparations.

All preparations were severely myopathic at their junctional regions, the nonjunctional regions being comparatively unaffected (table 8.7) ie. leupeptin had no significant effect on the *in vitro* development of myopathy, when applied 30 minutes after ECO administration *in vivo*. This may suggest that ECO-induced myopathy is not mediated by CANP, that leupeptin failed to penetrate the sarcolemma, or that its application was too late to effectively ameliorate the myopathy; although preparations were exposed to leupeptin 30 minutes after an injection of ECO *in vivo*, the actual access of leupeptin to the neuromuscular synapse will have been delayed by the diffusion of the drug through the tissue. Possibly, therefore, myopathy had been instigated before leupeptin had reached the neuromuscular synapse. It was decided to investigate an earlier application of leupeptin, prior to ECO administration *in vitro*.

8.4.2.2. In phrenic nerve-diaphragm preparations exposed to 500nM ECO *in vitro*.

Contraction records.

Leupeptin had no effect on the contractions elicited in normal Lileys saline nor on the usual patterns of contraction elicited by ECO *in vitro* (8.3.2.1.2) suggesting that leupeptin does not interfere with neuromuscular transmission in any way.

Table 8.8 % Procion staining showing the effect of 25μM leupeptin when administered before and during stimulation in 500nM ECO: the results represent the mean ± standard error of 6 experiments.

	% Procion staining	
	500nM ECO.	25μM leupeptin in 500nM ECO.
junctional	6.63±0.88	6.07±0.89
nonjunctional	0.16±0.05	0.14±0.03

Using Students-paired-'t'-test no significant difference was found to exist in the myopathy developed between the two sets of preparations at either the junctional or the nonjunctional regions.

Histological examination of preparations.

All preparations were severely myopathic at their junctional regions, the nonjunctional regions being comparatively unaffected (table 8.8) ie. pretreatment of phrenic nerve-diaphragm preparations with leupeptin had no effect on the development of myopathy initiated by ECO in vitro. The result was disappointing in the light of evidence given by Leonard and Salpeter (1980), of 25μM leupeptin partially protecting extensor digitorum longus (EDL) muscles against myopathy normally induced by carbachol. However, in these experiments Leonard and Salpeter preincubated the EDL muscles in leupeptin for 2 hours before exposure to carbachol. Possibly therefore, in the current experiments, leupeptin did not have sufficient time to penetrate the diaphragm and effect the inhibition of CANP. Alternatively, since the diaphragm is thought to be the muscle most susceptible to agonist-induced myopathy (Ariens et al,1968), it is possible that leupeptin, which had only a partial protective effect on EDL muscles anyway, was not effective at all in the protection of the diaphragm. It would be interesting to investigate the effect on ECO-induced myopathy, of longer preincubation periods in leupeptin and also to actually measure CANP activity of diaphragms treated with ECO since Toth et al., (1983), did show CANP to be significantly elevated in rat diaphragms following exposure to DFP/Wolfatox poisons.

There have however, been several reports of the failure of leupeptin to protect against Ca^{2+}-activated myopathies; in 1978

Duncan et al preincubated isolated cutaneous pectoris muscles of the frog in various concentrations of leupeptin, for times varying from 15 to 90 minutes before application of the ionophore A_{23187}, previously shown to induce muscle necrosis (Statham et al, 1976; Publicover et al, 1977). In none of these experiments did leupeptin show even partial protection against myopathy development (Duncan et al, 1978). Similarly, Enomoto and Bradley (1977) failed to produce evidence of any benefit conveyed by leupeptin against murine muscular dystrophy.

Such results suggest either that leupeptin cannot easily penetrate intact sarcolemmal membranes despite reports that it does so (Libby & Goldberg, 1978), or that CANP plays no part in the degeneration of muscle induced by A_{23187}, murine muscular dystrophy, or ECO. Possibly a different mechanism entirely is involved in the implementation of myopathy.

8.5 SUMMARY OF THE RESULTS OF THE INVESTIGATION INTO THE ROLE OF CALCIUM IN THE DEVELOPMENT OF ECO-INDUCED MYOPATHY.

The need for extacellular Ca^{2+} for the development of ECO-induced myopathy has been confirmed by two experiments:-

a) The reduction of extracellular Ca^{2+}, 30 minutes after ECO administration *in vivo*, greatly reduced the subsequent *in-vitro* development of myopathy (8.2.2.1).

b) The *in vitro* application of 10^{-5}M diltiazem, prior to and during ECO administration *in vitro*, prevented the development of myopathy (8.3.2.1.2).

It was thought that the *in vivo* development of myopathy might be ameliorated by preventing entry of Ca^{2+} into the diaphragm. However, when applied prophylactically *in vivo*, a range of membrane ion channel blockers failed to prevent Ca^{2+}-inflx into the diaphragm, and consequently myopathy developed as usual: diltiazem was the only drug that did reduce Ca^{2+} influx at the junctional region of the diaphragm but myopathy was still severe. Higher doses of the ion channel blockers may have been more successful but would undoubtably have caused unacceptable side effects in the mice. The results were disappointing in the light of the evidence that diltiazem prevented the *in vitro* development of ECO-induced myopathy, and highlight, once again, the problem of the extrapolation of results from *in vitro* experiments to an *in vivo* situation. Finally, an attempt was made to reduce the development of ECO-induced myopathy using leupeptin, an inhibitor of CANP (8.4.2.1) which has been implicated in the myopathic process (8.4.2.1). Again, this was unsuccessful, possibly due to inadequate access of leupeptin. However, it is possible also that CANP is not involved in the development of ECO-induced myopathy at all.

CHAPTER 9

THE REQUIREMENT FOR NERVE STIMULATION AND A FUNCTIONAL ACETYLCHOLINE RECEPTOR (AChR) IN THE DEVELOPMENT OF ECO-INDUCED MYOPATHY.

9.1. INTRODUCTION.

It is well accepted that motor neurones play an important role in the maintenence of the functional and structural integrity of skeletal muscle fibres, and numerous investigators have confirmed that the induction of muscle fibre necrosis, by inhibition of AChE, is a neurally mediated process, and that by prior sectioning of the appropriate nerve, the acute development of necrosis can be reduced, if not prevented (Ariens et al., 1969; Fenichel et al., 1972; Kawabuchi et al., 1975; Laskowski et al., 1975; Wecker & Dettbarn, 1977; Hudson et al., 1978; Wecker et al., 1978; Salpeter et al., 1979). In the present study, the fact that ECO-induced myopathy was located largely at the junctional region of the diaphragm (6.3.2), adjacent to the nerve terminal, suggests that it may be neurally mediated. If this is true, then it is probable also that the ACh receptor (AChR) may be integrally involved. Certainly, a functional AChR has been shown to be necessary for the development of myopathies induced by the antiChE diisopropylfluorophosphate (DFP) and by ACh agonists (Ariens et al., 1968; Leonard and Salpeter, 1979, 1980).

An experiment was designed to determine the need for nerve stimulation and a functional AChR in:-

a) the *in vitro* development of myopathy induced by ECO *in vivo*: using the *in vivo/in vitro* technique (2.4).

b) the initiation and development of myopathy by the *in vitro* application of 500nM ECO to phrenic nerve-diaphragm preparations: using the *in vitro* technique (2.5).

9.2. EXPERIMENTAL DESIGN.

9.2.1. The requirement for nerve stimulation for the in vitro progression of myopathy in the diaphragms of mice injected with ECO for 30 minutes in vivo.

Seven mice were injected with ECO and killed 30 minutes later. Their phrenic nerve-diaphragm preparations were prepared according to the in vivo/in vitro technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in normal Lileys saline (A2) throughout the incubation period (2 hours 30 minutes). The contralateral preparation was incubated in normal Lileys saline, without stimulation, for the duration of the incubation period (2 hours 30 minutes). Finally, all preparations were assessed for myopathy using the Procion technique (2.7). Statistical comparison between the two sets of preparations was made using Students-paired-'t'-test.

9.2.2. The requirement for nerve stimulation for the development of myopathy in phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro.

Eight mice were killed and their phrenic nerve-diaphragm preparations prepared according to the in vitro technique described in section 2.4. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in 500nM ECO (in normal Lileys saline) for the duration of the incubation period (3

hours). The contralateral preparation was incubated, without stimulation, in 500nM ECO (in normal Lileys saline) for 3 hours. Finally, all preparations were assessed for myopathy using the Procion technique (2.7). Statistical comparison between the two sets of preparations was made using Students-paired-'t'-test.

9.2.3. The requirement for a functional AChR for the in vitro progression of myopathy in the diaphragms of mice exposed to ECO in vivo for 30 minutes.

Seven mice were used and their phrenic nerve-diaphragm preparations prepared according to the in vivo/in vitro technique described in section 2.5. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in normal Lileys saline (A2) throughout the incubation period (2 hours 30 minutes). The contralateral preparation was stimulated in 9μM tubocurarine (in normal Lileys saline) for the duration of the incubation period (2 hours 30 minutes). Finally, all preparations were assessed for myopathy using the Procion technique (2.7). Statistical comparison between the two sets of preparations was made using Students-paired-'t'-test.

9.2.4. The requirement for a functional AChR for the in vitro development of myopathy in phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro.

Six mice were killed and their phrenic nerve-diaphragm preparations prepared according to the in vitro technique described in section 2.4. For each mouse, one phrenic nerve-diaphragm preparation was stimulated in 500nM ECO (in normal Lileys saline) for the duration of the incubation period (3 hours). The contralateral preparation was stimulated in 9μM tubocurarine (in 500nM ECO in normal Lileys saline) for 3 hours. Finally, all preparations were assessed for myopathy using the Procion technique (2.7). Statistical comparison between the two sets of preparations was made using Students-paired-'t'-test.

9.3. RESULTS AND DISCUSSION.

9.3.1. The requirement for nerve stimulation for the in vitro progression of myopathy in the diaphragms of mice injected with ECO for 30 minutes in vivo (9.2.1)

9.3.1.1. Contraction records.

Stimulated preparations elicited strong contractions throughout the duration of the incubation period (figure 9.1), declining finally during the "wash" in Ca-reduced saline. Unstimulated preparations produced only small, spontaneous contractions throughout the incubation period (figure 9.1).

9.3.1.2. Histological examination of preparations.

Both the stimulated and unstimulated preparations were severely myopathic at their junctional regions, the nonjunctional regions being comparatively unaffected (table 9.1) ie. nerve stimulation is not necessary for the progression of myopathy in vitro following a 30 minute exposure to ECO in vivo. Possibly the action of nonquantal ACh or spontaneously released ACh (known to increase following ECO administration, S.Kelly, personal communication), was sufficient to support the development of myopathy in vitro despite absence of neuronal stimulation. Alternatively, myopathy might have already been instigated

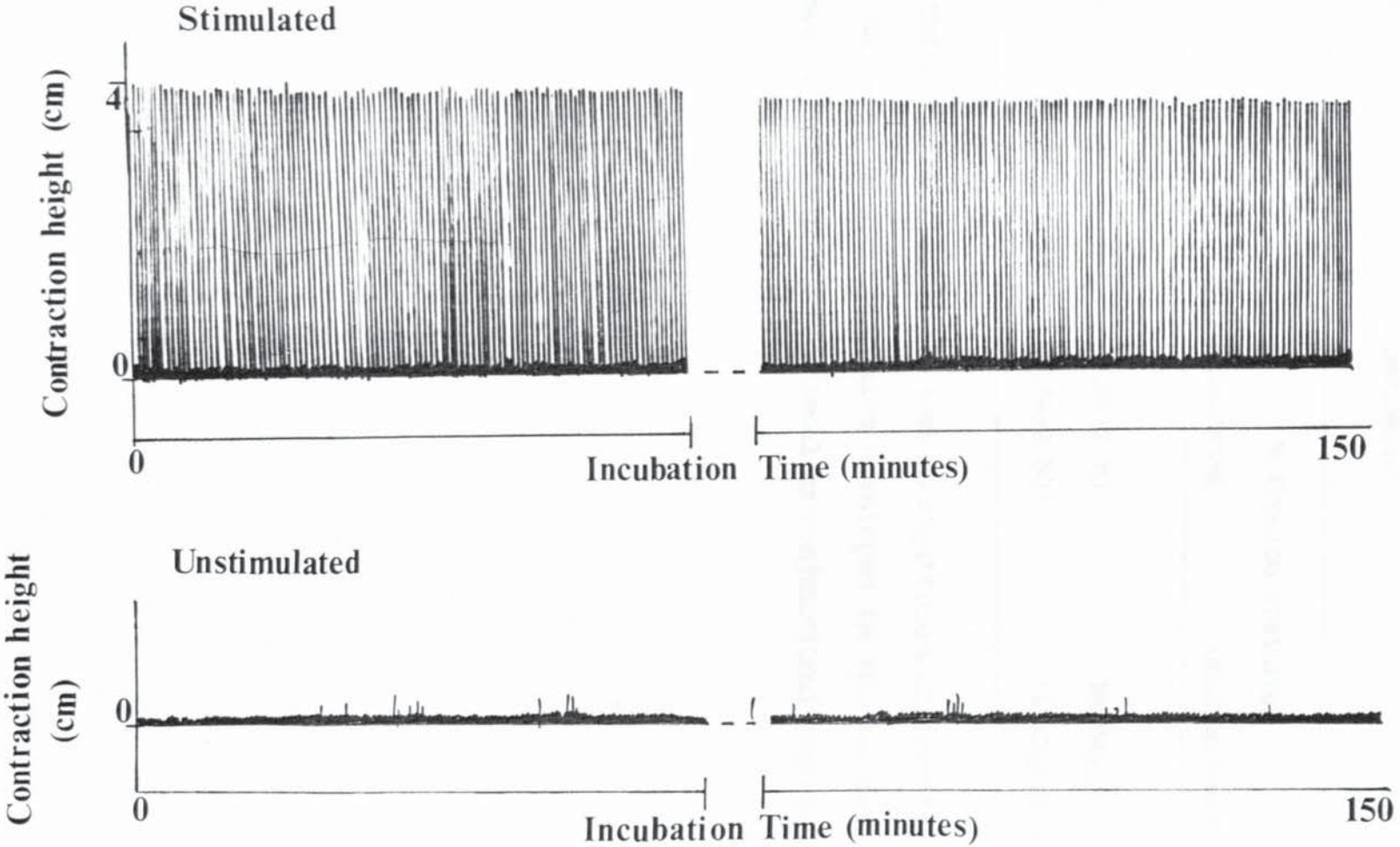


Figure 9.1. Stimulated preparations elicited strong contractions throughout the incubation period, but unstimulated preparations produced only small, spontaneous contractions throughout.

Table 9.1 % Procion staining of preparations stimulated/unstimulated *in vitro* following an initial 30 minute exposure to ECO *in vivo*:- the results represent the mean ± standard error of 7 experiments.

	% Procion staining	
	Stimulated	Unstimulated
junctional	19.01±5.70	16.34±1.70
nonjunctional	(2.54±2.30)	(1.32±0.24)

Using Students-paired-'t'-test no significant difference was found to exist in the myopathies developed in the two sets of preparations at either the junctional or nonjunctional regions.

during the 30 minute period *in vivo* such that the subsequent removal of nerve stimulation was ineffectual. However, it is possible that the development of ECO-induced myopathy might not be nerve mediated at all.

An experiment was designed to see if ECO-induced myopathy required nerve stimulation for its initiation *in vitro*. Such an experiment might also elucidate whether the myopathy could be induced *in vitro* by nonquantal and spontaneously released ACh alone.

9.3.2. The requirement for nerve stimulation for the in vitro development of myopathy in phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro (9.2.2)

9.3.2.1. Contraction records.

Application of ECO to stimulated preparations *in vitro* elicited a short-lived gradual potentiation of the contractions followed by a gradual decrease in contraction magnitude to a size much less than that originally induced by stimulation of the preparations in normal Lileys saline. Finally, the contraction magnitude increased again to a level greater than that induced prior to ECO exposure (figure 9.2).

