MECHANISMS OF ANTICHOLINESTERASE-INDUCED MYOPATHY AND ITS PREVENTION

by

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Summary

In the introduction a brief outline of the possible mechanisms involved in the process of cellular necrosis with particular emphasis on skeletal muscle necrosis after antiChE is discussed.

Ecothiopate (ECO), an antiChE, was shown to produce dose-dependent inhibition of both AChE and BuChE in diaphragm and blood of mice. Inhibition of AChE resulted in dose-dependent influx of calcium at the junctional region with the consequent development of morphological and biochemical alterations. Non-necrotising doses of ECO caused hypercontractions of varying severity, distorted end plate and slight elevation of serum creatine kinase (CK). Necrotising doses of ECO further caused contraction clumps, loss of striations and procion staining with high serum CK. The extent of ECO-induced myopathy depended on the entry of extracellular calcium rather than the degree of AChE inhibition. The essential Ca^{2+} mediated process(es) in ECO-induced myopathy was thought to be the generation of superoxide and superoxide-derived free radicals and/or lipid peroxidation. Mitochondria and xanthine oxidase may be the major contributors to the generation of superoxide. No evidence was found for the depletion of high energy phosphates.

ECO-induced myopathy could be successfully prevented by prior administration of pyridostigmine or various antioxidants. The most effective being Vit E or Vit E + N-acetylcysteine. Allopurinol or N-acetylcysteine alone were also effective. However, the use of a wide range of membrane end plate channel blockers or non-quantal release blockers were unsuccessful in the prevention of ECO-induced myopathy.

Keywords: Anticholinesterase, Ecothiopate, Calcium, Free radicals, Myopathy.

"Knowledge advances by steps , and not by leaps."

Macaulay, Essays: History.

Dedicated to Piklu, Riku, Tinu, Priya, Jisu, Bisu,.....
.....may this serve as a source of inspiration.

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ABBREVIATIONS

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

AA	Arachidonic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
antiChE	Anticholinesterase
AThCh	Acetylthiocholine
ATP	Adenosine 5'-triphosphate
BuChE	Butyrylcholinesterase
CANP	Calcium activated neutral protease
ChE	Cholinesterase
CK	Creatine kinase
CROC	Calcium-induced release of calcium
DFP	diisopropylfluorophosphate
DTNB	dinitrothiobisnitrobenzoate
ECO	Ecothiopate
EP	End plate
HCl	hydrochloric acid
IMP	Inosine 5'-monophosphate
J	Junctional region
NAC	N-acetylcysteine
NJ	Non-junctional region
NMJ	Neuromuscular junction
NMT	Neuromuscular transmission
OP	Organophosphorous anticholinesterase
PC	Phosphocreatine
PUFA	Polyunsaturated fatty acids
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
VIT E	Vitamin E

1.0 INTRODUCTION

1.1 Background to anticholinesterase compounds:

In view of the importance of myopathy in medicine, various preparations of skeletal and cardiac muscle have been utilised in the study of experimental myopathy, with a view of finding agents capable of reducing muscle damage. Organophosphorous anticholinesterase (OP) compounds have been shown to cause skeletal myopathy, as a consequence of inhibition of acetylcholinesterase (AChE), the enzyme responsible for the breakdown of acetylcholine (ACh). This initates a complex sequence of events culminating in the development of skeletal myopathy, which is separable from AChE inhibition and may be similar to the events occurring generally in cellular necrosis.

OP compounds such as TEPP have been known since the middle of the last century (De Clermont, 1854), but it was not until the middle thirties that a rigorous interest in their chemistry occurred. Gerhard Schrader, whose work and efforts in the synthesis and investigation of approximately 2000 OP compounds resulted in chemicals of benefit and potential danger to humanity (Schrader, 1952). Among the important compounds synthesised by him are the widely employed insecticides, parathion and paraxon. Tabun and sarin, because of their toxicity were regarded as potential warfare agents. OP compounds besides being of agricultural and industrial use, have been indispensable as pharmacological tools in studies of synaptic and neuroeffector transmission, and other physiological processes. OP compounds have also made contributions in medicine and are used clinically for the treatment of glaucoma and have been previously used in the management of myasthenia gravis (Foldes, 1959; Schaumann and Job, 1958).

The widespread handling of these compounds during the manufacture or in use as agricultural insecticides imposes a potential threat to the environment and a health hazard to humans. It is therefore important to study the mechanisms involved in anticholinesterase (antiChE)-induced myopathy.

1.2 Toxicity of Anticholinesterase in vivo:

The potential lethality of these compounds is due primarily to the inhibition of cholinesterase (ChE) systems, in particular AChE, the enzyme responsible for termination of transmitter action. Consequently the action of ACh released from nerve terminals, is prolonged and intensified and subsequently manifested in the signs of cholinesterase

poisoning. However, pharmacological evidence is accumulating to suggest that antiChE may have direct effects which are not dependent on AChE inhibition and/or on ACh accumulation (Albuquerque et al, 1984; Karczmar, 1984).

The effects of antiChE poisoning may be categorised as muscarinic, nicotinic and central. The muscarinic effects include bronchoconstriction, sweating, salivation and other increased exocrine secretions, nausea, vomiting, diarrhoea, bradycardia, hypotension, abdominal cramps, all signs which may be relieved by prompt administration of atropine (Grob, 1956; Durham and Hayes, 1962). The nicotinic effects, which usually occur after the muscarinic effects, are the consequences of AChE inhibition at skeletal neuromuscular junction. The nicotinic effects include muscular twitching, muscular fasciculation, increased fatigue and weakness of skeletal muscles which is increased by exertion (Durham and Hayes, 1962). Among the effects of antiChE agents on the central nervous system are anxiety, restlessness, impairment of memory, speech defects, convulsions and coma.

From a qualitative view the signs produced by an antiChE agent in various mammalian species, are essentially similar. However there are differences in the effects between antiChE agents depending on their propensity for central or peripheral actions.

1.3 AntiChE- induced skeletal myopathy :

It is generally accepted that exposure to antiChE agents produces alteration in normal biochemical and physiological processes involved in neuromuscular transmission (NMT) leading to the development of skeletal fibre necrosis in several striated muscles of which the diaphragm is the most severely affected (Ariens et al, 1969; Wecker and Dettbarn, 1976; Laskowski et al, 1975,1977; Wecker et al, 1978a,b; Salpeter et al, 1979; Meshul et al, 1985; Hudson et al, 1985, 1986; Gupta et al, 1987a,b; Gupta and Dettbarn, 1987).

AntiChE-induced disruption of the cytoarchitectural organisation of the muscle fibre surrounding the motor end plate has been consistently represented at the ultrastructural level by swelling and vacuolation of both sarcoplasmic reticulum (SR) and mitochondria, disarray of myofilaments, crystal formation, dissolution of Z-lines, loss of cytosolic enzymes with consequent increase in serum enzyme activity (Townsend, 1988; Hudson et al, 1985; Hudson et al, 1986; Meshul et al, 1985; Laskowski et al, 1975; Laskowski et al, 1977; Salpeter et al, 1979).

The ultrastructural disorganisation just described is not exclusive

to antiChE agents. In fact, a diverse range of agents including A23187, carbachol, caffeine, dinitrophenol, lindane and a wide spectrum of pathological conditions such as myocardial ischaemia, calcium paradox, oxygen paradox and certain muscular myopathies all show similar ultrastructural modifications, which are often very uniform, suggesting a similar mechanism or a final common pathway in the process of cellular necrosis.

The mechanisms involved in the process of antiChE-induced myopathy is poorly understood and the primary sequence of events responsible for the initiation of this process is still unknown. The mechanism(s) involved in cellular necrosis is a vast topic and a comprehensive overview is beyond the scope of this thesis. The following introduction represents a brief and necessarily restricted, overview of the very large literature concerning the possible mechanisms of cellular necrosis and its fascinating interrelationship with calcium. Consequently, the introduction begins with an analysis of the various mechanisms regulating calcium homeostasis in a typical eukaryotic cell. Furthermore, much of what follows is based on information derived from non-skeletal sources, where possible reference is made to skeletal muscle. This information is not necessarily irrelevant because of the lack of studies on skeletal preparation.

1.4 Importance of Calcium homeostasis :

Many important cellular functions, such as muscle contraction, cell division, motility, the secretion of neurotransmitters and hormones or enzyme activation depend on the transient changes in the free cytosolic calcium ion concentration, $[Ca^{2+}]_i$.

Intracellular calcium is held at a very low level, of the order of 10^{-7}M compared with the concentration in the extracellular fluid of about 10^{-3}M. The total calcium inside the cell is much higher than the 10^{-7}M, usually 0.1 to 1.0 m mol Kg^{-1}, and the bulk of this calcium is either bound to proteins, membranes or other cellular constituents, or most importantly it is sequestered inside intracellular organelles such as mitochondria, endoplasmic reticulum (SR in muscle), Golgi apparatus and nuclei (Irvine, 1986; England, 1986) (Fig 1.1).

The free Ca^{2+} concentration in the skeletal cytoplasm is tightly controlled by the uptake and release of the cation across the three major membrane systems which bound the cytoplasm: the plasmalemma, the inner mitochondrial membrane and the SR. Each of the major membrane, ER (SR in muscle), systems bounding the cytoplasm possess

Figure 1.1 The calcium balance of a typical eukaryotic cell. The figures are only approximate and are intended to indicate where Ca^{2+} is to be found in the cell (Campbell 1983).

distinctive pathways both for the uptake and release of the cation and the concerted action of all these pathways regulates $[Ca^{2+}]_i$.

The array of transport processes capable of moving Ca^{2+} into and out of the cytoplasm is quite formidable in its complexity and therefore important aspects of calcium transport processes are discussed.

1.4.1. Calcium regulation by plasma membrane :

The plasma membrane of the cell contains two mechanisms responsible for expelling Ca^{2+} from the cell. First, a Ca^{2+} translocating ATPase and the second a Na^+/Ca^{2+} exchanger.

1.4.1.1. Ca^{2+} Translocating ATPase :

The Ca^{2+} translocating ATPase was first isolated by Niggli et al (1979) from erythrocyte membrane, the ATPase has since been purified from a number of other plasma membranes, including those of heart (Caroni and Carafoli, 1981), nervous tissue (Hakim et al, 1982), skeletal muscle (Michalak et al, 1984) and smooth muscle (Wuytack et al, 1981). In all cases the enzymes appear to be very similar.

The calcium ATPase operates as a electroneutral, obligatory Ca^{2+} proton exchanger. The calcium ATPase splits ATP to derive energy. The energy enables it 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 $2H^+/Ca^{2+}$ (Carafoli and Zurini, 1982).

In the quiescent state, it has a very low affinity for Ca^{2+} (Km about 20μm). In the presence of calmodulin the enzyme becomes activated, it increases its affinity for Ca^{2+} by about 10 times (Km below 0.5μm) (Carafoli et al, 1986). Although it is usually assumed that calmodulin is the natural activator of the enzyme, it is important to emphasise that other compounds normally present in the ambient surroundings the ATPase, such as phosphatidyl serine, also shift the ATPase to the high affinity state. Acidic phospholipids (and long-chain unsaturated fatty acids) as well as limited proteolytic treatment with the trypsin has the same effect as calmodulin, shifting the enzyme to high Ca^{2+} affinity state (Carafoli et al, 1986).

1.4.1.2 Na^+ - Ca^{2+} Exchanger :

Sodium - Calcium counter transport represents one of a number of processes for transporting calcium ions across cellular membranes. The sodium-calcium exchanger is particularly interesting because it can

move Ca^{2+} in either direction across membranes, depending on the prevailing conditions, and therefore provides a link between mechanisms involved in sodium homeostasis and mechanisms in calcium homeostasis.

Since its discovery in invertebrate nerve (Baker et al, 1967) and cardiac muscle (Reuter and Seitz, 1968), sodium-calcium exchanger has been shown to be widespread, though by no means universal, in the plasma membranes of excitable cells. Information concerning Na^{+}-Ca^{2+} exchanger in the sarcolemma of vertebrate skeletal muscle is limited at present. Nevertheless, the exchanger has been demonstrated in frog and rabbit skeletal muscle and guinea pig diaphragm. Furthermore, the exchanger in rabbit skeletal muscle, like cardiac muscle and neurones, was capable of transmembranous Na^{+}-Ca^{2+} exchange (Gilbert and Meissner, 1982). In addition, a rather similar exchanger has been described in certain intracellular organelles, including some mitochondria (Crompton et al, 1977; Nicholls and Akermann, 1982).

Both chemical and electrical gradients drive the Na^{+}-Ca^{2+} exchanger of the excitable cell membrane. The system 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 et al, 1986). The system has also been shown to be electrogenic, importing 3 singly charged Na^{+} for every doubly charged Ca^{2+} exported (Reeves and Hale, 1984) and thus allowing the transport process to tap the resultant energy embodied in the membrane potential.

The roles played by the exchanger are still controversial. Under resting conditions, Ca^{2+} inflow and outflow through the exchanger is probably roughly in balance, but Ca^{2+} entry via the exchanger will be enhanced both by electrical depolarisation and by a rise in internal Na^{+}, and Ca^{2+} efflux by hyperpolarisation and a rise in internal free calcium.

Included in this second Ca^{2+} efflux category are a host of situations where Na^{+} - Ca^{2+} exchange contributes to the pumping of Ca^{2+} out of cells, following a period when cytosolic free Ca^{2+} has been elevated. Under these conditions the Na^{+}-Ca^{2+} exchanger is normally operating in parallel with the ATP-dependent Ca^{2+} pump and the exchanger seems to display a lower affinity for Ca^{2+} but much larger transport capacity than the ATP-dependent Ca^{2+} pump. The exchanger is well suited to effecting a swift reduction in free Ca^{2+} towards its resting level, with the ATP-dependent Ca^{2+} pump, where it is also present, probably completing the task and establishing a somewhat

lower free Ca^{2+} than can be achieved by the exchanger alone (Baker, 1986).

Because cytosolic free Ca is so low and because the exchanger is freely reversible, quite small alterations in exchange fluxes can bring about significant changes in cytosolic free Ca^{2+} which, in turn, may serve to modify Ca^{2+}-dependent processes in the cell. One much discussed example is tension generation in the heart (Mullins, 1981), where depolarisation may initially open both Ca channels and activate Na^{+}-Ca^{2+} exchange, but as free Ca^{2+} rises the direction of exchange may reverse and the resultant Na^{+}-Ca^{2+} exchange may have a dual role: pumping out Ca^{2+} and there by helping to terminate the contraction, and also providing a source of inward current, thereby helping to maintain the plateau depolarisation.

1.4.1.3 Calcium Channels :

Calcium channels are large membrane spanning glycoprotein molecules that allow controlled entry of Ca^{2+} from the extracellular fluid into cells. These channels were first demonstrated in skeletal muscle fibres of crustacea (Fatt and Ginsborg, 1958) and subsequently in many other cell types and in all excitable membranes (Hagiwara and Byerly, 1981; Reuter, 1983; Hagiwara, 1983).

The molecular structure of these channels not only determines which ions are allowed to pass through ie. their 'ion selectivity', but also the membrane potential-dependent opening and closing kinetics of the channel commonly called 'gating'. This gating process is primarily dependent on membrane potential and the probability of channels being open increases with depolarisation. However, this probability depends on other factors also; it is modulated by changes in $[Ca^{2+}]_i$, and may be altered by hormones, neural transmitters and neuropeptides, which generally act by way of intracellular second messengers (Reuter, 1983).

The surface membranes of excitable cells contain several types of Ca channel and these may be distinguished by their kinetic signature under single channel recording and by their voltage-dependence, unitary conductance, selectivity and pharmacology (Carbone and Lux, 1984; Nilius et al, 1985; Nowycky et al, 1985; Bean, 1985).

The most commonest and best characterised type of Ca-channel is the L(long) channel. This longer conducting, slowly inactivating channel, is only activated from relatively depolarised membranes (Reuter et al, 1982; Carbone and Lux, 1984; Bean, 1985). The second type is the T(transient) channel which is activated from quite negative membrane potentials and carries small transient currents (Carbone and

Lux, 1984). These two channel types have rather different sensitivities to pharmacological agents. ß-adrenoreceptor agonists seem to have an effect only on the L-type channels. Also, the blocking potencies of dihydropyridine derivatives, such as nimodipine or nitrendipine and Ca^{2+} ions, seem to be greater in L-type than in T-type channel (Reuter, 1986). A third type of channel N-channels, which is present in certain neurons and has the properties of neither L or T. This type requires very negative potentials for the removal of inactivation and is activated by strong depolarisations. Like T-type channels, the N-type channels does not seem to be sensitive to dihydropyridine compounds (Nowycky et al, 1985).

In neurones, cardiac and skeletal muscle and some secretory cells, voltage-dependent sodium channels, which generate nerve impulses, produce the depolarisation that stimulates Ca-channel opening (Stanfield, 1986). In general, the probability that Ca channels are open is influenced by the activity of other channel types affecting the membrane potential. Ca-channel opening will itself produce an element of self-reinforcing depolarisation, since Ca^{2+} entering will depolarise, open more Ca-channels and allow more entry.

In cardiac muscles, this effect sustains the long plateau of the action potential (Reuter, 1983). In other cases, this depolarising tendency is damped down by the opening of potassium channels, allowing an opposing efflux of K^+. Some of these K-channels are opened by Ca-entry itself (Meech and Standen, 1975).

The activation process depends on voltage and becomes faster and more complete the larger the depolarisation. But inactivation appears to depend only partly on voltage and is partly dependent on accumulation of calcium within the cell. Elevation of intracellular calcium reduces the probability of Ca-channel opening and inactivates Ca-channel opening while reduction in $[Ca^{2+}]_i$ enhances Ca-channel opening (Hagiwara, 1983). However, some classes of Ca-channel inactivate in a wholly voltage-dependent fashion and even in the majority of Ca-channels, where inactivation is Ca-dependent some additional voltage-dependent inactivation seems likely.

In all cells, Ca-channels co-exist with other channel types and furthermore, Ca-channels with different properties can co-exist in the same cell type (Nowycky et al, 1985). However, it is quite possible that only one channel type may be present in certain cells where it serves particular functions. It seems that T-type Ca-channels are the only predominant ones in an insulin-secreting cell line (Findley and Dunne, 1985). It has been hypothesised, that the T-type of Ca^{2+} channels could be involved in the rhythmic firing of neurones (Nowycky et al, 1985)

and specialised cardiac cells (Bean, 1985) while the L-type Ca-channels are important for the contractile activation of cardiac and possibly smooth muscle.

1.4.2 Calcium regulation by SR :

The sarcoplasmic reticulum (SR) of skeletal muscle is an internal organelle which is a highly specialised system of membraneous tubules. The membrane envelope of the terminal cisternae and the longitudinal tubules are rich in Ca^{2+}-transport ATPase (Franzini-Armstrong, 1980). The disposition Ca^{2+}-transport ATPase in native SR membranes is asymmetric, with most of the protein mass exposed on the cytoplasmic surface. These observations are consistent with the polarity of Ca^{2+} transport and release, which implies preferred orientation of the Ca^{2+} pump in the membrane (Martonosi, 1982).

The Ca^{2+} transport ATPase accounts for 30-80% of the protein content while phospholipid account for about 90% of the lipid content. furthermore, the ATPase and the Ca transport activity of SR is absolutely dependent on membrane phospholipid (Martonosi, 1982;1984).

A high affinity Ca^{2+} binding protein with a dissociation constant similar to that of troponin ($\approx$ 3μm) is assumed to be localised in the interior of SR. In addition, a glycoprotein is preferentially localised in the T-tubule. The functions of the high affinity Ca^{2+} binding protein and the glycoprotein are unknown.

Calsequestrin, an important protein constituent of the SR, is preferentially localised in the terminal cisternae of SR. This localisation is consistent with the presence of electron-dense material and Ca^{2+} deposition in the lumen of the terminal cisternae in intact muscle (Constantin et al, 1965). Calsequestrin is an acidic extrinsic membrane glycoprotein, able to bind about 10 moles of Ca^{2+}/10,000gm protein with an approximate dissociation constant of 0.5mM at physiological pH and ionic strength. In view of its Ca^{2+} binding capacity calsequestrin was suggested to participate in the binding of accumulated calcium within SR (Maclennon and Wong, 1971).

The transport of Ca^{2+} by SR is coupled to the hydrolysis of ATP through a Mg^{2+},Ca^{2+} activated ATPase. For each mole of ATP hydrolysed, two Ca^{2+} atoms are transferred across the membrane (Hasselbach, 1964). The process is reversible and permits the synthesis of 1 molecule of ATP for every Ca^{2+} atom released across the membrane, in agreement with the $2Ca^{2+}$/1ATP stochiometry of Ca transport. The

Ca^{2+} release is inhibited by ionised Ca^{2+} in the medium with a Ki of about 0.2μM.

SR membranes are characterised by relatively high permeability for anions and monovalent cations. The calcium storage capacity of SR increases 50 to 100 fold in the presence of Ca^{2+} participating anions such as oxalate. The physiological significance of the anion channels is not known. The large anion fluxes across SR may contribute to the buffering of osmotic and potential changes connected with the uptake and release of calcium during muscle activity (Martonosi, 1982;1984).

During excitation of muscle the depolarisation of the surface membrane spreads into the interior of the muscle fibres through the transverse (T) tubules, a system of tubular invaginations of the surface membrane, which form periodic junctions (triads and diads) with the SR (Franzini-Armstrong, 1980). At these junctions the depolarisation of T-tubules triggers the release of calcium from the SR. The T-tubule membrane is closely opposed to the terminal cisternae of the SR but is apparently structurally and electrically distinct from the SR membrane. The nature of the signal transmission at the triad has not been established but several possibilities are under consideration.

An initial step in the coupling process may be a voltage-dependent opening and closing of Ca-channels in the junctional region of SR, which is reflected in a voltage-dependent charge movement (Schneider and Chandler, 1973; Chandler et al, 1976; Schneider, 1986). According to this theory, Ca^{2+} release is localised to the junctional region and propagative depolarisation of SR is not required. Alternatively, a diffusible messenger might serve to transmit the signal from T-tubule to SR. It has recently been demonstrated that inositol 1,4,5-triphosphate is capable of activating calcium release in skinned skeletal muscle fibres (Vergara et al, 1985) and in isolated SR vesicles (Volpe et al, 1985). Furthermore, the terminal cisternae of skeletal muscle are much more sensitive to the action of inositol 1,4,5-triphosphate than the longitudinal tubules (Volpe et al, 1985). Whether the liberation and breakdown of inositol 1,4,5-trisphosphate and the re-synthesis of its precursor are sufficiently rapid to account for the kinetics of activation of calcium release in muscle remains to be determined. The release of Ca^{2+} from the SR may be regulated by the Ca^{2+} concentration in the cytoplasm (Endo, 1977; Fabiato and Fabiato, 1979; Endo et al, 1981).

Fabiato (1982) has shown that in both heart and skeletal muscle, that a rise in intracellular Ca above the resting physiological level can cause a further Ca induced release of Ca (CROC) from the SR, supporting similar findings of others (Ford and Podolski, 1970; Endo, 1977; Martonosi, 1984; Duncan and Smith, 1980).

Among the earliest morphological manifestations of various forms of muscular dystrophy are the 'vacuolation' and 'dilation' of SR and T-tubules (Martonosi, 1968,1982). Dystrophic mouse SR is characterised by a decreased rate of Ca^{2+} transport and Ca uptake capacity, a decrease in the maximum steady state concentration of phosphoenzyme intermediate, and a decrease in Ca^{2+} sensitive ATPase activity. These changes can not be regarded with certainty as primary manifestations of a genetic defect in the SR since changes in the ultrastructure of the surface membrane in muscle cells could result in these changes.

1.4.3 Calcium Regulation by Mitochondria :

The role of mitochondrial Ca^{2+} homeostasis is generally considered crucial in chronic elevation of intracellular calcium. The most dramatic feature of mitochondrial Ca^{2+} transport is the ability to accumulate almost limitless quantities of Ca^{2+}, with no deterioration in its bioenergetic properties, when the free Ca^{2+} concentration in its environment rises above 5μM (Nicholls and Crompton, 1980; Nicholls and Akerman, 1982). It is notable firstly that the activity of the Ca^{2+} uniporter shows a third power relationship with the external free Ca^{2+}, secondly that Ca^{2+} and phosphate form a complex in the matrix which does not affect the other properties of the mitochondrion and thirdly that the mitochondrion diverts all its respiratory capacity from ATP synthesis to Ca^{2+} accumulation when external Ca^{2+} is elevated, even hydrolysing external ATP under extreme conditions. Thus much of the experimental evidence indicates that the maximal rate and capacity of Ca^{2+} accumulation by mitochondria isolated from a variety of tissues far exceeds those of ER, SR or plasmalemma (Ash and Bygrave, 1977; Blaustein et al, 1980). All these properties are considered, by some authors (Carafoli and Crompton, 1978; Nicholls and Akerman, 1982; Nicholls, 1986; Ruigrok, 1985) to be important defence mechanisms by which a cell primarily attempts to control intracellular Ca^{2+} from rising to toxic levels.

In contrast, serious objections have been raised against the view that mitochondrial Ca^{2+} transport is important in establishing and regulating the cytosolic free Ca^{2+} concentration of normal 'resting' cells at 0.1-1μM. These objections are based in part on the observations that the Km for mitochondrial Ca^{2+} influx is significantly greater than 1μM and that the rate of mitochondrial Ca^{2+} influx-efflux cycling at < 1μM is extremely low (Blaustein et al, 1980). Furthermore the free Ca^{2+} within

the mitochondrial matrix of physiological muscle has been shown to be low (10^{-5}M) (Martonosi, 1984). However, a theoretical analysis of cellular Ca^{2+} transport kinetics such as that reported by Borle (1972,1981) and shared by Fry and Miller (1985) indicates that such arguments may not be valid.

It is generally agreed that Ca^{2+} enters mitochondria electrophoretically in response to the negative-inside membrane potential of about 150-180 mV developed across the inner membrane by electron transport. Calcium transported inward via uniport process in which each Ca^{2+} ion carries two net positive charges that are electrically compensated for by extrusion of two H^+ from the matrix (Akerman, 1978; Nicholls, 1978; Fiskum et al, 1979;Fiskum and Lehninger, 1982). This process has the effect of converting much of the transmembrane potential to a pH gradient, thus alkalinising the mitochondrial matrix. Matrix akalinisation can have deleterious effects on the structure and the permeability of the inner membrane and can inhibit further membrane potential-dependent Ca^{2+} influx. The profound rise in internal pH is normally prevented by the simultaneous influx of H^+ coupled to the influx of phosphate on the H^+-H_2PO_4 symporter (Coty and Pederson, 1975)) or by the inward diffusion of undissociated lipophilic free acid forms of an anion such as acetate.

Release of accumulated mitochondria Ca^{2+} in non-excitable tissues takes place through an electroneutral Ca^{2+}-$2H^+$ carrier (Fiskum and Lehninger, 1979;1982). Release of Ca^{2+} from mitochondria isolated from excitable tissues, such as heart, appears to occur by a electroneutral Ca^{2+}-$2Na^+$ antiport process (Crompton et al, 1977; Affolter and Carafoli, 1980).

In pathological circumstances, when Ca^{2+} loading becomes really excessive and especially in the absence of certain components, a generalised increase in the permeability of the inner mitochondria membrane develops, resulting in the efflux of Ca^{2+}, K^+, Mg^{2+} and adenine nucleotides (Hunter et al, 1976; Beatrice et al, 1980). The eventual Ca^{2+} overloading of mitochondria and subsequent release of stored Ca^{2+} may be the 'trigger' by which the initiation of rapid degradation of cellular components occurs (Wrogemann and Pena, 1976; Duncan, 1978).

Alterations in mitochondrial morphology have been observed in a great number of pathological and experimental conditions (see 1.5). Such processes are likely to occur in various forms of dystrophy since isolated mitochondria have been shown (Wrogemann et al, 1973;

Wrogemann and Nylen, 1978) to contain elevated concentrations of Ca^{2+} perhaps as high as 10-40 times that of normal mitochondria (Mezon et al, 1974).

1.4.4 Calcium regulation by soluble proteins :

Cells contain one or more of a family of homologous calcium binding proteins with one or more calcium binding sites which have an affinity for calcium in the micromolar range (Cohen, 1980; Robertson 1981;England, 1986). Thus Ca^{2+} regulation within the cell could be governed by soluble proteins found in the cytoplasm and within cellular organelles. However, the amount of Ca^{2+} that soluble proteins can bind is limited by the number of protein molecules and hence their capacity to regulate intracellular calcium would appear to be limited. However, more importantly, these proteins primarily act as transducers for relaying changes in intracellular calcium concentrations to the appropriate target systems. These responses can also be modulated by the actions of other second messengers and such interactions give the cell the ability to fine-tune its intracellular regulation to accommodate the wide range of stimuli to which it is subjected.

Examples of soluble Ca^{2+} binding proteins, important to skeletal muscle physiology are troponin, calmodulin and parvalbumins.

In striated muscle, the Ca^{2+} receptor protein for the control of the contractile system is troponin, a protein complex bound to the actin-containing thin filament which regulates the interaction of myosin and actin (Endo, 1977; Ebashi, 1980).

Calmodulin, a highly acidic protein with four calcium binding domains, when activated by calcium mobilises a protein kinase (phosphorylase kinase) which results in the activation of glycogen phosphorylase. This enzyme catalyses the breakdown of the glycogen to glucose which is then metabolised to provide energy for muscle contraction.

Cohen (1980) suggests that troponin could be a physiological regulator of phosphorylase kinase, enabling a tight coupling between muscle contraction and glycogenolysis.

Parvalbumins are virtually absent from cardiac and smooth muscles and are only present at high concentrations (> 40 μM) in fast white muscles. Parvalbumins have two divalent metal-ion binding pockets of structure, very similar to the non-specific binding sites of troponin, in that they have high affinity for both Ca^{2+} and Mg^{2+}. Thus, at physiological concentrations of Mg^{2+} the pockets will be occupied by this ion when the muscle is relaxed. During contraction, when intracellular, calcium rises to > 1μM, certain displacement of Mg^{2+}

might occur. However , the rate of dissociation of Mg^{2+} from parvalbumin is slow and it is unlikely that a significant amount of Ca^{2+} will be bound unless there is sustained tetanic contraction of the muscle (Robertson et al 1981). The actual function of these proteins are therefore unclear, although they could have a protective role in prolonged contraction by slowly reducing $[Ca^{2+}]_i$.

1.5 Calcium and Cellular Necrosis :

There have been many reports of increases in total tissue Ca^{2+} occurring under a variety of experimental and pathological conditions (Fig 1.2). Since the initial proposal of calcium-overload as a general causatory mechanism of cellular necrosis (Wrogemann and Pena, 1976), a number of diverse range of chemical agents whose common mode of action being the ability to cause a persistent rise in $[Ca^{2+}]_i$ by enhancing Ca^{2+} movements across biological membrane, have been shown to cause ultrastructural damage. These chemicals include antiChE inhibitors (Salpeter et al, 1979; Hudson et al, 1985;Townsend, 1988), calcium agonist Bay K 8644 (Howl and Publicover, 1987), acetylcholine receptor agonist carbachol (Leonard and Salpeter, 1979), A23187, a calcium ionophore (Statham et al, 1976; Publicover et al, 1978; Llados, 1985), caffeine (Duncan and Smith, 1980), 2,4-dinitrophenol (Duncan et al, 1980) and Lindane, an insecticide (Publicover et al, 1979).

Severe myocardial damage has been reported to occur when Ca^{2+} containing solution is re-admitted to hearts which have been perfused, for a short time period with a Ca^{2+} free solution, a phenomenon termed 'the calcium paradox' (Zimmerman and Hulsmann, 1966; Crevey et al, 1978; Ruigrok, 1985). This phenomenon appears to result from an uncontrolled uptake of Ca^{2+} across the sarcolemma following Ca^{2+} re-admission (Ruigrok, 1985). More recently a similar phenomenon has been observed in skeletal muscle (Armani et al, 1984; Soza et al, 1986).

In further support for the implication of elevated calcium in cell necrosis is the demonstration of increased muscle calcium in Duchenne muscular dystrophy (Bodensteiner and Engel, 1978; Cornelio and Dones, 1984) congenital muscular dystrophy (Cornelio and Dones, 1984) and other human muscular diseases (Oberc and Engel, 1977). Elevated calcium levels have also been reported in mitochondria isolated from the heart and skeletal muscle of dystrophic mice (Nylen and Wrogemann, 1983) and in experimentally damaged rodent skeletal muscle (Oberc and Engel, 1977).

Figure 1.2 Raised cell Ca^{2+} in cell injury (Campbell 1983).

The demonstration of Ca^{2+} accumulation in cellular injury provides little information regarding the problem of whether a rise in total cellular calcium is the cause or the consequence of cellular necrosis. The elevation of intracellular calcium may be due to influx from extracellular space or from the release of intracellular stores or from a combination of both mechanisms. It is difficult to delineate to what extent the increased rise in intracellular calcium is the cause of, or the result of myopathy.

There is growing evidence to suggest that at least in certain pathological conditions that early defective sarcolemmal membrane (ie. membrane leakiness) leads to the rapid elevation of intracellular calcium that results in ultrastructural damage (Munsat et al, 1973; Mokri and Engel, 1975; Duncan, 1978; Ruigrok, 1985).

Recently, Duncan and Jackson (1987) reported that low levels of Ca, trigger rapid and characteristic myofilament damage in chemically skinned skeletal muscle cells. Furthermore, chemically-induced hepatic cell death and damage to metabolically poisoned myocytes do not depend on extracellular calcium, thus cell damage seems to be a cause and not a consequence of a rise in free calcium (Cobbold and Bourne, 1984;Fariss and Reed, 1985).

There is evidence (Leonard and Salpeter, 1979) to suggest that in antiChE induced myopathies, at least free intracellular calcium may be causative and in fact Toth et al (1981) and Townsend (1988) both have demonstrated cytochemically and biochemically respectively, the uptake of Ca^{2+} prior to the development of myopathy at the junctional region of the diaphragm exposed to OP cholinesterase inhibitors.

Since Ca^{2+} plays such a pivotal role in regulating cellular activity, it is to be expected that agents which interfere with the homeostasis of Ca^{2+} and impair the ability of the cell to maintain $[Ca^{2+}]_i$ at physiological levels, will have a profound and deleterious effect upon cellular metabolism.

Ca^{2+} alone is unable to promote ultrastructural damage in washed, isolated frog myofibrils (Statham et al, 1976), suggesting that it must operate via Ca^{2+} activated cytoplasmic factor(s).

1.6 Calcium activated neutral protease and cellular necrosis:

Calcium activated neutral protease (CANP), a thiol endopeptidase was first extracted from rabbit skeletal muscle by Huston and Krebs (1968) and subsequently demonstrated in a variety of vertebrate tissues

(Busch et al, 1972; Reddy et al, 1975; Reville, 1976; Dayton et al, 1976,a,b; Ishiura et al, 1980; Inomata et al, 1985). CANP are stimulated directly by Ca^{2+} and are active over the physiological pH range.

Differential centrifugation studies have demonstrated that CANP are either free in the muscle cell cytoplasm or absorbed to myofibrils, and are not located in membrane-enclosed particles, such as lysosomes (Reville et al, 1976). It may be noted that in some muscle cells some CANP is specially localised on the Z line (Ishiura et al, 1980).

CANP isolated from porcine skeletal muscle (Dayton et al, 1976a) was found to have little proteolytic effect on myosin, actin or α-actinin but would degrade tropomyosin, troponin, C-protein and M-protein. When CANP was incubated with intact myofibrils, it was shown that α-actinin was released from the Z-line and troponin, tropomyosin, C-protein and M-protein were degraded. These observation supports the earlier findings of Busch et al (1972) that CANP was capable of complete removal of Z-lines.

Because of the unique selectivity and localisation of CANP, it would appear that CANP could play a significant role in the disassembly of the myofibril during the initial stages of cellular necrosis. However, since this enzyme requires Ca^{2+} in the millimolar range, ie. requiring a 100 fold increase from resting concentration for maximal activation, its role in the rapid degradation of myofibril appears limited (Imahori, 1982).

However, Mellgren (1980) proposed the existence of another type of CANP which requires micromolar Ca^{2+} (μ-CANP) for maximal activity, which was later described by others from various sources (Inomata et al, 1985; Kamakura, 1985). The relationship between these two types of CANP is not clear but evidence is provided by Imahori (1982) that μ-CANP can be derived from autolysis of m-CANP, suggesting that m-CANP may serve as a proenzyme of μ-CANP, providing a regulatory mechanism for CANP.