Unstimulated preparations however elicited no large contractions but did occasionally contract spontaneously during the incubation period following ECO administration (figure 9.2).

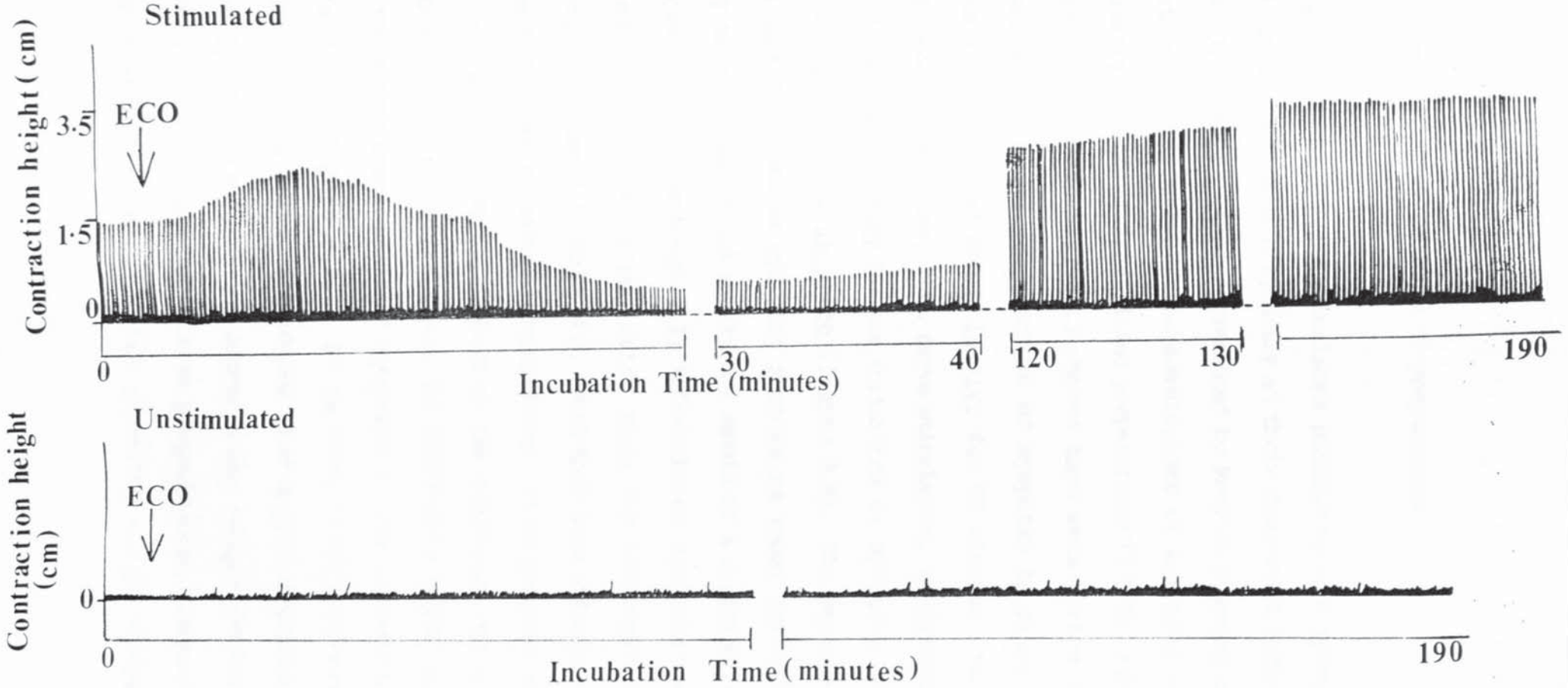


Figure 9.2. Application of ECO to stimulated preparations elicited a short-lived potentiation of contractions, followed later by a sustained period of contaction enhancement. Unstimulated preparations however, elicited only spontaneous contractions throughout the incubation period despite application of ECO.

9.3.2.2. Histological examination of preparations.

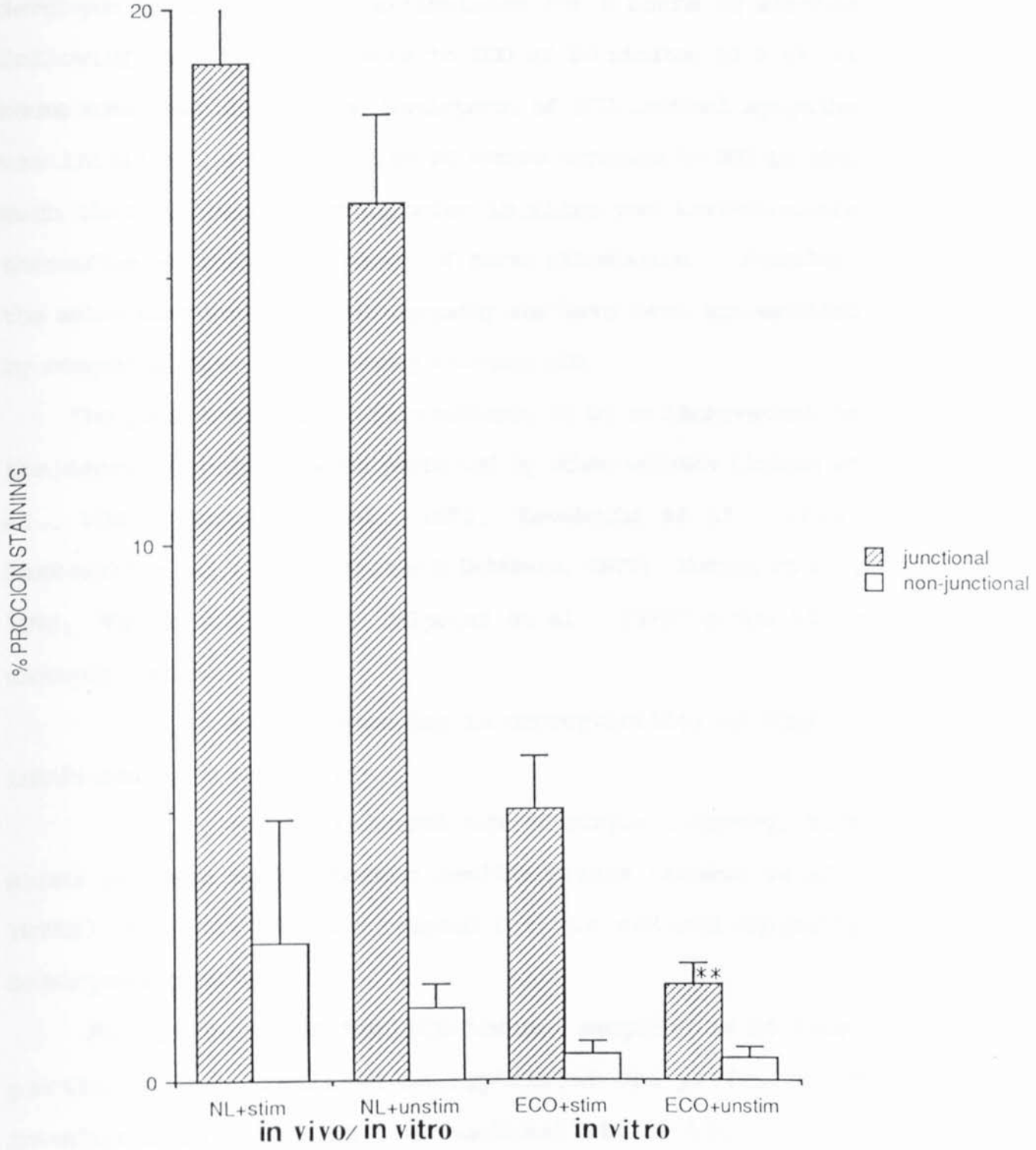
Application of ECO to stimulated phrenic nerve-diaphragm preparations in vitro induced myopathy at their junctional regions (table 9.2), myopathy being represented by Procion staining and hypercontractions. However, the development of myopathy was significantly reduced in unstimulated preparations ($P<0.01$: table 9.2). Previous experiments (9.3.1) showed that stimulation was not necessary for the progression of myopathy in vitro in diaphragms already exposed to ECO in vivo for 30 minutes (table 9.1). It seems therefore, that nerve stimulation, which evokes release of ACh, is necessary for the initiation of myopathy, but not for its progression in vitro (figure 9.3). The experiment suggests that ECO-induced myopathy must be at least partially neurally mediated and is not simply the result of a direct effect of the drug on the diaphragm. If ECO-induced myopathy was independent of nerve stimulation then the unstimulated preparations, incubated in 500nM ECO, would have been affected to the same degree as stimulated preparations. It is possible that ECO may have a slight direct effect on the diaphragm which is exacerbated by nerve stimulation, an hypothesis which could explain the slight development of myopathy in the unstimulated preparations (figure 9.3). It is more likely however, particularly in the light of evidence that muscle necrosis is mediated by ACh release in the neuromuscular synapse (Wecker et al., 1978b), that in the unstimulated preparations, nonquantal plus spontaneously released ACh promoted slight myopathy

Table 9.2 % Procion staining of stimulated and unstimulated preparations exposed to 500mM ECO *in vitro*:- the results represent the mean ± standard error of 8 experiments.

	% Procion staining	
	Stimulated	Unstimulated
junctional	5.60±1.00	1.77±0.39**
nonjunctional	0.49±0.22	0.42±0.20

** indicates that, using Students-paired-'t'-test, a significant difference exists, at the 1% level, between the two sets of preparations ie. the unstimulated preparations were significantly less myopathic than their stimulated counterparts.

Figure 9.3. The effect of nerve stimulation on the in vitro development of myopathy in preparations already exposed to ECO in vivo for 30 minutes, and in preparations exposed to ECO in vitro:- the results represent the mean ± standard error of at least 7 experiments.



** indicates that a significant difference exists, at the 1% level, between the stimulated and unstimulated preparations exposed to ECO in vitro.

development and may also have maintained the progression of myopathy following the removal of nerve stimulation 30 minutes after ECO administration *in vivo* (9.3.1).

However, owing to the extensive nature of the myopathy developed in preparations unstimulated for 2 hours 30 minutes following an *in vivo* exposure to ECO of 30 minutes (9.3.1), it seems more feasible that the development of ECO-induced myopathy was initiated during the first 30 minute exposure to ECO *in vivo* such that its further progression *in vitro* was irreversible thereafter by subsequent removal of nerve stimulation. Possibly, the subsequent development of myopathy may have been exacerbated by nonquantal plus spontaneously released ACh.

The present experiment is considered to be an improvement on the denervation experiments performed by other workers (Ariens et al., 1969; Fenichel et al., 1972; Kawabuchi et al., 1975; Laskowsli et al., 1975; Wecker & Dettbarn, 1977; Hudson et al., 1978; Wecker et al., 1978; Salpeter et al., 1979) since it is uncomplicated by

a) the decrease in susceptability of AChE to inhibition by OP agents, or

b) the increased rate of enzyme recovery, both shown to occur in dennervated hemidiaphragms (Wecker et al., 1978b), and both possible reasons for the reduced myopathy development observed.

Having established that ECO-induced myopathy is at least partially nerve mediated an experiment was performed to investigate the requirement of a functional AChR (9.2.3).

9.3.3. The requirement for a functional AChR in the development of myopathy in the diaphragms of mice exposed to ECO in vivo for 30 minutes.

9.3.3.1. Contraction records.

Control preparations, stimulated in normal Lileys saline exhibited strong contractions throughout the incubation period (figure 9.4), declining finally during the "wash" in Ca-reduced saline.

Preparations stimulated in the presence of tubocurarine demonstrated a gradual decline of the ECO-induced contractions, stopping altogether, within 20 minutes of exposure to tubocurarine (figure 9.4).

9.3.3.2. Histological examination of preparations.

Both sets of preparations were severely myopathic at their junctional regions, the nonjunctional regions being comparatively unaffected (table 9.3) ie. application of tubocurarine did not affect the progression of ECO-induced myopathy in normal Lileys saline. The result suggests either that myopathy may have been initiated during the first 30 minute period in vivo, such that it was irreversible by inactivation of AChRs, or that the AChRs are not involved in the development of ECO-induced myopathy at all. The second alternative seems unlikely in the light of evidence

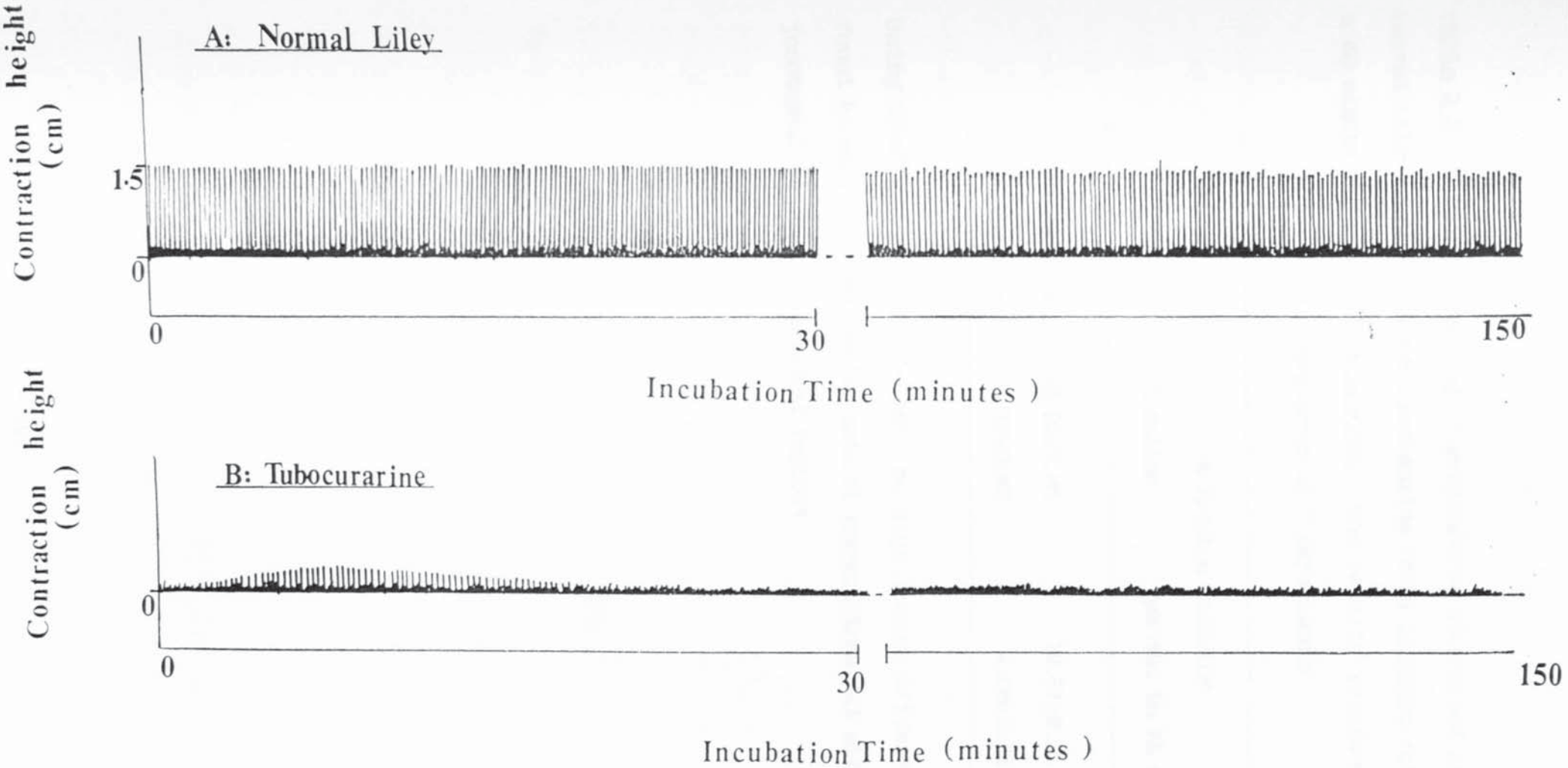


Figure 9.4. A. Control preparations stimulated in normal Lileys saline exhibited strong contractions for 2 hours 30 minutes, following a 30 minute exposure to ECO in vivo. B. Preparations stimulated in the presence of tubocurarine, following a 30 minute exposure to ECO in vivo, demonstrated a gradual decline of the contractions, stopping altogether, within 20 minutes of exposure to tubocurarine.