However, there is still considerable debate concerning the role played by CANP in the rapid degradation of myofilaments. In favour for the role of CANP are the findings that CANP activity is significantly increased in Duchenne muscular dystrophy, Becker dystrophy (Kar and Pearson, 1976) and in the muscles of dystrophic hamsters and mice (Neerunjum and Dubowitz, 1979; Klamut, 1983) but essentially normal in limb girdle dystrophy and other certain denervating diseases (Kar and Pearson, 1976). Furthermore, exposure to antiChE, DFP, showed significant elevation of CANP activity in the junctional region (Toth et al, 1981).

Further support stems from the observation that leupeptin, an

inhibitor of CANP and some lysosomal cathepsins, has been reported to delay the degeneration of dystrophic chicken muscle both in vivo (Stracher and McGowan, 1978) and in tissue culture systems (McGowan et al, 1976) and to prevent protein degradation in rat skeletal and cardiac muscle (Libby and Goldberg, 1978, 1980).

In contrast, Nonaka et al (1982) reported that administration of leupeptin to dystrophic chickens produced no significant effect upon the number of necrotic myofibres. Similarly, Enomoto and Bradly (1977) failed to produce evidence of any benefit conveyed by leupeptin against murine muscular dystrophy. Likewise there have been several reports of the failure of leupeptin to protect against Ca^{2+} activated myopathies: A23187 induced myofilament degradation in frog skeletal muscle in vitro, was not reduced by leupeptin (Duncan et al, 1979) nor were any protection seen in damaged induced by low levels of Ca in chemically skinned cells (Duncan,1987). Gerhard and Schneider (1980) reported that the Ca^{2+} stimulated increase in protein degradation observed in intact muscle after treatment with A23187 could not be prevented with mersalyl, a powerful inhibitor of CANP.

Such results suggest that leupeptin cannot easily penetrate intact sarcolemmal membranes despite reports that it does so (Libby and Goldberg, 1978) or that CANP is not involved in the degradation of the myofilament apparatus.

1.7 Lysosomal system and cellular necrosis :

Changes in proteolysis is now well documented under a variety of physiological and pathological conditions including various myopathies (Bird, 1975; Goldberg and St. John, 1976). The mechanism of degradation of cellular proteins, whether lysosomal or non-lysosomal, or both remains to be elucidated. It has been firmly established that the lysosomal apparatus and its full complement of acid hydrolases are present as well as functional, in all types of muscle cells which therefore suggests that the lysosomal system is perhaps involved in cellular necrosis (Bird, 1975).

The lysosome has been identified as a cellular organelle that contains a wide spectrum of proteases, including exopeptidases and endopeptidases together with other hydrolytic enzymes. Endopeptidases cleave peptide bonds 1 or 2 residues from the ends of the polypeptide chains whereas the exopeptidases attack the C-termini of polypeptide chains.

Of the endopeptidases, cathepsins B, D, H and L, purified from rat

skeletal muscle, will degrade native or purified F-actin and M-myosin (Schwartz and Bird, 1977; Bird et al, 1980). In particular, the degradation of myosin and actin by cathepsin D was more extensive than that by cathepsin B. Both of these enzymes have been visualised by electron microscopy to reside within muscle lysosomes (Bird et al, 1978). A recent study by Sohar et al (1979) showed that purified lysosomal cathepsins H and L had a greater specific activity against myosin than cathepsin B (5X and 10 X respectively).

Schwartz and Bird (1977) have made calculations based on purification data, of the possible in vivo significance of the concentrations of cathepsin B and D in skeletal muscle. At pH 5.0, cathepsin B and D could theoretically degrade all of the native myosin in 4 to 9 days. When one considers the possible enhancement of degradation by non-lysosomal 'mix' enzymes, denaturation and the additional contribution of the lysosomal exopeptidases, the physiological significance of these enzymes in myofibrillar protein degradation becomes quite apparent.

There is now substantial evidence in support of the hypothesis that lysosomal enzymes are involved in the degradation of myofibrillar proteins both in normal and pathological tissues (Iodice et al, 1972; Bird, 1975; Kar and Pearson, 1976; Libelius et al, 1981; Bird et al, 1980; Silver and Etlinger, 1985). Further support, has been provided by studies which have shown that inhibitors of lysosomal enzymes can delay atrophy and degeneration (McGowan et al, 1976), preserve the general architecture of the muscle with little fatty lipid infiltration (Stracher et al, 1978) and decrease protein degradation while protein synthesis remained unchanged (Libby and Goldberg, 1978) in normal and dystrophic muscle. Perhaps the most convincing evidence that lysosomes are involved in myofibrillar protein degradation is the demonstration of myofibrillar fragments within lysosomes (Li, 1980) and the observation that radiolabeled myofibrillar proteins become associated with isolated muscle lysosomes (Gerhard and Schneider, 1979).

The exopeptidases, such as cathepsin C and A, do not appear to be important in the degradation of myofibrillar proteins, however , the breakdown products of myofibrillar apparatus by endopeptidases appear to be good substrates for exopeptidases and thus suggest that exopeptidases act synergistically with endopeptidases (Iodice, 1966).

From the preceding sections it can be appreciated that muscle contains a variety of proteases active in a wide pH range. Many of these reside in lysosomal structures and a majority of these enzymes are increased in dystrophic and other diseased muscles. Many investigators agree that in most tissues, proteolysis probably involves both lysosomal

and non-lysosomal mechanisms. However considerable debate continues on the initiation of and the relative importance of these mechanisms. Consequently, it has been postulated that non-lysosomal protease (CANP) may be involved in the initial rapid breakdown of myofilament apparatus, after which the partially degraded myofibrils are catabolised completely by the lysosomal proteases (Busch et al, 1972). This appears more attractive, firstly by the fact that skeletal muscle cells have a relatively low lysosomal content and therefore low proteolytic activity (Bird and Carter, 1980) and secondly, whatever process initiates myofilament damage is also likely to damage other membrane systems such as the Golgi or lysosomal system thereby initiating the release of their content.

1.8 Oxygen radicals, Lipid peroxidation and cellular necrosis :

Molecular oxygen is both benign and malign. On the one hand it provides enormous advantages and on the other hand it imposes a universal toxicity. This toxicity is largely due to the intermediates of oxygen reduction such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), as well as the molecular oxygen in the excited state termed singlet oxygen ($^1O_2$). These reactive activated species of oxygen (collectively termed oxygen radicals) induce devastating cytotoxicity leading to cellular damage. Oxygen radicals are capable of destroying proteins, lipids, polysaccharides, nucleic acids and other biomolecules as well as killing bacteria and animal cells in culture. (Halliwell, 1984; Fridovich, 1976; Fridovich, 1983; Clark et al, 1985). It is the O_2^- and OH· that cause most damage. Unlike the hydroxyl ion, the hydroxyl radical is fearsomely reactive, combining with most molecules found in vivo at near diffusion-controlled rates (Floyd, 1983; Halliwell, 1984).

Oxygen radicals are essential to many normal biological processes and yet are highly destructive if not tightly controlled. A number of enzymes including xanthine oxidase, aldehyde oxidase, dihydro-ortic dehydrogenase and a variety of flavin dehydrogenases, all produce some O_2^- during their catalytic cycle (see Fridovich, 1976). Organelles such as mitochondria (Forman and Boveris, 1982; Boveris and Chance, 1973; Boveris and Cadenas, 1982; Loschen et al, 1974), chloroplast (Asada and Kiso, 1973), endoplasmic reticulum (Boveris et al, 1972), microsomes (Howco et al, 1980) and nuclei (Bartoli et al, 1977) have been shown to generate O_2^-. It is clear from the foregoing that there are

Figure 1.3 Formation and detoxification of reactive oxygen species in biological systems. SOD, superoxide dismutase, GSH reduced glutathione, GSSG, oxidised glutathione, GSH-PX glutathione peroxidase, GR glutathione reductase, ASC ascorbic acid (Clark et al, 1985).

Figure 1.4 The peroxidation of polyunsaturated lipids (Blake et al, 1987).

numerous potential sources of O_2^- in any given cell. Consequently aerobic cells have evolved an elaborate defence system consisting of superoxide dismutase, catalase and glutathione peroxidase collectively serving the indispensible role of detoxifying endogenous oxygen radicals. (Figure 1.3).

Lipid fluidity confers upon membranes several properties which are essential to the correct functioning of the cell. Lipid peroxidation has been broadly defined by Tappel as oxidative deterioration of polyunsaturated fatty acids. The polyunsaturated fatty acids (PUFA) in lipids are among the biological target molecules that are most easily attacked by free radicals (Mead, 1976; Tappel, 1980; Demopoulos, 1973). Lipid peroxidation can be initiated by enzymes which catalyse the formation of lipid hydroperoxides such as soyabean lipoxygenase, by other radicals or by substrates which secondarily give rise to radicals, as well as by certain types of chelated iron-oxygen complexes.

Initiation of peroxidation in a membrane or PUFA is due to the abstraction of hydrogen, of which the hydrogen in the divinyl methane structure, present in all PUFA is particularly susceptible (Mead, 1976; Demopoulos, 1973). The lipid radical thus formed will then react with molecular oxygen, at the head of a chain of reactions resulting in the breakdown of PUFA structure and the formation of lipid hydroperoxides (Figure 1.4). Lipid hydroperoxides are stable under physiological conditions until they come into contact with transition metals such as iron. These metals or their complexes cause lipid hydroperoxides to decompose in very complicated ways, producing radicals that can continue the chain reaction of lipid peroxidation, as well as producing cytotoxic aldehydes and hydrocarbon gases (Halliwell, 1984). The many products of lipid peroxidation such as hydroperoxides or their aldehyde derivatives can inhibit protein synthesis, block macrophage actions and alter enzymic activity. One series of aldehydes the 4-hydroxyalkenal series are highly cytotoxic and 4-hydroxynonenal (the major product of this series) alone exhibits a number of these cytopathological effects (Benedetti et al, 1980, 1981). These compounds with their cytotoxic capability, could then transmit the toxic effects of membrane lipid peroxidation throughout the cell.

The consequence of lipid peroxidation go far beyond the mere deterioration of membrane PUFA chains, which in itself de-stabilises membrane structure. The biochemical changes produced are many and varied including swelling and lysis of mitochondria and damage to mitochondrial electron transport (Narabayashi et al, 1982).

Lipid peroxidation has been linked directly or indirectly to many toxicities, deficiencies and abnormal states. Lipid peroxidation has been

implicated with ageing, carcinogenosis, atherosclerosis , inflammation, rheumatoid arthritis, a group of mental disorders, known collectively as neuronal ceroidlipofuscinoses and a peripheral arterial disease 'intermittent claudication' (see Clark et al, 1985; Barber and Bernheim, 1967).

Furthermore, in recent years considerable evidence has accumulated suggesting that oxygen radicals are responsible for the cellular injury during ischemia (Aoki et al, 1988; Rao et al, 1983; Roy and McCord, 1983), calcium paradox (Julicher et al , 1984a,b), the oxygen paradox (Gauduel and Duvelleroy, 1984; Myers et al, 1985), reperfusion induced arrhythmias (Woodward and Zakaria, 1985). In majority of these studies, the use of wide range of free radical scavengers have been shown to reduce the extent of myocardial necrosis (Walker et al, 1987; Woodward and Zakaria, 1985; Myers et al, 1985; Gauduel and Duvelleroy, 1984; Akizuki et al, 1984; Chambers et al, 1985).

Whether, antiChE-induced myopathy involves oxidative damage is a matter of speculation, suffice to say that favourable conditions are present in the cellular environment for oxidative damage to take place. In this regard Hasan and Ali (1980) have shown that OP pesticide dichlorvos caused dose-dependent inhibition of AChE coupled with dose-dependent increase in the rate of lipid peroxidation of various brain regions. Similar findings were reported by Spoerri and Glees (1979) following intoxication with triorthocresylphosphate (TOCP) in the hen.

Possible sources of oxygen radicals after exposure to antiChE include the univalent reduction of O_2 and the production of H_2O_2 and O_2^- by the mitochondrial electron transport system (Boveris and Chance, 1973; Boveris and Cadenas, 1982; Nohl and Hegner, 1987). This system might be particularly vulnerable when mitochondrial electron transport system is uncoupled perhaps due to excessive Ca^{2+} uptake. In addition, or alternatively, a rise in $[Ca^{2+}]_i$ might promote the conversion of the cytosolic enzyme xanthine dehydrogenase to xanthine oxidases (Packer, 1985). Xanthine oxidase using O_2 as an electron acceptor, generates H_2O_2 and O_2^- (Kellog and Fridovich, 1975), a process which has a particular significance in the ischemic heart and during the calcium paradox (Meerson et al, 1982; Myers et al, 1985; Schrader, 1985) with great medical implications. A further possibility is Ca^{2+} activated phospholipases and lipid peroxidation (see below).

Duchenne muscular dystrophy is similar to the myopathy that occurs with Vit. E deficiency, in many animals (Witting, 1980; Tappel, 1980; Barber and Bernheim, 1967). An increase of some of the protective enzymes has been demonstrated in muscle from patients with muscular

dystrophy, including DMD, suggesting enhanced free radical generation (Elbrink et al, 1987). Furthermore, muscle from DMD patients (Kar and Pearson, 1979) and experimental animals show functional disarray consistent with oxidant damage (Omaye and Tappel, 1974).

1.9 Depletion of high energy phosphates and cellular necrosis:

The entry of Ca^{2+} into the myoplasm will activate many energy requiring processes including myofilament contraction (Webber and Murray, 1973). Increased elevation of intracellular calcium forces the mitochondria and SR to maintain Ca^{2+} homeostasis by sequestering the excessive amounts of this ion. This is an energy consuming process and occurs in preference to ATP formation (Fiskum and Lehninger, 1982) ie. uncoupling oxidative phosphorylation. Consequently the finite stores of glycogen and adenine nucleotide pool become depleted. This has fatal consequences since Ca^{2+} pumps of the SR, mitochondria and sarcolemma will be disrupted, and by a process of negative feedback, raise the intracellular calcium further. Hence, cellular injury could be as a consequence of cessation of aerobic metabolism and the metobolic adjustments the myofibrils make to changes in their environment. A further consequence of uptake of calcium by mitochondria, in exchange for protons, may be the induction of localised reductions in pH_i and the activation of lysosomal enzymes (Rudge and Duncan, 1984; Gerbert et al, 1971).

The rapid depletion of high energy phosphate is of particular significance in myocardial ischemia and during the calcium paradox (Allison et al, 1977; Crevey et al, 1978; Jennings and Hawkins, 1980; Schafer and Tan, 1985; Ruigrok, 1985). The ultrastructural alterations occurring in myocardial ischemia has been suggested to be due to the direct consequence of high energy phosphate depletion (Jennings and Hawkins, 1980), with significant changes occurring in the first 180 sec. of ischemia (Braasch et al, 1968; Jones et al, 1976; Jennings et al, 1978).

With OP-induced increased duration of muscle activity the energetic state of the hyperstimulated muscle fibre would be comparable to the metabolic situation of muscle in ischemia (Dettbarn, 1984). Swelling and vacuolation of mitochondria is generally recognised as an early feature of OP poisoning (Townsend, 1988; Hudson et al, 1986; Meshul et al, 1985). Recently, Gupta and Dettbarn (1987a) have shown significant reduction in phosphocreatine (PC), ATP, coinciding with appearance of necrotic lesions, following acute doses of DFP and soman. Furthermore, in vitro exposure to A23187 in the presence and absence

of calcium, in doses which induce ultrastructural damage, results in significant decline in CP and ATP of diaphragm tissue (Sugden, 1980).

Alterations in the levels of high energy phosphate compounds has been proposed to occur in the muscles of dystrophic mice and hamsters (Wrogemann et al, 1973; Nylen and Wrogemann, 1983). Several studies have shown decreased ATP, CP content in dystrophic muscles (Elbrink, 1987). A one year clinical trial of allopurinol showed elevation in the concentration of ATP, CP and total adenylate in skeletal muscle (Thomson, 1985). Whether the efficacy of allopurinol is due to the stimulation of the 'purine salvage' pathway, stimulation of adenylate synthesis or the prevention of free radical damage is yet to be resolved.

A further consequence of high energy phosphate depletion is the suggestion that endogenous phospholipases, which are present in the plasmalemma of the sarcolemma (Franson et al, 1978) are activated when cellular ATP levels are low, perhaps because membrane proteins can no longer be phosphorylated. In fact, a relationship of membrane susceptibility to hydrolysis to the level of ATP in cell has been demonstrated, showing that ATP depletion in erythrocytes facilitate phospholipid hydrolysis of the cell membrane by exogenous phospholipase C (Gazitt et al, 1975).

1.10 Phospholipases, membrane permeability and cellular necrosis:

The maintenance of the integrity of membrane phospholipids is essential for cellular homeostasis (Van den Bosch, 1974; Weglicki, 1980). Activation of phospholipase activity, a membrane bound enzyme, at upper physiological or pathophysiological concentrations of Ca^{2+} (Peters,1986), releases free fatty acids, including arachidonic acid (AA), and lysophospholipids from lipid membranes. These substances are powerful membrane-perturbing agents and may produce many detrimental effects on membrane function (Weglicki, 1980; Peters,1986).

The involvement of calcium in the cellular metabolism of AA is two fold. First, calcium appears to be importantly involved in enzymatic reactions that generate free AA from membrane phospholipid, and secondly metabolites generated both in the course of mobilisation of AA from phospholipids and from the oxidative metabolism of free AA may affect the influx of calcium into cells and/or the mobilisation of internal calcium pools (Feinstein and Shaafi, 1983; Knapp et al, 1977; Moskowitz et al, 1982).

These 'putative' ionophores include lysophospholipids, phosphatidic acid, prostaglandin endoperoxides, thromboxane A_2, leukotrienes and have been shown to cause permeability changes in various membrane (Sheran et al, 1982; Taylor and Morris, 1983; Peters, 1986; Bakhle, 1983; Blackwell and Flower, 1983). Thus enhancement of phospholipase activity may directly disturb the membrane organisation leading to membrane leakiness and cellular necrosis.

The observations that inhibitors of phospholipase activity or lipoxygenases have proved effective in reducing the characteristic enzyme efflux from skeletal muscle treated with Ca^{2+} mobilising drugs suggest that phospholipase activity and the lipoxygenase pathway is involved in membrane disruption (Schanne et al, 1980; Jackson et al, 1984; Duncan and Jackson, 1987).

Several investigators (Barker and Brin, 1975; Braughler et al, 1985) have provided evidence that there is synergism between a Ca^{2+} stimulated increase in the phospholipase activity and a calcium-stimulated increase in the peroxidation of membrane lipids in membrane damage. This is not surprising since AA cascade pathway involves radical mediated reactions as well as the production of free radical species.

1.11 Choice of antiChE and preparation :

Ecothiopate, has been used as a pharmacological tool to investigate the mechanism(s) involved in skeletal muscle necrosis because :

(i) its effects are characteristic of antiChE
(ii) it lacks CNS effects, therefore complication from CNS are avoided.
(iii) it is more stable than for eg. DFP and therefore much safer to use.
(iv) this agent has been much used in this laboratory.

All experiments were done in mouse diaphragm because :

(i) of the many skeletal muscles investigated by others, this is the most affected.
(ii) the necrosis is initially localised to the junctional region, therefore in many instances, the non-junctional region enabled comparisons to be made between the two regions.

1.12 Aims:

The possible mechanisms involved in skeletal myopathy appear to be numerous, complex and often speculative. Thus rational or specific therapies are not likely to emerge without some understanding of these processes. The purpose of this investigation is to study the role of AChE inhibition and calcium in ecothiopate-induced myopathy. The role of high energy phosphates and superoxide were also investigated.

2.0 MATERIALS AND METHODS

2.0 Animals :

Albino mice used were 6 months old adult males, usually ex - breeders and weighing 40-50g. The animals were acclimatized for at least 1 week prior to use. The animals were allowed free access to water and to pelleted breeding diet.

At 6 months, mice are adult and the characteristics of their neuromuscular junction is stable for several months thereafter (Banker et al, 1982; Kelly, 1978).

In all experiments, whenever possible, controls were drawn from the same batch of mice.

2.1 Administration of drugs :

Ecothiopate iodide was used as Ecothiopate Eyedrops BNF (Phospholine iodide, Ayerst laboratories). This consists of a dry powder of 12.5mg ecothiopate iodide and 40mg potassium acetate and a diluent composed of 0.5% chlorobutanol, mannitol, boric acid and sodium phosphate.

The dry powder was dissolved in 3.2 ml of distilled water to give a stock of $1x10^{-2}$ M ecothiopate. This was further diluted with distilled water to $1x10^{-4}$ M stock solution and stored at -20°C in freezer. The 10^{-4} M ecothiopate stock solution in use was stored at 4°C for a maximum of two weeks.

A solution for injection was made from the ecothiopate stock with addition of atropine sulphate ($1.4x10^{-4}$ M) and sodium chloride to 0.9%. This injection shall be referred to as ECO.

All injections were given subcutaneously, between the shoulder blades of unanaesthetized mice, in a dose of 0.1ml per 20g body weight i.e ecothiopate 500 n mole Kg^{-1} plus 700 n mole Kg^{-1} atropine referred to as ECO. However mice used as controls were injected with 700 n moles Kg^{-1} atropine sulphate in normal saline.

All other drugs were also made up in normal saline and administered subcutaneously unless otherwise stated.

2.2 Preparation of hemidiaphragm :

Mice were killed by a blow on the head and section of cervical spinal cord. After removing the skin over the chest, the thorax was opened along the left and right side of the sternum thus exposing the

thoracic cavity completely and allowing the removal of the whole diaphragm from the animal.

Once isolated from the animal the diaphragm was immediately placed in physiological saline (see appendix) and was then divided into right and left hemidiaphragm preparations by section of the medial tendon, and the ribs trimmed back to the costal margin.

The procedure described above was essentially that of (Bulbring, 1946).

2.3 Histological methods.

2.3.1 Buffers :

Phosphate buffers have been mostly used as they are the most 'physiological', non-toxic to cells in culture and mimic certain components of extracellular fluid. Their only disadvantage is that precipates are more likely to occur during fixation than with other buffers. Most of the buffers used are based on Sorensen's which is a mixture of monobasic and dibasic sodium phosphates (see appendix). Other buffers were used if they appeared superior eg. acetate buffers.

2.3.2 Fixatives :

The requisite qualities of the ideal fixative are preservation from osmotic damage and shrinkage, amounting to avoidance of all consequent changes in morphology (Jones, 1973). These criteria cannot be satisfied completely but nearest approach to them is by the use of formaldehyde, glutaraldehyde or acrolein. Of these, the first two have been employed in this study. The choice of either of these fixers was governed by the type of microscopical technique.

2.3.2.1 Formaldehyde :

Formaldehyde 4% in 0.2M acetate buffer at pH 4 was routinely used to fix specimens for one hour to be viewed under UV light. The addition of acetate buffer to the formaldehyde has been reported and was found to minimise the autofluorescence of muscle cells (Stretton and Kravitz, 1973).

2.3.2.2 Glutaraldehyde :

Since cells remain osmotically active during glutaraldehyde fixation, the osmolarity of the fixative was chosen carefully. The osmolarity of glutaraldehyde fixatives was adjusted by altering the osmolarity of the buffer and not the fixative agent which has almost no

osmotic effect.

The fixative was freshly prepared from 25% glutaraldehyde (BDH). This was diluted to 5% by 0.1 M pH 7.4 phosphate buffer. Fixation was for 24 hours for specimens to be inspected by electron microscopy, or to be teased out for light microscopical examination of single fibres or of small bundles of fibres. The pH was kept below 7.5 to prevent polymerization of the glutaraldehyde and the loss of reactive groups (Sabatini et al, 1963).

2.3.3. Procion yellow staining :

Procion yellow MX 4R (ICI), a dichlorotriazinyl dye, was used as a vital stain for locating and quantifying necrotic muscle cells. Entry into normal cells is prevented by impermeability of lipid membranes to the dye, and binding of the dye to subcellular organelles presumably prevents leakage from damaged cells (Flanagan et al, 1974).

2.3.3.1. Procedure :

Hemidiaphragms were pinned to pieces of dental wax via the costal margin of the ribs and the central tendon. The pins were carefully placed such that fibres were not damaged but were aligned straight with some stretching. The preparation was slightly raised from the dental wax thus ensuring free access of fluid to all sides of the muscle. The piece of dental wax was then immersed for 1 hour at room temperature in 0.1 % procion yellow made up in physiological saline and washed at intervals of 10 minutes, for 30 mins, and then fixed for approximately 1 hour in 4% formaldehyde in 0.1M acetate buffer at pH 4.0 and subsequently stained for cholinesterase.

2.3.3.2 Quantification of damage by procion:

A morphometric point-counting technique was used to quantify the extent of procion staining in hemidiaphragm preparation. Such methods are indirect and essentially probabilistic ways of obtaining numerical information about anatomical structures and entail the use of test grids superimposed repeatedly and randomly on the object.

In this study, an eye piece graticule of 121 points, intersections of an 11x11 grid, was placed at regular fields of view over each hemidiaphragm. Magnification being such that the grid measured 0.62 mm^2. Epifluorescence illumination was used, the incident beam was violet (490-500nm) and wavelengths less than 520nm were filtered out of the visible beam.

Fields of view were selected with the aid of the mechanical stage, advanced at first 1mm along the length of the muscle fibres until either

the tendon or the costal margin was reached and then advanced 1mm across the fibre, until the whole of the hemidiaphragm was inspected.

For each field, the number of intersections of the grid coincident with a yellow fluorescing structures was counted. Any field which contained an end plate on the grid was designated as being in the junctional region. Other fields were designated as non-junctional.

The junctional region extended 0.62 mm maximum on either side of a band of end plates. Similarily the non-junctional region could extend close to the junctional region, thus a particular procion yellow stained contraction clump lying within 0.62 mm of the end plate of that cell may be counted either in junctional or in non-junctional region depending upon how the grid falls relative to the end plate.

2.3.3.3 Expression of results :

The no. of procion points as a percentage of the total no. of points for each preparation reflects the % surface area occupied by procion stained structure.

A comparison was made between the % procion staining in the junctional and in the non-junctional regions by estimating separately the % procion for each region. Therefore results were routinely expressed as the % procion yellow staining at the junctional and non-junctional region respectively. This conveys a better picture as to the site of necrosis as damage progresses from junctional to non-junctional region.

2.3.4. Histochemical localisation of cholinesterase:

The method of Karnovsky and Roots (1964) was used for the histochemical localization of cholinesterase. The advantages of the method are: colour is produced directly at the site of enzymatic activity, sites of low activity more easily detected and the development of the stain is under visual control.

2.3.4.1 Principle :

The basis of the method is that thiocholine liberated by the hydrolysis of acetylthiocholine by the cholinesterase reduces ferricyanide to ferrocyanide which combines with Cu^{2+} ions to form the insoluble copperferrocyanide (Hatchett's Brown). The Cu^{2+} ions in the medium are complexed with citrate to prevent formation of copper ferricyanide.

2.3.4.2 Procedure :

After washing in distilled for a few minutes, fixed hemidiaphragms were trimmed from the ribs, excess connective tissue, nerve and the original cuts were removed. Each preparation was then

incubated at room temperature in a freshly prepared staining medium , made up in the order they are listed in the appendix.

The staining medium had an apple green colour and the sites of cholinesterase activity finally appeared as patches of brown stain. Untreated preparation took approximatly 1 hour to stain while those of ecothiopate treated taking 60-90 minutes. Individual solutions were kept in the refrigerator at 4^0C for only a few weeks.

All specimens were washed in distilled water, neatly trimmed and then finally mounted entire in glycerol jelly on glass microscope slides.

2.3.5 Measurements of end plate dimensions :

In those experiments where dimensions of the end plates (EP) were measured, preparations were stained in the presence of $5x10^{-5}$ M ethopropazine hydrochloride. Optimum staining was judged to be when the majority of EP were at the initial stage of the staining process. At this point they had a clear and shiny appearance, with a distinct outline when viewed under the microscope. Over-stained end plates had an intense copper ferrocyanide precipitate, which in some cases had started to diffuse into the surrounding tissue. This diffusion made the outline blurred and measurement impossible.

2.3.5.1 Procedure :

The whole preparation was scanned in a similar manner to that described for procion counting but with the following changes:

i) The preparation was moved in the same direction throughout the counting procedure. Thus the slide was never moved backwards. This ensured that the same EP was not counted twice.

ii) The dimensions of width and length for every end plate in field of view which matched the criteria listed below was measured. This enabled personal selection to be eliminated.

2.3.5.2 Criteria :

i) Every EP measured must be on a muscle fibre. The outline of the muscle fibre must be clearly visible and in focus at the same time as the end plate.

ii) The outline of the EP must be distinct and defined. This is dependent to a large extent on the staining. If the stain diffuses out of the EP, the outer edges become blurred and the accuracy of the measurements reduced.

iii) The end plate must be flat on the muscle fibre (in the plane of focus). EP which were wrapped around the muscle fibre or

overlapping other EP were not measured.

Although each hemidiaphragm contains numerous EP, the likelihood of an EP being both ideally stained and meeting the criteria was found to be small, in comparison to the size of the total population.

At each dose, EP from more than one animal were counted, in an attempt to eliminate any variation between individual animals.

2.3.5.3 Measurements :

All measurements were made using a graduated scale placed in the 10x eye piece of Zeiss microscope. All specimens were viewed under tungsten light, with a x 100 objective in oil immersion. The microscope was calibrated using a stage micrometer. All results are quoted in μm.

2.3.6 Histochemical detection of superoxide :

The reduction of nitroblue tetrazolium (NBT) to yield blue deposits of formazan has been widely used as a probe of superoxide (O_2^-) generation in chemical and biological systems (Halliwell, 1984; Repine et al, 1979). The reliability of the technique is based on the following assumptions:

(i) NBT can accept electrons from various reductants, including O_2^-.

(ii) Superoxide dismutase (SOD), an enzyme that enhances the dismutation rate of O_2^-, inhibits the O_2^- mediated NBT reduction by a competitive process Beauchamp and Fridovich (1971).

2.3.6.1 Principle :

NBT is an electrophilic dicationic electron acceptor from electron donors. The complete reduction of NBT chloride to diformazan requires four electrons and four protons according to the equation :

$$NBT^{2+}\,2Cl^- + 4e + 4H^+ \longrightarrow \text{Diformazan} + 2\,HCl$$

The reaction proceeds in two steps, yielding the two electron reduced form of NBT (monoformazan) as a stable intermediate as indicated in the equation:

$$NBT^{2+}\,2Cl^- + 2e + 2H^+ \longrightarrow NBT^+\,Cl^{-1} + HCl$$

$$NBT^+Cl^{-1} + 2e + 2H^+ \longrightarrow \text{Diformazan} + HCl$$

In the oxidized form, NBT chloride is a yellow compound soluble in aqueous mixtures. Its reduction to formazan is accompanied by disappearance of the positive charges, resulting in a substantial decrease in solubility.

2.3.6.2 Procedure :

Fresh hemidiaphragms were incubated in NBT (0.5 mg/ml) made up in physiological saline containing 50 µg/ml catalase for 15 minutes in presence or absence of 50 µg/ml SOD in two baths of 40 ml capacity and maintained at $37^0C \pm 1^oC$.

After staining all preparations were briefly rinsed in distilled water and pinned to dental wax via the costal margin of the ribs and the central tendon, then immediately fixed in either of the fixers and subsequently stained for cholinesterase.

2.4 Biochemical methods :

2.4.1 Biochemical estimation of cholinesterase activity :

The activity of ChE in homogenates of skeletal muscle was estimated by the method of Ellman et al (1961) with necessary minor modifications. In comparison to other methods, the biochemical method of Ellman offers simplicity, sensitivity, convenience and has been widely used which makes this method suitable for the routine determination of ChE activity. A further advantage of this method is its suitability for samples with small amounts of enzyme. The main disadvantage is that the method lacks specificity where both AChE and ChE are concerned. Furthermore, the mean value obtained represents the activity of heterogenous population of structures and this does not convey very much topographical information on enzyme distribution of either of these enzymes, the same applies to just about any recognised method.

2.4.1.1 Principle :

The enzyme activity is measured by following the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accomplished by the continuous reaction of the thiol with 5,5-dithiobis-2-nitrobenzoate ion to produce the yellow anion 5-thio-2-nitro benzoic acid.

Acetylthiocholine ----------> Thiocholine + Acetate

Thiocholine + DTNB ----------> Yellow colour

The rate of colour production is measured by rate of change of absorbance at 412 nm and the reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentrations used does not inhibit the enzymic hydrolysis.

2.4.1.2. Preparation of samples :

2.4.1.2.1. Diaphragm:

Hemidiaphragms were pinned to Sylgard in a petri dish. Excess blood was removed by gently rubbing along the length of the whole muscle fibre squeezing the blood out of the vessels. Excess connective and adipose tissue were removed and the phrenic nerve was cut close to the muscle.

The central strip of the entire hemidiaphragm containing nerve terminal branches was cut approximately 3mm width and weighing approximately 25mg and put into glass vials containing 2mls of ice cold 0.1M phosphate buffer pH 8.0. The muscles were blotted dry, weighed and homogenised with a Potter-Elvehjem homogeniser for 1 minute using a glass receptacle and Teflon pestle. The homogeniser and pestle was washed with 2 ml of buffer and added to the homogenate. To the 4ml combined homogenate, 1ml of $2.5x10^{-4}M$ ethopropazine hydrochloride was added and the whole sonicated for 30 sec in a MSE soniprep 150 at medium frequency and at position 5. The clear homogenate was finally centrifuged at 3000rpm for 15 min in a Beckman T J bench centrifuge at 4°C. The supernatent was removed for assay of ChE activity.

During homogenization and sonication the samples were kept on ice.

2.4.1.2.2.Blood:

Mice were anesthetized with 2% halothane in an equal mixture of N_2O and O_2. The femoral artery of either limb was carefully exposed and severed. 100µl of blood was quickly transferred to 15.9 ml of ice cold 0.1M phosphate buffer pH8.0 with the aid of a Gilson pipette. The process is rapid and therefore problems with clotting are not encountered and anticoagulants are not needed. To the 16ml of diluted sample, 4 ml of $2.5x10^{-4}M$ ethopropazine were added, and kept on ice

until ready for assay.

2.4.1.3. Instrumentation :

The rate of hydrolysis of acetylthiocholine by blood or homogenate of skeletal muscles was determined using a Pye Unicam SP8000 UV recording spectrophotometer equipped with the following accessories:

(i) Unicam SP830 automatic cell changer.

(ii) Unicam SP874 constant temperature cell housing.

(iii) Unicam SP8005 programme controller, enabling the absorbance of a sample at fixed wavelength (412nm) to be recorded continuously for periods of 5 seconds and measurements repeated at preselected time intervals of 1 minute.

2.4.1.4. General procedure :

The selective inhibitors of AChE (E.C. 3.1.1.7.) and ChE (E.C.3.1.1.8) were preincubated with samples for at least 30 min before enzymes activities were determined.

AChE activity was determined in presence of ethopropazine a selective inhibitor of ChE (Bayliss and Todrick, 1956) using acetylthiocholine iodide ($3x10^{-3}M$) as substrate.

ChE activity was determined in presence of BW 284C51 ($3x10^{-5}M$) a selective inhibitor of AChE (Austin and Berry, 1953) using butrylthiocholine iodide ($3x10^{-2}M$) as substrate. Assays of all samples were in duplicate.

In this method there are two blanks to be subtracted from the assay of the sample. At pH 8.0, there is an appreciable non-enzymic hydrolysis of the substrates. This is measured by the substrate blank for each substrate used. The second blank, referred to as the test blank, represents the release of endogenous thiols at 412nm from the cells and the absorbance of other materials in the sample. The test blank is determined simultaneously with each sample.

2.4.1.4.1. Assay of substrate blank :

Four plastic disposable cuvettes of 3.0ml capacity and 10mm light path were preheated and maintained at 30°C. To two of these, 3mls of distilled water was added. To the remainder, 1ml of phosphate buffer pH 8.0, 1ml of DTNB and 1ml of substrate were added, contents were mixed using a plastic rod and transferred into the sample compartment. The water blanks were transferred into the reference compartment of the spectrophotometer. The change in absorbance was recorded for at least 5 minutes.

2.4.1.4.2. Assay of test and sample blank :

To each of four cuvettes, 1ml of the sample and 1ml of DTNB solution were added. Into two of the cuvettes (tissue blank) 1ml of distilled water was added and into each of the two remaining cuvettes (test samples) 1ml of substrate was added. After mixing the contents the tissue blanks were transferred into the reference compartment and the test samples was transferred into the sample compartment.

In all cases, the sequence in which the reagents were added was enzyme, DTNB and substrate: the reaction being always started off by the addition of the substrate and the rate of change in absorbance was monitored for at least 5 minutes.

2.4.1.5. Calculations :

Beers law (or Beer-Lambert law) relates the amount of absorption to the nature of absorbing species. In solution the number of molecules of absorber in the light path is proportional to the product of the concentration C and the light path length L. The absorption law is written in terms of absorbance, A, as in :

$$A = \frac{\log Io}{I_T} = ECL$$

where,

A = the absorbance.
Io = the intensity of the incident light.
I_T = the intensity of the transmitted light.
E = the extinction coefficient.
C = the concentration of the solution.
L = the pathlength of the cell.

Calculation of the enzyme unit for hemidiaphragm in each sample:
The molar extinction coefficient for the DTNB ion is 1.36×10^4 litres/mole/cm, since from Beer-Lambert,

$$E = \frac{A}{CL}$$

therefore because C is measured in moles/litre and L in centimetres then the units for E are given as follows,

$$E = \frac{A}{\frac{\text{moles} \times \text{cm}}{\text{litre}}} = \frac{A \times \text{litres}}{\text{moles} \times \text{cm}} = A \text{ litres/mole/cm}$$

For the purpose of these experiments E is required in terms of millilitres rather than litres, therefore:-

$$E = 1.36 \times 10^4 \times 10^3 = 1.36 \times 10^7 \text{ ml/mole/cm.}$$

It is also likely that the amount of enzyme activity will be in the order of nanomoles rather than moles, thus:-

$$E = 1.36 \times 10^7 \times 10^{-9} = 1.36 \times 10^{-2} \text{ ml/n mole/cm.}$$

Since the extinction coefficient is known the rate of hydrolysis can be converted to absolute units in the following equation:-

$$\text{Rate of hydrolysis} = \frac{\Delta \text{ absorbance (A)/min}}{1.36 \times 10\text{-}2} \text{ n mol/min/ml}$$

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A}{\text{min} \times \cancel{\text{cm}}} \times \frac{\text{n mol} \times \cancel{\text{cm}}}{\text{ml}} = \Delta A \text{ n mol/min/ml}$$

If the concentration of the tissue sample is X mg/ml, the cuvette volume Vml and the volume of the tissue sample in the cuvette is Yml s then the following applies:-

$$\text{Rate of hydrolysis} = \frac{\Delta A \times V}{1.36 \times 10^{-2} \times X \times Y} \text{ n mol/min/mg}$$

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A \times \text{n mol}}{\text{min} \times \cancel{\text{ml}}} \times \frac{\cancel{\text{ml}}}{\frac{\text{mg} \times \cancel{\text{ml}}}{\cancel{\text{ml}}}} = \Delta A \text{ n mol/min/mg}$$

Hence for these studies, where V=3ml and Y=1ml the equation can be abbreviated to:-

$$\text{Rate of hydrolysis} = \frac{\Delta A \times 220.59}{X} \text{ n mol/min/mg}$$

Calculation of the enzyme unit for whole blood in each sample:

For this case 5µl of sample was used in each assay. This is a 1 in 200 dilution. Thus in this case Y=1/200 and V=3ml, therefore:-

$$\text{Rate of hydrolysis} = \frac{\Delta A \times 3 \times 200}{1.36 \times 10^{-2}} = \Delta A \times 44118 \text{ n mol/min/ml}$$

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A \times \text{n mol} \times \cancel{\text{ml}}}{\text{min} \times \cancel{\text{ml}} \quad \text{ml}} = \Delta A \text{ n mol/ min/ml}$$

This, however, tends to give rather large figures for the rate, hence a conversion to µmolar units from nmolar gives numbers which are easier to handle. Thus the equation reads:-

$$\text{Rate of hydrolysis} = \Delta A \times 44.118 \ \mu \text{ mol/min/ml}$$

2.4.1.6. Expressions of results:

The AChE and ChE activities in blood and tissues was calculated and routinely expressed as µmole of acetylthiocholine hydrolysed min^{-1} ml^{-1} and nmol of acetylthiocholine hydrolysed min^{-1} mg^{-1} respectively. These were routinely abbreviated to µmol min^{-1} ml^{-1} and nmol min^{-1} mg^{-1}.

2.4.2 Biochemical determination of creatine kinase activity :

Creatine kinase activity of serum was assayed using assay kits purchased from Sigma. Initially the 46-UV and later the 47-10 UV kits was used. Both kits were identical but were packaged differently and were based on the optimized method recommended by the Scandinavian Society for clinical chemistry and clinical physiology (1979). The optimized method was based on the modification by Rosalki (1967) of the original technique devised by Oliver (1955).

The method has the advantage of convenience over most of the

older methods. The reactions are followed continuously in the spectrophotometer and the rates catalysed by small amounts of tissue can be measured with ease. With this procedure it is possible to measure enzymic activities in tissue homogenates and extracts diluted 2,000 to 20,000 times, when effects due to endogenous substances are negligible.

2.4.2.1 Principle:

Creatine kinase (C.K., C.P.K., ATP:Creatine N-Phosphotransferase, E.C.2.7.3.2) catalyses the reversible phosphorylation of ADP by phosphocreatine to form ATP and free creatine. The auxiliary enzyme hexokinase (H.K.) catalyses glucose phosphorylation by ATP to produce ADP and glucose -6-phosphate (G-6-P).

ADP + Creatine phosphate ----------> Creatine + ATP

ATP + Glucose ----------> ADP +G-6-P

The glucose-6-phosphate is oxidized to 6-phosphogluconate with the concomitant production of NADPH. The rate of NADPH formation, measured at 340 nm, is directly proportional to serum CK activity.

G-6-P +$NADP^+$ ----------> 6-Phosphogluconate + NADPH + H^+

2.4.2.2 Preparation of sample:

Blood samples was obtained in exactly the same manner as described in section 2.4.1.2.2 except that 1 ml of blood was usually transferred into Eppendorf tubes. Within 2 hours after collection, the blood was centrifuged in a bench centrifuge to remove blood cells, tissues and debris. The sera were transferred to new clean Eppendorf tubes and stored in the refrigerator at 4°C until ready for assay which was normally performed on the day of collection and without fail within 24 hours. Since red cells are practically devoid of CK, slight haemolysis does not affect serum CK (Rosalki, 1967). The CK activity in serum was reported by Sigma to be stable for up to 14 days refrigerated or frozen.

2.4.2.3 Instrumentation:

The manual procedure as described in both the assay kits was used to determine the rate of NADPH formation. The rate of NADPH formation, measured at 340nm and at 30°C was determined using the Beckman ACTA V uv-visible , digital reading and recording spectrophotometer. The spectrophotometer was equipped with

thermoregulated rectangular cell holder with 3 cuvette compartments, any of which can be brought into the path of the sample beam manually. The thermoregulated cell holder is kept at 30°C. The change in absorbance with time was recorded on the chart recorder using a chart speed of 1.5 inches 5 minutes^{-1}. At the end of each assay, the temperature of each reaction mixture was checked with a mercury thermometer.

2.4.2.4 Manual procedure for 46-UV assay kit:

To reagent A, 6.5 mls of reagent B was added and gently mixed until the contents of reagent A had dissolved. 3 mls of this reaction mixture was transferred into a glass cuvette and maintained at 30°C. Two additional cuvettes with distilled water also prewarmed, one of which was placed in the reference compartment and the other in any one of the 3 thermoregulated cuvette compartment of Beckman.

At the beginning of each assay the spectrophotometer was zeroed using the two distilled water blanks. The cuvette containing the prewarmed reaction mixture was then placed in either of the two thermoregulated cuvette compartment and manually placed in the path of the sample beam. A steady reading of the absorbance of this solution against distilled water was obtained before 50 µl of serum sample was added. This was then immediatly mixed with a plastic rod and the rate of NADPH was recorded.

In all assays, using this kit, glass cuvettes of 10mm light path and 3.0 ml capacity was used.

2.4.2.5 Manual procedure for 47-10 UV assay kit:

The procedure was identical to above but with the following exception: reaction mixture was made using 10 ml of distilled water to the reagent and 1.0ml of reaction mixture together with 20 µl of sample was used. All assays was carried out using glass cuvettes of 1.0 ml capacity and 10 mm light path.

2.4.2.6 Calculation:

For 46-UV assay kit:

The following equation was used to determine the CK activity in U/L

$$CK(U/L) = \frac{A/5min \times V \times 1000 \times TCF}{6.22 \times v \times 5}$$

Where V = Total reaction volume (3.05ml)
1000 = Conversion of activity ml^{-1} to activity L^{-1}
TCF = Temperature correction factor where TCF at 30°C is 1
v = Volume of sample
5 = Conversion of absorbance /5min to absorbance/min
6.22 = Absorption Coefficient of CK

This may be abbreviated to :

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

This factor is rounded off from 1961

For 47-10 UV assay kit:
The following equation was used to determine the CK activity in U/L

$$CK\ (Ul\text{-}1) = \frac{A/min \times TV \times 1000}{6.22 \times LP \times SV}$$

Where A/min = Change in absorbance per minute at 340 nm
TV = Total volume (ml)
SV = Sample volume (ml)
6.22 = Millimolar absorptivity of NADH at 340 nm
LP = Light Path
1000 = Conversion of units per ml to units per litre

Which may be abbreviated to:

$$CK(UL^{-1}) = \frac{A/min \times 1.02 \times 1000}{6.22 \times 0.02}$$

$$= A/min \times 8200$$

2.4.2.7 Expression of results:

The results were routinely expressed in international unit per litre (Ul^{-1}), where one international unit (U) of an enzyme will transform 1 µmol of substrate per minute.

2.4.3. Biochemical determination of calcium in tissues :

Many techniques are available for the determination of calcium. Compared with other techniques, atomic absorption spectrophotometry

(AAS) offers sensitivity, simplicity and convenience and which is relatively free from interferences. Also, the instrumental procedures are simple and rapid which makes the AAS the analytical tool of choice for the routine determination of calcium in biological tissues. However the major disadvantage is that the method measures total calcium and provides no information on the content of ionised calcium or on its intracellular distribution.

2.4.3.1 Principle:

AAS is an analytical method for the determination of elements based upon the absorption of radiation by free atoms. Interactions of atoms with various forms of energy results in three very closely related spectroscopic phenomena which may be used for analytical purposes - emission, absorption and fluorescence.

This absorption of radiant energy and its quantitative correlation with the concentrations of metal ions originally present in a sample solution serves as the basis of analytical AAS.

The production of atoms from a chemical compound requires the absorption of energy. This energy is usually supplied in the form of heat from a flame.

2.4.3.2. Destruction of organic matter:

The destruction of organic matter that work satisfactorily when applied to substances of one type become troublesome when used for materials having quite a different composition. Many investigators are convinced that the ideal method for ashing biological material has yet to be devised. Despite the many variations, almost all the methods for the destruction of organic matter fall into one of the two main classes.

2.4.3.2.1 Dry Ashing:

Usually accomplished by heating the sample to a relatively high temperature, usually between 400°C and 700°C, with atmospheric oxygen serving as the oxidant. Chemical compounds may be sometimes added to aid the process.

2.4.3.2.2 Wet Digestion:

In wet digestion the temperature is much lower, liquid conditions are maintained throughout and the oxidation is carried out by various oxidizing agents in solutions, usually oxidizing acids.

In this study, destruction of organic matter in skeletal muscles was carried out by concentrated hydrochloric acid (Aristar,BDH). From preliminary experiments, this agent was found to be satisfactory and

furthermore the level of calcium in the acid as an impurity was much lower than for eg. concentrated nitric acid. The level of calcium in HCl is 0.05 ppm as compared to 0.5 ppm in HNO_3, furthermore the absorbance signal of calcium is depressed greater by nitric acid than hydrochloric acid in the presence of lanthanum (Monder and Sells, 1967).

2.4.3.3. Preparation of diaphragm:

Hemidiaphragms for determination of calcium were incubated in calcium free physiological saline for 15 minutes at room temperature, while gassed, in order to remove extracellular calcium. The preparations was then pinned onto pieces of sylgard via costal margin of the ribs and central tendon. The pins were carefully placed, such that fibres was not damaged but was aligned straight with some stretching. The pinned preparations were then immediately placed in acetone and after several changes of acetone, left for at least couple of hours in acetone.

Whilst viewing the preparation in acetone in a petri dish with a dissecting microscope, the ribs, tendon and the cuts in the original dissection was removed. Excess fat and connective tissue and the phrenic nerve was also removed. Under appropriate illumination the intramuscular branch of the phrenic nerve could be seen traversing the axis of the muscle fibre. Muscles associated with the main intramuscular branch of the phrenic nerve and extending about 1-2 mm on either side represents the junctional region. By incising as close as possible either side of this area the junctional region of each hemidiaphragm was removed as a strip and the two sections above and below the junctional strip was removed as representative of non-junctional regions, in a manner described by Hebb et al (1964).

The junctional and non-junctional muscle segments were placed separately in conical bottomed polystyrene tubes. The acetone dried samples were separately weighed, the weight of the junctional and the non-junctional segments of a single hemidiaphragm were usually between 2-5 mg. To each tubes, 0.1mls of 11.3 M hydrochloric acid was added and left overnight for digestion. The tissue digest was dried on a laboratory hot plate at 50°C-60°C in a fume cupboard for couple of hours and the residue then redissolved in 0.8 ml of 0.1 M hydrochloric acid containing 0.1% lanthanum chloride. This solution also served as an acid blank.

2.4.3.4 Instrumentation:

The samples were routinely assayed on a Perkin-Elmer model 560 atomic absorption spectrophotometer equipped with a standard narrow-slot nebulizer burner with air-acetylene flame. Air pressure, air flow,gas flow and height above the burner slot were adjusted for maximum

absorption as recommended in the Perkin-Elmer manual on analytical methods. Rate of sample intake to the nebulizer was 6-7 ml min^{-1}. Distilled water was sprayed through the flame. The machine was standardised using 2 point standardising procedure employing 4ppm calcium as the upper limit and 0.2 ppm as the lower limit. Samples were assayed against acid blank and this was sprayed between every sample and machine zeroed each time to correct for base line drift.

2.4.3.5. Calculation :

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 contents of the tissues ($nmolmg^{-1}$) using the expression below :

$$\text{Calcium content (nmol mg}^{-1}) = \frac{25 \times V \times R}{W}$$

Where 25 = nmol ml^{-1} equivalent of 1ppm $CaCl_2$

V = Total Volume of sample (0.8ml)

R = Calcium concentration reading in ppm of the sample

W = Weight of the dry tissue.

The above expression was used to separately determine the junctional and non-junctional calcium content.

Calculation of Calcium accumulation at junctional region of the hemidiaphragm:

The junctional pieces will contain NJ parts of muscle cells as well as the EP. To make allowance for NJ uptake at the junctional region and to determine the EP specific calcium uptake the following assumptions are made:

i)That in all animals of same sex, strain, age and approximately same weight, the no. of cells in diaphragm, their diameter and the thickness of diaphragm are the same.

ii) Accumulation of calcium occurs evenly along the length of the muscle cells.

iii) The presence of the end plate does not selectively influence the accumulation of calcium in the NJ tissues of both regions.

The amount of Ca taken up at the end plate was calculated as the difference between the Ca content of the junctional portion and the Ca content of the same weight of NJ portion.

$$Ca^{2+} \text{ accumulation at junctional region (nmol mg}^{-1}) = \frac{J_W (J_C - NJ_C)}{W}$$

Where W = combined dry weight of J and NJ
J_C = J Calciùm content
NJ_C = NJ Calcium content
Jw = Junctional Weight

2.4.3.6 Expressions :

Results were expressed regularily as the accumulation of calcium at the Junctional region ie nmol mg^{-1} dry weight .

2.4.4 Biochemical determination of high energy phosphate compounds:

High pressure liquid chromatography (H.P.L.C), the newest of the chromatographic techniques is capable of precise, accurate, rapid separation of complex mixtures. H.P.L.C. is therefore suitable for the accurate quantitative analysis of high energy phosphate compound in skeletal muscles (Lush et al, 1979; Hearse, 1984).

2.4.4.1 Principle :

Chromatography of any kind depends on the differing interactions between each component in a mixture and the elements of its environment. In H.P.L.C., a sample dissolved in a solvent is passed down a column packed with solid particals. Under the correct conditions interactions between the sample and the solid phase cause some components to travel down the column slower than others, ie different volumes of solvent are required to elute the compounds.

2.4.4.2. Mobile phase :

The following mobile phases was used in the separation of high energy phosphate compounds from skeletal muscles. These were found to be optimal as regards to reasonable resolution, separation and retention times.

Mobile Phase A: For the separation of ATP, ADP, AMP and IMP.

0.2 M	NaH_2PO_4
0.025 M	Tetrabutylammonium hydroxide
10%	Methanol

Adjusted with 6 M NaOH to pH 5.8

Mobile phase B: For the separation of creatine and creatine phosphate.

0.067 M	NaH_2PO_4
0.008 M	Tetrabutylammonium hydroxide

Adjusted with concentrated phosphoric acid to pH 3.0.

The individual components of the mobile phase were taken from those illustrated by LKB in their booklet on 'Chromatographic Techniques'

2.4.4.3.Preparation of samples :

It is well known that nucleotides must be rapidly extracted from tissues to prevent changes due to ischaemia which lower the ATP/ADP ratio from about 5 to 15 down to 1 or below. To avoid this rapid freezing in liquid nitrogen followed by acid extraction are usually employed.

To achieve maximum recovery, procedures were designed such that all stages of the extraction procedures ie, powdering, extraction, centrifugation and neutralization was performed in one vessel.

Muscles frozen in liquid nitrogen were quickly weighed in a hole drilled in a block of expanded polystyrene which both prevented frost formation and acted as an insulator. The frozen muscle was transferred into a precooled test tube and manually ground to a powder using a loose-fitting, precooled pestle. To the ground powder 0.5 ml of 5% (w/v) trichloroacetic acid containing 0.65% (w/v) Na H_2P0_4 and 10% (v/v) methanol was added and quickly homogenised using Potter-Elvehjem homogeniser. Following homogenisation, homogenate was allowed to extract for 30 minutes at ice cold temperature. After 30 minutes, 2.5 mls of a mobile phase was added followed by centrifugation at 3,000 rpm for 10 minutes at 4°C using a Beckman bench centrifuge, model TJ. The clear supernatent was neutralized to pH 5.8 with approximatly 30 µl of 6M NaOH and finally filtered through Millipore GSWP (0.22 µm). Samples were either immediately assayed or stored at -20°C in suitable

aliqouts.

All samples whether fresh or thawed were further diluted 1:2 with the appropriate mobile phase prior to assay.

All dilutions were essential as this ensured that all samples were similar in composition to the mobile phase and that interference due to the build up of TCA anion was avoided. The inclusion of methanol in the extraction media prevented nucleotide interconversion (Lush et al 1979).

2.4.4.4 Instrumentation:

H.P.L.C. analyses were under taken using a system constructed from a Gilson piston pump model 302 coupled with Gilson 802 manometric module which is connected to the Rheodyne injection valve and a reversed-phase stainless steel shandon-type column (10cm X 4.6 mm ID) packed with hypersil-ODS (5µm) stationary phase. Cecil CE212 variable wavelength ultraviolet absorption monitor employing a continuous deturium source was used as the mode of detection.

In all cases, 100µl volume of injection was used for the analysis of both groups of high energy phosphates with a flow rate of 1 ml /min with a run time of approximately 15 min. The absorbance of phosphocreatine and creatine was monitored at 210nm and of ATP, ADP, AMP and IMP at 254nm.

2.4.4.5 Peak identification:

Eluent peaks were positively identified using the following methods:

i) Comparison of retention data of unknown to that of standard solutions; The retention data are characteristics of the sample mobile phase if all other operating conditions remain the same.

ii) Use of internal and external standards; There are several ways in which internal standards may be used. In one, the internal standard is added to the solution before the chromatography process. This method will compensate for errors made in the preparation of the solution. On the other hand, the internal standard may be injected separately, immediately before or after the injection of the sample. In this study, external standards were employed. In this process, known quantity of standard compounds were injected separately, immediately before or after the injection of the sample.

iii) Spiking with external standards; In liquid chromatography it is possible to add to the sample a known quantity of a compound that is thought to be present. Therefore ,this peak will increase in size by a given amount. This procedure has been found to be

practical in characterizing peaks of close retention time such as adenine nucleotide separation. In this study, spiking with a single external standard as well as various combination of standards were used.

The combined use of these methods enabled the positive identification of the various eluent peaks in extracts of skeletal muscle and further indicated that there are no recognisable interferences by unknown peaks.

2.4.4.6 Calculation:

The content of high energy phosphate compounds in skeletal muscle were calculated using the expression below:

$$\text{Compound } (\mu\text{mol } g^{-1}\text{wt}) = \frac{V_e}{1000} \times \frac{H_e \times C_s}{H_s} \times \frac{2}{W} \times 1000$$

where
- V_e = Total volume of extract (ml)
- $\frac{V_e}{1000}$ = Conversion from concentration to mole
- H_e = Peak height of extract (mm)
- H_s = Peak height of standard (mm)
- C_s = Concentration of standard (M)
- W = Weight of frozen tissues (mg)
- 2 = dilution factor (prior to assay)
- 1000 = Conversion to a gm of equivalent tissue

2.4.4.7 Expression :

All results were expressed as μmoles per gram of frozen muscles. This is routinely abbreviated as μ mol g^{-1}.

2.5 Statistical analysis :

The results are routinely presented as mean ± 1 SD, unless otherwise stated. Statistical significance between means of various groups where analysed using a non-parametric test, Mann-Whitney (see apendix). A probability of $P < 0.05$ was considered statistically significant. *, **, ***, denotes significance at $P<0.05$, $P<0.02$, $P<0.002$ respectively.

3.0 DISTRIBUTION OF CHOLINESTERASES IN MOUSE DIAPHRAGM AND EVALUATION OF CHOLINESTERASE ASSAY

3.0 Introduction :

Amongst the varied methods for the determination of ChE activity, the biochemical method of Ellman is the most widely used and tested. Unfortunately the method lacks specificity as regards to ChE. The resultant thiocholine hydrolysis could be attributable to AChE, BuChE or esterases or a combination of these (table 3.1). Furthermore, the use of thioesters of choline as substrates which are hydrolysed faster than ACh (Hobbiger and Lancaster, 1971) and the use of crude preparations containing mixtures of enzymes such as that found in homogenates further adds to the complication.

The objective of experiments described in this section are to evaluate:

1) The extent of acetylthiocholine and butyrylthiocholine hydrolysis attributable to AChE, pseudocholinesterases and non specific esterases.

2) The effect of an antiChE, ecothiopate given in vivo on the extent of acetylthiocholine and butyrylthiocholine hydrolysis by blood and diaphragm.

3) The validity of the technique. In particular its usefulness as an indicator of junctional AChE in the synaptic cleft.

ChE were distinguished from non-specific esterases using eserine. AChE were distinguished from pseudocholinesterase by the use of appropriate combination of 'specific' substrates and 'specific' inhibitors in the correct concentrations derived from experiments by others using purified enzymes.

The name ChE has long been used to cover both AChE and pseudocholinesterases. Hence, in agreement with Silver (1974), I propose to continue the use of ChE(s) to cover these enzymes. The non-specific types will be referred to as pseudocholinesterases or will be named more explicitly according to their substrate preferences eg. Butyrylcholinesterase (BuChE).

CHARACTERISTICS	AChE	PSEUDO-ChE
Nomenclature	Specific ChE 'True ChE' 'E-type' ChE I AChE	Non-Specific ChE 'Pseudo-ChE' 'S-type' ChE II BuChE
E.C.	Acetylcholine hydrolase EC3.1.1.7	Acylcholine acylhydrolase EC3.1.1.8
General distribution	Erythrocytes, Nerve tissues, Muscles	Blood, serum Glands, Pancreas
Optimum pH	7.5-8.0	8.5
Optimum Substrate concentration	3mM	10mM
Inhibition by excess substrate	+	–
Substrate specificity: preferred substrate	ACh	BuCh or PrCh
Activity towards:		
MeCh	+	–
BuCh	–	+
BzCh	–	+
Inhibitors:		
Eserine	Inhibited by 10^{-5}M	Inhibited by 10^{-5} M
Iso-OMPA	Resistant	Susceptible
Ethopropazine	Resistant	Susceptible
BW284C51	Susceptible	Resistant

Table 3.1: Characteristics of the two main acetylcholine hydrolysing enzymes (Augustinsson, 1948; Silver, 1974).

3.1 Experimental Design:

3 hours after atropine injection, tissues were removed as described in section 2.4.1.2. Hemidiaphragm were separated into junctional and non-junctional regions, as described in section 2.4.3.3 and separate homogenate produced. Each region was assayed in the presence and absence of eserine, a 'specific' inhibitor of ChE (Silver , 1974), BW284C51, a 'specific' inhibitor of AChE (Austin and Berry, 1953), ethopropazine, a 'specific' inhibitor of pseudocholinesterase (Bayliss and Todrick, 1956; Klingman et al, 1968).

Assays of both regions were also done after exposure to 500 n mol Kg^{-1} ECO in vivo for 3 hours.

In all cases, activity was determined using both acetylthiocholine and butyrylthiocholine as substrates.

3.2 Calculation of total hemidiaphragm End Plate specific activity (EPSA):

In the junctional region of the diaphragm a tiny portion (< 1%) of each fibre is specialised as an end plate (EP), but in many other respects the junctional and non-junctional regions of each muscle fibre are similar. For this reason, it is assumed that non-junctional ChE mg^{-1} of muscle is the same in the junctional and non-junctional regions of the diaphragm and evaluated the ChE specifically associated with EP (EPSA) by subtracting non-junctional ChE activity mg^{-1} from total ChE activity mg^{-1} in the junctional region.

$$EPSA = \frac{(J_a - NJ_a)\, J_W}{NJ_W + J_W}$$

where:

J_a = Junctional activity.
NJ_a = Non-junctional activity.
J_W = Junctional weight.
NJ_W = Non-junctional weight.

3.2.1 Estimation of EPSA of the junctional strip ($EPSA_J$):

The junctional strip is composed of the end plate component and the non-junctional component. The inhibitory effects on the junctional

strip after various inhibitors could be due to inhibition of either or both components. In order to quantitatively assess the effect of the various inhibitors on these components, it is necessary to evaluate the end plate specific activity of a mg of junctional strip ($EPSA_J$). In this regard, the direct comparison of EPSA calculated previously is inappropriate as this represents the EPSA of hemidiaphragm based on the total weight of both regions which are approximately of the same weight. Hence the previous EPSA values were converted to junctional end plate specific activity ($EPSA_J$) by multiplying by the total hemidiaphragm weight divided by junctional weight.

3.3 Results:

In the diaphragm of albino mice , almost all the end plates occur in the middle of the muscle fibres in close proximity to the main nerve branches, so, that the muscle can be divided by dissection into junctional and non-junctional regions. Furthermore, the kinetic properties of the ChE in the synapses was shown not to be altered drastically by homogenisation (Miledi et al, 1984).

3.3.1 To determine the extent of acetylthiocholine and butyrylthiocholine hydrolysis attributable to ChE:

In the absence of any inhibitors (see figure 3.3), the activity of the junctional strip is composed of the combined activity of the end plate specific activity of the junction plus the activity of the non-junctional strip. Alternatively, the activity of the non-junctional strip is the result of the absence of the $EPSA_J$ from the junctional activity.

Susceptibility to inhibition by eserine is one of the distinctive characteristics of ChE and has been used to distinguish ChE from those eserine resistant esterases. For most vertebrate species a concentration of 1×10^{-4} M is more than adequate to inhibit all activity in tissues in vitro (Silvers, 1974).

Eserine (5×10^{-5} M) reduced acetylthiocholine hydrolysis of homogenates of diaphragm by 2.69 n mol min^{-1} mg^{-1} (98%) in junctional region and by 1.38 n mol min^{-1} mg^{-1} (96%) in non-junctional region while the activity of blood was reduced by 1.41 μ mol min^{-1} ml^{-1} (97%) (see table 3.2). The 2.69 n mol min^{-1} mg^{-1} reduction in the junctional region appear to be due to virtually complete inhibition of both EPSAj and the non-junctional component. The residual activity of both region being the same (see figure 3.3). Thus essentially, in both regions and in both tissues, virtually all of the acetylthiocholine

SUBSTRATE & TISSUE	ACTIVITY				
	INHIBITORS				
	NONE	ESERINE	BW284C51	ETHOP	ECO
AcThCh (NJ)	1.45 ±0.20	0.06 ±0.11	0.52 ±0.09	0.21 ±0.08	0.30 ±0.12
N	16	12	12	17	14
AcThCh (J)	2.73 ±0.33	0.05 ±0.02	0.69 ±0.12	1.46 ±0.32	0.45 ±0.12
N	16	12	12	17	14
AcThCh (Blood)	1.46 ±0.29	0.05 ±0.10	0.62 ±0.08	0.97 ±0.12	0.32 ±0.07
N	13	6	9	19	8
BuThCh (NJ)	1.44 ±0.31	0.14 ±0.04	1.36 ±0.16	0.21 ±0.10	0.48 ±0.10
N	15	12	23	19	16
BuThCh (J)	1.61 ±0.20	0.18 ±0.05	1.52 ±0.13	0.23 ±0.10	0.61 ±0.14
N	15	12	23	19	16
BuThCh (Blood)	1.56 ±0.21	0.44 ±0.09	1.43 ±0.21	0.49 ±0.09	0.64 ±0.09
N	18	6	17	19	5

Table 3.2 The effect of various inhibitors on the hydrolysis of acetylthiocholine (ACThCh) and butrylthiocholine (BuThCh) by blood and non-junctional (NJ) and junctional (J) region. Blood and diaphragm activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. Values are mean ± 1SD, where N = no. of hemidiaphragms.

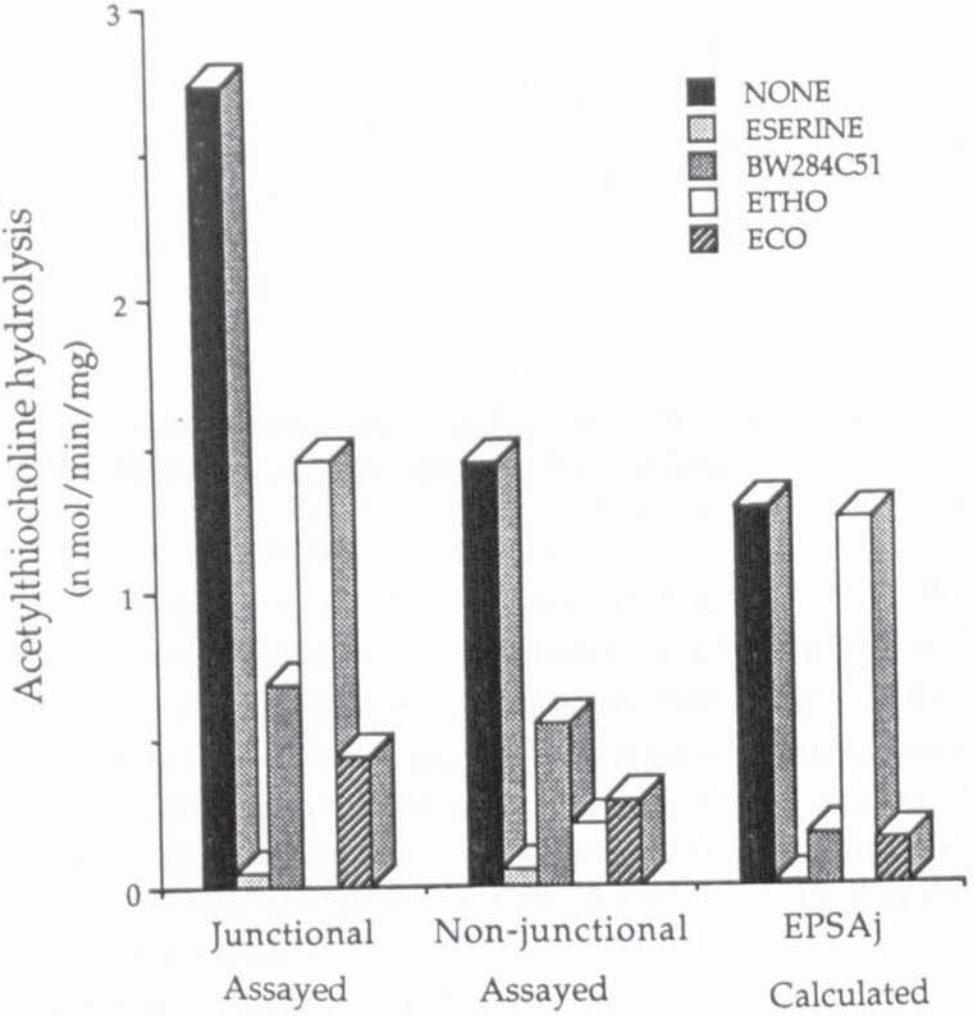


Figure 3.3 The effect of various inhibitors on the calculated end plate specific activity ($EPSA_J$) and on the acetylthiocholine hydrolysis of the junctional and non-junctional region.

hydrolysis was mediated via ChE rather than non-specific esterases eg. aliesterases, arylesterases.

When butyrylthiocholine is used as a substrate, eserine reduced butyrylthiocholine hydrolysis by 1.42 n mol min^{-1} mg^{-1} (88%) in junctional region and by 1.30 n mol min^{-1} mg^{-1} (90%) in non-junctional region while the activity of blood was reduced by 1.12 μ mol min^{-1} ml^{-1} (72%) (see table 3.2). Thus approximately 10-12% of both regions of hemidiaphragm and 28% of blood butyrylthiocholine hydrolysis can be attributed to non-specific esterases resistant to eserine, which in the hemidiaphragm appears to be evenly distributed along the length of the fibre.

3.3.2 To determine the extent of acetylthiocholine and butyrylthiocholine hydrolysis attributable to AChE:

To determine how much of the ChE activity was due to specific AChE the inhibitor BW284C51 was used.

In homogenates, in the presence of 3×10^{-5} M, BW284C51 acetylthiocholine hydrolysis was reduced by 2.04 n mol min^{-1} mg^{-1} (73%) in junctional region and by 0.89 n mol min^{-1} mg^{-1} (62%) in non-junctional region while blood activity was reduced by 0.84 μ mol min^{-1} ml^{-1} (58%) (see table 3.2). Unlike eserine, the 2.04 n mol min^{-1} mg^{-1} reduction in the junctional strip appears to be due primarily to the reduction of $EPSA_J$ in addition to some reduction in the non-junctional component (see figure 3.3).

Although the greater majority of acetylthiocholine hydrolysis is attributable to specific AChE, the residual activity (27-40%) appears to be mediated by esterases other than AChE. It was previously concluded that non-specific esterases were not involved in acetylthiocholine hydrolysis, hence it is suggested that the residual activity is mediated by pseudocholinesterases.

In the presence of BW284C51, butyrylthiocholine hydrolysis was reduced by 0.082 n mol min^{-1} mg^{-1} (5-6%) in both regions whereas only 0.128 μ mol min^{-1} ml^{-1} (8%) of blood butyrylthiocholine is reduced. This suggests that hydrolysis of butyrylthiocholine by AChE in blood and hemidiaphragm is insignificant.

3.3.3 To determine the extent of acetylthiocholine and butyrylthiocholine hydrolysis attributable to pseudocholinesterases:

To determine how much of the ChE activity was due to pseudocholinesterases, the inhibitor ethopropazine was used.

In the presence of 5×10^{-5}M ethopropazine, acetylthiocholine hydrolysis of homogenates of hemidiaphragm was reduced by 1.27 n mol min^{-1} mg^{-1} (47%) in junctional region and by 1.23 n mol min^{-1} mg^{-1} (85%) in non-junctional region while the activity of blood was reduced by 0.49 μ mol min^{-1} ml^{-1} (34%) (see table 3.2). In contrast to eserine and BW284C51, the reduction of 1.27 n mol min^{-1} mg^{-1} in the junctional strip appears to be due solely to the non-junctional component, the $EPSA_J$ appear to be unaffected (see figure 3.3) suggesting that the ChE at EP is AChE.

The results show that substantial acetylthiocholine hydrolysis is mediated by ethopropazine sensitive ChE, possibly in part by pseudocholinesterase. This is in contradiction to the general findings that pseudocholinesterase hydrolyses AChE substrates at a low rate or not at all (Adams , 1949; Augustinson 1948; Augustinson and Nachmanssohn, 1949) and furthermore the optimum substrate concentration is usually much higher. However it is possible that hydrolysis of the synthetic thiocholine esters by pseudocholinesterases becomes significantly faster under the present circumstances (Hobbiger and Lancaster, 1971) or/and the hydrolysis of acetylthiocholine may be dramatically influenced by myosincholinesterases, depending on the prevailing conditions. Kover, Kovacs and Kong (1957) and Kover and Kovacs (1957) have shown that depending on the source of enzyme and experimental conditions myosincholinesterase behaves in certain respects like AChE and at other times like pseudocholinesterase.