Table 9.3 % Procion staining of preparations stimulated in normal Lileys saline(NL)/9μM tubocurarine (dtc) *in vitro* following a 30 minute exposure to ECO *in vivo*:- the results represent the mean ± standard error of 7 experiments.

	% Procion staining	
	NL saline	9μM dtc in NL saline
junctional	18.92±3.86	19.61±5.21
nonjunctional	1.16±0.52	1.08±0.29

Using Students-paired-'t'-test, no significant difference was found to exist between the two sets of preparations, at either the junctional or the nonjunctional regions.

that the AChR is integrally involved in the development of other antiChE-induced myopathies (Ariens et al., 1968; Salpeter et al., 1979; Leonard and Salpeter, 1979, 1980).

Although preparations were exposed to tubocurarine 30 minutes after ECO administration, the actual access of the receptor blocker to the neuromuscular synapse is likely to have been delayed by the diffusion of the drug through the tissue. Infact the contraction records indicate that complete neuromuscular block was not obtained until approximately 20 minutes after application of tubocurarine (9.3.3.1). Possibly therefore, the development of ECO-induced myopathy had been instigated before tubocurarine actually reached the neuromuscular synapse.

An earlier administration of tubocurarine was investigated in order to determine whether the complete block of AChR in phrenic nerve-diaphragm preparations, prior to exposure to ECO, would affect the subsequent development of myopathy in vitro.

9.3.4. The requirement for a functional AChR for the in vitro development of myopathy in phrenic nerve-diaphragm preparations exposed to 500nM ECO in vitro (9.2.4)

9.3.4.1. Contraction records.

Application of ECO to stimulated preparations elicited a gradual potentiation of the contractions followed by a gradual decrease in contraction magnitude to a size much less than that originally induced by stimulation of the preparations in normal

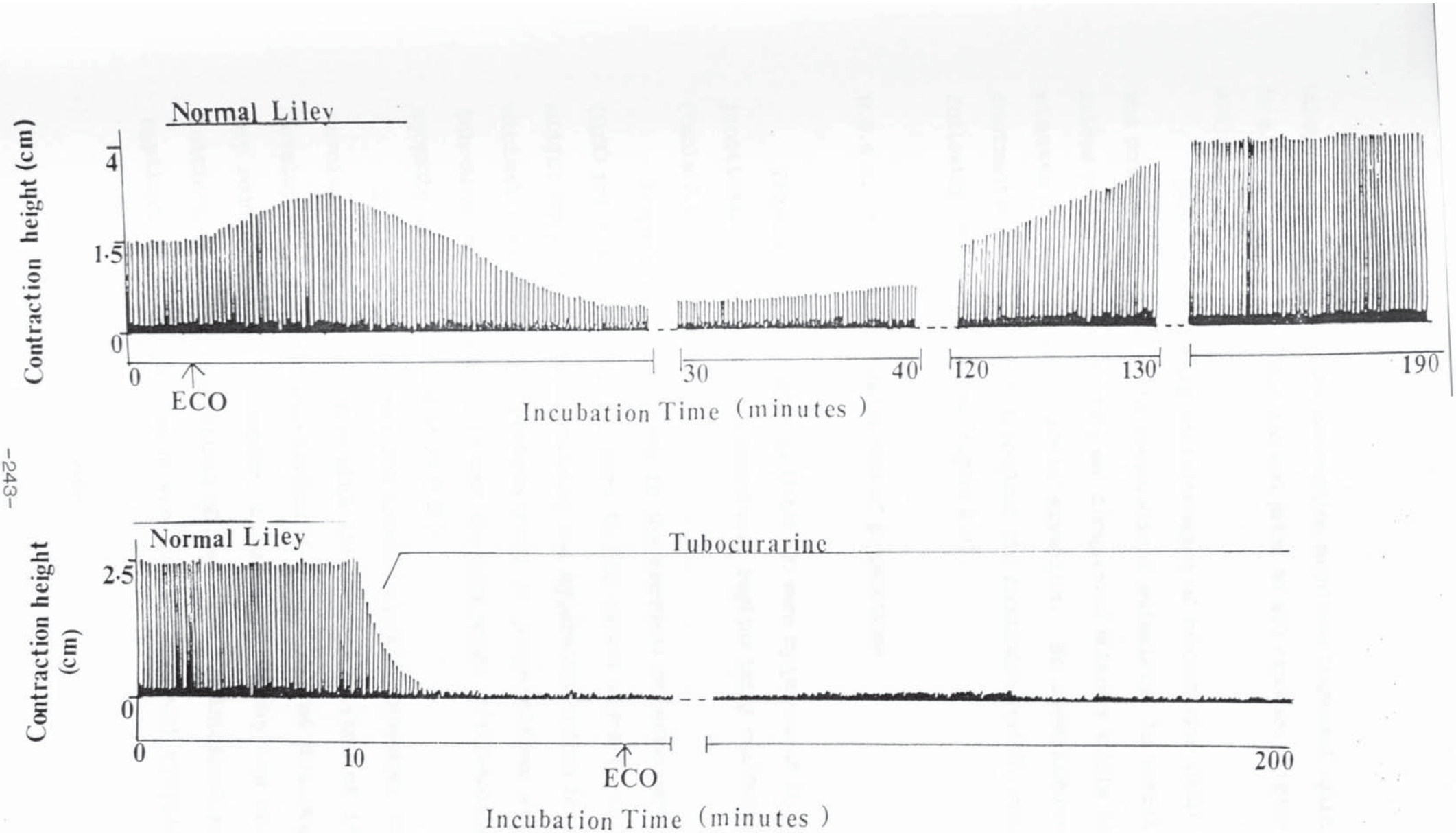


Figure 9.5. A. Application of ECO to preparations in normal Lileys saline elicited a short-lived potentiation of contractions, followed later by a sustained period of contraction enhancement. B. Application of tubocurarine to preparations stimulated in normal Lileys saline caused a rapid decline in contraction magnitude, dissappearing entirely within 20 minutes. No spontaneous contractions were recorded even after ECO administration.

Lileys saline. Finally, the contraction magnitude increased again to a level greater than that induced prior to ECO exposure (figure 9.5).

However, following administration of tubocurarine (9μM), the contractions elicited in preparations stimulated in normal Lileys saline decreased rapidly and disappeared entirely within 20 minutes of tubocurarine administration. No spontaneous contractions were recorded throughout the incubation period, even following ECO administration (figure 9.5).

9.3.4.2. Histological examination of preparations.

Preparations stimulated in 500nM ECO were myopathic at their junctional regions, the nonjunctional regions being unaffected (table 9.4).

Preparations stimulated in the presence of tubocurarine (9μM) prior to and during exposure to ECO showed signs of only slight myopathy, Procion staining and hypercontractions being minimal (table 9.4) ie. pretreatment of preparations with tubocurarine significantly reduced the development of ECO-induced myopathy in vitro (table 9.4: $P < 0.01$).

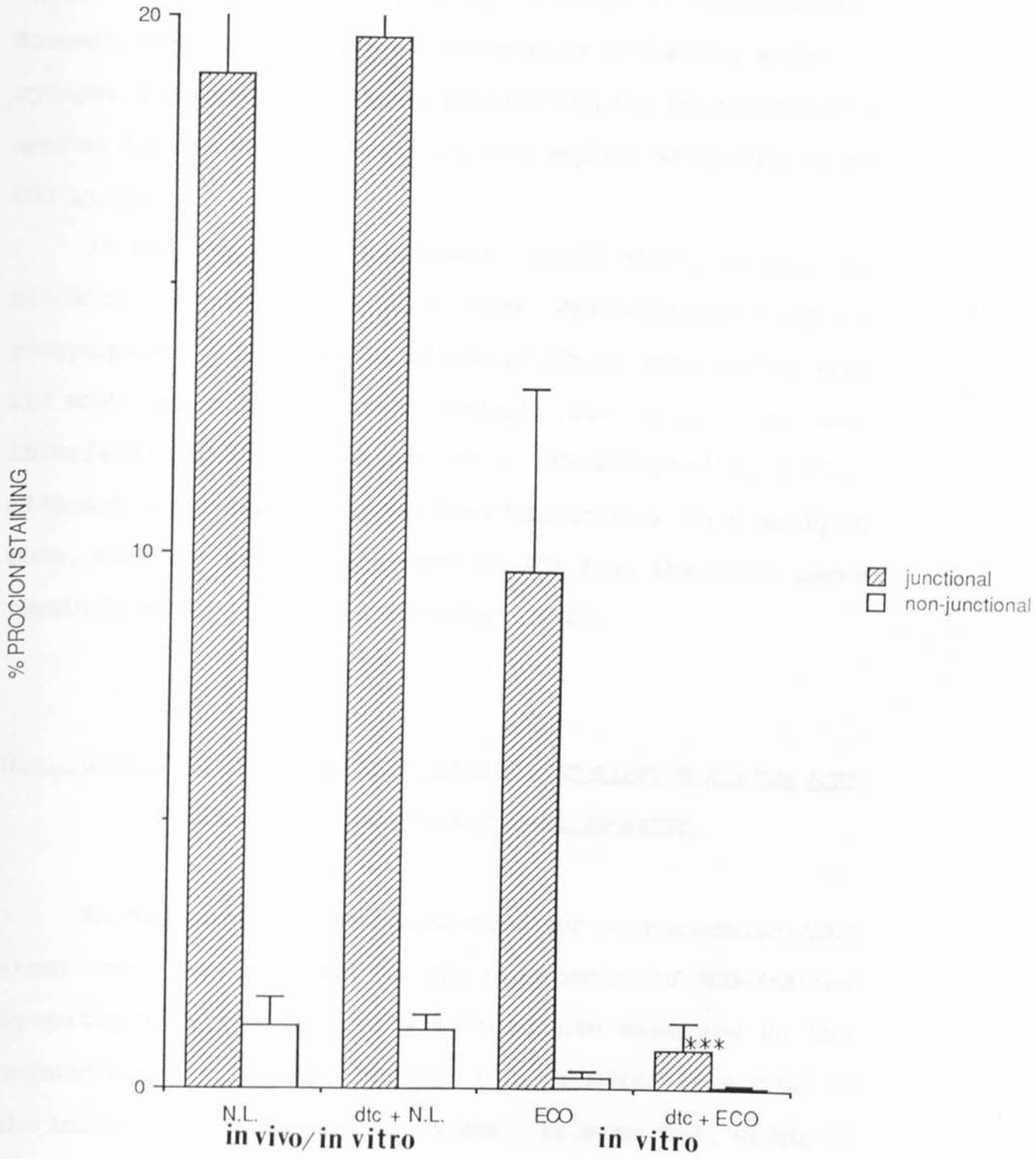
It is concluded that ECO-induced myopathy requires the involvement of a responsive AChR for the initiation of its development, and that inactivation of the receptor at this stage may reduce myopathy. However, once the myopathy has been initiated (in this case within 30 minutes of the intoxication), application of tubocurarine was ineffective and myopathy

Table 9.4 % Procion staining of phrenic nerve-diaphragm preparations stimulated for 3 hours in 500nM ECO, with or without added tubocurarine (9μM):- the results represent the mean ± standard error of 6 experiments.

	% Procion staining	
	500nM ECO	9μM tubocurarine in 500nM ECO
junctional	9.70±3.40	0.71±0.53***
nonjunctional	0.22±0.09	0.06±0.02

*** indicates that, using Students-paired-'t'-test, a significant difference exists, at the 0.1% level, between the results of the two sets of preparations ie. tubocurarine, when administered prior to ECO significantly reduced subsequent myopathy development.

Figure 9.6. The effect of d-tubocurarine (dtc) on the *in vitro* development of myopathy in preparations exposed to ECO *in vivo* for 30 minutes (ie. the *in vivo/ in vitro* experiments) and those exposed to ECO *in vitro* (ie. the *in vitro* experiments):- the results represent the mean ± standard error of at least 6 experiments.



*** indicates that a significant difference exists, at the 0.1% level, between the preparations treated with dtc plus ECO *in vitro* and those treated with only ECO *in vitro*.

developed as usual (figure 9.6). Again this could be explained by the suggestion that myopathy might have been instigated during the 30 minute period *in vivo* such that its further progression *in vitro* became irreversible by application of tubocurarine. However, the delay of access of tubocurarine to the neuromuscular synapse does provide a viable explanation for its inefficiency against the development of myopathy when applied 30 minutes after ECO *in vivo*.

It has been suggested (Bowman, 1980) that, as well as blocking the postsynaptic AChR sites, 9uM tubocurarine may act presynaptically, reducing the release of ACh by interfering with its mobilisation from the nerve terminal. Such an effect may also interfere with the development of an ACh-mediated myopathy, although other reports suggest that tubocurarine, in a paralytic dose, does not affect the output of ACh from the motor nerve terminals at all (Fletcher & Forrester, 1975).

9.4. SUMMARY OF THE INVOLVEMENT OF NERVE STIMULATION AND THE ACHR IN THE DEVELOPMENT OF ECO-INDUCED MYOPATHY.

Neither phrenic-nerve stimulation nor neuromuscular-AChR involvement were required for the progression of ECO-induced myopathy *in vitro* following a 30 minute exposure to the organophosphate *in vivo*. However, both parameters were vital to the initiation of the myopathic process. It seems that, within 30 minutes after ECO administration, the subequent progression of

myopathy becomes irreversible by the removal of nerve stimulation or AChR involvement. It is unlikely that, within the first 30 minutes *in vivo*, sufficient Ca^{2+} enters the muscle to initiate severe myopathy since an earlier experiment (8.2.2.1) demonstrated that the reduction of extracellular Ca^{2+}, 30 minutes after ECO administration did infact reduce the development of myopathy (8.2.2.1). However, in the case of the nerve stimulation experiments, it is possible that the nonquantal plus spontaneously released ACh (9.3.1.1) may have allowed Ca^{2+} influx to continue and promote the development of a more extensive myopathy. In the case of AChR involvement however, tubocurarine blocked all contractile response to nerve stimulation, both stimulated and spontaneous (9.2.2.1): thus, further entry of Ca^{2+} may therefore have been prevented. However, when tubocurarine was applied to the bath, 30 minutes after ECO administration *in vivo* , the 20 minute delay of neuromuscular block may have allowed further Ca^{2+} influx, sufficient to initiate the development of a severe myopathy. Indeed, previous experiments have shown that a 60 minute exposure to extracellular Ca^{2+}induced severe myopathy (8.3 & 8.4), hence the total 50 minute period before tubocurarine actually blocked the AChRs may have allowed a comparable influx of Ca^{2+} and hence induced a comparable degree of myopathy.

An alternative explanation for the development of severe myopathy in preparations exposed to ECO for 30 minutes *in vivo*, prior to removal of nerve stimulation or AChR involvement, may be

that, following a 30 minute exposure to ECO, the Ca^{2+} channels became "locked" in a permanently open state thereby allowing a continual influx of Ca^{2+} (Kuba et al., 1974) despite the removal of neuronal stimulation or the blocking of AChRs. However, assays of the Ca^{2+} content of the preparations would be necessary to confirm the continual influx of Ca^{2+} following neuromuscular block or the removal of nerve stimulation and infact Katz & Miledi (1975) refuted the idea of "locked open" Ca^{2+}-channels.

CHAPTER 10

TREATMENT OF ECO-INDUCED MYOPATHY.