An interesting observation noted by Hall (1973) was the lack of substrate inhibition by the 4S molecular form of AChE, a feature common to pseudocholinesterase, yet the 4S was susceptible to inhibition by BW284C51 and actively hydrolysed methylcholine, features characteristics of AChE. It is therefore possible that the intracellular release of 4S during homogenization, which although hydrolysed acetylthiocholine might also have been susceptible to ethopropazine. This could account for the extensive inhibition of acetylthiocholine hydrolysis in the non-junctional and junctional region.

These discrepancies, mentioned above, may further be examples of species differences or they could reflect a difference in the behaviour of purified versus crude enzymes.

When butyrylthiocholine was used as a substrate, ethopropazine reduced junctional butyrylthiocholine hydrolysis by 1.37 n mol min^{-1} mg^{-1} (85%) and in non-junctional by 1.23 n mol min^{-1} mg^{-1} (86%) and blood by 1.07 μ mol min^{-1} ml^{-1} (68%) (see table 3.2). These results are essentially similar in magnitude to those obtained with eserine. The results suggest that approximately 85-88% of the butyrylthiocholine hydrolysis of the diaphragm regions is attributable to pseudocholinesterase (BuChE) the remainder being attributable to non-specific esterase. Thus essentially all BuChE activity whether intracellular or extracellular is inhibited by ethopropazine.

3.3.4 To determine the effect of ecothiopate on acetylthiocholine and butyrylthiocholine hydrolysis:

Exposure to 500 n mol Kg^{-1} ECO in vivo for 3 hours resulted in acetylthiocholine hydrolysis reduction by 2.28 n mol min^{-1} mg^{-1} (84%) in junctional region and by 1.15 n mol min^{-1} mg^{-1} reduction in the non-junctional region (88%) (see table 3.2). The 2.28 n mol min^{-1} mg^{-1} reduction in the junctional strip appear to be due to combined reduction of $EPSA_J$ and the non-junctional component. The results are similar to eserine, though less pronounced (see figure 3.3).

Butyrylthiocholine hydrolysis was reduced by 1.00 n mol min^{-1} mg^{-1} (63%) for junctional and by 0.961 n mol min^{-1} mg^{-1} (67%) for non-junctional region. Blood acetylthiocholine and butyrylthiocholine hydrolysis was reduced by 1.14 μ mol min^{-1} ml^{-1} (78%) and 0.92 μ mol min^{-1} ml^{-1} (60%) respectively.

The reductions in acetylthiocholine hydrolysis are greater than those obtained with BW284C51 presumably because of inhibition of pseudocholinesterase (BuChE) by ECO. The results suggest that ECO is non-specific as regards to inhibition of AChE and BuChE. ECO appears to be as effective as BW284C51 for AChE but less effective than ethopropazine for BuChE.

3.3.5 To determine the effect of various inhibitors on the end plate specific activity (EPSA):

Although AChE and BuChE can be measured using a combination of selective substrate and inhibitors, the extent of EPSA of hemidiaphragm cannot be measured. EPSA was therefore estimated using the formula in section 3.2.

Table 3.4 shows the EPSA calculated after various inhibitors. In the absence of any inhibitor, using acetylthiocholine as substrate, this value is 0.59 n mol min^{-1} mg^{-1}, this represents 28.4% of

	End Plate Specific Activity (EPSA)				
	INHIBITORS				
SUBSTRATE	NONE	ESERINE	BW284C51	ETHOP	ECO
AcThCh	0.59	0.01	0.09	0.58	0.07
	±0.14	±0.01	±0.08	±0.14	±0.06
N	16	12	12	17	14
BuThCh	0.08	0.02	0.08	0.01	0.06
	±0.12	±0.02	±0.09	±0.04	±0.05
N	15	12	23	19	16

Table 3.4 The effect of various inhibitors on the calculated end plate specific activity (EPSA). End plate specific activity is in nmol $min^{-1}mg^{-1}$. Values are mean ± 1SD, where N = no. of hemidiaphragms.

hemidiaphragm acetylthiocholine hydrolysis mediated by eserine sensitive ChE. Whereas in the presence of ethopropazine, the value is 0.58 and represents 70% of hemidiaphragm acetylthiocholine hydrolysis, mediated by AChE as EPSA.

After treatment with eserine, BW284C51 and ECO, EPSA is reduced by 99%, 86% and 89% respectively. Thus these inhibitors virtually inhibit all end plate specific ChE activity. In the presence of ethopropazine, EPSA is reduced by only 2%. Thus the results taken as a whole suggest that the ChE responsible for the EPSA is primarily AChE.

3.3.6 Can AChE and BuChE activity be accurately measured?

The preceding sections have highlighted the fact that no substrate are absolutely specific for either AChE or BuChE particularly when using homogenates as a source of enzymes. In its unmodified form, The biochemical method of Ellman, by solely relying on the substrate specificity can not accurately estimate AChE or BuChE. However, a combination of 'specific' substrate and 'specific' inhibitor in the correct concentrations can allow the accurate estimation of AChE and BuChE.

Therefore AChE can be accurately estimated using acetylthiocholine as substrate in the presence of ethopropazine. Under these circumstances, the EP AChE is unaffected and contribution from BuChE and non-specific esterases eliminated.

Similarly, BuChE can be estimated using butyrylthiocholine in the presence of BW284C51. In this instance, 88-90% of the activity is solely attributable to BuChE, the remainder being attributable to non-specific esterases and contribution from AChE eliminated.

3.4 Discussion:

In agreement with other investigations (Denz, 1953; Augustinsson, 1948; Teravainen, 1967; Hall, 1973), these experiments show in mouse diaphragm as in other vertebrate muscles, the existence of several types of enzymes capable of hydrolysing choline esters. AChE, BuChE and non-specific esterases are not only found in the junctional region, but are distributed throughout the muscle fibres. Neither the function nor the subcellular localization of ChE in non-junctional regions of muscle is known.

In the absence of inhibitors, the junctional strip accounted for 65% of the hemidiaphragm ChE activity, similar to the 60% reported by Miledi et al, (1984). However, in the presence of ethopropazine the junctional strip accounted for 87% of hemidiaphragm AChE activity, the

activity in junctional region presumably reflecting the AChE at the motor end plate. Evidence is also provided which suggests that under the conditions used in theis study, PseudoChE in mouse diaphragm homogenates are capable of hydrolysing acetylthiocholine to a significant extent.

Therefore in studies where AChE inhibition is to be correlated with other parameters or compared with other agents, it is imperative that inhibitors for PseudoChE are employed. In their absence, using any substrate, the activity measured will be that of ChE in general. Under these circumstances the AChE inhibition by OP compounds will be underestimated, the magnitude of which will be dependent upon the nature of the OP compound.

The calculation that 28% of the hemidiaphragm ChE and 70% of hemidiaphragm AChE is end plate specific is a relatively crude one which ignores any differences other than the presence of end plates which might exist between junctional and non-junctional regions. The EPSA values of 28%, for total ChE calculated in this study, differ from those of 40 % reported by Hall, (1973) and 56% reported by Younkin et al, (1982). Although there are a number of differences in the methodology, neither of these two studies employed any 'specific' inhibitors for PseudoChE and therefore relied on the specificity of the substrate. Hence, these studies may have falsely overestimated the EPSA. It is also possible that the higher rate of hydrolysis of the thioesters employed in this study, may have underestimated the EPSA.

In interpreting this data it is important to recognise that cellular elements specific to the EP region of the diaphragm include intramuscular phrenic nerve axons, nerve terminals of the phrenic nerve and Schwann cells, as well as end plates. It has long been known from histochemical staining that external AChE is concentrated at NMJ, but the possibility that some of the external EP specific enzyme measured in this study is located outside the immediate end plate region cannot be ruled out. The available evidence suggests that phrenic nerve contains only 5-10% of the AChE in the end plate region (Davy and Younkin, 1978; Salpeter, 1967) so it is reasonable to propose that most of the intracellular component of the EP specific AChE is within muscle fibres.

AChE may be estimated utilising either homogenates or with intact preparations (Mittag et al, 1971). A question of great importance and controversy is which of these methods reflects a good indication of junctional AChE. Some workers have suggested that the data obtained with intact preparations are more relevant to the physiological function of the enzyme at the synapses than data obtained with homogenates

(Mittag et al, 1971; Lund et al, 1977). However, there are several reports showing that intact muscles hydrolyse ACh at a much slower rate than their homogenates (Marnay and Nachmansohn, 1938; Mittag et al, 1971; Lund et al, 1977). Marnay and Nachmansohn (1938) suggested that with assays on intact muscles the relative slowness of the enzymatic hydrolysis of external ACh is due to its slow diffusion into the muscle, and Hobbiger (1976) suggested that assays on intact muscle, the ChE activity in the synapses is greatly underestimated by hindered diffusion of external ACh into the synaptic cleft. On the other hand, in the mammalian muscle the difference in ChE activity between intact and homogenized preparations has been attributed mainly to the liberation of intracellular esterases by the homogenization.

In order to assess the validity of the AChE technique described earlier, the effects of different degree of AChE inhibition after ECO was correlated with the shape of the miniature end plate current. If the reciprocal of the time to half amplitude (THA) recorded extracellularly with a conventional microelectrode is plotted against the % inhibition of AChE activity, a linear relationship is found. Furthermore, this relationship holds true for a number of tertiary and quarternary antiChE compounds (Bamforth et al, 1988). The physiological relevance of a particular AChE technique may be dependent to a large extent on the physiological parameter measured ie. THA (Bamforth et al, 1988), twitch height (Mittag et al, 1971) or extracellular end plate potentials (Ferry and Marshall, 1973). According to Mittag et al (1971), 33% inhibition of external AChE inhibition shows no twitch enhancement while a similar level of AChE inhibition showed 25% increase in THA. Nevertheless, these correlations with THA and AChE inhibition provide further support for the view of Miledi et al, (1984) that AChE activity in homogenates gives a less distorted picture of synaptic AChE activity than that measured in whole muscles.

3.5 Summary:

(1) The existence of several types of enzymes capable of hydrolysing choline esters was identified biochemically in both diaphragm and blood of albino mice.

(2) Both BuChE and non-specific esterase were found to be evenly distributed along the length of the fibres without any evidence of focal concentration.

(3) BuChE was shown to be capable of hydrolysing acetylthiocholine to a significant extent.

(4) Non-specific esterase was shown to be capable of hydrolysing BuThCh but not AcThCh.

(5) In the presence of ethopropazine, only AChE activity is measured and in the presence of BW284C51 and butyrylthiocholine, 88-90% of the activity is due to BuChE and the remainder attributable to non-specific esterase.

(6) AChE was found to be focally concentrated at junctional region of which 85% represents end plate specific activity.

(7) Ecothiopate was found to be non-selective as regards to BuChE being as effective as BW284C51 but less effective than ethopropazine.

(8) AChE measured by this method provides a good indication of synaptic AChE activity.

3.6 Cholinesterase method in the rest of the thesis:

Since the junctional strip contains most of the diaphragm AChE activity and the fact that this region provides a good indication of synaptic AChE activity, AChE and BuChE will be determined subsequently on junctional strips using selective substrates and inhibitors.

4.0 DESCRIPTION OF MYOPATHY BASED ON CHANGES IN BIOCHEMICAL AND MORPHOLOGICAL PARAMETERS

4.0 Introduction:

There is considerable information on the time-dependent changes in morphological and biochemical parameters after a single acute doses of various antiChEs (Townsend, 1988; Gupta and Dettbarn, 1987; Gupta et al 1985,1987a,b; Wecker et al, 1978a,b; Laskowski et al, 1977,1975). Despite this, the mechanism of antiChE-induced myopathy still remains uncertain perhaps because subtle changes at low doses have been overlooked. In this regards, the recent reports of Hudson et al, (1986, 1985), have shown considerable ultrastructural changes in both presynaptic and postsynaptic structures with doses and AChE inhibition which would not cause cell necrosis. Therefore it is considered that a detailed dose-dependent study on the subtle biochemical and morphological changes may provide additional information which may shed new light on the mechanism of antiChE-induced myopathy.

The main purpose of this section is to show:

1) Dose-dependent changes in various biochemical parameters.
2) Dose-dependent changes in various morphological parameters.
3) The relationship between AChE and BuChE inhibition and changes in biochemical and morphological parameters.
4) To establish the dose and factor(s) which govern the threshold for these changes.

4.1 Experimental Procedure:

Mice were divided into 8 groups of 6 or more mice. Each group received only one of the following treatments:

Group 1: A dose of 700 n mol Kg^{-1} Atropine
Group 2: A dose of 25 n mol Kg^{-1} ECO
Group 3: A dose of 50 n mol Kg^{-1} ECO
Group 4: A dose of 100 n mol Kg^{-1} ECO
Group 5: A dose of 200 n mol Kg^{-1} ECO
Group 6: A dose of 300 n mol Kg^{-1} ECO
Group 7: A dose of 400 n mol Kg^{-1} ECO
Group 8: A dose of 500 n mol Kg^{-1} ECO

Therefore 3 hours after the single injection of drug, the mice were anaesthetised. The diaphragm was removed. One hemidiaphragm was

used for procion staining (see 2.3.3) and the other for assay of diaphragm AChE activity (see 2.4.1). Allocation of right and left hemidiaphragms was alternate. Blood from the femoral artery was removed for the determination of CK and ChE activity (see 2.4.1).

To assess significant correlation between some pairs of data, linear regression analysis was performed (see appendix).

4.2 Results and Discussion :

4.2.1 Behavioural changes after ECO:

Mice exposed to doses of ECO of up to 200 n mol Kg^{-1} showed no behaviour different from atropinised controls. A sign of gross antiChE toxicity was evident in mice exposed to 400 or 500 n mol Kg^{-1} ECO with time to fasciculation (mean ±1SD) of 16.9 ± 1.41 (min) and 13.7 ±0.50 (min) respectively.

All animals exposed to these high doses of ECO, initially exhibited increased salivation and lacrimation, simultaneous with rapid fasciculations and tremors of superficial muscle groups. As intoxication progressed ie. between time to fasciculation and 30 mins, majority of animals displayed no additional behavioural responses (usually the survivors). Others (usually those that died before 30 min post injection) exhibited periods of chronic contractions of the fore and hind limb muscles, continued gross muscle fasciculations increasing in intensity, frequency and duration. By 2-3 hours all animals appeared to be sedated and gross muscle fasciculations began to decline and by 3 hours appeared normal. Those that died showed no procion staining of diaphragm but showed severe hypercontraction at end plate with extremely distorted end plates as shown in Plate 4.1. A dose of 500 n mol Kg^{-1} ECO was estimated to be approximately LD_{10}.

Animals exposed to 300 n mol Kg^{-1} ECO, on majority of occasions also exhibited behavioural changes including fasciculations with a mean time to fasciculations of 24.2 ± 3.58 (mins). In all cases the severity and the duration were considerably reduced and by about 2 hours, the animals appeared to be normal. No deaths were recorded at this dose.

Considerable individual variation was observed in the behavioural response of mice during the 30 mins following a single injection.

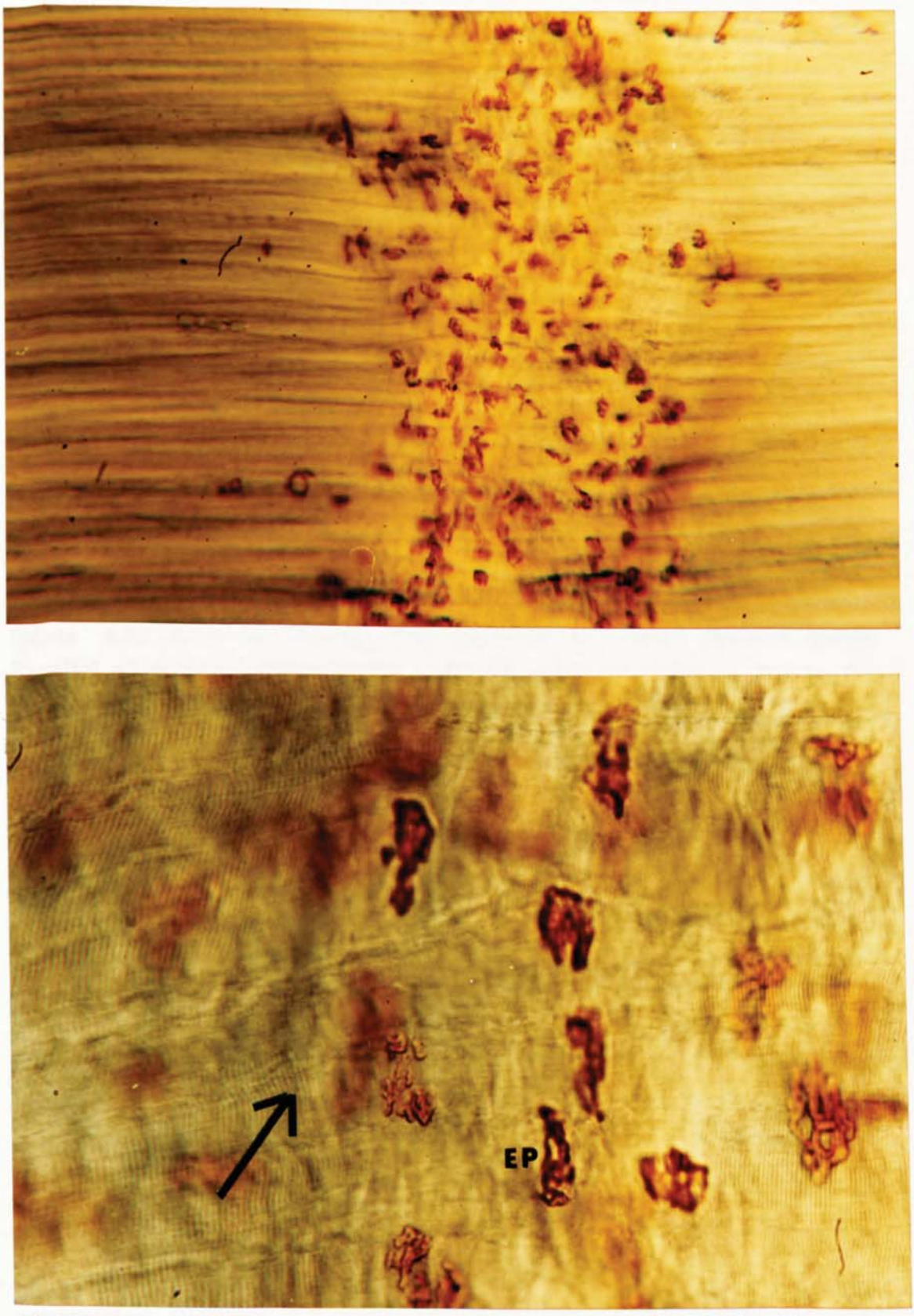


Plate 4.1 : Hemidiaphragm exposed to 500 n mol Kg^{-1} ECO for 20 minutes in vivo. Unsectioned whole preparation. Fixed and then stained for ChE (end plates are represented by patches of brown stain). Tungsten illumination. Calibration 100 µm.

Top: Note the severe hypercontraction of the junctional region.

Bottom: Note the severe distorted end plates (EP) and the distortion of striations due to hypercontractions ( ).

4.2.2 Morphological changes after ECO:

Hypercontraction is a marked, excessive, and presumably unphysiological contraction of myofibrils, reflected by abnormally short sarcomeres as seen in plate 5.3.8. Hypercontractions can be quite focal, involving only a few sarcomeres on one side of muscle fibre, or can extend for hundreds of micrometers, forming a large block of hypercontracted sarcomeres. It can be multifocal within a fibre, causing stretching or even tearing of the muscle fibre.

The term hypercontraction used in this study is used to denote areas of marked contraction, sufficient not to obscure the sarcomere pattern. However, where sarcomere pattern is completely obscured or contracted segments show stretching or/and tearing of myofibrils, the term contraction clump will be used (see plate 5.10).

Diaphragm from atropinised mice under tungsten illumination (plate 4.2) show the characteristic features of normal skeletal fibres: Parallel alignment of fibres with the characteristic striation due to the repeated array of actin and myosin myofilaments. All cells and sarcomeres resemble one another. Approximately half way along each fibre is an end plate (EP), stained brown. Photographs of representative preparations from various doses of ECO are shown in plates 4.3 - 4.9, viewed under tungsten and UV illumination. The native fluorescence of normal fixed muscle is sometimes bright green but usually weak green, indicative of lack of procion penetration. Contraction clumps stained with procion fluorescence bright yellow. Inter-fibre connective tissue and the central tendon also stain with procion yellow.

Exposure to 25 n mol Kg^{-1} ECO resulted in no morphological differences compared to atropinised mice. The earliest dose-dependent abnormality was the presence of distorted EP with 50 n mol Kg^{-1} ECO and subsequently the appearance of hypercontraction with a dose of 100 n mol Kg^{-1} ECO. As the dose was further increased, the severity and the frequency of EP deformations and hypercontraction increased. Significant contraction clumps were evident with 300 n mol Kg^{-1} ECO or greater and increased rapidly with increasing dose. The appearance of contraction clumps, at a given dose was variable, majority were in chains of contraction clumps, some were detached from fibres while others appeared granular.

In all preparations the non-junctional region was relatively unaffected and large proportion of the fibres were unaffected from gross damage.

Plates 4.2-4.9: This series of plates comprises photographs of individual hemidiaphragms from mice given various doses of ECO in vivo for 3 hours. Unsectioned whole preparation. Vital staining with procion yellow, fixed and then stained for ChE (end plates are represented by patches of brown stain). Top: Tungsten illumination. Bottom: Ultraviolet illumination. Calibration 100 μm.

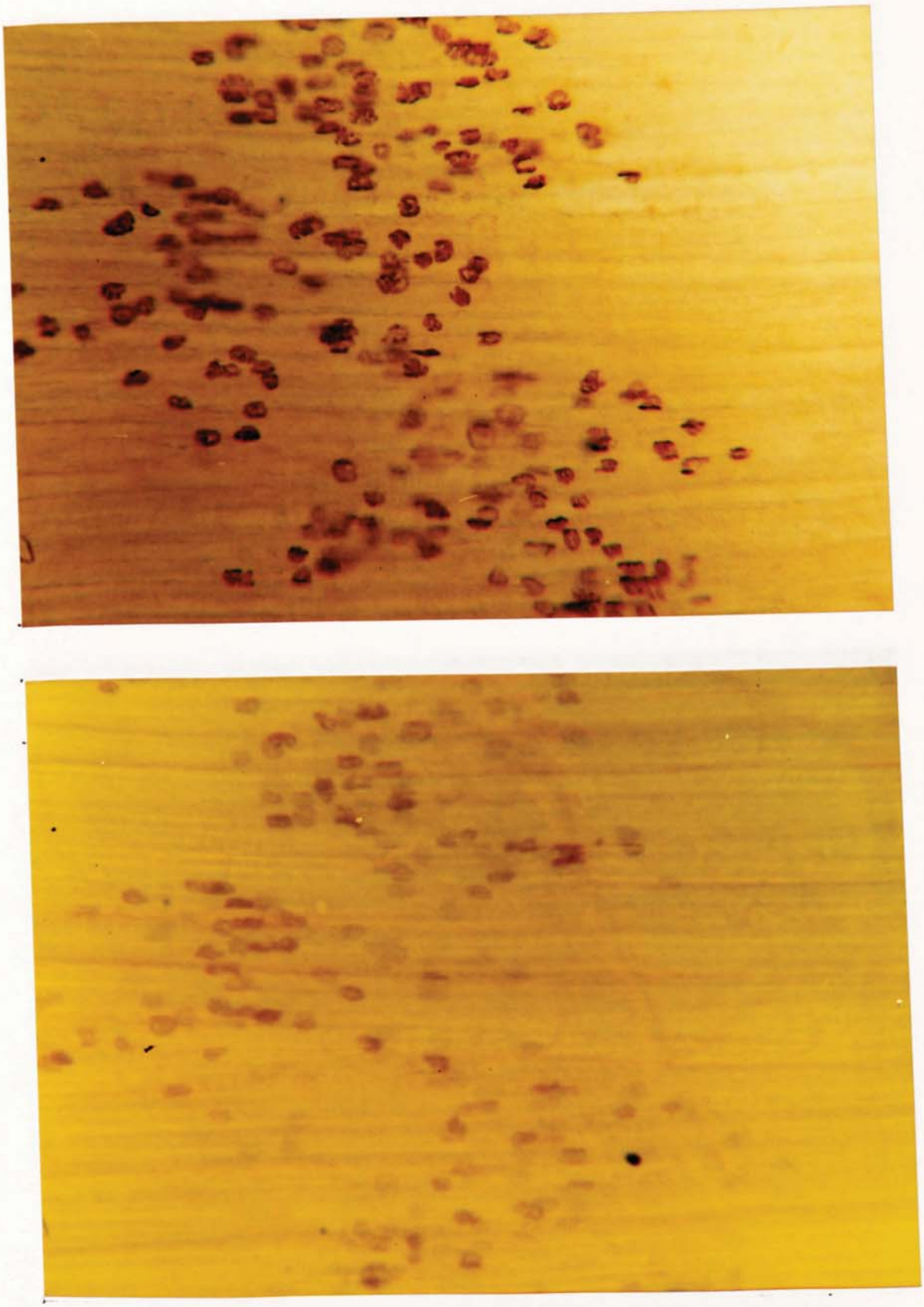


Plate 4.2: Mouse given atropine only. Note muscle fibres run parallel to each other and fluoresce green in UV light. Also procion has stained connective tissue but has not penetrated the fibres themselves.

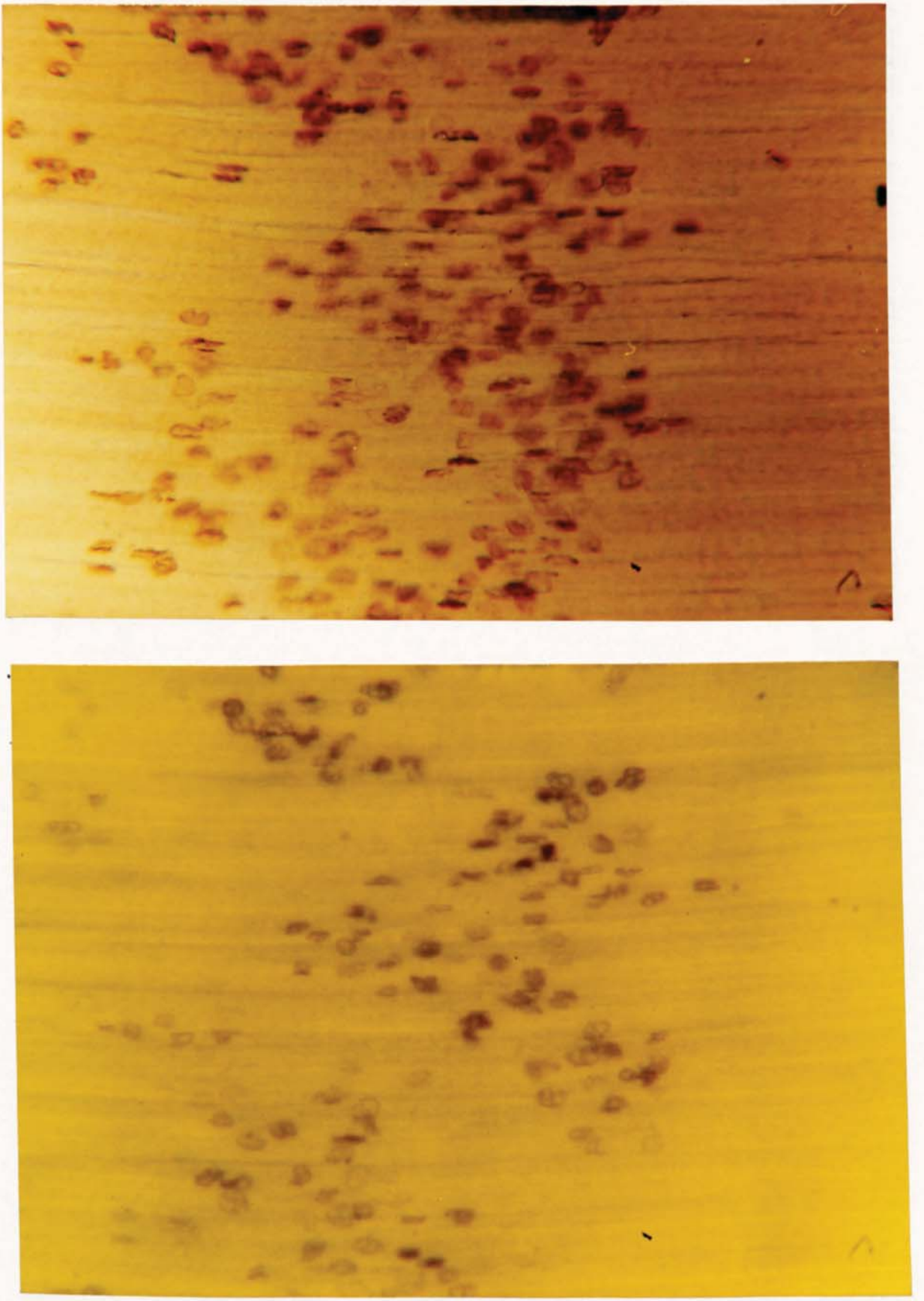


Plate 4.3: Mouse given 25 n mol Kg^{-1} ECO. Hemidiaphragm is similar to that from atropinised mouse shown in plate 4.2.

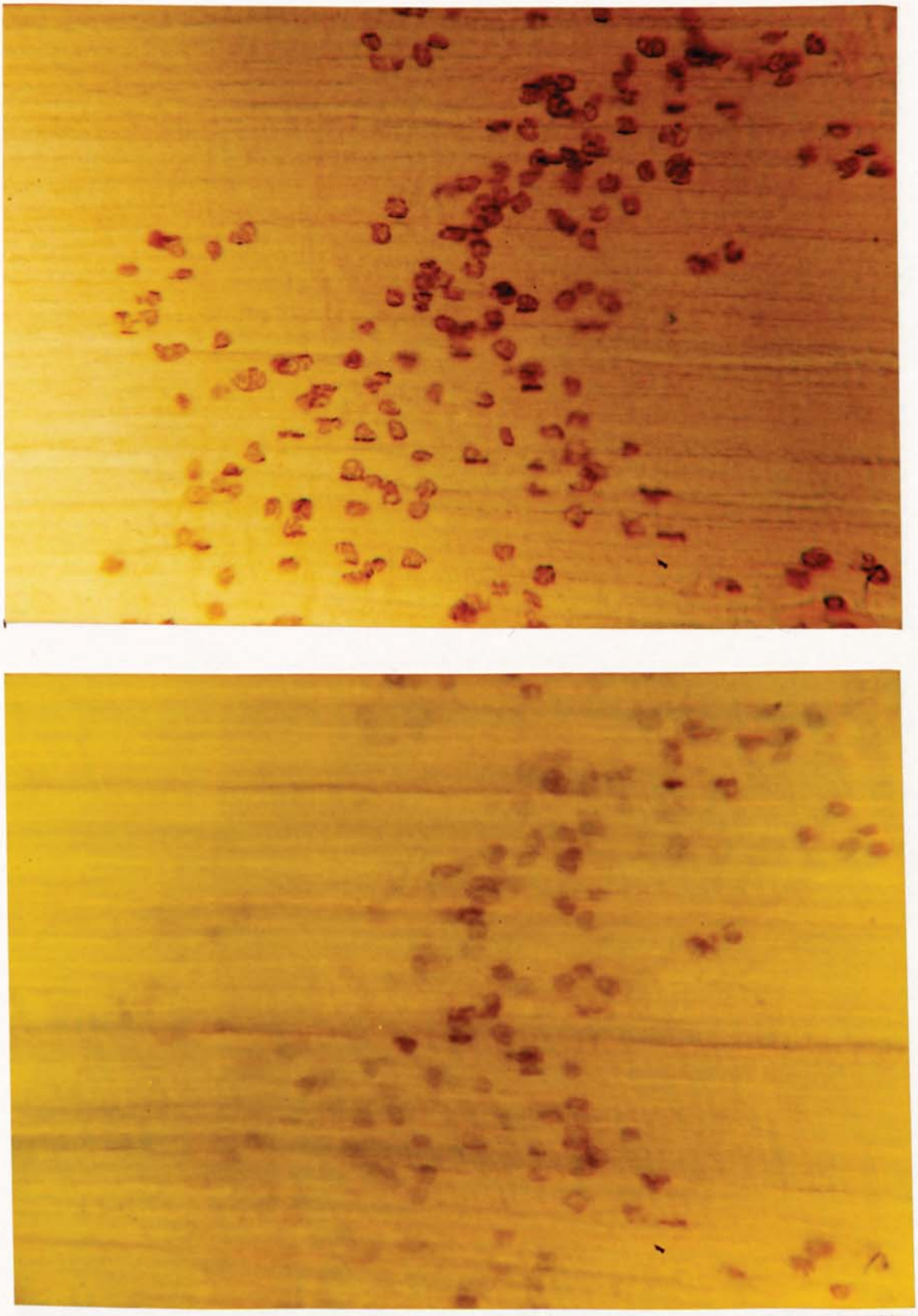


Plate 4.4: Mouse given 50 n mol Kg^{-1} ECO. Hemidiaphragm is similar to that from atropinised mouse shown in plate 4.2.

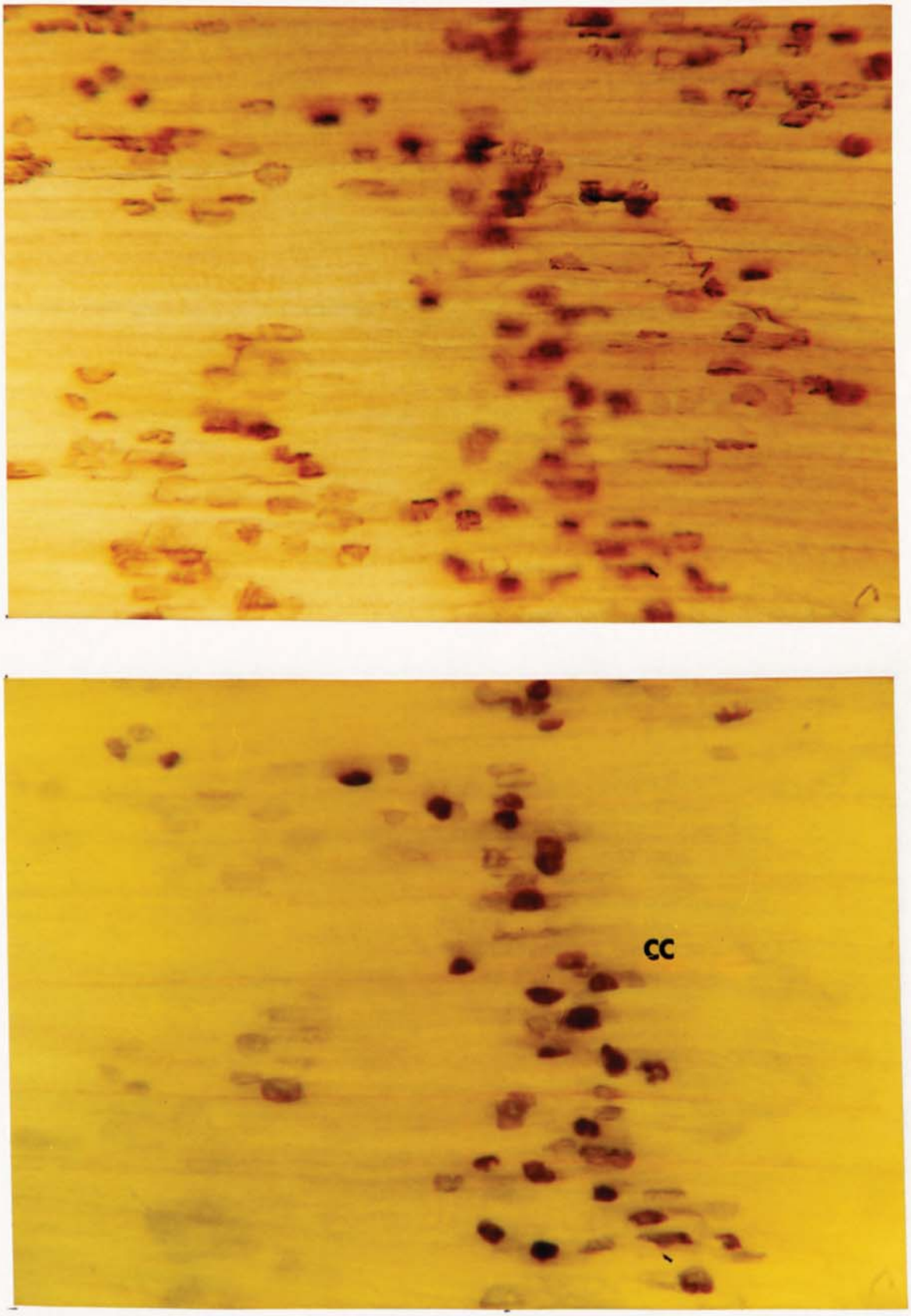


Plate 4.5: Mouse given 100 n mol Kg^{-1} ECO. Hemidiaphragm shows the appearance of contraction clumps (CC) stained with procion and fluorescing yellow in UV light.

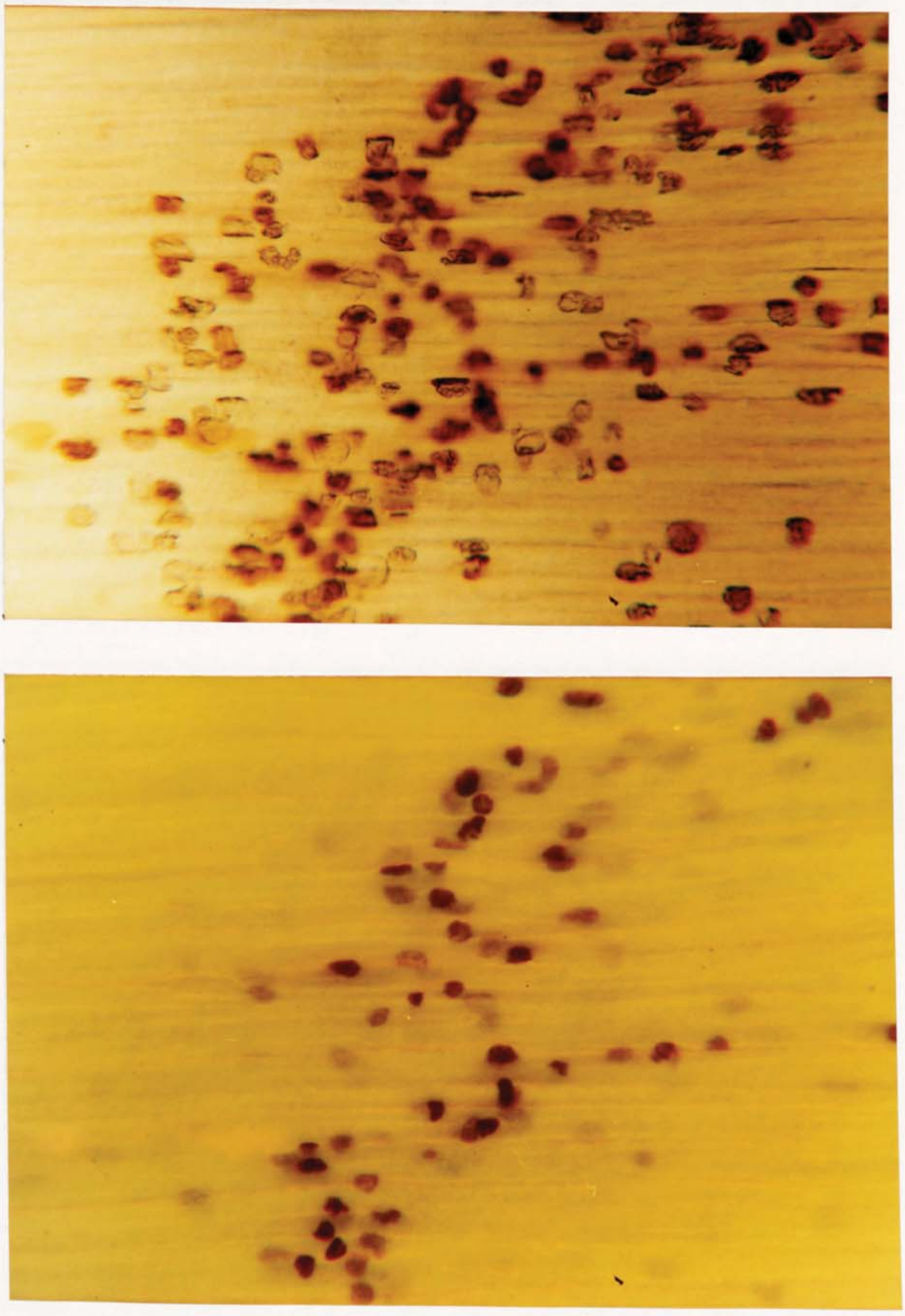


Plate 4.6: Mouse given 200 n mol Kg^{-1} ECO. Hemidiaphragm is similar to that from 100 n mol Kg^{-1} ECO shown in plate 4.5.

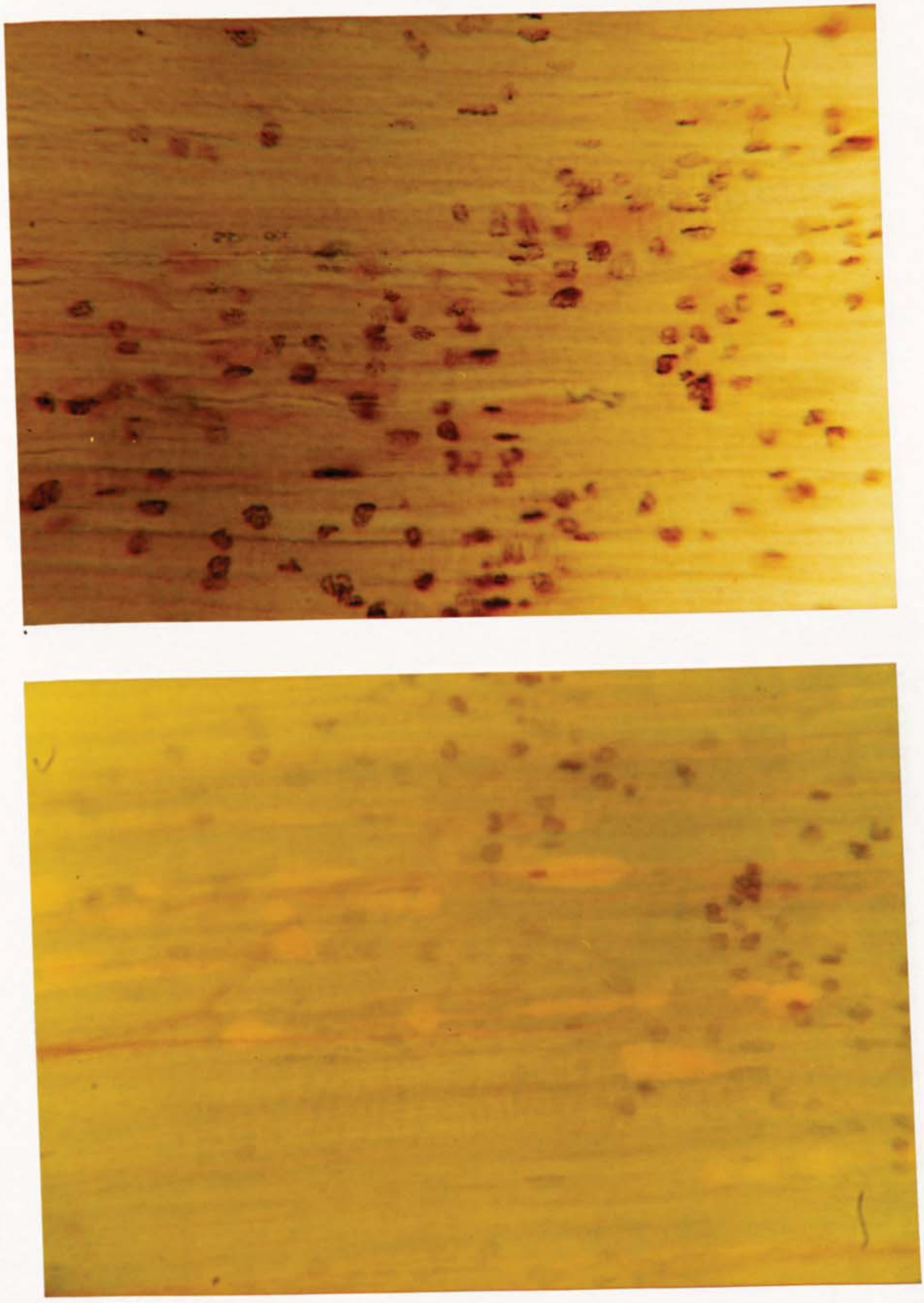


Plate 4.7: Mouse given 300 n mol Kg^{-1} ECO. Hemidiaphragm shows procion yellow staining in the junctional region. Note the is staining more extensive than 200 n mol Kg^{-1} ECO as shown in plate 4.6.

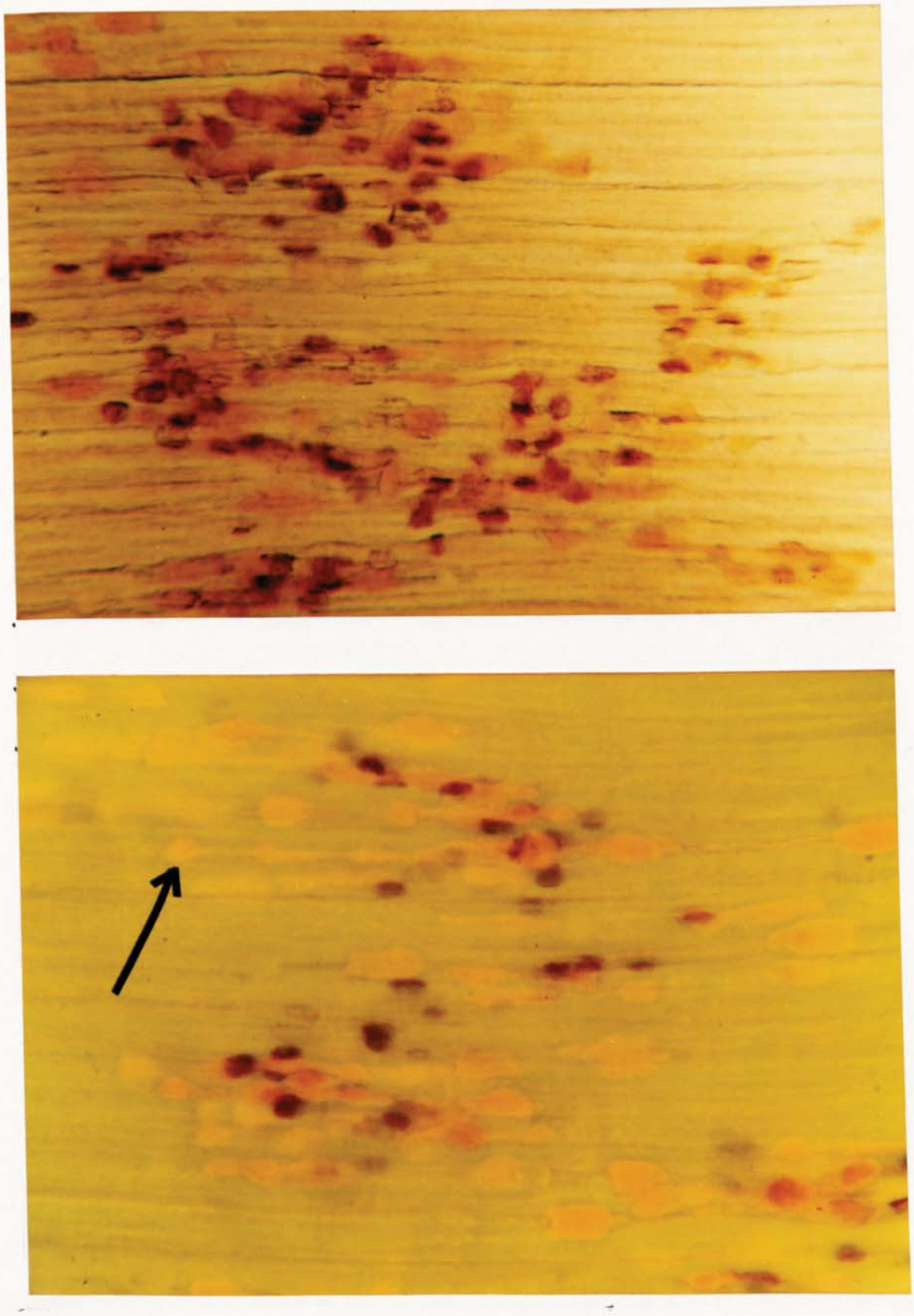


Plate 4.8: Mouse given 400 n mol Kg^{-1} ECO. Hemidiaphragm showing procion staining which is more extensive than 300 n mol Kg^{-1} ECO (Plate 4.7). Note the appearance of chains of contraction clumps (↑).

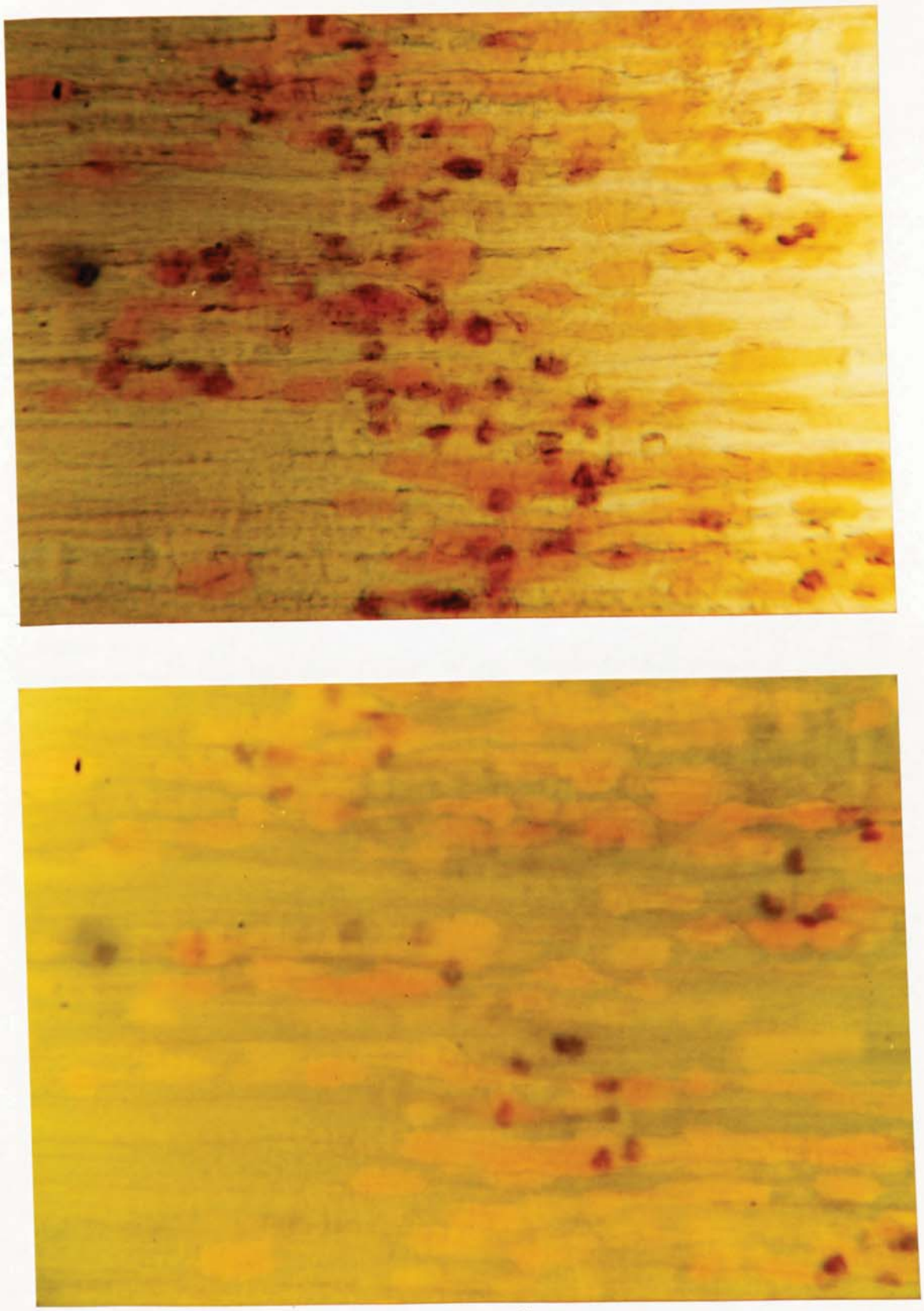


Plate 4.9: Mouse given 500 n mol Kg^{-1} ECO. Hemidiaphragm showing procion staining which is more extensive than 400 n mol Kg^{-1} ECO (Plate 4.8).

4.2.3 Changes in AChE and BuChE after ECO:

The effect of a single variable dose of ECO on AChE and BuChE activity in blood and diaphragm is presented in table 4.10. AChE and BuChE in both tissues was inhibited by a dose-dependent manner with a correlation coefficient for diaphragm of 0.94 (P=0.0061) and 0.97 (P=0.0043) respectively. For blood correlation coefficient of 0.86 (P=0.028) and 0.99 (P=0.0063) for AChE and BuChE respectively.

Figure 4.11 shows that AChE activity of both tissues was inhibited to a similar extent for a particular dose . Significant inhibition of AChE is produced by a dose of 25 n mol Kg^{-1} and a maximum of approximately 88% and 77% in diaphragm and blood respectively was produced with a dose of 300 n mol Kg^{-1} or greater.

Unlike inhibition of AChE, inhibition of BuChE with low doses, appeared to be more pronounced in blood than in diaphragm eg. a dose of 50 n mol Kg^{-1} inhibited blood BuChE activity by 44% whereas only 14% of diaphragm activity was inhibited (table 4.10 and figure 4.12). At these doses, the detoxification of ECO by non-specific binding,the inhibition of BuChE and possibly also carboxylesterase in blood and other tissues, reduces the amount of free ECO available for BuChE inhibition in diaphragm. Hence, maximum inhibition of blood BuChE activity was achieved with 200 n mol Kg^{-1} ECO while maximum inhibition of diaphragm activity is achieved with 400 n mol Kg^{-1}.

The onset of fasciculation appears at a dose of ECO (300nmol Kg^{-1}) associated with a critical level of approximately 88% AChE inhibition. The variation in the behavioural changes of groups of animals that have similar inhibition of AChE may be due to the rate at which AChE is inhibited. The data for time to fasciculation would tend to support this possibility.

In order to investigate this possibility, groups of animals were given doses of 200 n mol Kg^{-1}, 300 n mol Kg^{-1} and 400 n mol Kg^{-1} ECO and 20 mins, 30 mins and 60 mins after ECO administration, blood and diaphragm AChE were determined.

Table 4.13 shows the effect of ECO doses on AChE inhibition after various times. The results clearly show that at all the times tested, AChE is inhibited to the same extent in both tissues. Particularly in the case of with 300 n mol Kg^{-1} and 400 n mol Kg^{-1} ECO, where there is a difference in the behavioural changes, a critical level of AChE inhibition is reached within 20 mins and maintained at this level for at least 3 hours. It is possible that the rate of onset may differ at an earlier time point ie. 10 mins. This is unlikely since the distribution of ECO in vivo is unlikely to change dramatically in 10 mins leading to differential rates of AChE

ECO	AChE Activity				BuChE Activity			
Dose nm/kg	Blood	% Inhib	Diaphragm	% Inhib	Blood	% Inhib	Diaphragm	% Inhib
None	0.96 ±0.18	—	1.42 ±0.25	--	1.43 ±0.21	—	1.52 ±0.13	--
N	42		55		17		23	
25	0.61** ±0.10	36.7	0.88** ±0.09	38.0	--	--	--	--
N	4		5					
50	0.53*** ±0.09	45.1	0.85*** ±0.10	40.5	0.80*** ±0.03	44.1	1.32* ±0.07	13.7
N	15		8		6		4	
100	0.37*** ±0.10	61.3	0.57*** ±0.11###	59.6	0.73*** ±0.07	48.7	1.12** ±0.20##	26.4
N	8		8		6		4	
200	0.33*** ±0.09	65.8	0.33*** ±0.08###	77.0	0.54*** ±0.09	62.4	0.89*** ±0.10###	41.9
N	8		8		6		4	
300	0.31*** ±0.15	67.4	0.16*** ±0.02###	88.6	0.55*** ±0.03	61.5	0.54*** ±0.04#	64.4
N	7		4		6		4	
400	0.27*** ±0.06	71.8	0.18*** ±0.04	87.3	0.54*** ±0.07	62.6	0.42*** ±0.14	72.2
N	9		9		6		4	
500	0.25*** ±0.10	74.0	0.14*** ±0.06	90.3	0.47*** ±0.05	67.0	0.40*** ±0.11	73.6
N	13		18		5		9	

Table 4.10 The effect of various doses of ECO 3hours in vivo on AChE and BuChE activity of blood and diaphragm. AChE and BuChE activity of blood and diaphragm are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. % inhib. = % inhibition. Values are mean ± 1SD, where N = no. of hemidiaphragms. *,**,*** denotes results which are significantly different from those of atropine group at 5%, 2%, 0.2% levels respectively. #, ##, ### denotes results which are significantly different between values immediately above at the 5%, 2%, 0.2% levels respectively.

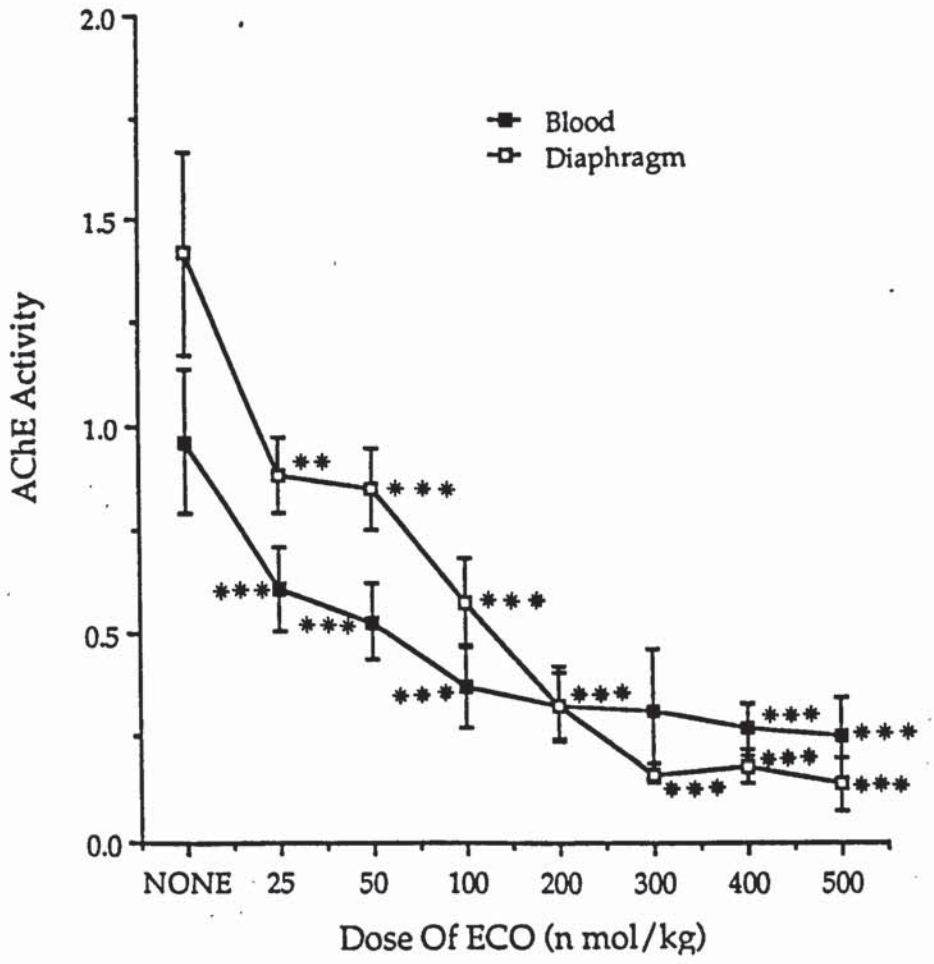


Figure 4.11 The effect of various doses of ECO 3hours in vivo on blood and diaphragm AChE activity. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. Results are mean ± 1SD. **,*** denotes results which are significantly different from those of atropine group at2% and 0.2% levels respectively. (NB. for full statistical evaluation see table 4.10).

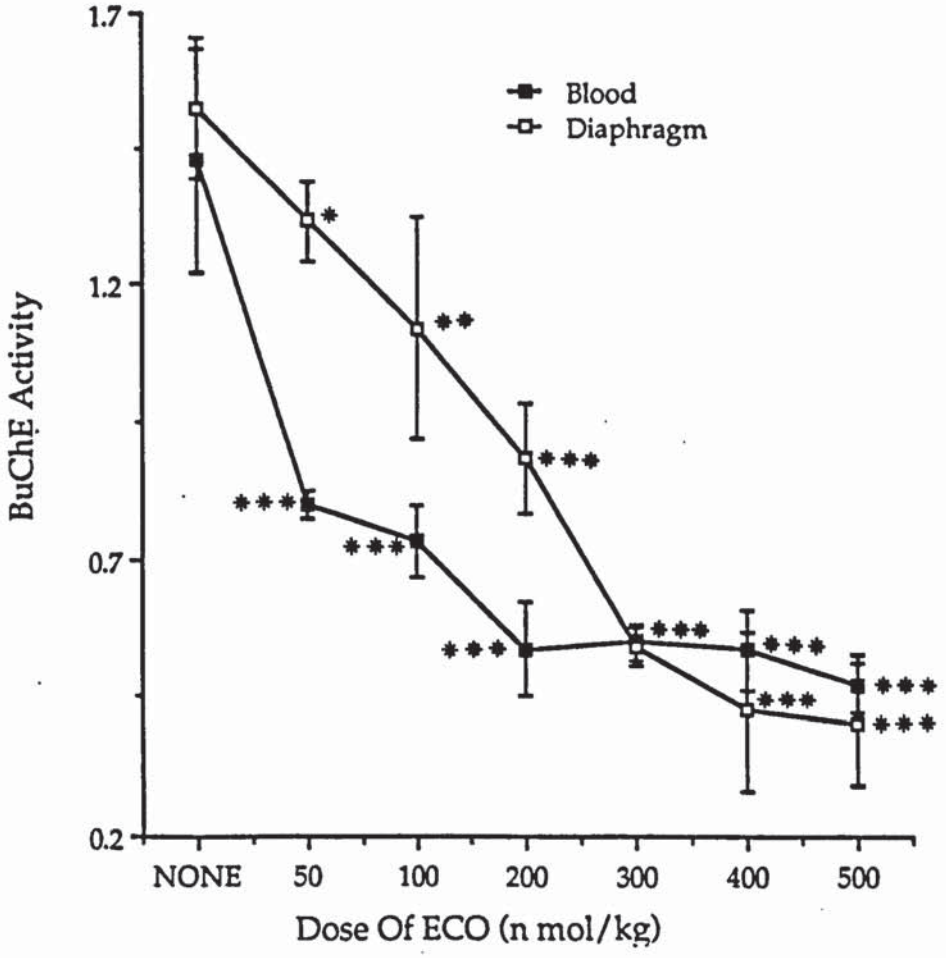


Figure 4.12 The effect of various doses of ECO 3hours in vivo on blood and diaphragm BuChE activity. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. Results are mean ± 1SD. *,**,*** denotes results which are significantly different from those of atropine group at 5%, 2%, 0.2% levels respectively. (NB. for full statistical evaluation see table 4.10).

Time (mins)	DOSES OF ECO (n mol Kg^{-1}) 200 Blood	200 Diaphragm	300 Blood	300 Diaphragm	400 Blood	400 Diaphragm
20	--	--	--	0.21 ±0.05	--	0.20 ±0.04
N				5		6
30	0.25 ±0.04	0.24 ±0.05	0.31** ±0.06	0.19 ±0.04	0.28 ±0.06	0.16 ±0.03
N	4	8	4	4	4	4
60	0.30 ±0.05	0.24 ±0.06	0.28 ±0.09	0.18 ±0.09	0.29 ±0.07	0.14 ±0.05
N	4	4	7	11	8	14
180	0.33 ±0.09	0.33 ±0.08	0.31 ±0.15	0.16 ±0.02	0.27 ±0.06	0.18 ±0.04
N	8	8	7	4	9	9

Table 4.13 The effect of various doses of ECO on AChE activity of blood and Diaphragm at various times after ECO administration. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. Values are mean ± 1SD, where N = no. of hemidiaphragms. **, denotes significant difference from a dose of 200 n mol Kg^{-1} ECO group at the 2% level.

inhibition or reaction proceedes during preparation of assay.

It is possible that the differences in the severity of behavioural changes may be dependent upon the maximal inhibition of AChE coincident with maximal inhibition of BuChE. BuChE may contribute to the removal of ACh released quantally or non-quantally at the synaptic junction. This action may be sufficient to keep many neuronal axons below the threshold for initiation of antidromic activity. Under these circumstances antidromic activity is likely to be more pronounced for higher doses and this may explain the behavioural differences between the doses. Certainly the behavioural observations and enzymic activity are consistent with this idea.

4.2.4 Changes in end plate shape after ECO:

Normal and distorted end plates of varying severity is depicted in plate 4.14. The dimensions, width(w) and length(l), of each end plate (EP) were measured and the individual EP ratio calculated as w/l. The distribution of individual EP ratio for various doses of ECO are presented in table 4.15 and illustrated in histograms in figure 4.16.

For atropinised controls, the end plate ratio is distributed normally (Chi^2 test) about the mean value of 0.61 (figure 4.16). As the dose of ECO is increased, there is a progressive shift in the population to the right and the distribution becoming positively skewed until a new broad distribution is formed about the mean value of approximately 1.0 with a dose of 400 n mol Kg^{-1} ECO or greater.

The data suggests that up to 300 n mol Kg^{-1} ECO, undistorted end plates coexist with distorted end plates of varying severity and as the dose is increased, more and more normal EP become affected until most EP are affected with a dose of 400 n mol Kg^{-1} ECO or greater, giving rise to a new population with different characteristics.

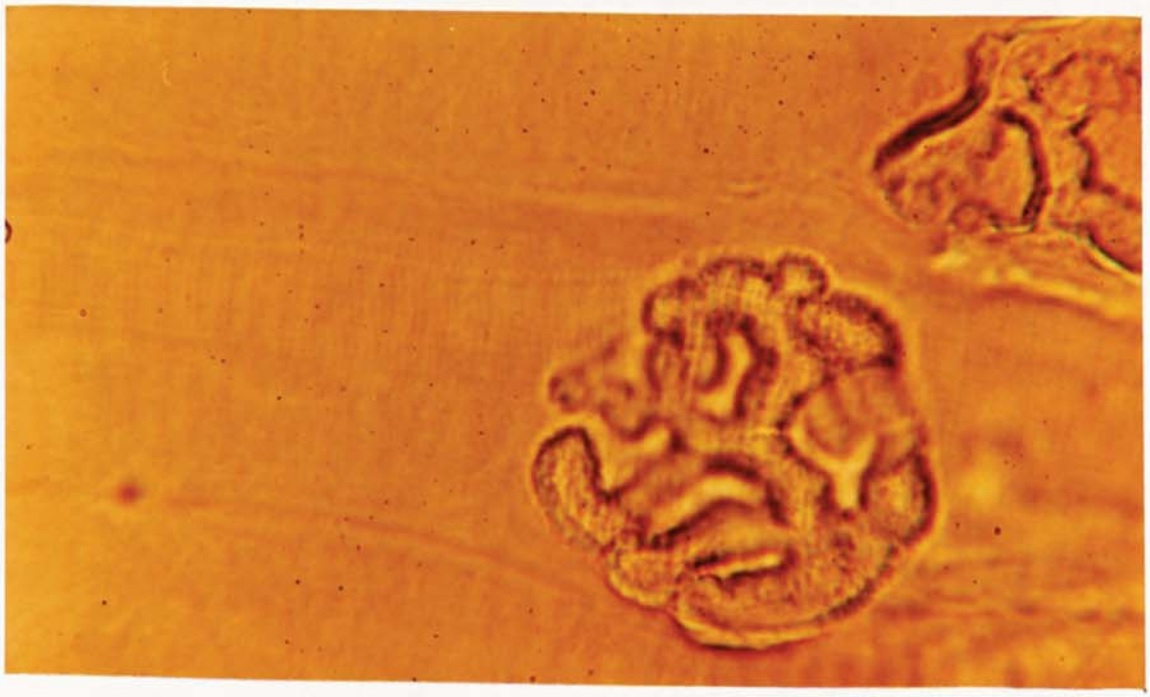
This progressive dose-dependent shift in population can be clearly seen in figure 4.17 with a correlation coefficient of 0.89 ($P=0.008$). Significant changes occur at 50 n mol Kg^{-1} ECO and this may be the threshold dose at which the changes in end plate shape become apparent. The figure also illustrates that by 300 n mol Kg^{-1} ECO, the EP ratio begins to level off. At higher doses than this, there are a significant number of severely distorted EP as previously shown in plate 4.1. The higher values of mean end plate ratio at these doses could be due to the inclusion of some of these end plates. In this technique, the very severely distorted EPs are not recorded, because of the difficulty in meeting the criteria, hence the severity of EP deformations is underestimated at these doses.

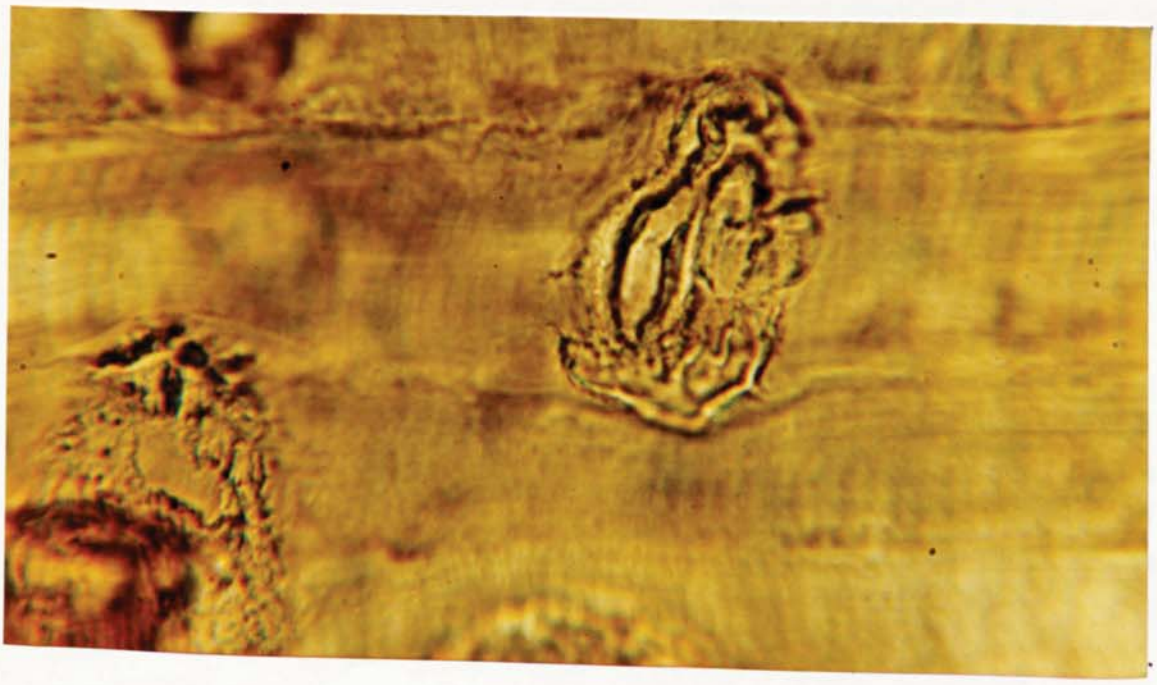
Plate 4.14: Unsectioned whole preparation. Vital staining with procion, fixed and then 'lightly' stained for cholinesterase. Tungsten illumination. Calibration 10μm.

Top: Note normal end plate of hemidiaphragm from mouse given atropine only.

Middle: Note 'rounded' end plate of hemidiaphragm from mouse given 50 n mol Kg^{-1} ECO for 3 hours in vivo.

Bottom: Note 'very distorted' end plate of hemidiaphragm from mouse given 100 n mol Kg^{-1} ECO for 3 hours in vivo.





DOSE OF ECO (n mol kg-1)	END PLATE PARAMETERS WIDTH (μm)	LENGTHS (μm)	RATIO
None	21.3 ± 4.8	35.2 ±6.8	0.61 ±0.1
N	66/2	66/2	66/2
25	21.1 ± 3.0	33.3 ±4.1	0.64 ±0.1
N	36/3	36/3	36/3
50	32.5*** ±9.7###	41.5*** ±10.4###	0.80*** ±0.2###
N	70/2	70/2	70/2
100	26.6*** ±5.3###	31.1*** ±5.7###	0.88*** ± 0.2##
N	70/2	72/2	72/2
300	25.4*** ±4.5	28.5*** ±4.5##	0.91*** ±0.2
N	95/5	95/5	95/5
400	31.1*** ±4.8###	35.5*** ±5.2###	0.98*** ±0.2##
N	62/4	62/4	62/4
500	27.4*** ±4.4###	27.8*** ±5.4###	1.01*** ±0.2
N	57/4	57/4	57/4

Table 4.15 The effect of various doses of ECO, 3 hours in vivo on various parameters of end plate. Values represent mean ± 1SD, where N = no. of observations/no. of hemidiaphragms. *** denotes significant difference from atropine group at the 0.2%. ##, ### denotes significant difference between values immediately above at 2% and 0.2% respectively.