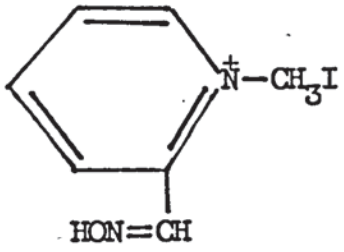
10.1. INTRODUCTION.

Progressive myopathies have been observed with administration of OP antiChEs such as DFP (Toth et al., 1983) and paraoxon (Ariens et al., 1969; Fenichel et al., 1974; Laskowski et al.,1975,1977; Wecker et al., 1978a&b) to rats. Intoxication with these compounds can cause death by respiratory paralysis (Grob & Harvey, 1953; Durham & Hayes, 1962), and since OP compounds are used in everyday life, it is necessary to have access to antidotes or prophylactics against their toxic effects. The most successful therapeutic agents for OP poisoning have been shown to be the oximes and hydroxamic acids (Durham & Hayes, 1962) whilst, prophylactically, several carbamates, reversible inhibitors of AChE have been shown to be effective against OP intoxication (Berry & Davies, 1970; Dirnhuber & Green, 1978; French et al, 1979; Dirnhuber et al, 1979) .

10.1.1. The use of oximes in the treatment of OP poisoning.

Oximes have been shown to successfully reactivate AChE if applied soon after its inhibition by an OP compound (Lipson et al., 1969; Fischer, 1970; Murtha et al., 1970; Laskowski & Dettbarn, 1977; Wecker et al., 1978a). Oximes act by making a neucleophillic attack on the phosphorous atom of the enzyme-inhibitor complex: the OP moiety is split off and hydrolysed, then the oxime residue undergoes a further reaction to regenerate the active enzyme (Durham & Hayes, 1962).

Pyridine-2-aldoxime methiodide (2PAM) is one of the most effective oximes in the treatment of OP poisoning. 2PAM is a white, odourless, crystalline solid of high water solubility (Lipson et al., 1969; Durham & Hayes, 1962) and low mammalian toxicity (Durham & Hayes, 1962). It has been found to prevent the development of myopathies induced by paraoxon (Laskowski et al., 1977; Wecker et al, 1978a &b), parathion, phosdrin (Brachfeild & Zavon, 1965) and DFP (Ariens et al., 1968, 1969) but is relatively ineffective against soman (Loomis & Salafski, 1963; Heilbron & Tolegen, 1965; Murtha et al., 1970).



Pyridine-2-aldoxime methiodide (2PAM): (Grob & Johns, 1958).

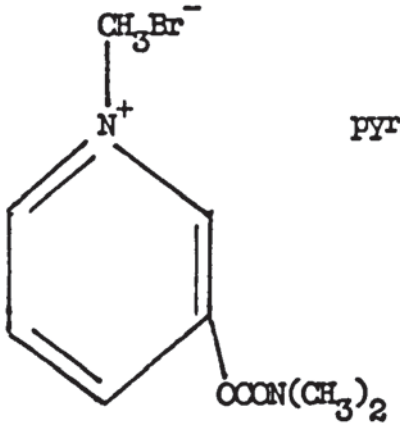
Jewel and Lehman (1958) showed that 2PAM, when administered simultaneously with ECO in vivo, completely protected mice against 23x the lethal dose: the oxime diacetylmonoxime provided no protection, whilst atropine was effective against the muscarinic but not the nicotinic effects of ECO. However, no study has been made, to the author's knowledge, to assess the therapeutic effectiveness of 2PAM against myopathy induced by ECO.

10.1.2. The use of carbamates in the prophylaxis of OP poisoning.

Carbamates, the reversible inhibitors of cholinesterase,

have been implemented in the prevention of OP poisoning (Dirnhuber & Green, 1978; Gordon et al., 1978; Wecker et al., 1978a&b; Dirnhuber el al., 1979; French et al., 1979). The beneficial action of carbamates is due to their ability to reversibly inhibit AChE (Berry & Davies, 1970; Dirnhuber & Green, 1978), and to the fact that tissues contain more AChE than is necessary for normal functioning (Hobbiger, 1976). Once AChE has been inhibited by a carbamate the irreversible binding of an OP is no longer possible (Berry & Davies, 1970). Eventually, the carbamate dissociates from the enzyme, freeing it from inhibition completely (Durham & Hayes, 1962; Berry & Davies, 1970). For a carbamate to prevent the binding of OP to the AChE molecule it must be administered prior to the OP. However, the carbamate should not be administered too far in advance of the OP since, once the carbamate-AChE complex begins to dissociate, the AChE is no longer protected from irreversible OP poisoning.

One of the more commonly applied carbamates is pyridostigmine bromide which does not pass the blood-brain barrier (Birtley et al, 1966).



pyridostigmine bromide

Pyridostigmine bromide is used clinically in the treatment of myaesthenia gravis and has also been shown to be effective in preventing the neuromuscular block normally produced by soman (Dirnhuber & Green, 1978; French et al., 1979; Dirnhuber et al., 1979) and against soman poisoning (Berry & Davies, 1970; Gordon et al., 1978). It has been shown to reduce the myopathy normally induced by other OP compounds (Dirnhuber et al., 1979).

The aims of the experiments described in the present chapter were:-

1. To investigate the therapeutic effectiveness in vivo, of 2PAM in the treatment of ECO-induced myopathy in the diaphragms of mice.

2. To investigate the prophylactic effectiveness in vivo, of pyridostigmine bromide in the prevention of ECO-induced myopathy in the diaphragms of mice.

10.2. EXPERIMENTAL DESIGN.

10.2.1. The therapeutic effectiveness in vivo, of 2PAM against ECO-induced myopathy.

Eight groups of 6 mice were used:-

Group a: injected with atropine only (2.2).

Group b: injected with 30mgKg^{-1} 2PAM (in 0.9% NaCl).

Group c: injected with ECO (+ atropine) (2.2).

Group d: injected with ECO (+ atropine) (2.2) followed, 15 minutes later, by 2PAM (30mgKg^{-1}).

Group e: injected with ECO (+ atropine) (2.2) followed, 30 minutes later by 2PAM (30mgKg^{-1}).

Group f: injected with ECO (+ atropine) (2.2) followed, 45 minutes later by 2PAM (30mgKg^{-1}).

Group g: injected with ECO (+ atropine) (2.2) followed, 60 minutes later by 2PAM (30mgKg^{-1}).

Group h: injected with ECO (+ atropine) (2.2) followed, 75 minutes later by 2PAM (30mgKg^{-1}).

NB. All injections were administered subcutaneously between the shoulder blades.

Mice were anaesthetised 3 hours after ECO administration (a time when myopathy has been shown to be extensive: 3.3.2), and blood was obtained from the femoral artery for estimation of serum CK (2.10). Those mice given atropine or 2PAM only were anaesthetised 3 hours after their single injections.

Mice were killed whilst anaesthetised, and their diaphragms were removed and cut into hemidiaphragms (2.3). One hemidiaphragm was assayed for Ca^{2+} influx at the junctional region (2.9) and the other was assessed for myopathy using the Procion technique (2.7).

Statistical comparisons were made between the eight groups of mice using Students-unpaired-"t"-test and, in this way, the therapeutic effectiveness of 2PAM against ECO-induced myopathy was evaluated.

10.2.2. The prophylactic effectiveness in vivo of pyridostigmine bromide against ECO-induced myopathy.

Eight groups of mice were used:-

Group a: injected with atropine only (2.2).

Group b: injected with 100ugKg^{-1} pyridostigmine bromide (made up in 0.9% NaCl saline).

Group c: injected with ECO (+ atropine) (2.2).

Group d: injected with pyridostigmine bromide (100ugKg^{-1}) then, 15 minutes later, by ECO (+ atropine) (2.2).

Group e: injected with pyridostigmine bromide)100ugKg^{-1}) then, 1 hour later, by ECO (+ atropine) (2.2).

Group f: injected with pyridostigmine bromide ($100\mu gKg^{-1}$) then, 2 hours later, by ECO (+ atropine) (2.2).

Group g: injected with pyridostigmine bromide ($100\mu gKg^{-1}$) then, 4 hours later, by ECO (+ atropine) (2.2).

Group h: injected with ECO (+ atropine) (2.2) then, 15 minutes later by pyridostigmine bromide ($100\mu gKg^{-1}$)

All injections were administered subcutaneously, between the shoulder blades, and the dose of pyridostigmine bromide ($100\mu gKg^{-1}$) was chosen because it has been reported to be "sign free" and effective in the prophylactic treatment of OP-induced myopathy (Dirnhuber et al, 1979).

3 hours after ECO administration, (when myopathy is known to be extensive: 3.3.2), mice were anaesthetised and blood was obtained from the femoral artery for analysis of serum CK (2.10). Mice administered atropine or pyridostigmine bromide only were anaesthetised 3 hours after their respective single injections. Mice were killed under anaesthesia, and their diaphragms were removed and cut into hemidiaphragms (2.3). One hemidiaphragm was assayed for Ca^{2+} influx at the junctional region (2.9) and the other was stained with Procion and assessed for myopathy using the Procion technique (2.7).

Students-unpaired-"t"-test was used to compare the eight groups of mice and the prophylactic effectiveness of pyridostigmine bromide against ECO-induced myopathy was evaluated.

10.3. RESULTS AND DISCUSSION.

10.3.1. The therapeutic effectiveness in vivo, of 2PAM against ECO-induced myopathy

10.3.1.1. Behavioural response.

An injection of ECO provoked overall ataxia plus body tremor due to the fasciculations of individual muscles including the diaphragm. However, subsequent administration of 2PAM ($30mgKg^{-1}$) within 30 minutes of ECO, terminated such effects within 10-15 minutes and the mice resumed a normal pattern of behaviour. 2PAM, administered later than 30 minutes after ECO, became progressively less effective, a delay of greater than 60 minutes no longer relieving the behavioural effects of ECO. Administration of 2PAM, or atropine only, caused no noticeable behavioural changes in mice.

10.3.1.2. Histological examination of hemidiaphragms.

Diaphragms of mice injected with ECO developed severe myopathy at the junctional region only, the nonjunctional region being comparatively unaffected (table 10.1). However, when 2PAM ($30mgKg^{-1}$) was administered 15 minutes after ECO (ie. at the onset of tremor), the subsequent development of myopathy was prevented (table 10.1). Procion staining at the junctional region of the diaphragm increased gradually with increasing delay of 2PAM

Table 10.1. The therapeutic effectiveness of 2PAM on the development of ECO-induced myopathy: the results represent the mean ± standard error of 6 experiments.

	% Procion staining		Serum CK	Calcium influx
	junctional	nonjunctional	(IUL^{-1})	at J ($nmol\,mg^{-1}$)
Atropine	$0.15\pm0.11^{***}$	$0.13\pm0.04^{***}$	$64\pm18^{***}$	$0.547\pm0.058^{**}_{+}$
2PAM only	$0.66\pm0.49^{***}$	0.28 ± 0.22	$157\pm77^{***}$	$0.69\pm0.033^{**}$
2PAM 15′	$0.63\pm0.24^{***}$	0.28 ± 0.11	$148\pm43^{***}$	$1.21\pm0.160_{++}$
2PAM 30′	$1.50\pm0.79^{***}$	0.60 ± 0.23	$183\pm45^{***}$	$0.985\pm0.240_{++}$
2PAM 45′	$1.60\pm0.43^{***}$	0.22 ± 0.14	$1018\pm146^{*}_{+++}$	$1.670\pm0.370_{+++}$
2PAM 60′	$2.80\pm1.00^{***}$	$0.11\pm0.04^{*}$	$1060\pm176^{*}_{+++}$	$1.140\pm0.110_{+++}$
2PAM 75′	$5.05\pm0.85^{*}_{+++}$	0.26 ± 0.04	$1615\pm388_{++}$	$1.300\pm0.170_{++}$
ECO	$11.50\pm3.20_{+++}$	0.46 ± 0.19	$3923\pm565_{++}$	$2.440\pm0.900_{+++}$

***, **, * indicate results that are significantly different from those pertaining to mice injected with ECO only, at the 0.1%, 1% and 5% levels respectively.

+++, ++, + indicate results that are significantly different from those pertaining to mice injected with 2PAM only, at the 0.1%, 1% and 5% levels respectively.

2PAM 45′ indicates 2PAM administered 45 minutes after ECO.

administration (table 10.1), but became significantly increased ($P<0.01$) only when 75 minutes were allowed to elapse between ECO and 2PAM injections. However, a delay of 75 minutes still significantly reduced the severity of myopathy induced by ECO alone ($P<0.0.5$: table 10.1). The severity of the myopathy was directly related to the time interval between injections ie. the duration of AChE inhibition. Treatment with 2PAM following ECO administration did not effect the % Procion values at the non-junctional region of the diaphragm, reflecting the very limited spread of the myopathy away from the junctional region rather than a lack of effect of 2PAM itself.

10.3.1.3. Serum CK activity

Administration of 2PAM alone caused no significant Procion staining of the diaphragm and did not significantly affect serum CK activity in mice (table 10.1). However, when administered within 30 minutes of ECO, 2PAM did prevent the elevation of serum CK normally induced by ECO. Serum CK activity then increased with increasing delay of 2PAM administration until finally, a delay of 75 minutes caused elevation of serum CK to a level not significantly different from that induced by ECO only.

The results suggest that serum CK activity was significantly increased before Procion staining of the diaphragm; delays of 45 minutes and 60 minutes respectively caused significant elevation of serum CK ($P<0.01$ in each case), yet no significant increase in Procion staining. Perhaps CK had leaked from the muscles via some

route not involving damage to the sarcolemma, possibly via open ion channels in the membrane. Infact, it has been suggested (Suarez-Kurtz, 1983) that CK leakage may result from prolonged depolarisation of the neuromuscular junction or from an increase in the Ca^{2+} concentration of the sarcoplasm both of which are known to occur during ECO-intoxication. Alternatively, it is possible that CK had been lost from tissues other than the diaphragm, possibly the heart, brain or other skeletal muscles.

10.3.1.4. Calcium influx at the junctional region of the diaphragm

Administration of 2PAM alone caused a slight influx of Ca^{2+} at the junctional region of the diaphragm (table 10.1) and failed to reduce the large Ca^{2+} influx caused by ECO (table 10.1). Assuming that Ca^{2+} influx occurred prior to reactivation of AChE by 2PAM and that access of 2PAM to AChE takes 10-15 minutes from the time of its administration (10.3.1.1), it is suggested that the influx of Ca^{2+} must have occurred within the first 30 minutes of exposure to ECO supporting the hypothesis that Ca^{2+} influx is an early result of the inactivation of AChE.

It seems that there exists a requirement, in the development of ECO-induced myopathy, for the persistence of AChE inactivation, of at least 75 minutes which is independent of its ability to promote further Ca^{2+} influx into the diaphragm.

The diminishing effectiveness of 2PAM against OP-induced myopathies may be due to the onset of an irrevocable situation such that the development of myopathy had been initiated to the point of no return. However, if this were the case, one would expect an all-or-nothing effect of 2PAM administration at various times after ECO , and not the graded response observed. An alternative and more plausible explanation for the diminishing effectiveness of 2PAM may be explained by the "ageing" of the AChE-OP complex (Lipson et al., 1969; Durham & Hayes, 1962). Ageing involves a dealkylation reaction during which an ionised hydroxyl group is attatched to the phosphorous atom of the phosphorylated enzyme. The result is that the inhibited enzyme will no longer yield to nucleophilic attack which is the basis of oxime reactivation (Lipson et al., 1969). Hence the longer the delay of 2PAM administration, the less likelihood of AChE reactivation and the greater the extent of myopathy development. The lack of effectiveness of 2PAM against soman poisoning is beleived to be due to the rapid ageing of the soman-AChE complex (Loomis & Salafski, 1963; Murtha et al., 1970). An interesting extension to the current work would be to measure the AChE activity of ECO-treated diaphragms during progressively delayed 2PAM therapy. In this way we could obtain a clearer indication of the speed of the ageing process of the ECO-AChE complex.