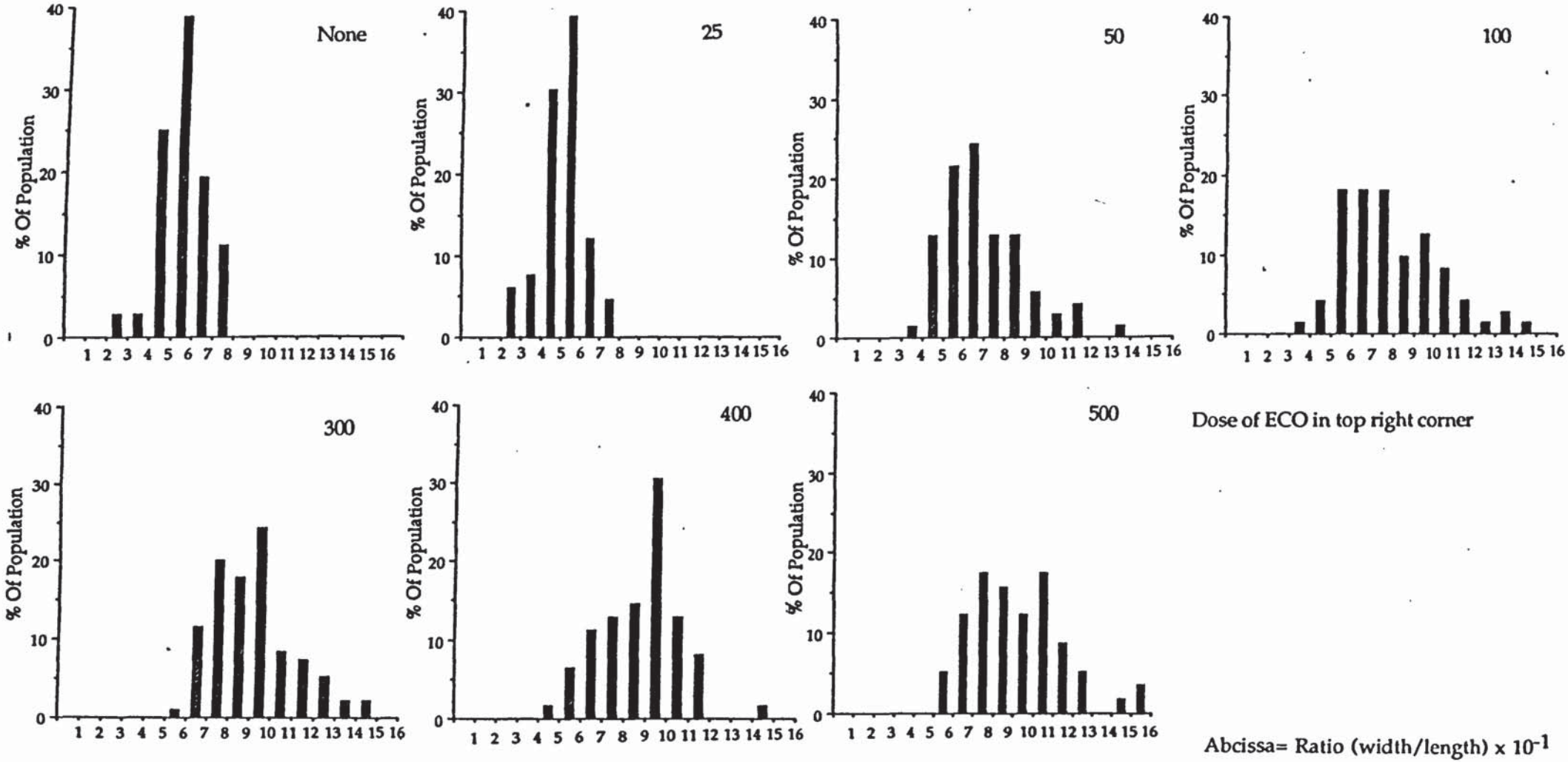


Figure 4.16 The effect of various doses of ECO 3 hours in vivo on the distribution of end plate ratio (width/length). (NB. for full statistical evaluation see table 4.15).

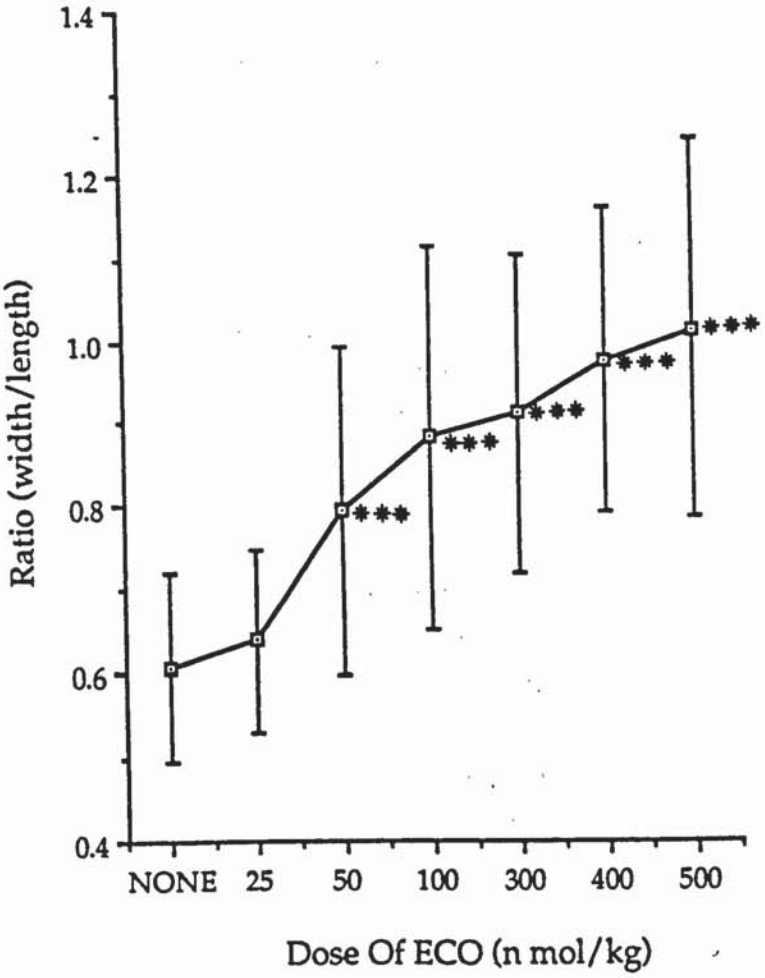


Figure 4.17 The effect of various doses of ECO 3 hours in vivo on the end plate ratio (width/length) (NB. for full statistical evaluation see table 4.15).

The factors involved in end plate distortion are uncertain. Since T-tubular and SR swelling are an early feature of OP-induced myopathy (Townsend, 1988; Laskowski et al, 1977,1975) it is possible that end plate distortion may involve changes in volume either as a result of proteolysis and.or early ionic shifts. Alternatively, if sarcomeric contraction is isovolumetric under these circumstances, persistent hypercontractions, may be responsible for change in EP shape.

4.2.5 Changes in procion staining after ECO:

The % procion yellow staining at the junctional region, 3 hours after single variable doses of ECO is shown in table 4.18. The extent of % procion yellow staining at junctional region is increased with AChE inhibition as well as behavioural changes. Thus, greater the AChE inhibition and greater the severity of fasciculations the greater the % procion yellow staining. Hence, a dose of 300 n mol Kg^{-1} ECO or greater, resulted in significant % procion yellow staining (table 4.18 and figure 4.19). The % procion yellow staining at non-junctional region follows a similar profile to that of the junctional region, however the degree of staining was considered to be insignificant, usually less than 1% and never extensive (figure 4.19).

With low doses of ECO, it is possible that the initiation of damage was delayed until after 3 hours. The results in table 4.20 show the extent of % procion yellow staining and AChE activity at 3 hours and 24 hours after 100 n mol Kg^{-1} ECO. The results show that the extent of procion yellow staining at 24 hours is smaller than at 3 hours and not significantly different from controls at both time points studied. AChE activity recovered in both tissues, the rate of recovery being faster in blood.

In view of these results, it is unlikely that myopathy appeared between 3 and 24 hours because (i) myopathy is always associated with behavioural changes, which did not appear to be the case with 100 n mol Kg^{-1} ECO, (ii) myopathy appears when AChE is inhibited in a very narrow critical range which is significantly different from the inhibition produced with 100 n mol Kg^{-1} ECO, (iii) the critical stage for myopathy appears to be the first few hours after AChE inhibition, at a later time the development of myopathy is unlikely due to adaptation and recovery.

It has been reported that the extent of the muscle fibre necrosis was dependent upon a critical level and duration of AChE inhibition (Wecker et al, 1978a,b). The results in table 4.18 shows significant variation in the extent of % procion yellow staining in groups of

DOSE OF ECO (n mol Kg^{-1})	% AChE INHIBITION (Diaphragm)	% PROCION YELLOW STAINING (Junctional)	(Non-Junctional)
None		0.16	0.28
	-----	±0.20	±0.29
N		9	9
25	38	0.14	0.21
		±0.15	±0.27
N		7	7
50	40.5	0.47	0.78
		±0.95	±1.40
N		11	11
100	59.6	0.90	0.33
		±1.61	±0.34
N		7	7
200	77.0	0.95	0.31
		±1.26	±0.49
N		9	9
300	88.6	3.51^{***}	0.20
		$\pm 2.03^{\#\#}$	±0.17
N		6	6
400	87.3	14.53^{***}	0.88
		$\pm 5.95^{\#\#\#}$	±1.22
N		9	9
500	90.3	18.22^{***}	2.02
		±8.17	±2.74
N		23	23

Table 4.18 The effect of various doses of ECO 3 hours in vivo on the extent of procion yellow staining. Values represent mean ± 1SD, where N = no. of hemidiaphragms. **, *** denotes significant difference from those of atropine group at the 2% and 0.2% respectively. ##, ### denotes significant difference between values immediately above at 2% and 0.2% respectively.

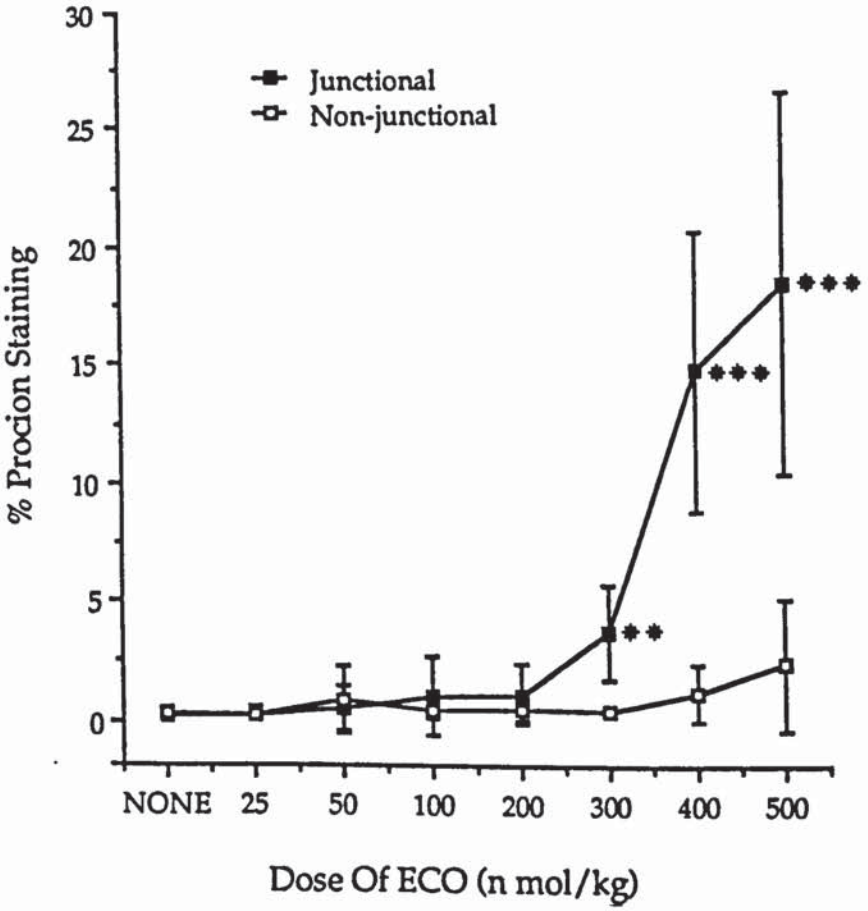


Figure 4.19 The effect of various doses of ECO 3 hours in vivo on the extent of procion yellow staining in the junctional and non-junctional region. Values represent mean ± 1SD. **, *** denotes significant difference from those of atropine group at the 2% and 0.2% respectively. (NB. for full statistical evaluation see table 4.21).

	AChE ACTIVITY		% PROCION YELLOW STAINING	
Time	Blood	Diaphragm	Junctional	Non-junctional
None	0.96	1.42	0.16	0.28
	±0.18	±0.25	±0.20	±0.29
N	42	55	9	9
3 hrs	0.37***	0.57***	0.90	0.33
	±0.10	±0.11	±1.61	±0.34
N	8	8	7	7
24 hrs	0.80***	0.83***	0.19	0.26
	±0.20###	±0.22##	±0.36	±0.19
N	8	8	8	8

Table 4.20 The effect of 100 n mol Kg^{-1} ECO on AChE activity of blood and diaphragm and procion yellow staining at 3 and 24 hours after ECO administration. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively. Values are mean ± 1SD, where N = no. of hemidiaphragms. *** denotes significant difference from those of atropine group at 0.2% level. ##, ### denotes significant difference between 3 hours 24 hours at 2% and 0.2% respectively.

animals that have similar levels and duration of AChE inhibition and this suggests that in addition to the above factors an additional factor(s) governs the extent of myopathy.

The extent of % procion yellow staining could be governed by the rate at which AChE is inhibited. This possibility was previously shown to be unlikely as maximum inhibition was evident at 20 mins with doses greater than 300 n mol Kg^{-1} ECO. The AChE activity in this study reflects the activity of the heterogeneous structures in solution. It is possible that in the intact preparation the rate and the extent of AChE inhibition in the individual motor EP, particularly at high doses, may well vary considerably (Toth, et al, 1981).

Contributions from BuChE to the termination of the synaptic ACh was previously suggested as an explanation for the initiation and extent of antidromic activity expressed in the whole animal as fasiculations. If indeed, this is the case, then the extent of % procion yellow staining could be governed by the extent of antidromic activity, akin to those of paraxon (Laskowski and Dettbarn, 1979; Ferry, 1988) or perhaps by changing the release of ACh due to different firing patterns (Misulis et al, 1987). A Similar mechanism of drug action has been repeated for other inhibitors. ECO has been shown to initiate antidromic activity in doses similar to those employed in this study (Ferry, 1988) and changes in firing patterns (Bamforth, personal communication). The selective inhibition of particular motor units by ECO cannot be ruled out.

The selective procion staining at junctional region suggests loss of local sarcolemmal integrity (Bradley and Fulthorp, 1978). It is possible that the procion yellow staining observed in the non-junctional region could be due to diffusion of procion from junctional region. This appears unlikely since diffusion of procion would be restricted due to binding to intracellular proteins (Flanagan et al, 1974). Therefore, the extremely small degree of procion staining seen at low doses either at junctional or non-junctional regions presumably reflect experimentally induced damage or part of natural turnover.

The reason for the prominent staining of clumped myofibrils is not clear. The condensation of myofibrils may induce 'holes' or 'tears' or some physiochemical changes of membrane biochemistry probably increases the concentration of basic groups in the myofibrils which acts as mordants for the dye. This process would not be disadvantageous to the study, since it would tend to localise the dye in the region of the sarcolemmal defects through which it entered the muscle fibre.

The procion technique measures the % area of a hemidiaphragm showing yellow fluorescence of the procion dye. As the dye is capable of

penetrating 10 to 15 fibres deep (Bradley and Fulthorp, 1978), the overlap of two or more procion stained damaged fibre would be recorded by this technique as one. The extent of this underestimation is difficult to quantify. Underestimation is likely to be more frequent in extensive myopathy with high doses and longer exposure times and probably insignificant with low doses and shorter exposure time . Hence, as suggested by Townsend (1988), procion technique at the early stages of myopathy provides a better estimate of the extent of myopathy.

4.2.6 Changes in serum creatine kinase activity after ECO:

Figure 4.22 shows the effect of various doses of ECO on serum CK elevation (table 4.21). Extensive elevation of CK is correlated with critical level of AChE inhibition, severe behavioural changes, EP deformations and % procion yellow staining at junctional region. The profile being more or less identical to the procion yellow staining suggesting that loss of sarcolemmal integrity is the cause of serum CK elevations.

The elevation of CK activity in serum is a consequence of the effects of ECO on the animal as a whole. Contributions from brain can be excluded since ECO is unlikely to penetrate the blood brain barrier. Since skeletal muscle is a rich source of CK and since 40% of the adult body mass is of skeletal muscle, it is suggested that the resultant serum CK elevation is attributable to peripheral skeletal muscle. It has been shown that after ECO administration, significant CK was depleted from junctional region while the CK level in non-junctional region remained the same (Townsend, 1988). In the same study, it was also suggested that the amount of CK depletion from the diaphragm could contribute to a significant proportion of the serum CK activity. Therefore, CK elevations estimated in this study is also suggested and assumed to be mainly from the diaphragm.

The basal level of CK measured in serum from atropinised controls could result from local skeletal damage caused during dissection. Alternatively, this level might be due to controlled processes of cell turnover. The translocation of intracellular enzymes to the extracellular space is a normal physiological phenomenon (Opie, 1979).

The slight, though significant elevations in serum CK activity after 50-200n mol Kg^{-1} ECO could be taken as evidence for loss of this control and perhaps may be exacerbated under these conditions. Alternatively, it is possible that disturbances in transmembrane gradients resulting in net cellular accumulation of sodium, calcium and cellular leakage of K^+, ie. ionic shifts contribute to the promotion of cellular water uptake (Leaf, 1973; Hillis and Braunwald, 1977) and may

DOSE OF ECO (n mol Kg^{-1})	% AChE INHIBITION (DIAPHRAGM)	CREATINE KINASE (U/l)
None	-----	55 ±38
N		20
50	40.5	105** ±29
N		8
100	59.6	104** ±27
N		8
200	77.0	111** ±40
N		8
300	88.6	433*** ±101###
N		5
400	87.3	2070*** ±537###
N		11
500	90.3	3910*** ±1720###
N		32

Table 4.21 The effect of various doses of ECO 3 hours in vivo on serum CK activity. Values are mean ± 1SD, where N = no. of hemidiaphragms. **, *** denotes significant difference from those of atropine group at 2% and 0.2% level respectively. ### denotes significant difference between values immediately above at 0.2% level.

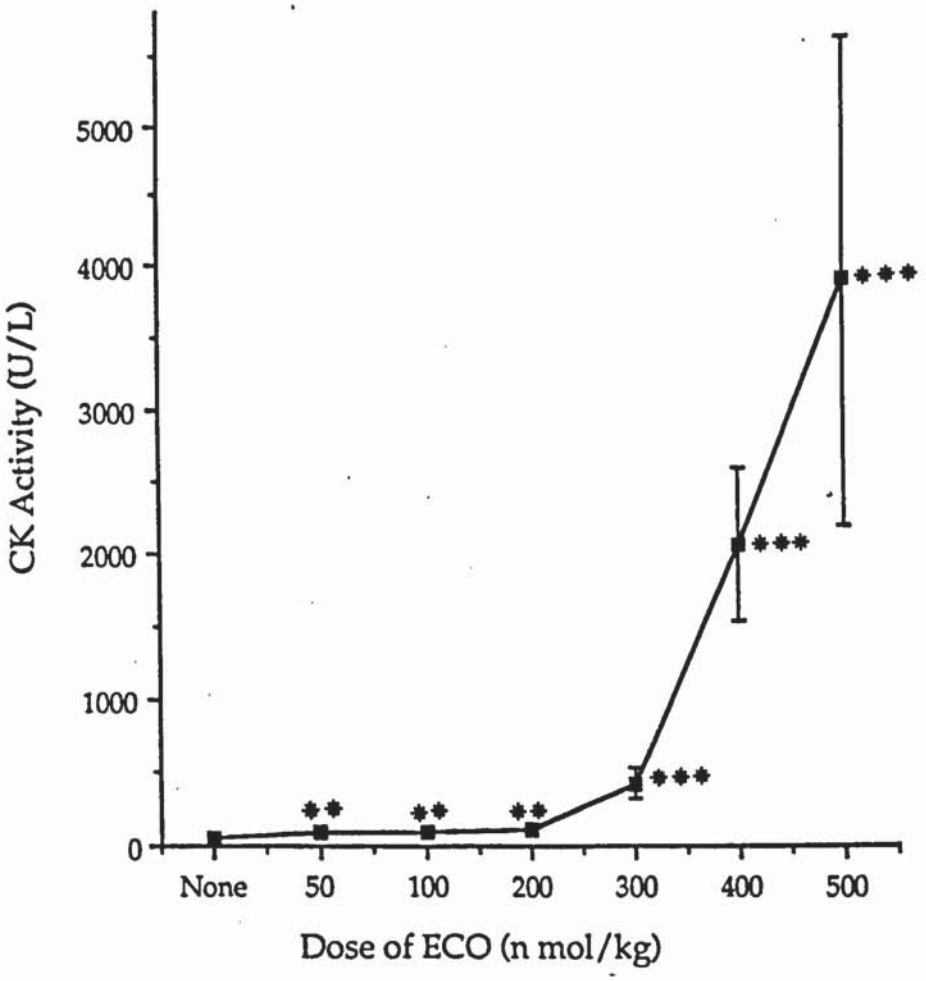


Figure 4.22 The effect of various doses of ECO 3 hours in vivo on serum CK activity . Values represent mean ± 1SD. **, *** denotes significant difference from those of atropine group at the 2% and 0.2% respectively. (NB. for full statistical evaluation see table 4.21).

lead to membrane swelling and may induce adverse changes in the membrane permeability ie. membrane 'leakiness'. Of these, the latter possibility appears more feasible since (i) inspection of EP morphology suggests possible involvement of marked volume changes at low doses (ii) ultrastructural studies suggested that there was evidence of early cellular edema and swelling (Townsend, 1988) (iii) during ischemia or hypoxia a rise in cytoplasmic enzyme was not accompanied by leakage of membrane bound enzyme (Hearse, 1980) (iv) if transmembrane fluxes are related to energy supplies, then a time when energy supplies are severely limited there is unlikely to be an increase in the activity of energy-requiring vesiculation process. It is more likely that the shortage of energy and the consequent disruption of cellular homeostasis results in the development of abnormal membrane permeability.

While membrane stretching and macromolecular swelling may be adequate to explain the early phases of enzyme leakage it is unlikely to be able to explain the more extensive loss of CK at higher doses. At the higher doses, it is suggested that significant formation of 'holes' occur in the cell membrane leading to cellular emptying and procion penetration (Michell and Coleman, 1979). This possibility is supported by studies with erythrocyte membranes (Seeman, 1974) which have revealed that under certain conditions, such as hypotonic haemolysis, small slits (20-100nm long) appear in the erythrocyte membranes. Of particular interest is the observation that due to the remarkable resealing properties of the cell membrane, these slits may exist for only a few seconds. The results of higher doses of ECO are consistent with this possibility. Increased enzyme leakage associated with increased doses could than be explained by increasing durations of slit opening and eventually by the loss of membrane sealing properties.

It is also suggested that with a dose of 300 n mol Kg^{-1} ECO, the loss of membrane resealing property becomes the critical factor and at this stage it seems most likely that the enzyme leakage threshold represents the onset of irreversible damage caused by some critical change in membrane biochemistry.

4.3 Summary:

Exposure to low doses ECO manifested in dose-dependent AChE and BuChE inhibition, changes in EP shape, hypercontractions and release of creatine kinase, changes which were essentially reversible in nature. At higher doses (300 n mol Kg^{-1} or greater) resulted in critical levels of AChE and BuChE inhibition, gross behavioural changes, loss of

sarcolemmal integrity and creatine kinase release, changes which were irreversible.

Description of myopathy therefore can be made in terms of changes in morphological or biochemical parameters whether reversible or irreversible. To define myopathy is more difficult, as it may include changes which can range from mild and reversible to severe and irreversible or changes which are transient. In this respect the definition suggested by Townsend, (1988) is apt. Myopathy was defined and used as a term to describe any abnormal state which may reside within a muscle fibre. It may be very mild, consisting of slight abnormality such as changes in EP shape, or gross such as procion staining, indicating necrosis.

5.0 MECHANISMS INVOLVED IN ECOTHIOPATE-INDUCED MYOPATHY

5.1 ECO-induced myopathy as a consequence of calcium elevation:

5.1.1 Introduction :

A question of great importance and an area of spirited controversy is the relative importance of extracellular and intracellular calcium in the process of cellular necrosis (see general introduction). Schanne et al (1979) demonstrated that cultured rat hepatocytes, exposed to various membrane active toxins were killed more rapidly in the presence of extracellular Ca^{2+} rather than in its absence. Observations which have been subsequently supported by others (Farber, 1981). In contrast, Fariss and Reed (1986) have concluded that 'extracellular Ca^{2+} plays no specific role in cytotoxic injury', and Cobbold and Bourne (1984) have reported that free $[Ca^{2+}]_i$ in metabolically poisoned myocytes is remarkably stable and that severe injury to the cell occurs before $[Ca^{2+}]_i$ rises above 1-3 $x10^{-7}$ M.

There is growing evidence that expression of antiChE induced myopathy is dependent upon extracellular calcium (Leonard and Salpeter, 1979; Townsend, 1988). Leonard and Salpeter (1979) proposed that the myopathy is mediated by an excess Ca^{2+} influx and that the muscle damage could be prevented by removing Ca^{2+} from the incubating medium, or by blocking the ACh receptor with α-bungarotoxin, suggesting that extracellular calcium as being the important source of calcium-induced necrosis.

It is possible that in these studies, the administration of acute doses of antiChE resulted in early modifications in membrane structure leading to membrane 'leakiness' which could have resulted in calcium entry and hence expression of myopathy.

The main objectives of this section are to evaluate whether:

1) ECO-induced myopathy is a consequence of calcium elevation?
2) calcium elevation precedes myopathy?
3) the degree of calcium entry can govern the extent of necrosis?

5.1.2 Experimental procedure :

Mice were divided in 7 groups of 6 or more mice. Each group received only one of the following treatments.

Group 1:	700 n mol Kg^{-1} atropine only
Group 2:	50 n mol Kg^{-1} ECO
Group 3:	100 n mol Kg^{-1} ECO
Group 4:	200 n mol Kg^{-1} ECO
Group 5:	300 n mol Kg^{-1} ECO
Group 6:	400 n mol Kg^{-1} ECO
Group 7:	500 n mol Kg^{-1} ECO

3 hours after the single administration of the drug, mice were killed and diaphragms removed. One hemidiaphragm was used for determination of calcium as described in section 2.4.3 and the other hemidiaphragm was used for the determination of AChE activity (see 2.4.1) or procion staining (see 2.3.3). Allocation of right and left hemidiaphragm was alternate. The determination of AChE activity and procion staining was simply to confirm whether AChE inhibition and the extent of myopathy were similar to earlier findings.

5.1.3 Results and discussions :

The effect of single variable doses of ECO on calcium accumulation in diaphragm is presented in table 5.1.1. Calcium accumulation at the junctional region is increased in a dose-dependent manner (figure. 5.1.2), with significant calcium accumulation occurring with a dose of 100 n mol Kg^{-1} ECO. Further, significant accumulation occurring with increasing dose, until a plateau level is obtained with a dose of 400 n mol Kg^{-1} ECO (table 5.1.1).

The elevation in calcium accumulation appears to be solely due to the increased influx of calcium at the junctional region and not at just any point along the muscle fibre (table 5.1.1). In contrast, the calcium content of the non-junctional region remained unaltered with increased doses of ECO (figure. 5.1.3).

It is possible that the extent of calcium accumulation is underestimated as a possible consequence of calcium efflux during the 'wash' period in the calcium free saline. However, this appears unlikely for two reasons. Firstly, it has been shown by Townsend (1988) that the calcium content of NJ and J regions were not significantly different between 'washed' and 'unwashed' preparations. Secondly, the calcium concentration of Ca^{2+} free saline, as determined by AAS, was found to

DOSE OF ECO	% AChE INHIBITION	% PROCION STAINING	CALCIUM (n mol mg^{-1}) CONTENT		ACCUM
(n mol Kg^{-1})	(Diaphragm)	(J)	(NJ)	(J)	(J)
None	00.0	0.16	1.80 ±0.52	2.15 ±0.51	0.15 ±0.10
N			47	47	47
50	40.5***	0.47	1.71 ±0.54	2.05 ±0.51	0.16 ±0.07
N			20	20	20
100	59.6***	0.90	1.13 ±0.22	1.72 ±0.39	0.26* ±0.16
N			10	10	10
200	77.0***	0.95	1.48 ±0.41	3.59*** ±0.67###	0.97*** ±0.29###
N			10	10	10
300	88.6***	3.51***	1.51 ±0.34	4.53*** ±0.96#	1.32*** ±0.31##
N			20	20	20
400	87.3***	14.53***	1.75 ±0.92	6.86*** ±1.69###	2.19*** ±0.59###
N			20	20	20
500	90.3***	18.22***	1.74 ±0.91	6.44*** ±2.66	2.07*** ±0.80
N			25	25	25

Table 5.1.1 The effect of various doses of ECO 3 hours in vivo on the calcium content of the junctional (J) and non-junctional (NJ) region and the extent of calcium accumulation at the junctional region. Values represent mean ± 1SD, where N = no. of hemidiaphragms. *,*** denotes significant difference from those of atropine group at 5% and 0.2% level respectively. #,##,### denotes significant difference between values immediately above at 5%, 2%, 0.2% levels respectively.

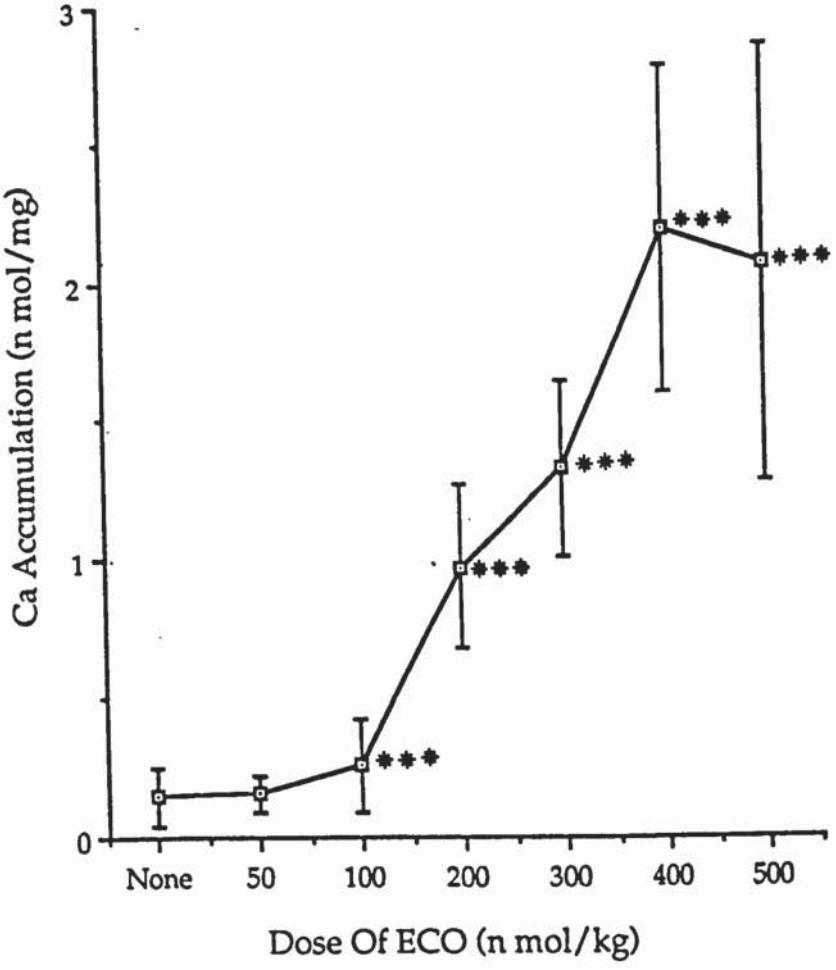


Figure 5.1.2 The effect of various doses of ECO, 3 hours in vivo on the extent of junctional calcium accumulation. Values represent mean ± 1SD. *,*** denotes significant difference from those of atropine group at 5% and 0.2% level. (NB. for full statistical evaluation see table 5.1.1).

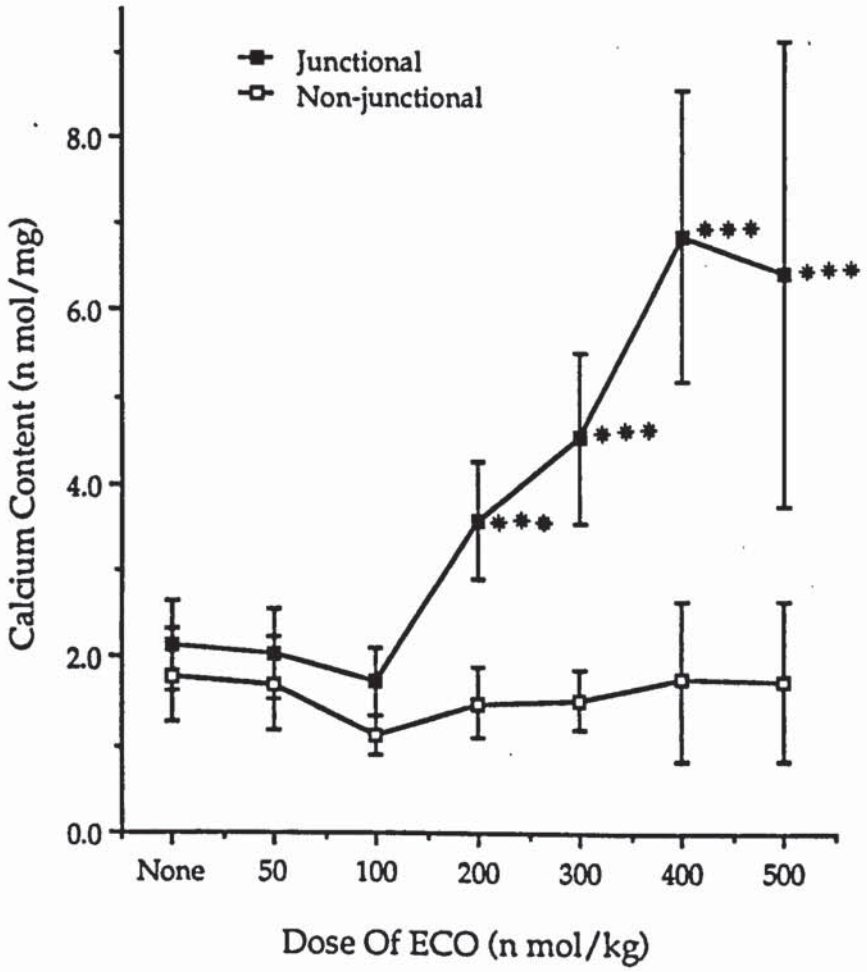


Figure 5.1.3 The effect of various doses of ECO, 3 hours in vivo on the extent of junctional and non-junctional calcium content. Values represent mean ± 1SD. *** denotes significant difference from those of atropine group at 0.2% level. (NB. for full statistical evaluation see table 5.1.1).

be in the range of 3-5 x 10^{-6} M, thus a strong electrochemical gradient would still prevail, therefore negating Ca^{2+} efflux. It is more probable, that the calcium content of both regions is elevated as a result of binding of calcium to extracellular structures. However, if this is the case, then as long as the binding is uniform throughout the fibre, then the calcium accumulation calculated would remain relatively unaffected (See 2.4.3.5).

The fact that the NJ calcium content is not elevated even when junctional calcium content is significantly elevated which together with the fact that calcium content in 'washed' and 'unwashed' preparations remained the same, would strongly suggest that calcium, once in, is rapidly bound by intracellular proteins and cellular organelles (see 1.4). However, while much of this elevated calcium may be buffered, the appearance of significant number of hypercontractions, even at a low dose of ECO (100 n mol Kg^{-1}) suggest that much of it may also be ionised and that cytosolic Ca^{2+} concentration must be greater than 10^{-7}M. The persistence of hypercontractions at these low doses, may be due to either the entry of calcium per se or perhaps due to CROC (Duncan and Smith, 1980; Fabiato, 1982).

The results have clearly shown that administration of non-necrotising doses of ECO induces an early significant accumulation of calcium at the junctional region of the diaphragm. This occurs prior to any evidence of myopathy as indicated by the lack of procion yellow staining (table 5.1.1), further supporting the view that non-necrotic cells are capable of buffering extensive fluctuations in calcium. In necrotising doses, it is not known if elevation of Ca^{2+} exists in fibres prior to necrosis, in necrotic fibres or in damaged fibres. The significant increase in Ca^{2+} and procion yellow staining between 300-400 n mol Kg^{-1} ECO (table 5.1.1 and table 4.18) would tend to suggest that Ca^{2+} is retained in damaged fibres, possibly in mitochondria as electron dense deposits. Numerous mitochondria in damaged fibres, have been shown to contain electron dense deposits, presumably as crystalline hydroxyapatite (Townsend, 1988).

The source of this elevated calcium is most probably extracellular in origin. In contrast to cardiac and smooth muscle, where Ca^{2+} entry through sarcolemmal Ca^{2+} channels is clearly important for contraction, contraction of skeletal muscle has generally been found to be unaffected by removing external Ca^{2+} or by blocking its entry. Moreover, voltage-clamp recordings of Ca^{2+} currents in skeletal muscle over the last decade or so have tended to confirm that the currents play little part either electrically, in the formation of the action potentials, or

in the control of internal Ca^{2+} levels; the channels activate so slowly (10s to 100s of ms) on depolarisation that only a tiny fraction of channels opens during an action potential and Ca^{2+} entry is probably miniscule (Bean, 1986). Hence, in common with the findings of others (Evans, 1974; Leonard and Salpeter, 1979), the elevation of calcium in this study is most likely via open end plate channels during the prolonged transmitter action. These EP channels have been shown to open in the presence of ACh (Miledi, 1977) and allow the exchange of Na^+, K^+ and Ca^{2+} ions (Takeuchi, 1963) in the soleplate region of the muscle concerned. A possible contributing factor in the net calcium gain during ECO exposure may be associated, in part, with a loss in the ability of cells to remove calcium from the cytosol. The Na^+/ K^+ - ATPase activity in sarcolemma isolated from rabbit hearts is reduced by 75% in the calcium paradox (Lamers et al, 1984).

The relative contribution of the various mechanisms of ACh release, ie. nerve evoked, spontaneous quantal and non-quantal, in the elevation of junctional calcium content is difficult to ascertain. Significant contribution could arise from the non-quantal release of ACh since the amount of ACh released non-quantally is about 10 times that released quantally in unstimulated diaphragm (Miledi et al, 1983). Which in the presence of AChE inhibitors acts postsynaptically to an extent equivalent to about 10^{-7} M ACh (Vyskocil et al, 1983). Therefore some authors are of the opinion that the non-quantal release of ACh is an important source of agonist action and could play a major role in the initiation of cellular necrosis (Ferry, personal communication). In contrast, the source of calcium responsible for the rise in intracellular calcium which initiates the development of hypercontractions, is difficult to speculate. It could be as a direct consequence of calcium entry per se, or as a consequence of CROC or a combination of these processes.

It was shown in section 4.7 that non-necrotising doses of ECO while not significantly increasing procion yellow staining, did result in slight but significant increase in CK activity of blood (table 4.24). If this elevation in CK activity can be taken as an indication of alterations in sarcolemmal membrane ie. membrane 'leakiness', perhaps as a consequence of phospholipid hydrolysis (see 1.10), then calcium elevation could be as a consequence of membrane 'leakiness'. As the dose is increased, membrane 'leakiness' worsens to a state of irreversibility leading to the rapid uncontrolled entry of calcium. This rapid uncontrolled entry of Ca^{2+} may be the 'trigger' mechanism by which cellular necrosis is initiated. This is consistent with the growing evidence that cellular injury in a number of experimental and

pathological conditions proceed via two stage process, involving initially membrane disruption followed by rapid calcium entry. Such a scheme has been suggested to occur in the calcium paradox (Rudge and Duncan, 1984; Ruigrok, 1985), oxygen paradox (Myers et al, 1985), hepatoxicity by toxins (Schanne et al,1979; Farber, 1981) and reperfusion injury (Jennings et al, 1978).

It was shown in section 4.6 (see table 5.1.1) the extent of procion yellow staining varied significantly in groups of animals having similar levels of AChE inhibition. The results of this section provides convincing evidence that myopathy is mediated by calcium entry. Hence, experiments were done to investigate whether the amount of calcium entry in groups of animals having similar AChE, could govern the extent of cellular necrosis.

Animals were administered with 300 n mol kg^{-1} and 400 n mol Kg^{-1} ECO, and 30 mins and 3 hours after ECO administration calcium accumulation was determined.

The calcium content of junctional region and therefore calcium accumulation at both time point was significantly higher for 400 n mol Kg^{-1} ECO (table 5.1.4) (figure 5.1.5) suggesting that the extent of Ca^{2+} entry was greater for the higher dose. Thus the results are consistent with the hypothesis that the extent of myopathy could indeed be governed by the degree of calcium entry.

What factors influence the rate of Ca entry is uncertain. It could be the direct effect of ECO on the membrane or membrane component, perhaps by activating phospholipases or alternatively, ECO may induce changes in physiological processes in the presynaptic apparatus and therefore indirectly influence the actions of ACh on the postsynaptic structure.

DOSE OF ECO (n mol Kg-1)	CALCIUM (n mol mg^{-1}) CONTENT (NJ)	CONTENT (J)	ACCUMULATION (J)
300 (30 mins)	1.81 ±0.59 10	3.65 ±0.90 10	0.80 ±0.24 10
400 (30 mins)	1.51 ±0.37 10	5.48*** ±0.85 10	1.52*** ±0.27 10
300 (3 hours)	1.51 ±0.34 20	4.53 ±0.96 20	1.32 ±0.31 20
400 (3 hours)	1.75 ±0.92 20	6.86*** ±1.69 20	2.19*** ±0.59 20

Table 5.1.4 The effect of necrotising doses of ECO on the calcium content of junctional (J), non-junctional (NJ) and the extent of junctional calcium accumulation (J) at (a) 30 mins and (b) 3 hours after ECO administration. Values are mean ± 1SD, where N = no. of hemidiaphragms. *** denotes significant difference between the two doses at 0.2% level.

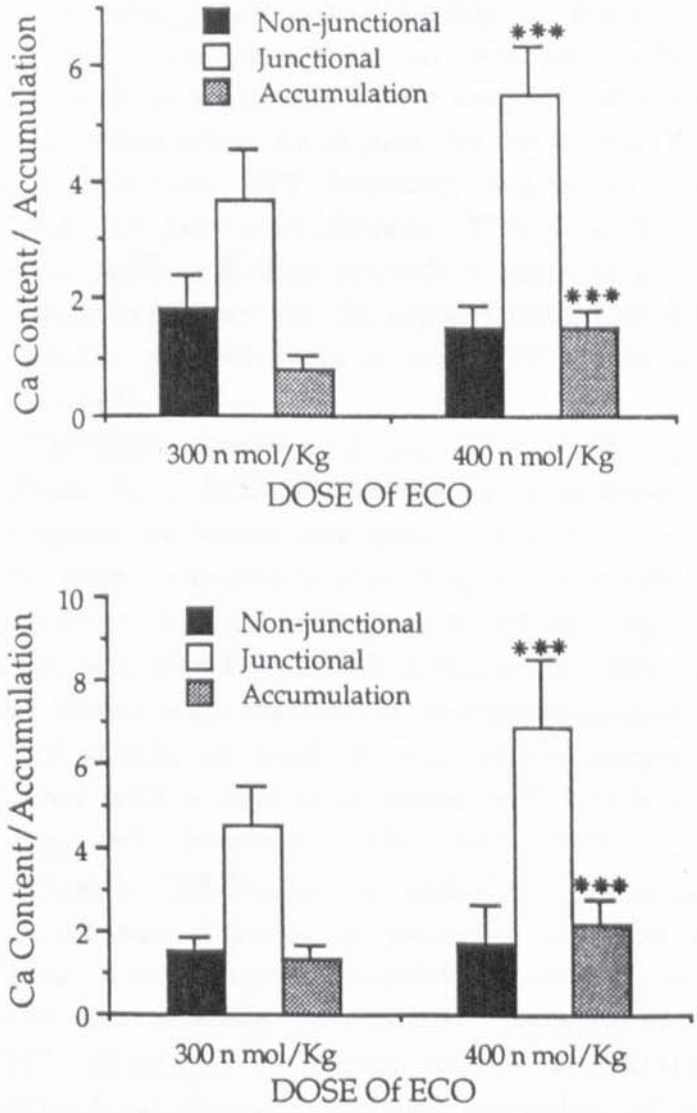


Figure 5.1.5 The effect of 300 and 400 n mol Kg^{-1} of ECO on the calcium content of junctional (J), non-junctional (NJ) and the extent of junctional calcium accumulation (J) at (Top:) 30 mins and (Bottom:) 3 hours after ECO administration. Calcium content/accumulation is n mol mg^{-1}. Values represent mean ± 1SD. *** denotes significant difference between the two doses at 0.2% level.

5.2 ECO-induced myopathy as a consequence of high energy phosphate depletion :

5.2.1 Introduction :

As a high energy phosphate donor, ATP serves as the immediate source of energy for many biological reactions. Consequently, its concentration in tissues is of interest, whether under optimal conditions, or in situations where energy demands are intensified, such as muscle contraction or myocardial ischemia. When energy demands exceed production, ATP decreases and under extreme conditions, the total adenylate pool may decrease. Therefore, the sum of the adenylates as well as ATP itself may provide a measure of the viability of tissues. The immediate donor for the replenishment of ATP is phosphocreatine (PC). Under physiological stress, ATP levels are maintained at the expense of PC.

Cellular necrosis as a consequence of depletion of high energy phosphate have been implicated in a number of experimental and pathological conditions (see general introduction, 1.9). In particular, the ultrastructural alterations occurring in myocardial ischemia are a direct consequence of high energy phosphate depletion (Jennings, 1978; Jennings and Hawkins, 1980; Jones et al, 1976). Energy metabolism is closely linked with membrane maintenance and function (Opie, 1979) and cell death, at least in myocardial ischemia of dog, is closely correlated with a decline of tissue ATP to about 2µmol/g dry weight (Jennings, 1978; Jennings and Hawkins, 1980).

Duncan (1987) reported rapid Ca triggered damage in less than 120 sec in skinned muscles in presence of 3mM ATP, suggesting that depletion of high energy phosphate is unlikely to be the direct cause of myofilament damage. In contrast, exposure to skeletal muscles with A23187 in vitro, in doses which are known to induce major ultrastructural changes, showed depletion of ATP and PC (Sugden, 1980).

Whether, OP-induced cellular necrosis results from depletion of high energy phosphate is questionable, though the evidence for this is accumulating. Dettbarn (1984) reported that 2 hours following exposure to paraoxon or DFP, glycogen levels in liver and muscles were reduced by 50%. Since glycogen is the source of glucose in anaerobic glycolysis, the results could be indicative of increased ATP demand. Recently, Gupta and Dettbarn (1987a) showed significant depletion of PC, ATP, which was accompanied by marked increases in ADP and AMP, in skeletal muscles of rats intoxicated with acute doses of DFP and soman, at a time coinciding with the appearance of necrotic lesions. The report

also mentioned that subchronic doses of DFP (0.5 mg Kg^{-1}) did not cause significant alterations in adenine nucleotides.

Although these studies have shown casual links between depletion of high energy phosphate and myopathy, the studies failed to provide evidence of whether the changes observed were the cause or consequence of myopathy, possibly because of complications by the presence of both necrotic and non-necrotic fibres.

The main objectives of this section are to evaluate whether:

1) ECO-induced myopathy is associated with depletion of high energy phosphates?
2) depletion of high energy phosphates causes necrosis?
3) depletion occurs with non-necrotising doses?

5.2.2 Experimental procedure :

Mice were either administered atropine or 500 n mol Kg^{-1} ECO. 3 hours after drug administration, mice were anaesthetised and the whole diaphragm with the rib cage intact was quickly excised, and immediately placed in liquid nitrogen. While thawing, the diaphragm was gently separated from the ribs and central tendon and again transferred back into liquid nitrogen. The frozen diaphragm was then prepared for assay of high energy phosphates as described in section 2.4.4.3.

In later experiments, it became necessary to evaluate the high energy phosphate content of the junctional region. In these experiments, the isolated diaphragm was transferred into a petri dish containing physiological saline and hemidiaphragms produced by the section of the medial tendon. Each hemidiaphragm was pinned to a piece of sylgard block as described in section 2.3.3.1 and immediately placed in a vertical organ bath for 15 mins, while being gassed and maintained at 37°C ± 0.5°C. After 15 mins. incubation, the junctional region was quickly excised and immediately frozen in liquid nitrogen and subsequently prepared for assay of high energy phosphates as described in section 2.4.4.3.

5.2.3 Results and discussion :

The separation of purine nucleotides from a mouse diaphragm extract and from a mixture of standards is illustrated in figure 5.2.1. Figure 5.2.2 demonstrates the separation of PC and creatine. Both compounds are eluted during the first 5 min. Though creatine is retained for a very short period of time, it is clearly separated and interference from the front peak eliminated by dissolving the standards in the appropriate mobile phase.

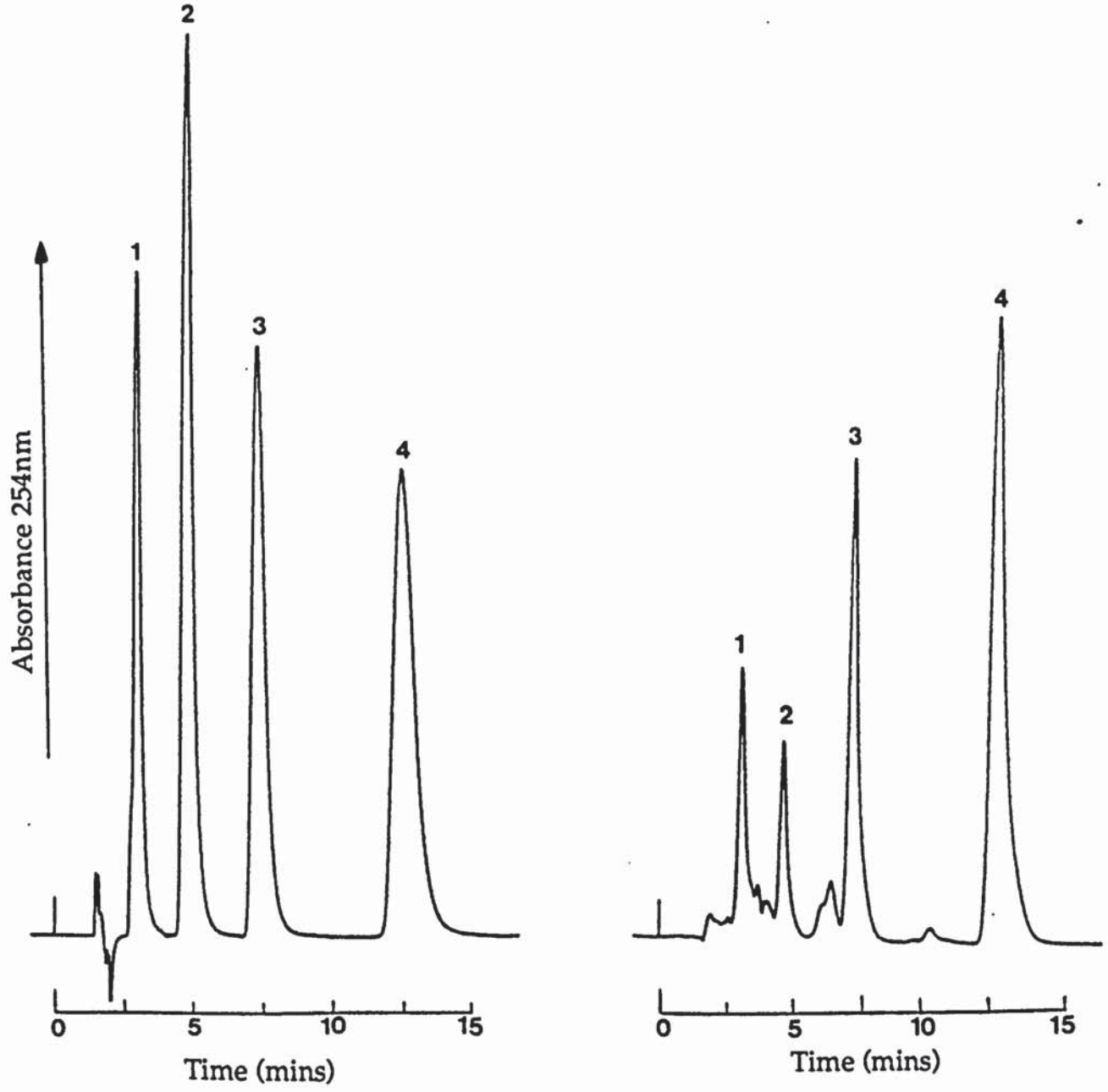


Figure 5.2.1 Separation of purine nucleotides. The left chromatogram shows the separation of a standard mixture of purine nucleotides (μM); 1=inosine 5'-monophosphate (20.8), 2=adenosine 5'-monophosphate (21.0), 3=adenosine 5'-diphosphate (21.3), 4=adenosine 5'-triphosphate (20.1). Right chromatogram shows the separation of purine nucleotides of an anoxic mouse diaphragm extracted in low levels of trichloroacetic acid.

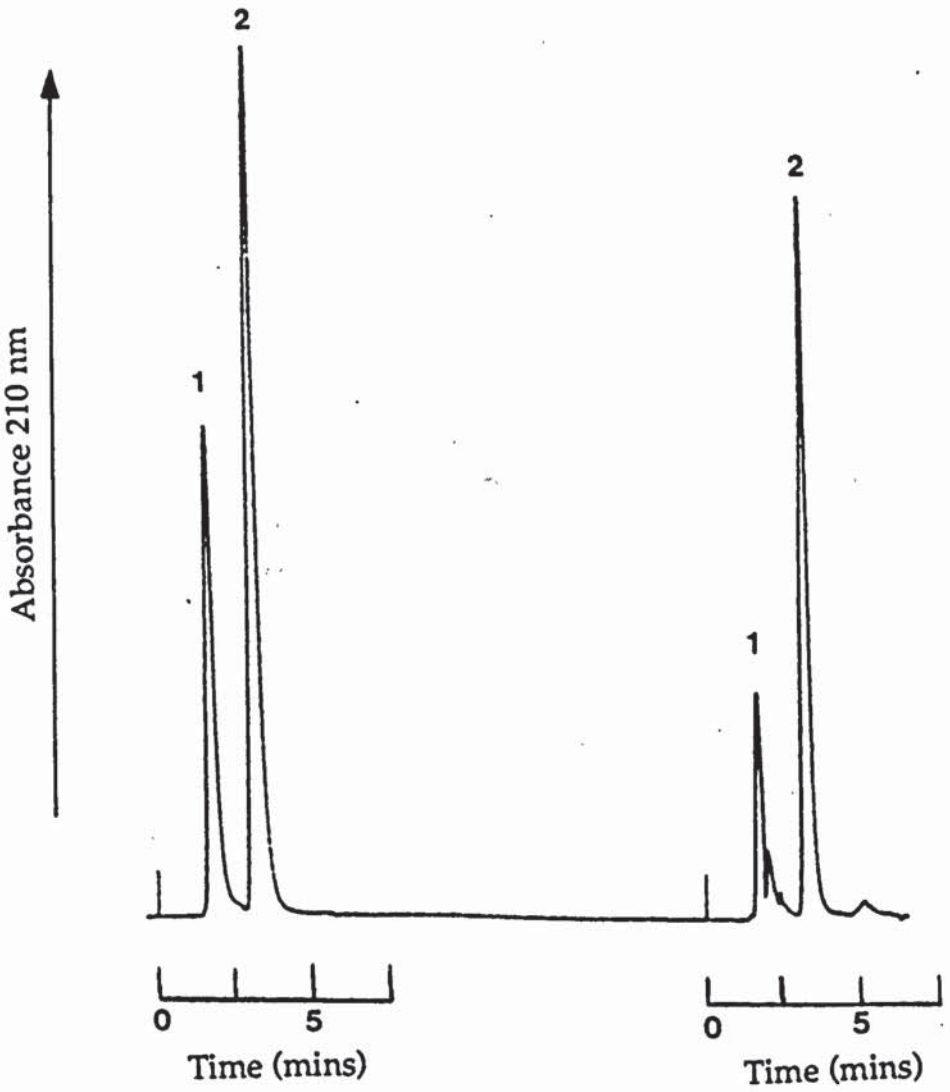


Figure 5.2.2 Separation of creatine and phosphocreatine. The left chromatogram shows the separation of a standard mixture of creatine and phosphocreatine (μM); 1=creatine (34.3) and 2=phosphocreatine (20.9). Right chromatogram shows the separation of creatine and phosphocreatine from a mouse diaphragm extract.

The effects of single dose of ECO (500 n mol Kg^{-1}) for 3 hours in vivo on the contents of diaphragm purine nucleotides and PC is presented in table 5.2.3. The data shows no significant changes in the levels of any of the purine nucleotides or in PC after ECO administration. Furthermore, no significant changes in either of the ATP/ADP or ATP/AMP ratio was found (table 5.2.3). There was no significant difference in the values of energy charge as calculated by ATP + 1/2 ADP/(ATP+ADP+AMP) between control and ECO treated mice. Energy charge of less than 0.85 is indicative of overall demand for ATP. These results are in direct contrast to the findings of Gupta and Dettbarn (1987).

The failure to detect changes in high energy phosphates, particularly in respect of PC and ATP, suggested possible flaws in methodology and/or in instrumentation. It is generally regarded and as reported by Lush et al (1979) that in extracts of fresh muscle an ATP/ADP ratio greater than 6 indicates minimal degradation of ATP during the extraction and is therefore considered satisfactory. In this study the ATP/ADP ratio was approximately 5, although lower than that reported by Lush et al (1979), is considerably better than that reported by Gupta and Dettbarn (1987) of 3.69 for controls and is similar in magnitude to those reported by others (Jennings et al, 1978). Thus the extractant as well as the extraction procedure employed in this study appear to be satisfactory.

The lack of any changes in adenine nucleotides could be due to nucleotide interconversion as a consequence of the combined effects of myokinase and creatine kinase, despite the presence of high ATP/ADP ratio (Lush et al,1979). However, this appears unlikely because firstly, no significant changes were observed in either of ATP/ADP or ATP/AMP ratios (table 5.2.3) suggesting no changes in any of the individual nucleotide pool and secondly, the inclusion of methanol in the extraction medium was reported by Lush et al (1979) to be a rapid and effective way inactivating myokinase, hence elimination of nucleotide interconversion.

It is possible that the presence of endogenous materials liberated during homogenisation, strongly absorb at 254 nm with retention times similar to the nucleotides in question. In order to investigate this possibility, the ATP contents of the same extracts as those in table 5.2.3 were determined by the bioluminescent method using luciferin and luciferase system (1243 - 107 ATP assay kit, LKB Wallac). The ATP levels of both the atropine and ECO treated groups are higher than that obtained with H.P.L.C. perhaps because interferences from endogenous materials were minimised since samples were diluted 150 - 200 fold

PARAMETER	TREATMENT IN VIVO (3 HRS) ATROPINE (700 n mol Kg-1)	ECO (500 n mol Kg-1)
ATP	3.89	4.10
	±0.33	±0.63
N	7	7
ADP	0.80	0.78
	±0.10	±0.13
N	7	7
AMP	0.30	0.23
	±0.05	±0.04
N	7	7
IMP	1.60	1.47
	±0.20	±0.54
N	7	7
ATP/ADP	4.88	5.13
	±0.45	±0.53
N	7	6
ATP/AMP	13.5	17.8
	±2.84	±4.70
N	7	6
Energy charge	0.86	0.88
PC	13.8	15.3
	±4.32	±3.01
N	7	6
Creatine	15.7	20.0
	±1.95	±4.25
N	7	7

Table 5.2.3 The effect of 500 nmol Kg^{-1} ECO 3 hours in vivo on the levels of high energy phosphates in diaphragm.Values of ATP, ADP, AMP, IMP, PC, Creatine are μ mol g^{-1} frozen weight and are represented as the mean ± 1SD, where N = no. of hemidiaphragms.

METHOD	LEVELS OF ATP (μ mol g^{-1}) ATROPINE (700 nmKg-1)	ECO (500 nmKg-1)
H.P.L.C.	3.89 ±0.33 7	4.10 ±0.63 7
Bioluminescent (luciferin-luciferase system)	4.36 ±1.32 7	5.15 ±1.39 7

Table 5.2.4 The levels of ATP in diaphragm as determined by the H.P.L.C. and bioluminescent method of the same extracts, as in table 5.2.3. Values of ATP is in µmol g^{-1} frozen weight and represented as the mean ± 1SD, where N = no. of hemidiaphragms.

prior to assay (table 5.2.4). However, the ATP levels of the ECO treated groups as determined by the bioluminescent method was not significantly different from the atropine controls, in fact the ATP levels of the ECO treated group was marginally higher, suggesting that ECO administration does not change diaphragm ATP levels.

Furthermore, if diaphragms were extracted with TCA of less than 2.5% and/or preparations deliberately made anoxic, then the levels of adenine nucleotides showed characteristic changes (see figure 5.2.1). There is significant reduction in ATP, ATP/ADP and ATP/AMP ratios with significant concomitant elevations in both ADP and AMP levels (table 5.2.5). The results provide additional reassurance that no inherent flaws in both the methodology and instrumentation.

It was shown in the preceding section 5.1 that considerable calcium elevation occurs in the diaphragm of ECO treated mice. The possibility that mitochondria are forced to sequester Ca^{2+} ions in preference to ATP synthesis exists. In this case cellular necrosis could be initiated as a consequence of ATP depletion (see 1.9). It would then be expected that the majority of fibres which are affected after a necrotising dose of ECO, as indicated by changes in EP shapes and the presence of numerous hypercontraction, would also result in changes in the levels of high energy phosphates. The failure to detect any changes in the content of high energy phosphates after a necrotising dose of ECO strongly suggests that ECO-induced myopathy is not a consequence of high energy phosphate depletion. Alternatively, it is possible that necrosis occurs in only those fibres which show decline in ATP below a certain level. Under these circumstances, the failure to detect any changes is perhaps not surprising since the % procion yellow staining (an index of myopathy) in the whole hemidiaphragm, 3 hours after 500 n mol Kg^{-1} ECO, is very small, less than 5%. In comparison, the % procion yellow staining at the junctional region 3 hours after 500 n mol Kg^{-1} ECO is approximately 18% (see table 4.18). Saks et al (1978) have shown that no change in the ATP level is found in living muscle except during extreme exhaustion, whether diaphragms treated with 500 n mol Kg^{-1} ECO for 3 hours in vivo, can be compared to extreme exhaustion is debatable. Although pesticide induced increased duration of muscle activity has been suggested by Dettbarn (1984) to be comparable to ischemia, such generalisation is questionable.

In order to maximise the detection of small changes, if any, in high energy phosphates, attempts were made to look at compounds of high energy phosphates in the junctional region of the hemidiaphragm. In this regards, the procedure of <u>in vivo</u>/<u>in vitro</u> was used as described

PARAMETER	TREATMENT	
	ATROPINE (3 hrs) (700 n mol Kg-1)	ANOXIC DIAPHRAGM
ATP	3.89	2.35**
	±0.33	±0.70
N	7	7
ADP	0.80	2.64***
	±0.10	±0.62
N	7	7
AMP	0.30	0.93***
	±0.47	±0.32
N	7	7
IMP	1.60	1.37
	±0.20	±0.28
N	7	7
ATP/ADP	4.88	0.92***
	±0.45	±0.30
N	7	7
ATP/AMP	13.5	2.97***
	±2.84	±1.62
N	7	7
Energy charge	0.86	0.62***

Table 5.2.5 The effect of anoxia and low levels of trichloroacetic acid on the levels of diaphragm adenine nucleotides. Values of ATP, AMP, IMP are in μ mol g^{-1} frozen weight and represented as mean ± 1SD, where N = no. of hemidiaphragms. **, *** denotes significant difference from those of atropine group at 2% and 0.2% levels respectively.

in section 5.2.2.

3 groups of mice were injected subcutaneously with either 700 n mol Kg^{-1} atropine or 500 n mol Kg^{-1} ECO or 200 n mol Kg^{-1} ECO and 3 hours after drug administration diaphragms were removed and prepared for assay as described in section 5.2.2.

The level of adenine nucleotides and PC following exposure to atropine or a necrotising or non-necrotising dose of ECO is presented in table 5.2.6. As can be seen from the results, no significant changes in any of the high energy phosphates could be detected. The ATP/ADP and ATP/AMP ratios as well as the energy charge were not significantly different from atropine controls. Furthermore, the values are similar to those obtained in vivo (table 5.2.3).

To further substantiate the view that myopathy is not associated with a decline in high energy phosphate compounds, a group of mice were treated with 500 n mol Kg^{-1} ECO for 12 hours in vivo before analysis were performed. Exposure to 500 n mol Kg^{-1} ECO for 12 hours in vivo is known to produce maximum myopathy with 40% procion yellow staining (Townsend, 1988). The levels of high energy phosphates 12 hours after ECO exposure is presented in table 5.2.7. The results further indicate that there were no significant changes in the levels of any of the purine nucleotides even when there was maximum myopathy. However, in contrast to the earlier findings, extensive myopathy was associated with significant reduction in PC and creatine (40% and 34% reduction respectively). A significant reduction in PC is indicative of a demand for ATP and therefore changes in ATP levels would have been expected. The ATP values compared to atropine controls was lower but not statistically significant. The significant reduction in the AMP levels is questionable, since no other changes in purine nucleotides were observed. It is possible that the reduction in AMP levels is a consequence of 'leakage' from cells since all the high energy phosphate compounds show slight reductions in the values compared to atropine controls (Table 5.2.7).

The results taken as a whole strongly suggest that myopathy is not a consequence of high energy phosphate depletion and furthermore is not associated with the development of myopathy, though CP was reduced with extensive myopathy, findings which are in direct contrast to that reported by Gupta and Dettbarn (1987). The conclusions in this section are consistent with that reported by Duncan (1987).

PARAMETER	TREATMENT IN VIVO / IN VITRO ATROPINE (700 n mol Kg-1)	ECO (500 n mol Kg-1)	ECO (200 n mol Kg-1)
ATP	4.15	4.07	4.34
	±0.30	±0.85	±0.50
N	15	8	8
ADP	0.83	0.77	0.87
	±0.09	±0.12	±0.12
N	15	8	8
AMP	0.30	0.34	0.35
	±0.07	±0.08	±0.04
N	15	8	8
IMP	1.77	1.75	1.68
	±0.30	±0.33	±0.11
N	15	8	8
ATP/ADP	5.00	5.28	5.05
	±0.32	±0.56	±0.42
N	15	8	8
ATP/AMP	14.6	12.2	12.7
	±3.10	±1.59	±1.90
N	15	8	8
Energy charge	0.87	0.86	0.86
PC	10.4	10.7	12.6
	±2.70	±1.46	±1.79
N	15	8	8
Creatine	19.8	15.9	19.4
	±4.23	±2.81	±2.00
N	15	8	8

Table 5.2.6 The effects of necrotising and non-necrotising doses of ECO 3 hours in vivo on the levels of high energy phosphates in diaphragm. Values of ATP, ADP, AMP, IMP, PC and creatine are μ mol g^{-1} frozen weight and represented as the mean ± 1SD, where N = no. of hemidiaphragms.

PARAMETER	TREATMENT	
	ATROPINE (700 n mol Kg-1)	ECO (500 n mol Kg-1)
ATP	4.15	3.86
	±0.30	±0.29
N	15	6
ADP	0.83	0.80
	±0.09	±0.08
N	15	6
AMP	0.30	0.22*
	±0.07	±0.05
N	15	6
IMP	1.77	1.56
	±0.30	±0.18
N	15	8
ATP/ADP	5.00	4.83
	±0.32	±0.26
N	15	6
ATP/AMP	14.6	18.5
	±3.10	±4.36
N	15	6
Energy charge	0.87	0.87
PC	10.4	6.28***
	±2.70	±1.20
N	15	6
Creatine	19.8	13.0**
	±4.23	±1.87
N	15	6

Table 5.2.7 The effect of 500 n mol Kg^{-1} ECO, 12 hours in vivo on the levels of high energy phosphates in diaphragm. Values of ATP, ADP,AMP, IMP, PC and creatine are μ mol g^{-1} frozen weight and represented as the mean ± 1SD, where N = no. of hemidiaphragms. *, **, *** denote significant difference from those of atropine group at 5%, 2% and 0.2% levels respectively.

5.3. ECO-induced myopathy as a consequence of free radical generation.

5.3.1 Introduction:

Following phagocytosis, normal polymorphonuclear leukocytes develop a burst of oxidative metabolism, a process well known for generating powerful free radical species and rapidly reduce nitro blue tetrazolium (NBT) to blue formazan (Repine et al, 1979). The reduction of NBT has been clinically exploited in the detection of patients and carriers with chronic granulomatous disease (Repine et al, 1979). Furthermore, the reduction of NBT has been used as a chemical method for measuring rates of superoxide generation in aqueous solutions by enzyme generating systems eg. xanthine - xanthine oxidase (Clifford and Repine, 1984) or tissue generating systems eg. in homogenates of tissues from experimental and pathological sources (Oberley and Spitz, 1984).

It is clear from the general introduction (see 1.8) that O_2^- is a commonly encountered intermediate of oxygen reduction whether from enzymic oxidations, subcellular organelles or in intact cells, and that this free radical constitutes a threat to the chemical integrity of living cells. This threat may arise from the intrinsic and relatively selective reactivity of O_2^- or it may be due to generation of the vastly reactive OH· by iron catalysed reactions (Halliwell, 1984) and this threat may be exacerbated under various experimental and pathological conditions. Since oxidative free radicals are highly destructive and capable of rapid and extensive cellular damage this could thus represent a potential mechanism by which OP may induce myopathy. OP-induced myopathy (to the authors knowledge) has not been associated with oxidative damage, although OP has been shown to induce lipid peroxidation in various brain regions (Hasan and Ali, 1980).

Furthermore, the induction of early biochemical and morphological changes after OP administration provides favourable conditions in the intracellular environment for the generation of O_2^- and O_2^- derived free radicals.

The main objectives of this section are to evaluate whether :

1) O_2^- is generated after necrotising doses of ECO, detected histochemically as reduction of NBT to formazan (blue deposits)? The reduction of NBT to blue formazan has been widely used as a probe of O_2^- generation in chemical and biological systems. NBT is an electrophilic dicationic

compound which can easily accept electrons from electron donors. Its reduction to formazan is accompanied by the disappearance of the positive charges, resulting in a substantial decrease in solubility and the appearance of intense blue colour, visible as a dark blue precipitate within the cytoplasm.

2) O_2^- is generated at site of myopathy?

3) O_2^- generation is the cause or a consequence of myopathy.

5.3.2 Experimental Procedure :

Mice were administered subcutaneously with 500 n mol Kg^{-1} ECO and at various times after ECO exposure the diaphragms were removed and hemidiaphragms produced by section of the medial tendon. Hemidiaphragms were stained in NBT in the presence and absence of superoxide dismutase (SOD) as described in section 2.3.4. Following NBT staining, preparations were quickly rinsed and fixed and subsequently stained for ChE.

In some experiments, after NBT staining, preparations were stained in procion (for 30 mins only) (see section 2.3.3) and then fixed. In another experiment preparations were stained in NBT following exposure to non-necrotising doses of ECO.

5.3.3 Results and discussion:

In the present investigation, the reduction of NBT to formazan was used in combination with SOD to visualise the presence of the superoxide radical after OP administration.

Inspection of NBT stained hemidiaphragm after atropine exposure showed characteristic features of normal skeletal muscle as described in section 4.3 with no obvious changes in morphology. In contrast, a single exposure to a necrotising dose of ECO (500 n mol Kg^{-1}) resulted in deposition of blue formazan primarily associated with the junctional region in close proximity to EP and subsequently progressed along the length of the fibres to the NJ region as the duration of ECO exposure increased. The degree of formazan deposit and the intensity and spatial distribution of formazan were all dependent upon the duration of AChE inhibition (Plates 5.3.1 - 5.3.7).