The therapeutic action of the reactivator 2PAM has been explained in more than one way:-

a) Reactivation of AChE allows the enzyme to perform its primary function of destroying the accumulation of ACh in the neuromuscular synapse (Grob & Johns, 1958; Lipson et al., 1969).

b) By forming a complex of the oxime with AChE, thereby neutralising AChE before the OP reaches the active site of the enzyme ie. 2PAM itself has a slight inhibitory effect on AChE (Goyer, 1968, 1970; Lipson et al., 1969).

c) 2PAM may react with and inactivate the OP molecule (Durham & Hayes, 1962).

d) 2PAM may have a tubocurarine-like effect, so blocking neuromuscular transmission in the diaphragm (Goyer, 1970).

In the present study, since the oxime was never applied within 15 minutes after ECO, it is unlikely that it will have reacted with the OP. If 2PAM did inactivate ECO then presumably this event would occur after the maximal binding of ECO to AChE sites. Hence 2PAM would not have contributed to any prophylaxis against AChE inhibition. Similarly, inhibition of AChE by 2PAM could only have been very slight since administration of the oxime alone induced only slight Ca^{2+} influx at the junctional region of the diaphragm, no Procion staining, no change in serum CK and no change in behaviour. Finally, it is unlikely that 2PAM could have had a neuromuscular blocking action since, when administered to mice, 2PAM alone caused no behavioural changes indicative of neuromuscular block.

It seems most likely therefore, that 2PAM exerts its therapeutic effect against ECO-induced myopathy largely by its property to reactivate AChE.

The effectiveness of 2PAM therapy supports the suggestion that ECO acts via inhibition of AChE rather than by a direct effect on the diaphragm since, if ECO had been acting directly on the diaphragm, then the reactivation of AChE would have been ineffective against subsequent myopathy development.

10.3.2. The prophylactic effectiveness in vivo, of pyridostigmine bromide against ECO-induced myopathy.

10.3.2.1. Behavioural response.

Single injections of pyridostigmine bromide ($100\mu gKg^{-1}$) or atropine (2.2) alone evoked no noticeable behavioural changes in mice. An injection of ECO plus atropine however, caused overall ataxia plus body tremor due to the fasciculation of individual muscles. These effects were prevented when pyridostigmine was administered 15 minutes, 1 hour or 2 hours before ECO. When pyridostigmine was injected 4 hours before ECO the behavioural response to ECO was reduced but not prevented, and pyridostigmine administered 15 minutes after ECO had no affect on the behavioural response usually induced by ECO.

10.3.2.2. Histological examination of hemidiaphragms.

Acute administration of pyridostigmine bromide alone caused slight Procion staining ($P<0.05$) at the junctional region of the diaphragm suggesting that the dose used ($100\mu gKg^{-1}$) was not entirely sign-free as had been suggested (Dirnhuber & Green, 1979). However, table 10.2 shows that pyridostigmine, when administered within 2 hours of ECO, successfully prevented the subsequent development of severe myopathy although, as the period between injections increased, there was a corresponding increase in the % Procion staining (table 10.2). When administered 4 hours before ECO pyridostigmine was no longer effective and myopathy developed to a level not significantly different from that induced by ECO alone (table 10.2).

10.3.2.3. Serum CK activity.

Administration of pyridostigmine alone caused no elevation of serum CK in mice (table 10.2). However, when administered within 2 hours of ECO, pyridostigmine bromide prevented not only the development of severe myopathy, but also the elevation of serum CK activity normally induced by the intoxication (table 10.2). Again, as the period between injections increased, a corresponding increase in the serum CK activity occurred accompanying the progressive development of myopathy. Finally, when administered 4 hours before ECO, pyridostigmine bromide was no longer effective

Table 10.2 The prophylactic effectiveness of pyridostigmine bromide against against development of ECO-induced myopathy (n = at least 5 for each experiment).

	% Procion staining		Serum CK	Ca^{2+} influx at
	junctional	nonjunctional	(UL^{-1})	J ($nmolmg^{-1}$).
Atropine	$0.15\pm0.11^{***}_{\ddagger}$	0.13 ± 0.04	$56\pm35^{***}$	$0.547\pm0.058^{***}_{\ddagger}$
PB only	$0.73\pm0.23^{***}$	0.28 ± 0.13	$43\pm10^{***}$	1.096 ± 0.208
PB15'E	$1.37\pm0.33^{***}$	0.40 ± 0.13	$40\pm30^{***}$	$1.660\pm0.140_{\ddagger}$
PB1hE	$0.88\pm0.41^{***}$	0.45 ± 0.19	$53\pm19^{***}$	1.140 ± 0.086
PB2hE	$1.27\pm0.33^{***}$	0.16 ± 0.07	$92\pm35^{***}$	1.540 ± 0.202
PB4hE	$10.6\pm2.10_{+++}$	$1.20\pm0.40_{+}$	$183\pm55^{***}_{\ddagger}$	$2.757\pm0.359_{\ddagger}$
ECO	$11.5\pm3.20_{\ddagger}$	0.50 ± 0.18	$3923\pm1606_{\ddagger}$	$2.440\pm0.904_{\ddagger}$
E15'PB	$14.4\pm1.30_{+++}$	0.40 ± 0.14	$2646\pm49_{+++}$	$4.300\pm0.270^{***}_{+++}$

PB15'E indicates that pyridostigmine bromide was administered 15 minutes before ECO.

J, NJ indicates junctional, nonjunctional regions.

*** indicates that the result is significantly different from that pertaining to mice treated with ECO only, at the 0.1% level.

+++ and + indicates results that are significantly different from those pertaining to mice treated with pyridostigmine only at the 0.1% and 5% levels respectively.

and serum CK was elevated (with respect to pyridostigmine alone: ($P<0.05$: table 10.2) but was still significantly less than that induced by ECO alone ($P<0.001$: table 10.2). Thus pyridostigmine, when applied 4 hours prior to ECO, seemed to have reduced ECO-induced leakage of CK from the tissue, despite evidence of extensive myopathy in the diaphragm. Alternatively, since pyridostigmine is itself an antiChE, shown to induce Ca^{2+} influx (10.3.2.4) and indeed slight myopathy in the diaphragm (table 10.2), it may have also caused a slow but consistent leakage of CK from the tissues, possibly mediated by elevated iCa^{2+} (Suarez-Kurtz, 1983). Such a slight release of CK might not have been measureable due to continuous clearance of the enzyme from the blood; Subsequent exposure to ECO 4 hours later, might have exacerbated the myopathy, but, due to earlier leakage of CK induced by pyridostigmine, serum CK activity would not have been elevated to the degree expected 3 hour after ECO administration.

10.3.2.4. Calcium influx at the junctional region of the diaphragm.

Although pyridostigmine successfully prevented both the development of myopathy and the elevation of serum CK when administered up to 2 hours before ECO, it did not prevent influx of Ca^{2+} at the junctional region of the diaphragm (table 10.2) and infact, in none of the groups of mice pretreated with pyrido-

stigmine was Ca^{2+} influx into the diaphragm significantly different from that induced by ECO alone. Again, it seems that there exists a requirement for the persistence of AChE inactivation in the development of ECO-induced myopathy, which is independent of its ability to promote further Ca^{2+} influx into the diaphragm (10.3.1.4).

When pyridostigmine was administered 15 minutes after ECO, Ca^{2+} influx was significantly greater than that induced by ECO alone ($P<0.01$: table 10.2) yet there was no significant effect on the subsequent development of myopathy. In this instance pyridostigmine was probably not acting at the same AChE sites as ECO since binding of the carbamate would have been prevented by the irreversible binding of the OP. However, pyridostigmine may have been bound to enzyme sites different from those inactivated by ECO. Binding of pyridostigmine to such enzyme sites might be expected to increase Ca^{2+} influx and Procion staining at the junctional region, without affecting serum CK activity.

The results of this experiment imply that pyridostigmine is an effective prophylactic agent against ECO-induced myopathy in mice, provided that it is applied within 2 hours before the OP injection. However, it is of no therapeutic use when administered after ECO.

Pyridostigmine is itself an antiChE agent, which in a dose of 360μgKg^{-1}, has been shown to inhibit whole blood AChE by 60-70% and cause both pre- and post-synaptic morphological alterations to the diaphragm, soleus and EDL (Hudson et al, 1985).

Dirnhuber et al (1979) demonstrated that pretreatment of primates with a maximum "sign-free" dose of pyridostigmine bromide (200μgKg^{-1}) offered considerable protection against soman intoxication. They also stated that the protection was afforded by 25% of the "sign-free" dose. In the present study 100μgKg^{-1} pyridostigmine bromide has been shown to cause no behavioural, cholinergic symptoms, and only slight myopathy in the diaphragm. When administered within 2 hours of ECO, this dose relieved the gross symptoms and myopathy induced by the OP. The dose of pyridostigmine administered (100μgKg^{-1}) is known to cause a 20% depression of AChE in the diaphragm (S.Das- personal communication). Hence, ECO must be required to inhibit greater than 80% of the AChE present in the diaphragm in order to induce severe myopathy in this muscle (7.3.1), and it seems that a very low functional level of AChE (less than 20%) is necessary for the maintainence of normal muscular function. Dirnhuber and Green (1978) reported that 4 hours after pyridostigmine bromide administration, carbamoylation of AChE dropped to 14% (from approximately 55% at 30-40 minutes after administration) and was no longer effective in the reversal of the neuromuscular blockade normally induced by soman. It is suggested that a similar drop in carbamoylation occurred in the present study, 4 hours after

pyridostigmine administration, and that this enabled further phosphorylation of AChE to occur on administration of ECO such that greater than 80% of the enzyme was inactivated and myopathy was thus induced in the diaphragm.

It would be interesting to assay the AChE activities of diaphragms exposed to pyridostigmine for 15 minutes, 1 hour, 2 hours and 4 hours respectively to see what degree of reversible AChE inhibition is necessary to protect the diaphragm from myopathy induced by a subsequent exposure to ECO.

CHAPTER 11

GENERAL DISCUSSION.

11.1. THE NATURE AND PROGRESSION OF OF ECO-INDUCED MYOPATHY

The present study has established that ecothiopate iodide (ECO), when administered subcutaneously to mice, induced a progressive myopathy, begining at the junctional region of the diaphragm but spreading along the fibre with increasing time after ECO administration (3.3.2). The myopathy was preceeded and accompanied by a progressive Ca^{2+} influx (5.3) and was concomitant with loss of creatine kinase (CK) from the necrotic region (4.3) causing elevation of enzyme activity in the serum (Ferry & Townsend, 1986). The early manifestations of ECO-induced myopathy included the appearance of hypercontractions at the junctional regions of affected muscle fibres, within 20 mintutes of ECO administration, indicative that free Ca^{2+} had been elevated in the sarcoplasm (Podolski & Costantin, 1964; Huxley, 1973). These early signs of ECO-induced myopathy correspond with the "disorganised soleplate cytoarchitecture" observed by Hudson et al (1985) within 30 minutes of antiesterase administration to rats, and with dilatation of SR and mitochondria observed in the present study (6.3). The time course of ECO-induced ultrstructural damage was similar to that observed by Wecker et al (1978a&b) in a study on the effect of paraoxon on rat skeletal muscle although other investigators have reported a much later onset and relatively delayed progression of damage (Ariens et al., 1969; Toth et al., 1983). Generally there is a great deal of variation between reports of the time courses of both the development of OP-induced myopathy and its repair/regeneration. In 1968 Ariens et al

reported the beginings of regeneration of skeletal muscle 48 hours after DFP/paraoxon administration to rats, and regeneration was still incomplete 10 days after the intoxication. An even slower regeneration of myopathy was observed in the experiments of Toth et al (1983) in which DFP/Wolfatox-induced myopathy was still severe 72 hours after intoxication, a time when regeneration was practically complete in the ECO treated diaphragms of mice (3.3.2, 6.3.2.10). Such differences between OP-induced myopathies are likely to be due largely to variations in the drug, its route of administration, the magnitude of exposure, the species of animal used and the type of muscle under study. More active muscles such as the diaphragm have been shown to be more susceptible to attack by OP compounds (Ariens et al., 1969; Wecker et al., 1978b). However, Wecker et al (1978b) demonstrated that, even in the diaphragm, only a small percentage of muscle fibres were affected by paraoxon at a time when myopathy was most extensive, and the present study revealed also that, although the number of fibres involved in ECO-induced myopathy did increase with time, at no time were more than 40% of the fibres in the diaphragm affected (Table 6.2).

ECO-induced myopathy was represented, at the ultrastructural level, by dilatation of the SR and mitochondria plus dissolution of Z-disks at its least severe, and by gross sarcoplasmic destruction plus phagocytosis at its most severe (6.3.2). Such features are consistent with those found by other workers (Ariens et al., 1969; Laskowski et al., 1975, 1977; Wecker et al., 1978b; Toth et al., 1983), the early manifestations, at least,

implicating involvement of high levels of iCa^{2+} and possibly also of Ca^{2+}-activated neutral proteases (CANP). In ECO-treated diaphragms, the fact that necrosis appeared to be most severe at the soleplate region of fibres, suggests a close involvement of the nerve terminal in the development of myopathy.

11.2 THE REQUIREMENT FOR NERVE STIMULATION AND THE INVOLVEMENT OF A FUNCTIONAL AChR FOR THE DEVELOPMENT OF ECO-INDUCED MYOPATHY

The initiation of the development of ECO-induced myopathy was shown to be both nerve and AChR mediated (ie. dependent on the stimulated release of ACh from the nerve terminal and also on the integral involvement of the AChR; 9.3.2 & 9.3.4). Other workers have suggested that OP-induced myopathies are nerve-mediated (Ariens et al., 1969; Fenichel et al., 1972; Kawabuchi et al., 1975; Laskowski et al., 1975; Wecker & Dettbarn, 1977; Hudson, 1978; Wecker, 1978; Salpeter, 1979) and require the involvement of an intact AChR (Ariens et al., 1969; Leonard & Salpeter, 1979, 1980) but, what has not been reported before (to the author's knowledge) is that, in the case of ECO at least, once the myopathy has been initiated (within 30 minutes of ECO administration), the need for nerve stimulation and AChR involvement is no longer important and myopathy develops regardless (9.3.1, 9.3.3). It is possible, in the case of the nerve stimulation experiments, that nonquantal plus spontaneously released ACh maintained the development of myopathy *in vitro*, following the removal of nerve stimulation from preparations exposed to ECO *in vivo* for 30

minutes (9.3.1 & 9.3.2). In the case of AChR involvement, however, it is thought that the 20 minute delay of access of tubocurarine, when applied 30 minutes after ECO administration in vivo, may have allowed the influx of Ca^{2+} to continue in vitro (9.3.3 & 9.3.4) to a point where myopathy became irrevocable.

It is not thought that the development of myopathy observed was due to a direct action of ECO on the diaphragm. If this were the case then preparations incubated in 500nM ECO without stimulation, or in the presence of tubocurarine would probably have been severely myopathic.

11.3. THE RELATIONSHIP OF ECO-INDUCED MYOPATHY TO ACHE INHIBITION

The development of ECO-induced myopathy was shown to be preceeded by extensive inhibition of AChE in both the blood (63%) and the diaphragm (83%). However, the progression of myopathy was not dependent on increased AChE inhibition, and infact blood AChE activity had recovered within 12 hours of ECO administration, at a time when myopathy was most extensive. It is suggested that the consequential accumulation of ACh in the neuromuscular synapse (Katz & Miledi, 1973) is responsible for the influx of Ca^{2+} at the junctional region of the diaphragm (Ashley & Ridgeway, 1968; Evans, 1974; Miledi et al., 1977) which in turn is responsible for the development of myopathy.

There was only a 25% recovery of diaphragm AChE activity during the return of muscle morphology to normality, suggesting

that very little functional AChE is required for the normal operation of cell physiology (Hobbiger, 1976).

11.4. THE INVOLVEMENT OF CALCIUM IN THE DEVELOPMENT OF ECO-INDUCED MYOPATHY

The reduction of Ca^{2+}-influx into diaphragms exposed to ECO reduced subsequent myopathy development (8.5), suggesting that myopathy is the result rather than the cause of the elevated iCa^{2+}. However, influx of Ca^{2+} from extracellular fluid may not be the only source of elevated iCa^{2+}. Ca^{2+} may also have been released from the intracellular Ca-buffers, most notably the mitochondria and SR, both thought to buffer iCa^{2+} (Oberc & Engel, 1977). Prolonged depolarisation of the postsynaptic membrane, by the prolonged presence of ACh, may have promoted the release of Ca^{2+} from the SR (Endo, 1977) and similarly, a Ca^{2+}-induced release of Ca^{2+} (CROC) from the SR may have occurred (Ford & Podolski, 1970; Endo, 1977; Fabiato, 1982). Alternatively, ECO, by prolonging the halflife of ACh in the synapse, may have promoted elevation of intracellular Na^{+} which may, in turn, have initiated release of Ca^{2+} from mitochondria via the Na^{+}/Ca^{2+} transporter of the inner mitochondrial membrane. Elevation of iCa^{2+} by release from intracellular organelles would not have been detected by the Ca-assay used in the present study which measured

total iCa^{2+}, both bound and free. It seems probable therefore, that the source of the elevated Ca^{2+} observed in diaphragms treated with ECO, may be both extracellular and intracellular.

The fact that both the SR and mitochondria become swollen following treatment of muscle fibres with ECO suggests that at least some of the iCa^{2+} is bound and therefore unavailable for activation of cytosollic proteases. This would certainly account for the lack of efficacy of leupeptin observed in the present study (8.4) but the role of Ca^{2+} in the development of ECO-induced myopathy remains unexplained. One possibility is that, in mitochondria, the massive uptake of Ca^{2+} takes primacy over ATP production, an event already shown to occur in liver mitochondria (Lehninger, 1967). The result of this would be, an inhibition of cell respiration, accumulation of the breakdown products of cellular metabolism and ultimate poisoning of the muscle fibre. Concurrently, the lack of ATP production would negate expulsion of Ca^{2+} from the cell, free iCa^{2+} would increase and may activate intracellular enzymes, including CANP and accounting for the loss of Z-lines observed in ECO-induced myopathy (6.3). The appearance of large electron-opaque deposits, thought to be due to the precipitation of excess Ca^{2+} with inorganic phosphate (Greenawalt et al., 1964) within mitochondria, supports the idea that loading of mitochondria with Ca^{2+} may be a key factor for the development of ECO-induced myopathy.

The integral involvement of mitochondria in the development of ECO-induced myopathy is made more attractive by the fact that, in the ultrastructural study performed, it was noticed that those fibres which remained 'normal' within necrotic tissues, also contained a higher volume density (Vv) of mitochondria (6.3.3). Perhaps, by having more mitochondria, these fibres were more able to buffer the high prevailing concentrations of iCa^{2+} such that myopathy was not initiated.

It is suggested that the development of ECO-induced myopathy is a multifactoral event in which heavy loading of mitochondria with Ca^{2+} plays a key role whilst the activation of intracellular proteases plays, at most, a minor part.

11.5. THE PREVENTION OR TREATMENT OF ECO-INDUCED MYOPATHY

The present study has found two drugs capable of ameliorating ECO-induced myopathy *in vivo*. The first, the carbamate pyridostigmine bromide, was shown to be active prophylactically when administered within 2 hours of ECO, and was progressively less effective the longer in advance of ECO administration that it was applied (10.3.2). Carbamates have previously been used in the prevention of OP poisoning (Dirhuber & Green, 1978; Gordon et al., 1978; Wecker et al., 1978; Dirnhuber et al., 1979; French et al., 1979) and are thought to be effective due to their property to reversibly bind with AChE, thus preventing subsequent irreversible binding by ECO. The time dependency of carbamate

prophylaxis is thought to be due to the progressive hydrolysis of the AChE-carbamate complex with time, and was previously reported by Wecker et al (1978a) in the prophylaxis of paraoxon-induced muscle necrosis. The dose of pyridostigmine bromide administered in the current study ($100\mu gKg^{-1}$) causes 20% depression of AChE activity in the mouse diaphragm (S.Das; personal communication) suggesting that ECO must be required to inhibit greater than 80% diaphragm AChE to induce severe myopathy.

The second agent shown to ameliorate ECO-induced myopathy in vivo was pyridine-2-aldoxime methiodide (2PAM) which was successful when administered therapeutically to mice within 60 minutes of the exposure to ECO (10.2), the effectiveness of the therapy decreasing with increasing delay of 2PAM administration. It is thought that 2PAM acts largely by reactivating the AChE enzyme, permitting normal muscle function to ensue and implying that AChE inhibition is a triggering factor leading to an ACh-mediated muscle necrosis (Dettbarn, W-D, 1984). The decreased effectiveness of 2PAM, when applied at later times after ECO, is thought to be due to "ageing" of the ECO-AChE complex (Lipson et al., 1969) and has been reported previously (Wecker et al., 1978a&b) in the treatment of paraoxon-induced myopathy and by other workers (Murtha et al., 1070; Laskowski et al., 1977).

APPENDICES.

A1 SOURCE OF REAGENTS.

REAGENT	SUPPLIER
Acetic acid	Fisons
Acetone	BDH-AristaR
Acetyl-thiocholine iodide	BDH
Araldite CY212 resin	Emscope laboratories
Atropine sulphate	Sigma
BDMA accelarator	Agar scientific laboratories
Calcium chloride ($CaCl_2$)	BDH-AristaR
Copper sulphate ($CuSO_4$)	Hopkin & Williams LtD
Creatine kinase assay kit	Sigma
DDSA hardener	Emscope laboratories
Dextrose	Fisons-A.R. grade
Disodium hydrogen orthophosphate	Fisons-A.R. grade
Ditiazem	Sigma
Disopyramide phosphate	Sigma
Dithiobisnitrobenzoate	BDH
Ecothiopate iodide	Ayerst laboratories
Ethopropazine	Sigma
Formaldehyde	Fisons A.R. grade
Glutaraldehyde	BDH-AristaR
Glycerol jelly	BDH
Hydrochloric acid (10M)	BDH-AristaR

Lanthanum chloride ($LaCl_2$)	BDH-AristaR
Lead nitrate	BDH
Leupeptin	Sigma
Magnesium chloride ($MgCl_2$)	BDH
Mecamylamine	Sigma
Methanol	BDH
Potassium chloride (KCl)	BDH-AnalaR
Potassium ferricyanide	Hopkin & Williams LtD
Procainamide	Sigma
Procion yellow MX4R	ICI
Propylene oxide	BDH
Pyridine-2-aldoxime methiodide (2PAM)	Sigma
Pyridostigmine bromide	Sigma
Sodium acetate	Fisons SLR grade
Sodium hydrogen carbonate ($NaHCO_3$)	BDH-AnalaR
Sodium citrate	BDH-AnalaR
Sodium chloride (NaCl)	Fisons A.R.
Sodium hydroxide	BDH AnalaR
Sodium dihydrogen orthophosphate	BDH AnalaR
Toluidine blue	Emscope laboratories
Trisodium citrate	BDH AnalaR
d-tubocurarine	Sigma
Uranyl acetate	Emscope laboratories
Verapamil	Sigma

A2. COMPOSITION OF SALINE SOLUTIONS.

Table A2.1 Composition of Lileys physiological saline (pH 7.4) as modified by Krnjević and Miledi (1958).

	Final Concentration(mM)
NaCl	137
KCl	5
$CaCl_2$	2
$MgCl_2$	1
NaH_2PO_4	1
$NaHCO_3$	12
Dextrose	25

NB. $CaCl_2$ was added as 1.0M volumetric $CaCl_2$ solution (2ml per litre) when required, and the resulting saline was henceforth referred to as normal Lileys saline. When not required, the addition of $CaCl_2$ was omitted, resulting in a final calcium reduced saline (Ca-reduced saline) which, when assayed , was shown to contain 5×10^{-6}M ionised calcium (Ca^{2+}).

pH was adjusted to 7.4 using NaH_2PO_4 or $NaHCO_3$ and the final solutions (both normal Lileys and Ca-reduced salines) were gassed with carbogen ($95\%O_2:5\%CO_2$) before use.

A3. COMPOSITION OF STAINING SOLUTIONS.

A3.1. STAINS USED FOR LIGHT MICROSCOPY.

Table A3.1 Preparation of the cholinesterase stain (Karnovski and Roots, 1964).

1.Mix:_	0.06N sodium acetate	15.8ml
	0.1N acetic acid	0.5ml
	0.1N sodium citrate	3.6ml
2.Add:_	acetyl thiocholine iodide and dissolve	12.5mg
3.Add:_	30mM copper sulphate	2.5ml
	5mM potassium ferricyanide	2.5ml

The final solution is bright green and stable at room temperature for several hours.

A3.2 STAINS USED FOR ELECTRON MICROSCOPY.

Table A3.2. Composition of 30% uranyl acetate stain.

Uranyl acetate	30g	
		the solvent was passed through grade 50 filter paper.
Methanol	100ml	

The stain was stored in the dark at 4°C.

Table A3.3. Composition of lead citrate stain.

1. Mix:-	distilled water	16ml
	1.0M Tri-sodium citrate	3ml
	1.0M Lead nitrate	2ml
2. Add:-	1.0M Sodium hydroxide	4ml

Pass the final solution through a Millipore filter and store in the dark at $4^{o}C$.

A4. COMPOSITION OF EMBEDDING MEDIA USED FOR ELECTRON MICROSCOPY.

Table A4.1 Composition of araldite resin (prepared in a fume cupboard).

Araldite CY212 resin	50g	
DDSA hardener	50g	
		mix well
Dibutylphthalate plasticizer	2.5ml	
BDMA accelarator		

A5. COMPOSITION OF SOLUTIONS USED IN ASSAYS OF ACETYL-CHOLINESTERASE ACTIVITY.

Double distilled water was used and all solutions were kept on ice throughout the experiment.

A5.1. COLOUR REAGENT:-5-5 DITHIOBIS (2 NITRO-BENZOIC) ACID (DTNB). BDH.

A5.1.1. Stock Solution:- 0.01M, keeps for about a week at 4^{0}C.

Table A5.1 Composition of 0.01M DTNB stock solution.

DTNB	39.6mg
$NaHCO_3$	15.0mg
0.1M Phosphate buffer, pH 7.0	10.0ml

A5.1.2. Assay solution:- The stock solution was diluted 1:40 with distilled water for use in the assay.

Table A5.2 Composition of phosphate buffer, pH 7.0

0.2M $Na_2PO_4.12H_2O$	30.5ml
0.2M $NaH_2PO_4.2H_2O$	19.5ml
Distilled water	50.0ml

A5.2. SUBSTRATE- ACETYLTHIOCHOLINE IODIDE (BDH).

This was made up on the day of the assay such that a final cuvette concentration of 0.5mM was attained.

Table A5.3. Composition of acetylthiocholine iodide (ASCHI) substrate solution.

Acetylthiocholine iodide	21.7mg
Distilled water	50.0ml

A5.3. BUTYRYLCHOLINESTERASE INHIBITOR:- ETHOPROPAZINE. (BDH).

A solution of 1.0 M ethopropazine was made up on the day of each experiment.

Table A5.4 Composition of 1.0 M ethopropazine.

Ethopropazine	17.5mg
Distilled water	50ml

A5.4. PHOSPHATE BUFFER, pH 8.0.

Table A5.5. Composition of phosphate buffer, pH 8.0

0.2M Na_2HPO_4	47.4ml
0.2M NaH_2PO_4	2.6ml
Distilled water	50.0ml
	100ml buffer

NB. For 200 assays 4 litres of pH 8.0 phosphate buffer was adequate.

REFERENCES.

Aherne, W.A. and Dunhill, M.S. (1982). in Morphometry. (Arnold, E: publisher). Chapter 4. pp.33-45.

Almers, W. and M^{c}Cleski, E.W. (1984). Nonselective conductance in calcium channels of frog muscle: Calcium selectivity in a single-file pore. J. Physiol. 353, 585-608.

Ariens, A.T., Cohen, E.M., Meeter, E. and Wolthius, O.L. (1968). Reversible necrosis in striated muscle fibres of the rat after severe intoxication with various cholinesterase inhibitors. Industr. Med. Surg. 37, 532.

Ariens, A.T., Meeter, E., Wolthuis, O.L. and Von-Benthem, R.M. (1969). Reversible necrosis at the endplate region in striated muscles of the rat poisoned with cholinesterase inhibitors. Experientia 25, 57-59.

Arnani, M., Angelini, C., Cacciavillani, M. and Ausoni, S. (1984). Calcium paradox phenomenon in mammalian muscle; an experimental model for the study of pathogenesis of opaque fibres. Ital. J. Neurol. Sci. 3, 111-115.

Ashley, C.C. and Ridgeway, E.B. (1968). Simultaneous recording of membrane potential. Calcium transient and tension in single muscle fibres. Nature (Lond) 219, 1168-1169.

Baker, P.F. (1986). The sodium-calcium exchange system. in Calcium in the Cell. Wiley, Chichester. (Ciba Found. Symp. 122) pp5-22.

Banker, B.Q., Kelly, S.S. and Robbins, N. (1982). Neuromuscular transmission and correlative morphology in young and old mice. J. Physiol. 339, 355-375.

Bennet, M.B.L., Payton, B.W. and Pappas, G.D. (1969). Permeability and structure of junctional membranes at an electrotonic synapse. Science 166, 1641-1643.

Berry, W.K. and Davies, D.R. (1970). The use of carbamates and atropine in the protection of animals against poisoning by 1, 2, 2-Trimethylpropyl-methylphosphono-fluoridate. Biochem. Pharmac. 19, 927-934.

Birtley, R.D., Roberts, J.B., Thomas, B.H. et al., (1966). Excretion and metabolism of [^{14}C]-pyridostigmine in the rat. Brit. J. Pharmacol. 26, 393-402.

Bodensteiner, J.B. and Engel, A.G. (1978). Intracellular calcium accumulation in Duchenne muscular dystrophy and other myopathies. Neurology 28, 439-448.

Bodwell, C.E. and Pearson, A.M. (1964).
The activity of partially purified bovine catheptic enzymes on various natural and synthetic substrates. J. Food Science 29, 602-607.

Bowman, W.C. (1980). in Pharmacology of neuromuscular function (Wright, J. and Sons LtD: publishers). pp71-121

Brachfeld, J. and Zavon, M.R. (1965). Organic phosphate (phosdrin) intoxication. Report of a case and the results of treatment with 2PAM. Arch. Env. Health (Chicago). 11, 859-862.

Bradley, W.G. and Fulthorpe, J.J. (1978). Studies of sarcolemmal integrety in myopathic muscle. Neurology 28, 670-677.

Brandt, N.R., Caswell, A.H., Brunschwig, J.P. (1980). ATP-energised calcium pump in isolated transverse tubules of skeletal muscle. J. Biol. Chem. 255, 6290-6298.

Brierly, G. and Slauterback, D.B. (1964). An EM study of the accumulation of calcium and inorganic phosphate by heart mitochondria. Biochim. Biophys. Acta 82, 183-186.

Bulbring, E. (1946). Observations on the isolated phrenic nerve preparation of the rat. Brit. J. Pharmacol. 1, 38-61.

Busch, W.A., Stromer, M.H., Goll, D.E. and Suzuki, A. (1972). Calcium-specific removal of Z-lines from rabbit skeletal muscle. J. Cell Biol. 52, 367-381.

Carafoli, E. and Crompton, M. (1976). Calcium ions and mitochondria. in Calcium in biological systems. (Duncan, C, J., ed). Symp. Soc. Exp. Biol. 30, 89-115.

Carafoli, E. and Zurini, M. (1982). The Ca^{2+}-pumping ATP-ase of plasma membranes. Biochem. Biophys. Acta. 683, 279-301.

Carafoli, E., Zurni, M. and Benaim, G. (1983). The calcium pump of plasma membranes. in Calcium and the Cell. Wiley, Chichester (Ciba Found. Symp. 122) pp58-72.

Cisson, C.M. and Wilson, B.W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl-phosphate-induced delayed neurotoxicity:- altered AChE molecular forms and increased CPK activity in plasma. Toxicol. and App. Pharmacol. 64, 289-305.

Costantin, L. (1975). Contractile activation in skeletal muscle. Prog. Biophys. Molec. Biol. 29 (2), 197-224.

Crompton, M., Capano, M. and Carafoli, E. (1976). The sodium-induced efflux of calcium from heart mitochondria. A possible mechanism for the regulation of mitochondrial calcium. Eur. J. Biochem 69, 453-462.

Csillik, B. and Savay, G. (1963). Release of calcium in the myoneural junction. Nature (Lond.) 198, 399-400.

Cullen, M.J., Appleyard, S.T. and Bindoff, L. (1978). Morphologic aspects of muscle breakdown and lysosomal activation. Annals of the NY. Acad. of Sci. 317, 440-404.

Cullen, M.J. and Mastaglia, F.L. (1980). Morphological changes in dystrophic muscle. Brit. Med. Bull. 36 (2), 145-152.

Dawson, D.M. (1965). Efflux of enzymes from chicken muscle. Biochem. et Biophys. Acta 113, 144-157.

Dawson, D.M., Eppenberger, H.M. and Kaplan, N.O. (1967). The comparative enzymology of creatine kinase. ii. Physical and chemical properties. J. Biol. Chem. 242, 210-217.

Dayton, W.R., Goll, D.E., Stromer, M.H., Reville, W.J., Zeece, M.G. and Robson, R.M. (1975). Some properties of a calcium-activated protease that may be involved in myofibrillar turnover. in Proteases and Biological Control. (Reich, E., Rifkin, D, B. and Shaw, E. eds). pp.551-577.

Dayton, W.R., Goll, D.E., Zeece, M.G., Robson, R.M. and Reville, W.J. (1976). A calcium-activated protease possibly involved in myofibrillar turnover. Biochem. 15, 2150-2158.

Dayton, W.R. and Schollmeyer, J.V. (1980). Localisation of a calcium-activated neutral protease in skeletal muscle. J. Cell. Biol. 87, 267a.

Delesse, A. (1847). Procede mechanique pour determines la composition des roches (extrait). CR. Acad. Sci. (Paris). 25, 544

Dettbarn, W-D. (1984). Pesticide-induced muscle necrosis: mechanisms and prevention. Fund. Appl. Toxicol. 4, S.18.

Dirnhuber, P., French, M.C., Green, D.M., Leadbeater, L. and Stratton, J.A. (1979). The protection of primates against soman poisoning by pretreatment with pyridostigmine. J. Pharm. Pharmacol. 31, 295-299.

Dirnhuber, P. and Green, D.M. (1978). Effectiveness of pyridostigmine in reversing neuromuscular blockade produced by soman. J. Pharm. Pharmac. 30, 419-425.

Duncan, C.J. Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. Experientia. 34. Fax. 12, 1531-1672.

Duncan, C.J., Smith, J.L. and Helen Greenaway. (1978). Failure to protect frog skeletal muscle from ionophore-induced damage by the use of the protease inhibitor leupeptin. Comp. Biochem. Physiol. 63c, 205-207.

Durham, W.F. and Hayes, W.J. (1962). Organophosphorous poisoning and its therapy. Arc. Environ. Health 5, 27-44.

Ebashi, S. and Endo, M. (1968). Calcium ions and muscle contraction. Progr. Biophys. Mol. Biol. 18, 123-183.

Ebashi, S., Ohnishi, E., Maruyama, K. and Fujii, T. (1975). Molecular mechanism of regulation of muscle contraction by the calcium-troponin system. in Proteins of Contractile Systems. (Biro, E.N: ed). Vol. 31, 71-84.

Ellman, G.L., Courtney, K.D., Andres, V.Jr. and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Endo, M. (1977). Calcium release from sarcoplasmic reticulum. Physiol. Rev. 57, 71-108.

Endo, M., Tanaka, M. and Ogawa, Y. (1970). Calcium-induced release of calcium from sarcoplasmic reticulum of skeletal muscle fibres. Nature. (Lond.) 288, 34-36.

England, P.J. (1986). Intracellular calcium receptor mechanisms. Brit. Med. Bull. 42, 375-383.

Enomoto, A. and Bradley, W.G. (1977). Therapeutic trials in muscular dystrophy. III. Studies of microbial proteinase in murine dystrophy. Arch. Neurol., Chicago 34, 771-773.

Eppenberger, H.M. and Strehler, E. (1982). M-line proteins. Methods in Enzymology 85, 140.

Evans, R.H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm muscle. J. Physiol. 240, 517-533.

Fabiato, A. (1982). Calcium release in skinned cardiac cells: variations with species, tissues and development. Fed. Proc. 41, 2238-2244.

Fatt, P. and Ginsborg, B.L. (1958). Theionic requirements for production of action potentials in crustacean muscle fibres. J. Physiol. 142, 516-543.

Fenichel, G.M., Dettbarn, W-D. and Newman, T.M. (1974). An experimental myopathy secondary to excessive acetylcholine release. Neurology 24, 41-45.

Fenichel, G.M., Kibler, W.B., Olson, W.H. and Dettbarn, W.D. (1972). Chronic inhibition of cholinesterase as a cause of myopathy. Neurology 22, 1026-1033.

Ferry, C.B. and Helen Townsend. (1986). Anticholinesterase-induced myopathy and changes in creatine kinase in mice. J. Physiol. 373, 29P.

Finean, J.B., Coleman, R. and Michell, R.H. (1978). in Membranes and their Cellular Functions, 2nd ed. Blackwell, Oxford.

Fischer, G. (1970). Die azetylcholinesterase an der motorischen endplatte des ratten zwerchfells nach intoxikation mit paraoxon und soman bei applikation von oximen. Experientia 26, 402-403.

Fiskum, G. & Lehninger, A.L. (1982). Mitochondrial regulation of intracellular calcium. in. Calcium and Cell function. (Cheung, W.Y. ed) Acad. Press. 2 (Ch 2).

Fletcher, P. and Forrester, T. (1975). The effect of curare on the release of ACh from mammalian motor nerve terminals and an estimate of quantum content. J. Physiol (Lond). 251, 131-143.

Ford, L.E. and Podolski, R.J. (1970). Regenerative calcium release within muscle cells. Science 167, 58-59.

French, Mary, C., Janet Wetherell and White, P.D.T. (1979). The reversal by pyridostigmine of neuromuscular block produced by soman. J. Pharm. Pharmacol. 31, 290-294.

French, Mary, C., Janet Wetherell and White, P.D.T. (1983). The reversal by oximes and their de-oximinomethyl analogues of neuromuscular block produced by soman. European J. Pharmacol. 91, 399-409.

Freidman, I.A., Laufer, A.E. and Davies, A.M. (1969). Studies on lysosomes in rat heart cell cultures. ii. The effect of exogenous lysosomes. Experientia 25, 1092-1093.

Gilbert, J.R. and Meissner, G. (1982). Sodium-calcium exchange in skeletal muscle sarcolemmal vesicles. J. Membrane Biol. 69, 77-84.

Ginks, W.R., Sybers, H.D., Maroko, P.R., Covell.J.W., Sobel, B.E. and Ross, J.Jr. (1972). Coronary artery perfusion. ii. Reduction of myocardial infarct size at one week after the coronary artery occlusion. J. Clin. Invest. 51, 2717-2723.

Gordon, J.J., Leadbeater, L. and Maidment, M.P. (1978). Protection of animals against organophosphorus poisoning by pretreatment with a carbamate. Toxicol. and Applied Pharmacol. 43, 207-216.

Goudou, D. and Rieger, F. (1983). Recovery of acetylcholinesteras and of its multiple molecular forms in motor end-plate-free and motor end-plate-rich regions of mouse striated muscle after irreversible inactivation by an organophosphorus compound (methyl-phosphorothiolate derivative). Biol. Cell 48, 151-158.

Goyer, G.R. (1968). Action of 2PAM on neuromuscular transmission in vitro and in vivo. Can. J. Physiol. Pharmacol. 46 (5), 757-764.

Goyer, R.G. (1970). The effects of 2PAM on the release of ACh from the isolated diaphragm of the rat. J. Pharma. Pharmacol. 22, 42-45.

Greenawalt, J.W. and Carafoli, E. (1966). E.M. studies in the active accumulation of Sr^{2+} by rat liver mitochondria. J. Cell. Biol. 29, 37-61.

Greenawalt, J.W., Rossi, C.S. and Lehninger, A.L. (1964). Effect of active accumulation of calcium and phosphate on the structire of rat liver mitochondria. J. Cell Biol. 23, 21-38.

Grob, D. (1956). The manifestations and treatment of poisoning due to nerve gas and other organophosphorus anticholinesterase compounds. Arch. Int. Med. 98, 221-239.

Grob, D. and Harvey, A.M. (1953). The effects of nerve gas poisoning. Amer. J. Med. 14, 52-63.

Grob, D. and Johns, R.J. (1958). Use of oximes in the treatment of intoxication by anticholinesterase compounds in normal subjects. Amer. J. Med. 24, 497-518.

Harvey, A.L., Jones, S.V.P., and Marshall, I.G. (1984). Disopyramide produces non-comtetitive, voltage-dependent block at the neuromuscular junction. Br. J. Pharmac. 81, 169P.

Heilbronn, E and Tolegen, B. (1965). Toxogonin in sarin, soman and tabun poisoning. Biochem. Pharmacol. 14, 73-77.

Herzberg, O., Moult, J. and James, M.N.G. (1986). Calcium binding to skeletal muscle troponin C and the regulation of muscle contraction. in Calcium and the Cell. Wiley, Chichester (Ciba Found. Symp. 122) pp.120-144.

Hess, J.W., Mac Donald, R.P., Frederick, R.J., Jones, R.N., Neely, J. and Goss, D. (1969). Serum CK activity in disorders of heart and skeletal muscle. Ann. Int. Med. 61, 1015-1028.

Hobbiger, F. (1976). in Handbook of Experimental Pharmacology. Springer, Berlin, Heidleberg. NY. New series 42, 487-581.

Hudson, C.S. and Foster, R.E. (1984). Presynaptic effects of pyridostigmine on the neuromuscular junction of the rat diaphragm. J. Cell Biol. 99, 23a.

Hudson, C.S., Foster, R.E. and Kahng, M.W. (1985). Neuromuscular toxicity of pyridostigmine bromide in the diaphragm, EDL and soleus muscle of the rat. Fund. and Appl. Toxicol. 5, 5360-5369.

Hudson, C.S., Rash, J.E., Teidt, T.N. and Albuquerque, E.X. (1978). Neostigmine-induced alterations at the neuromuscular junction. J. Pharmacol. and Exper. Ther. 205, 340-356.

Huxley, A.F. (1971). The activation of striated muscle and its mechanical response. Proc. Roy. Soc. (series b) 178, 1-27.

Huxley, H.E. (197). Structural changes in actin- and myosin-containing filaments during contraction. Cild Spring Harbour Symp. Quant. Biol. 37, 361-376.

Huxley, H.E. and Jean Hanson. (1960). The structural basis of the contraction mechanism in *striated muscle.* Ann. NY. Acad. Sci. 81, 403-408.

Inomata, M., Hayashi, M., Nakamura, M., Imahori, K. and Kawashima, S. (1983). Purification and characterisation of a calcium-activated neutral protease from rabbit skeletal muscle, which requires Ca^{2+} of the μM order concentration. J. Biochem. 93, 291-294.

Iodice, A.A., Leong, V. and Weinstock, I.M. (1966). Separation of cathepsins A and D of skeletal muscle. Arch. Biochem. Biophys. 117, 477-486.

Irvine, R.F. (1986). Calcium transients: Mobilisation of intracellular calcium. Brit. Med. Bull. 42, (4), 369-374.

Ishiura, S., Sugita, H., Nonaka, I. and Imahori, K. (1980). Calcium-activated neutral protease. J. Biochem. 87, 343-346.

Jewel, H.A. and Lehman, R.A. (1958). Pharmacology of phospholine iodide- an alkyl-phosphothiocholine. Fed. Proc. 17, 381.

Karczmar, A.G. (1984). Acute and long lasting central actions of organophosphorous agents. Fund. Appl. Toxicol. 4, S1-17.

Karnovski, M.J. and Roots, L. (1964). A "Direct-colouring" thiocholine method for cholinesterases. J. Histochem. Cytochem. 12. 219-221.

Katz, B. and Miledi, R. (1973). The binding of ACh to its receptor, and its removal from the synaptic cleft. J. Physiol. 231, 549-574.

Katz, B. and Miledi, R. (1975). The nature of the prolonged endplate depolarisation in anti-esterase treated muscle. Proc. Royal Soc. Lond. 192, 27-38.

Kawabuchi, M., Osame, M., Watanabe, S., Igata, A. and Kanaseki, T. (1975). Myopathic changes at the endplate region induced by neostigmine methylsulfate. Experientia 32, 623.

Kjekshus, J.K. and Sobel, B.E. (1970). Depressed myocardial CK activity following experimental myocardial infarction in the rabbit. Circulation Res. 27, 403-414.

Koelle, G.B. and Steiner, E.C. (1956). The cerebral distributions of a tertiary and a quaternary anticholinesterase agent following intravenous and intraventricular injection. J. Pharmacol. Exp. Ther. 118, 420-434.

Krnjević, K. and Miledi, R. (1958). Motor units in the rat diaphragm. J. Physiol. 140, 427-439.

Kuba, K., Albuquerque, E.X., Daley, J. and Barnard, E.A. (1974). A Study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on the time course of endplate current in frog sartorius muscle. J. Pharmacol. Exp. Ther. 189, 499-512.

Laskowski, M.B. and Dettbarn, W-D. (1977). The pharmacology of experimental myopathies. Ann. Rev. Pharmacol. Toxicol. 17, 387-409.

Laskowski, M.B., Olson, W.H. and Dettbarn, W-D. (1975). Ultrastructural changes at the motor endplate produced by an irreversible ChE inhibitor. Exper. Neurol. 47, 290.

Laskowski, M.B., Olson, W.H. and Dettbarn, W-D. (1977). Initial EM abnormalities at the motor endplate produced by ChE inhibition. Exp. Neurol. 57, 13-33.

Lehninger, A.L., Carafoli, E. and Rossi, C.R. (1967). Advances in enzymol. 29, 259-320.

Leonard, J.P. (1981). Agonist-induced release of LDH from skeletal muscle: an assay of myopathy. Abstr. Soc. Neurosci. 7, 600.

Leonard, J.P. and Salpeter, M.M. (1979). Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. J. Cell Biol. 82, 811-818.

Leonard, J.P. and Salpeter, M.M. (1980). Calcium-dependent myopathy in response to carbamylcholine in normal and dystrophic mice. J. Cell Biol. 87, 70a.

Leonard, J.P. and Salpeter, M.M. (1982). Ca^{2+}-mediated myopathy at the neuromuscular junctions of normal and dystrophic mice. Exper. Neurol. 76, 121-138.

Libby, P. and Goldberg, A.L. (1978). Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. Science 199, 534-536.

Lievrement, M., Czajka, M. and Tazieff-Depierre, F. (1968). Etude in situ d'une fixation de calcium et de sa liberation à la jonction neuromusculaire. C.r.hebd. Seanc. Acad. Sci. Paris 267, 1988-1991.

Liley, A.W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. 132, 650-666.

Lipson, M.L., Holmes, J.H., Ellis, P.P. and Denver, M. (1969). Oral administration of pralidoxime chloride in echothiophate iodide therapy. Arch. Opthal. 82, 830-835.

Loomis, T.A. and Salafsky, B. (1963). Antidotal action of pyridinium oximes in anticholinesterase poisoning; comparative effects of soman, sarin and neostigmine on neuromuscular function. Toxicol. appl. Pharmac. 5, 685-701.

Luttgou, H.C. and Moisescu, G.D. (1978). Ion movements in skeletal muscle in relation to the activation of contraction. in Physiology of Membrane Disorders. (Andreoli, T.E. et al.: eds). Plenum publishing, NY, pp. 493-515.

M^{c}Gowan, E.B., Shafiq, A. and Stracher, A. (1976). Delayed degeneration of dystrophic and normal muscle cell cultures treated with pepstatin, leupeptin and antipain. Exper. Neurol. 50, 649-657.

Martinosi, A.N. (1984). Mechanisms of calcium release from sarcoplasmic reticulum of smooth muscle. Physiol. rev. 64, 1240-1320.

Miledi, R. (1972). Transmitter release induced by injection of calcium ions into nerve terminals. Proc. Royal Soc. Lond. (Biol). 183, 421-425.

Miledi, R. and Parker, I. (1981). Diltiazem inactivates acetylcholine-induced membrane channels in skeletal muscle fibres. Biomed. Res. 2, 587-589.

Miledi, R., Parker, I. and Schalow, G. (1977). Ca^{2+} entry across the postjunctional membrane during transmitter action. J. Physiol. (Lond.) 268, 32-33.

Muallem, S., Schoeffield, M., Pandol, S. and Sachs, G. (1985). Inositol triphosphate modification of ion transport in rough endoplasmic reticulum. Proc. Nat. Acad. Sci. USA. 82, 4433-4437.

Murtha, E.F., Fleisher, J.H., Torre, M.A. and Innerebrier, T.A. (1970). Reactivatability of soman-inhibited endplate ChE and neuromuscular transmission. Toxicol. Appl. Pharmacol. 16, 214-226

Nakada, K., Nakada, F., Ito, E. and Inoue, F. (1984). Quantification of myonecrosis and comparison of necrotic activity of snake venoms by determination of CK activity in mice sera. Toxicol. 22, 921-930.

Nakajima, Y. and Engel, W.K. (1973). Release of Ca^{2+} induced by depolarisation of the SR membrane. Nature New Biol. 246, 216-218

Namba, T., Nolte, C.T., Jackrel, J. and Grob, D. (1971). Poisoning due to organophosphorus insecticides- acute and chronic manifestations. Amer. J. Med. 50.(4), 475-492.

Neerjun, J.S. and Dubowitz, V. (1979). Increased calcium-activated neutral protease activity in muscles of dystrophic hamsters and mice. J. Neurol. Sci. 40, 105-111.

Newman, J.R., Virgin, J.B., Younkin, L.H. and Younkin, S.G. (1984). Turnover of AChE in innervated and dennervated rat diaphragm. J. Physiol. 352, 305-318.

Nicholls, D.G. (1986). Intracellular calcium homeostasis. Brit. Med. Bull. 42(4), 353-358.

Oberc, M.A.E. and Engel, W.K. (1975). Calcium localisation in fresh normal and abnormal muscle. J. Cell Biol. 67, 312A.

Oberc, M.A.E. and Engel, W.K. (1977). EM of mitochondria and Ca^{2+}-loading. Lab. Invest. 36, 566-577.

Oliver, I.T. (1955). A spectrophotometric method for determination of CPK and myokinase. Biochem. J. 61, 116-122.

Osserman, K.E., Cohen, E.S. and Genkins, G. (1961). Phospholine iodide: an anticholinesterase drug of new structure. Preliminary report in the treatment of myaesthenia gravis. in second internat. myaesthenia gravis symp. proc., Springfeild. ILL 196. pp.581-594.

Padykula, H.A. and Gauthier, G.F. (1963). Cytochemical studies of adenosine triphosphatases in skeletal muscle fibres. J. Cell Biol. 18, 87-107.

Paterson, Y. and Lawrence, E.F. (1972). Factors affecting creatine phosphokinase levels in normal adult females. Clin. Chim. Acta. 42, 131-139.

Park, D.C. and Pennington, R.J. (1967). Proteinase activity in muscle particles. Enzymol. Biol. Clin. 8, 149-157.

Park, D.C. and Pennington, R.J. (1967). Proteinase activity in muscle particles. Enzynol. Biol. Clin. (Basel) 8, 149-160.

Payton, B.W. (1969). Histological staining properties of Procion M4RS. J. Cell Biol. 45, 659-662.

Peachey, L.D. (1964). EM observations on the accumulation of divalent cations in intramitochondrial granules. J. Cell Biol. 20, 95-111.

Pearce, M.S., Pennington, R.J. and Walton, J.N. (1964). Serum enzyme studies in muscle disease. J. Neurol. Neurosurg. and Psychiat. 27, 96-99.

Podolski, R.J. and Costantin, L.L. (1964). Regulation by Ca^{2+} of contraction and relaxation of muscle fibres. Fedn. Proc. 23, 933-939.

Publicover, S.J., Duncan, C.J. and Smith, J.L. (1977). Ultrastructural changes in muscle mitochondria in situ, including the apparent development of internal septae associated with the uptake and release of Ca^{2+}. Cell Tiss. Res. 185, 373-385.

Putney, J.W.Jr. (1978). Stimulus-permeability coupling role of calcium in the receptor regulation of membrane permeability. Pharmacol. Rev. 30, 209-245.

Ramesh, C., Gupta, G.T., Patterson, T. and Dettbarn, W-D. (1986). Mechanisms of toxicity and tolerance to DFP. Toxicol. Appl. Pharmacol. 84, 541-550.

Rasmussen, H. and Barret, P.Q. (1984). Calcium messenger system: an integrated view. Physiol. Rev. 64, 938-984.

Reddy, M.K., Etlinger, J.D., Fischman, D.A., Rabinovitz, M.I. and Zak, R. (1975). Removal of Z-lines and a-actinin from isolated myofibrils by a Ca^{2+}-activated neutral protease. J. Biol. Chem. 250, 4278-4284.

Reddy, M.K., Rabinowitz, M. and Zak, R. (1983). Stringent requirement for Ca^{2+} in the removal of Z-lines and a-actinin from isolated myofibrils by Ca^{2+}-activated neutral proteinase. Biochem. J. 209, 635-641.

Reeves, J.P. and Hale, C.C. (1984). The stoichiometry of the cardiac Na-Ca exchange system. J. Biol. Chem. 259, 7733-7739.

Reimer, K.A., Low, J.E. and Jennings, R.B. (1977). Effect of the calcium antagonist verapamil on necrosis following temporary coronary artery occlusion in dogs, Circulation. 55, 581-587.

Reuter, H. (1986). Voltage-dependent mechanisms for raising intracellular free calcium concentration: calcium channels. in Calcium and the Cell. Wiley, Chichester (Ciba Found. Symp. 122) pp5-22.

Reville, W.J., Goll, D.E., Stromer, M.H., Robson, R.M. and Dayton, W.R. (1976). A Ca^{2+}-activated protease possibly involved in myofibrillar turnover. J. Cell Biol. 70, 1-8.

Rosalki, S.B. (1967). An improved proceedure for serum creatine kinase determination. J. Lab. Clin. Med. 69, 696-705.

Ross-Canada, J., Chizzonite, R.A. and Meltzer, H.Y. (1983). Retension of sarcoplasmic calcium inhibits development of the phencyclidine-restraint experimental myopathy. Expl. Neurol. 79, 1-10.

Ruigrok, T.J.C. (1985). The calcium paradox and the heart. in Control and Manipulation of Calcium Movement. (Parrat, J.R. ed. Rowen Press. NY).

Salpeter, M, M., Kasprzak, H., Feng, H. and Fertuck, H. (1979). Endplates after esterase inactivation in vivo: correlation between esterase concentration, functional response and fine-structure. J. Neurocytol. 8, 95-115.

Sandow, A. (1965). Excitation-contraction coupling in skeletal muscle. Pharmacol. Rev. 17, 265-320.

Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. (1979). Calcium dependence of toxic cell death. Science 206, 700-702.

Schauman, W. and Job, C. (1958). Differential effects of a quaternary ChE inhibitor, phospholine, and its tertiary analogue compound 217-AO, on the central control of respiration and on neuromuscular transmission. The antagonism by 217-AO of the respiratory arrest caused by morphine. J. Pharmacol. 123, 114-126.

Schneider, M.F. (1986). Voltage-dependent mobilisation of intracellular calcium in skeletal muscle. in Calcium and the Cell. Wiley, Chichester (Ciba Found. Symp. 122) pp.23-38.

Sherwin, A.L., Karpati, G. and Buloke, J.A. (1969). Immunohistochemical localisation of creatine phosphate in skeletal muscle. Proc. Natl. Acad. Sci. USA. 64, 171-175.

Siekevitz, P. (1972). Biological membranes: the dynamics of their organisation. Ann. Rev. Physiol. 34, 117-140.

Silver, A. (1974). The Biology of cholinesterases. North Holland Research Monographs: Frontiers of Biology Volume 36 (Neuberger, A. and Tatum, E, L: eds.).

Sobel, B.E. (1974). Quantification of myocardial damage. Triangle 13, N° 1. 1-10.

Squire, J. (1981). Muscle regulation: a decade of the steric blocking model. Nature 291, 614-615.

Stanfield, P.R. (1986). Voltage dependent calcium channels of excitable membranes. Brit. Med. Bull. 42, 359-367.

Statham, H.E., Duncan, C.J. and Smith, J.L. (1976). The effect of the ionophore A_{23187} on the ultrastructural and electrophysiological properties of frog skeletal muscle. Cell Tiss. Res. 173, 193-209.

Stracher, A. and M°Gowan, E.B. (1978). Muscular dystrophy: inhibition of degeneration in vivo with protease inhibitors. Science 200, 50.

Stretton, A.O.W. and Kravitz, E.A. (1968). Neuronal geometry: determination with a technique of intracellular dye injection. Science 162, 132-134.

Suarez-Kurtz, G. (1983). Enzyme release from skeletal muscle. Braz. J. Med. Biol. Res. 16, 283-290.

Sugita, H., Ishiura, S., Suzuki, K. and Imahori, K. (1980). Calcium-activated neutral protease and its inhibitors: in vivo effect on intact myofibrils. Muscle and Nerve 3, 335-339.

Suzuki, K., Tsuji, S. and Ishiura, S. (1981). Effect of Ca^{2+} on the inhibition of Ca^{2+}-activated neutral protease by leupeptin, antipain and epoxysuccinate derivatives. Febs. Lett. 136, 119-122

Tabti, N. (1987). Pharmacological characterisation of the calcium-insensitive intermittent ACh release at rat neuromuscular junction. Acta Physiol. Scand. 128, 429-436.

Takeuchi, N. (1963). Some properties of conductance changes at the endplate membrane during the action of ACh. J. Physiol. (Lond) 167, 128-140.

Tammelin, L.E. (1957). Dialkyl-phosphoryl-thiocholines, alkoxy-methyl-phosphorylthiocholines and analogous choline esters. Acta. Chem. Scand. 11, 1349.

Theussen, L., Jorgense, J.R., Kvistgaa, H.J., Sorensen, J.A., Vaeth, M., Jensen, E.B., Jensen, J.J. and Hagerup, L. (1983) Effect of verapamil on enzyme release after early intravenous administration in acute myocardial infarction. Brit. Med. J. 286, 1107-1108.

Toth, L., Karcsu, S., Poberai, M. and Savay, G. (1983). Histochemical evidence for the role of Ca^{2+}-activated neutral protease in the development of the substrate myopathy induced by organophosphorus compounds. Acta. Histochem. 72, 71-75.

Toy-Oka, T., Shimizu, T. and Masaki, T. (1978). Inhibition of the proteolytic activity of Ca^{2+}-activated neutral protease by leupeptin and antipain. Biochem. Biophys. Res. Commun. 82, 484-491.

Wachtel, R.E. (1987). Effects of verapamil and diltiazem on responses to acetylcholine. Brit. J. Pharmacol. 92, 561-566.

Walliman, T., Doetschman, T.C. and Eppenberger, H.M. (1983). Novel staining pattern of skeletal muscle M-lines upon incubation with antibodies against MM-creatine kinase. J. Cell Biol. 96, 1772-1779.

Walliman, T. and Eppenberger, H.M. (1983). A novel double-line staining pattern of skeletal muscle M-lines upon incubation with monovalent anti-M-creatine kinase antibodies. Experientia 39, 676a.

Walliman, T., Moser, H. and Eppenberger, H.M. (1983). The myofibrillar localisation of a creatine kinase isoenzyme specific to the M-line. Experientia 39, 676.

Walliman, T., Turner, D.C. and Eppenberger, H.M. (1975). Creatine kinase and M-line structure. in Proteins of Contractile Systems. (Biro, E.N.A: ed) 31, 119-124.

Walliman, T., Turner, D.C. and Eppenberger, H.M. (1977). Localisation of creatine kinase isoenzymes in myofibrils. i. Chicken skeletal muscle. J. Cell Biol. 75, 297-317.

Wecker, L. and Dettbarn, W-D. (1976). Paraoxon-induced myopathy: muscle specificity and ACh involvement. Exp. Neurol. 51, 281-291.

Wecker, L. and Dettbarn, W-D. (1977). Effects of dennervation on the production of an experimental myopathy. Exp. Neurol. 57, 94-101.

Wecker, L., Kiauta, T. and Dettbarn, W-D. (1978a). Relationship between acetylcholinesterase inhibition and the development of a myopathy. J. Pharmacol. Exp. Ther. 206, 97-104.

Wecker, L., Laskowski, M.B. and Dettbarn, W-D. (1978b). Neuromuscular dysfunction induced by AChE inhibition. Fed. Proc. 37, 2818.-2822.

Wecker, L., Mrak, R.E. and Dettbarn, W-D. (1986). Evidence of necrosis in human intercostal muscle following inhalation of an organophosphorus insecticide. Fund. Appl. Toxicol. 6 (1), 172-174

Weinbach, E.C. and Von Brand, T. (1965). The isolation and composition of dense granules from Ca^{2+}-loaded mitochondria. Biochem. Biophys. Res. Commun. 19, 133-137.

Welsch,F. and Dettbarn, W-D. (1971). Inhibition of cholinesterases of rat diaphragm muscle by organophosphates and spontaneous recovery of enzyme activity in vitro. Biochem. Pharmacol. 21, 1039-1049.

Westwick, J. and Poll, C. (1986). Mechanisms of calcium homeostasis in the polymorphonuclear leukocyte. Agents and Actions 19, (1/2), 80-86.

Woodhead, J.L. and Lowey, S. (1983). An in vitro study of the interactions of skeletal muscle M-protein and creatine kinase with myosin and its subfragments. J. Mol. Biol. 168, 831-846.

Wrogeman, K. and Pena, S.D.J. (1976). Mitochondrial Ca^{2+} overload: a general mechanism for cell necrosis in muscle disease. The Lancet 1, 672-673.

Wulfson, N.L., Smith, J.C. and Foldes, F.F. (1966). Acute phospholine intoxication after intracutaneous injection. Clin. Pharmacol. Ther. 7, 44-47.

Yomamoto, S. and Greeff, K. (1981). Effect of intracellular sodium on calcium uptake in isolated guinea-pig diaphragm and atria. Biochim. Biophys. Acta. 646, 348-352.

Zuurveld, Judith, G.E., Veerkamp, J.H. and Wirtz, P. (1985). Isolated myofibrers from rat skeletal muscle in suspension; cellular morphology and calcium homeostasis. Muscle & Nerve 8, 750-759.