The predominant feature 30 mins after ECO administration were formazan deposition with many hypercontractions and distorted EP, sometimes the formazan deposit was light and sometimes intense as depicted in plate 5.3.8. However, superimposed on these features, longer exposure revealed major disorganisation of contractile apparatus such as loss of striation and the appearance of greater number of contraction

Plates 5.3.1-5.3.7: This series of plates comprises photographs of individual hemidiaphragms from mice given 500 n mol Kg^{-1} ECO in vivo for various times. Unsectioned whole preparations. Vital staining in NBT, fixed and then stained for ChE (end plates and formazan deposits are represented by patches of brown and blue stain respectively). Tungsten illumination. Top: absence of SOD. Bottom: presence of SOD. Calibration 100 μm.

Plate 5.3.1: Hemidiaphragm of mouse given atropine only is similar to that of plate 4.2.

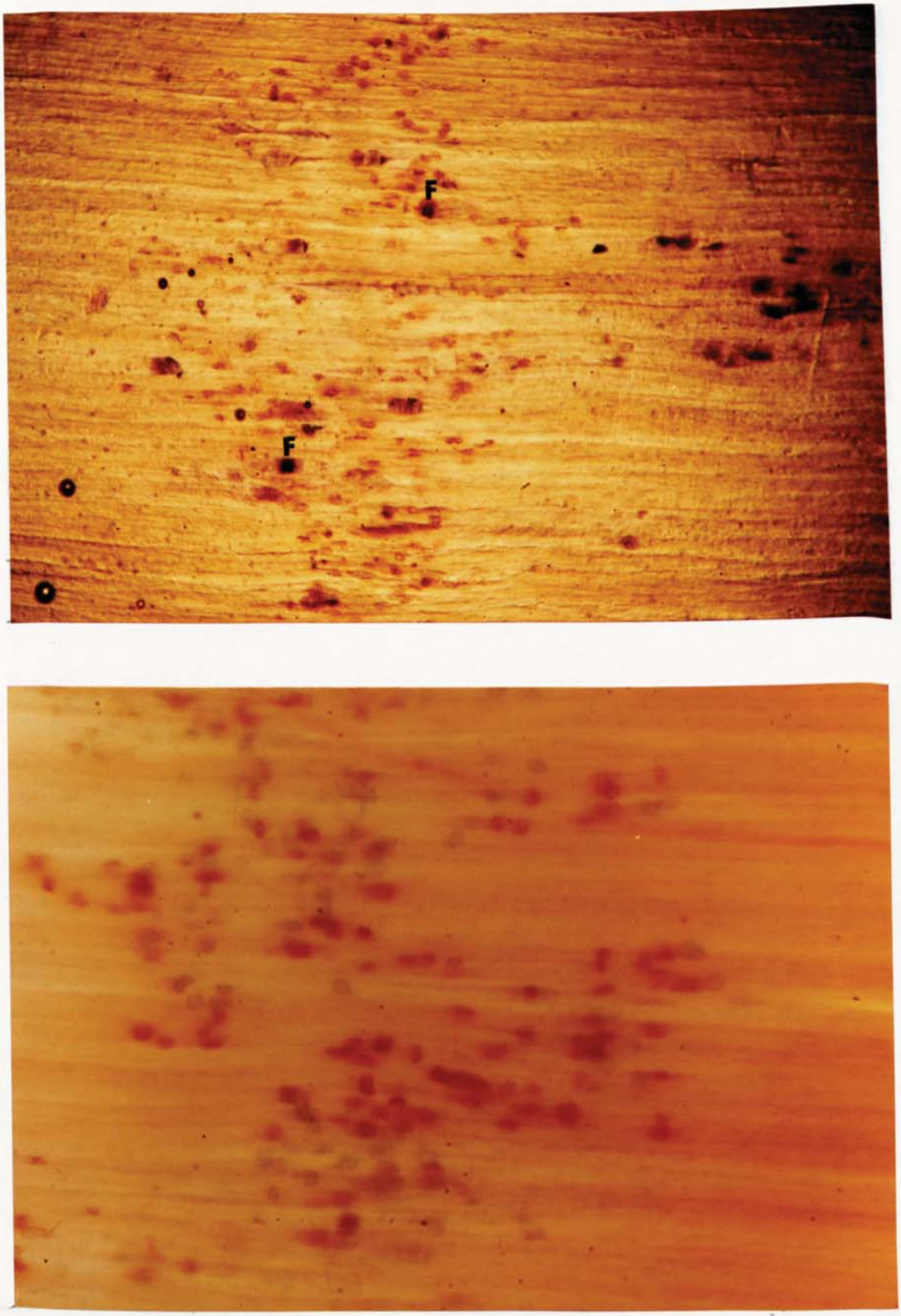


Plate 5.3.2: Hemidiaphragm taken from mouse 30 mins after ECO <u>in vivo</u>. Note the appearance of formazan (F) in close association with end plates. Also notice the reduction in formazan in the presence of SOD.

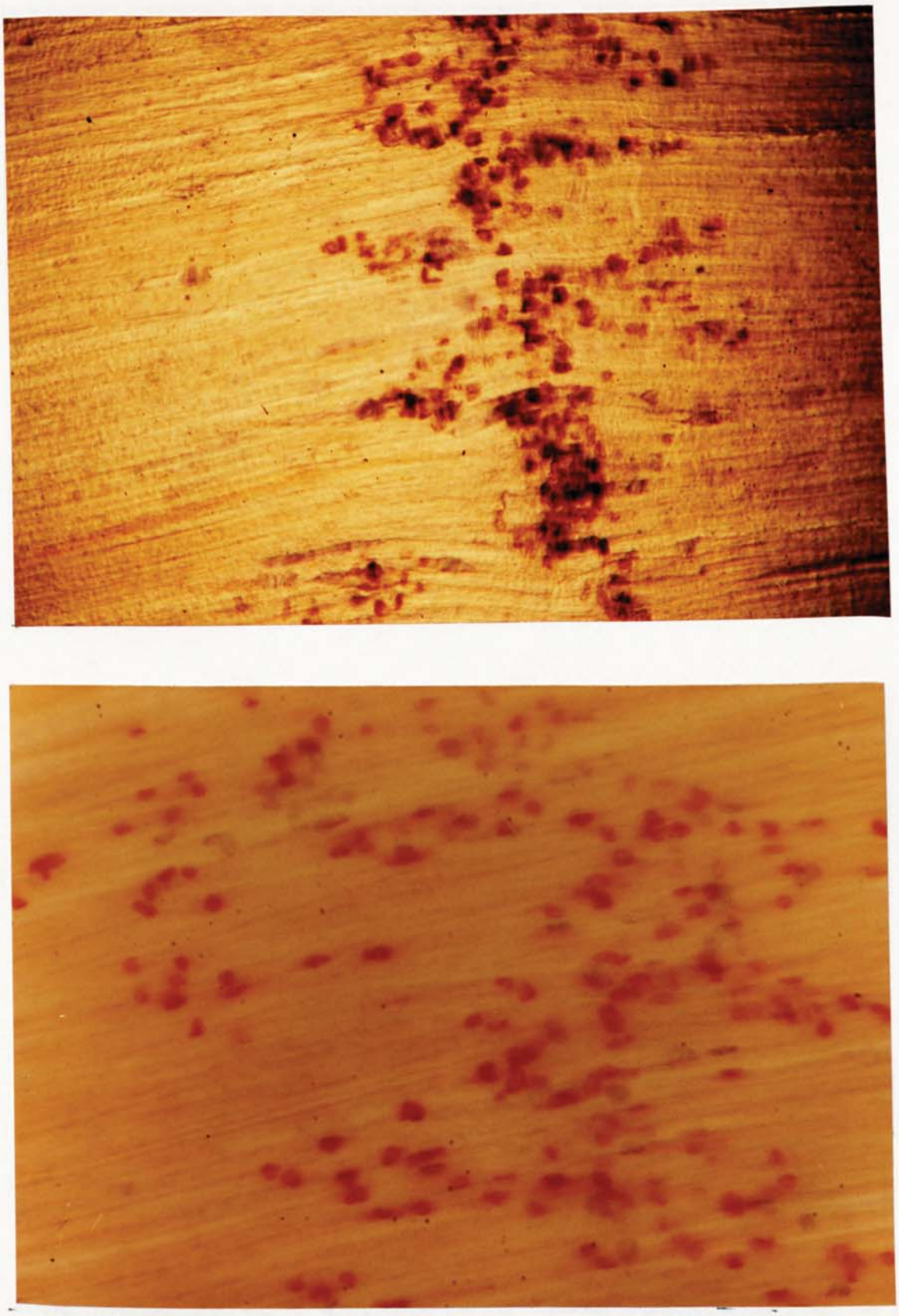


Plate 5.3.3: Hemidiaphram taken from mouse 60 mins after ECO in vivo. Note the appearance of formazan which is more frequent than in plate 5.3.2. Also notice the reduction in formazan in the presence of SOD.

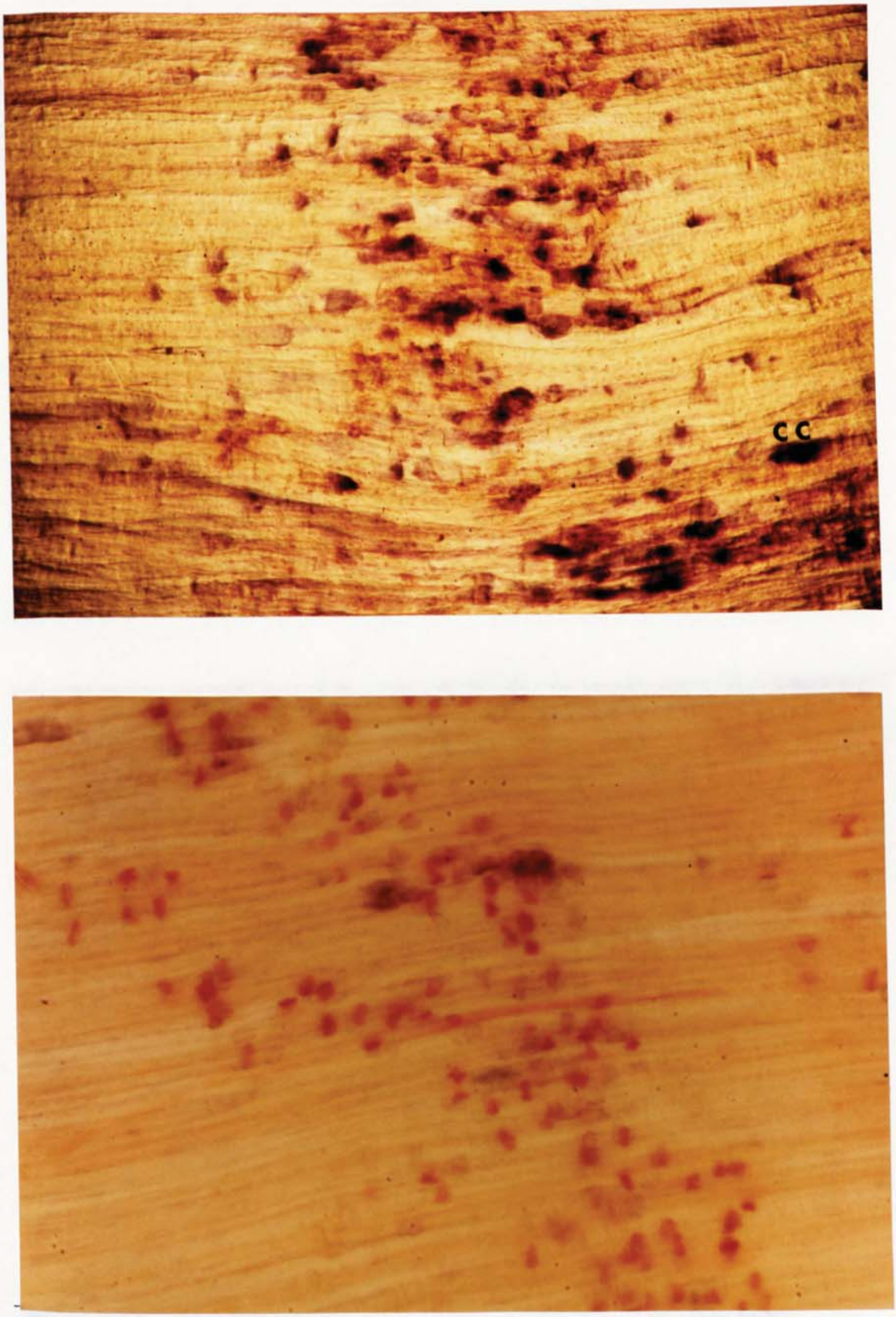


Plate 5.3.4: Hemidiaphram taken from mouse 2 hours after ECO in vivo. Note the greater spatial distribution and intensity of formazan than that shown in plate 5.3.3. Notice that the formazan is not only associated with end plates but is also found in the contraction clumps (CC) and there is still reduction in formazan in the presence of SOD.

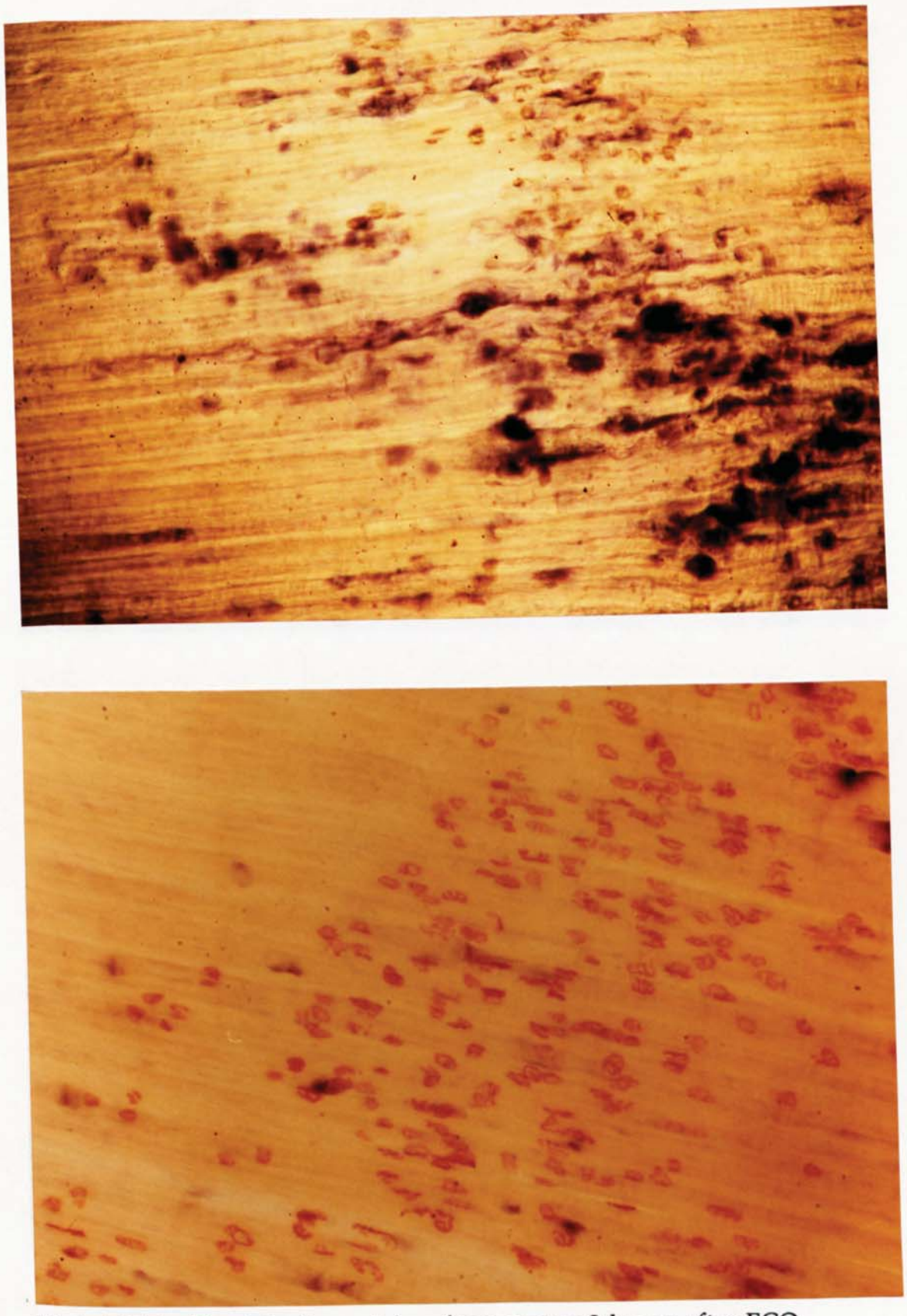


Plate 5.3.5: Hemidiaphram taken from mouse 3 hours after ECO in vivo. Note the greater spatial distribution and intensity of formazan than that shown in plate 5.3.4. There is still reduction in formazan in the presence of SOD.

Plate 5.3.6: Hemidiaphram taken from mouse 6 hours after ECO in vivo. Note the greater spatial distribution and intensity of formazan than that shown in plate 5.3.5. Notice the spread of formazan away from the junctional region. There is still reduction in formazan in the presence of SOD.

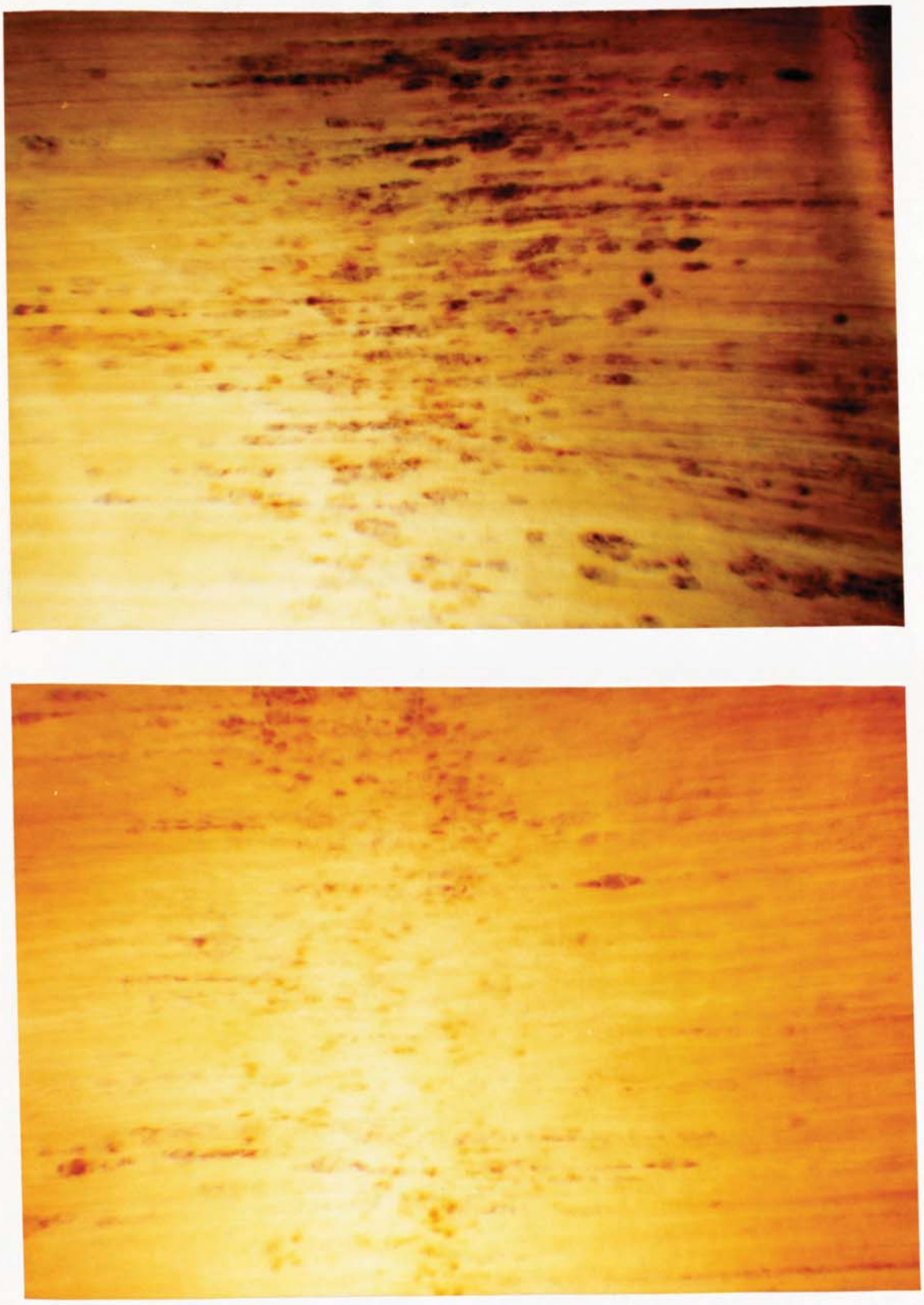


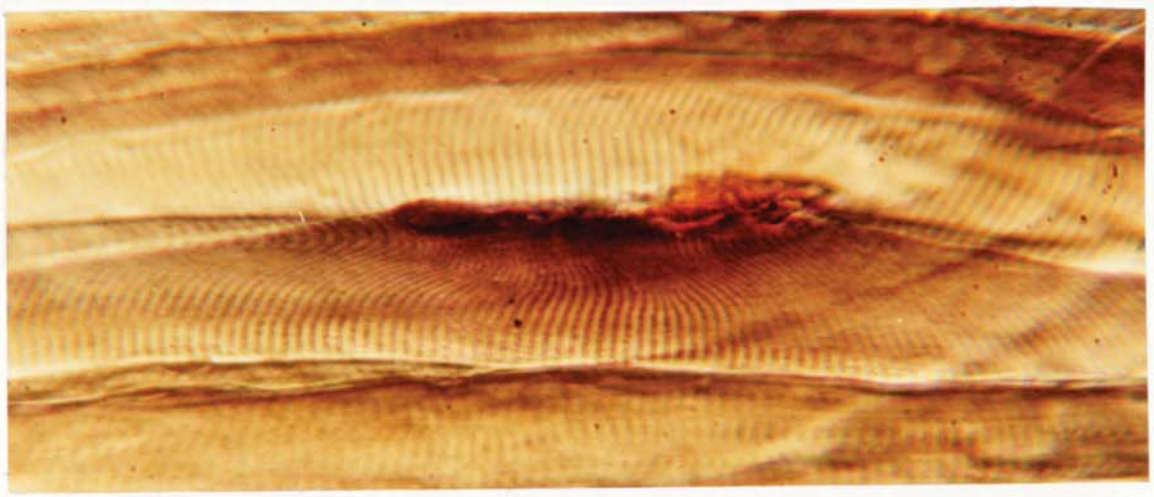
Plate 5.3.7: Hemidiaphram taken from mouse 24 hours after ECO in vivo is similar to plate 5.3.6. There is still reduction in formazan in the presence of SOD.

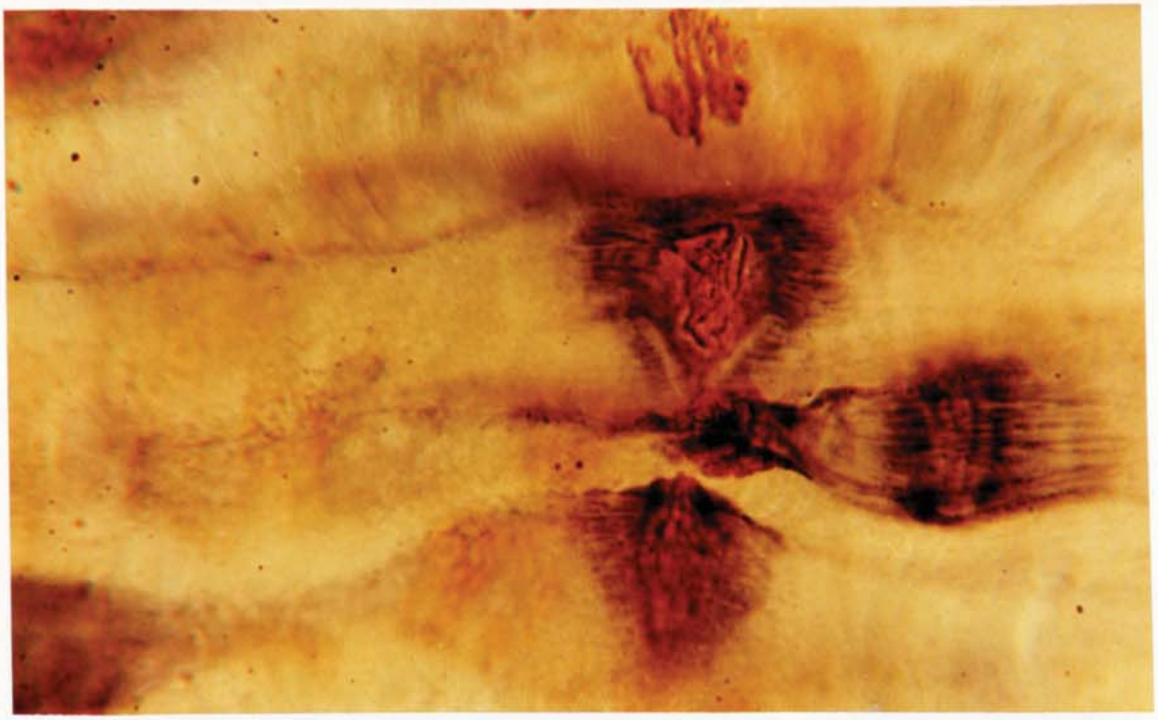
Plate 5.3.8: Photos of hemidiaphragms dissected 30 mins after 500 n mol Kg^{-1} ECO in vivo, note the association of formazan with various stages of myopathy. Unsectioned preparations. Vital staining in NBT, fixed and then stained for ChE (end plates and formazan deposits are represented by patches of brown and blue stain respectively). Nomarski optics. Calibration 100 µm.

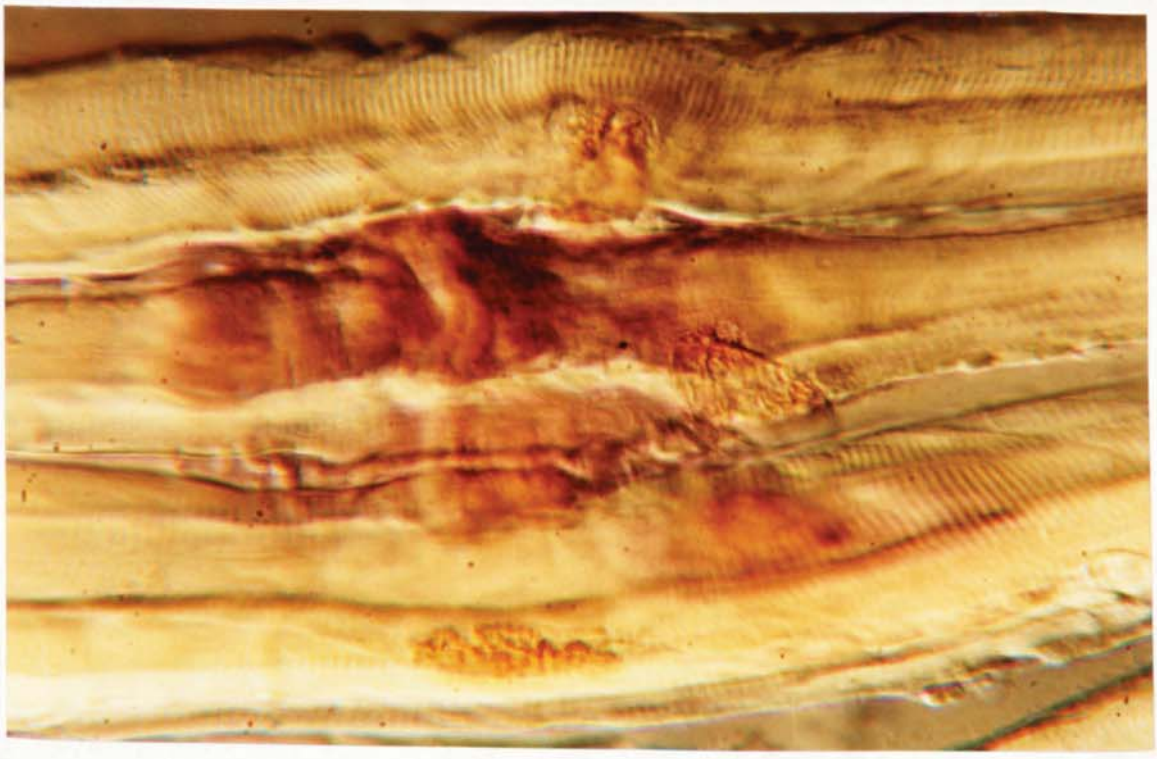
Top: Note misalignment of cross striations due to subsynaptic hypercontractions associated with slight deposits of formazan.

Middle: Note severe distortion of end plates associated with intense deposits of formazan.

Bottom: Teased preparation of junctional region showing from the top, a fibre with subsynaptic hypercontraction, a fibre with disorganised and swollen end plate region with formazan deposits and a normal fibre.







clumps stained with formazan (Plate 5.3.8).

Three hours after non-necrotising dose of ECO (200 n mol Kg^{-1}) formazan deposits were clearly evident in close association with hypercontractions and distorted EP. Although occasional contraction clump stained with formazan were evident, similar in magnitude to that observed with 500 n mol Kg^{-1} ECO for 30 mins, but considerably less frequent then other time points.

Experiments with SOD were carried out to exclude O_2^- independent reduction of NBT. The formation of formazan after ECO exposure was considerably reduced in the presence of SOD (50 μ g ml^{-1}) indicating that the majority of NBT reduction to formazan was specifically mediated via O_2^- (see plate 5.3.1 - 5.3.7). In all cases, myopathic features were still present even though formazan deposits were considerably reduced.

It is possible that the observed production of formazan is a consequence of non-specific toxicity of NBT. This probably appears unlikely in view of the fact that atropinised hemidiaphragms showed no morphological changes when incubated in NBT (see plate 5.3.1). Furthermore, the concentration and incubation period used were determined from preliminary studies and are similar to those used for diagnostic purposes.

Classically, NBT staining was used for the localisation of oxidative enzymes in mitochondria (Novikoff et al, 1961). It is therefore more probable that ECO exposure enhances the entry of NBT which activates mitochondrial transmembrane dehydrogenase and/or promotes electron flow, thereby increase O_2^- generation and formazan production.

The fact that SOD is inaccessible to biological membranes and because of the charged nature of the NBT molecule and the fact that exogenous application of SOD was capable of reducing formazan formation strongly suggests that much of the formazan deposits were predominantly extracellular.

In order to ascertain whether formazan deposits were intracellular or extracellular, transverse sections of the junctional region and longitudinal section of muscle fibre were prepared from hemidiaphragm treated with 500 n mol Kg^{-1} ECO. Inspection of the transverse sections of the junctional region revealed that most of the formazan granules were located within the cells though some were found to be extracellular (see Plates 5.3.9 and 5.3.10). Such findings suggest that O_2^- is generated intracellularly and both SOD and NBT were able to penetrate into the cellular environment perhaps as a

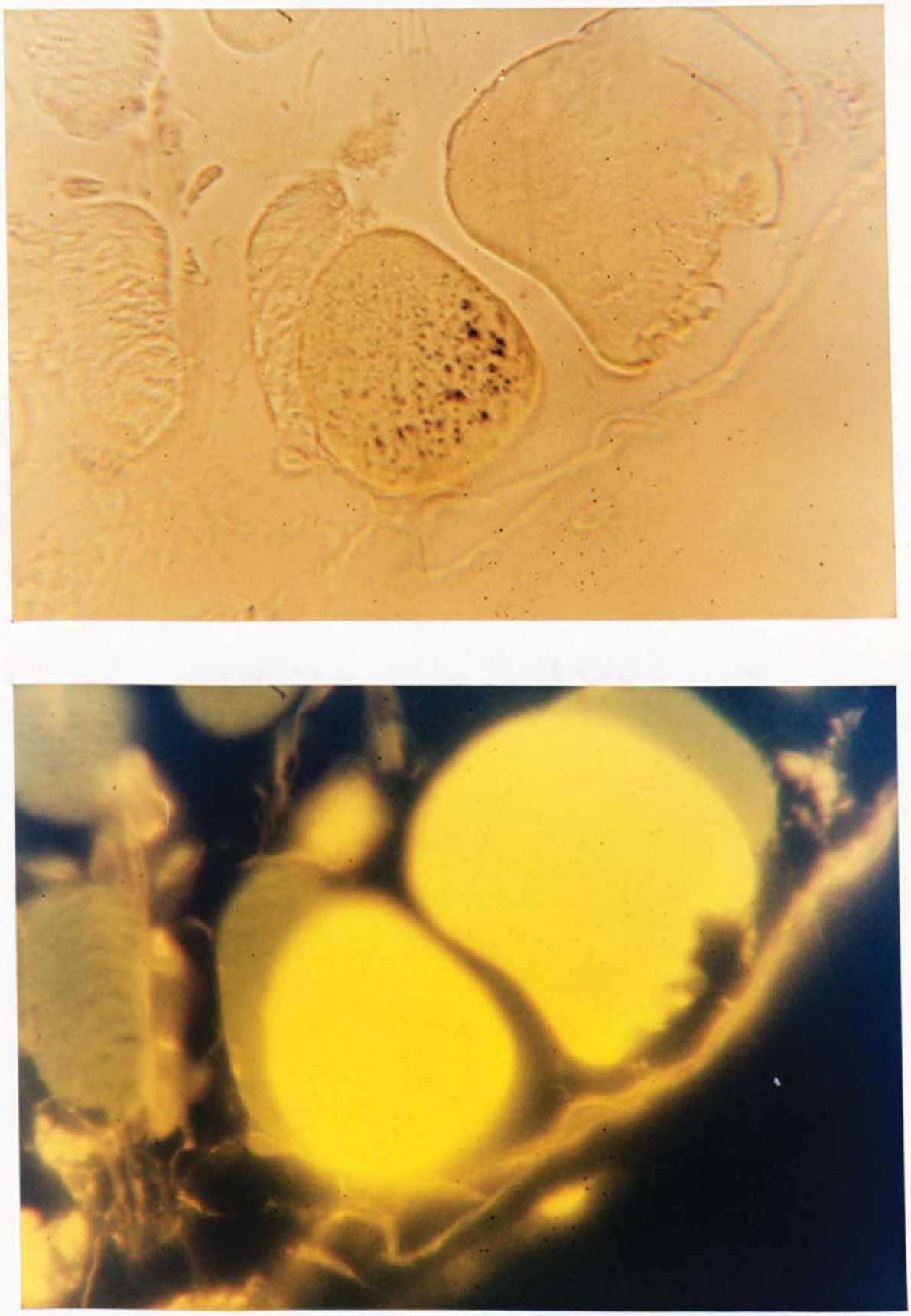
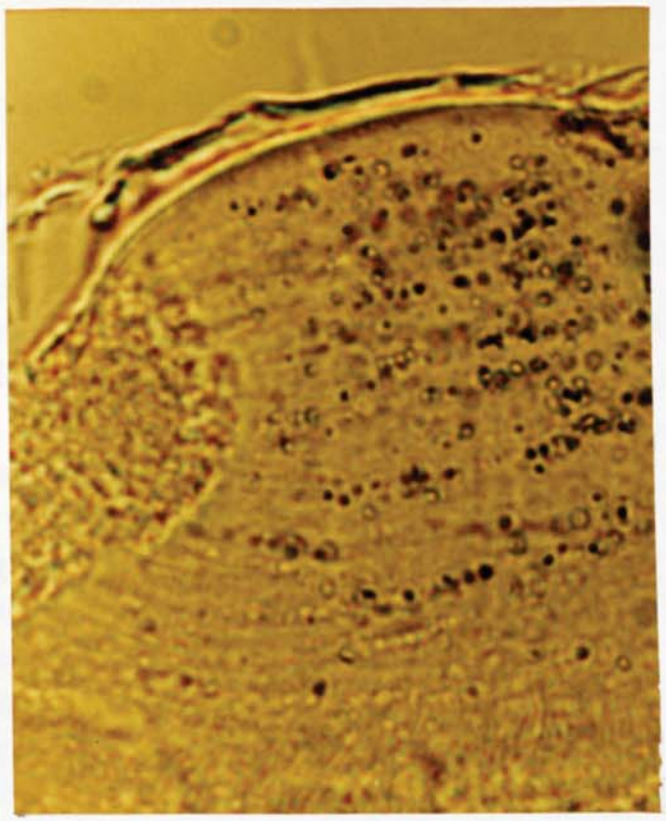


Plate 5.3.9: Transverse section of a hemidiaphragm of mouse exposed to 500 n mol Kg^{-1} ECO for 3 hours in vivo. Vital staining in NBT followed by procion staining and then fixed. Note the presence of formazan granules in only one of the procion stained fibre. Top: Tungsten illumination. Bottom: UV illumination. Calibration 10 µm.



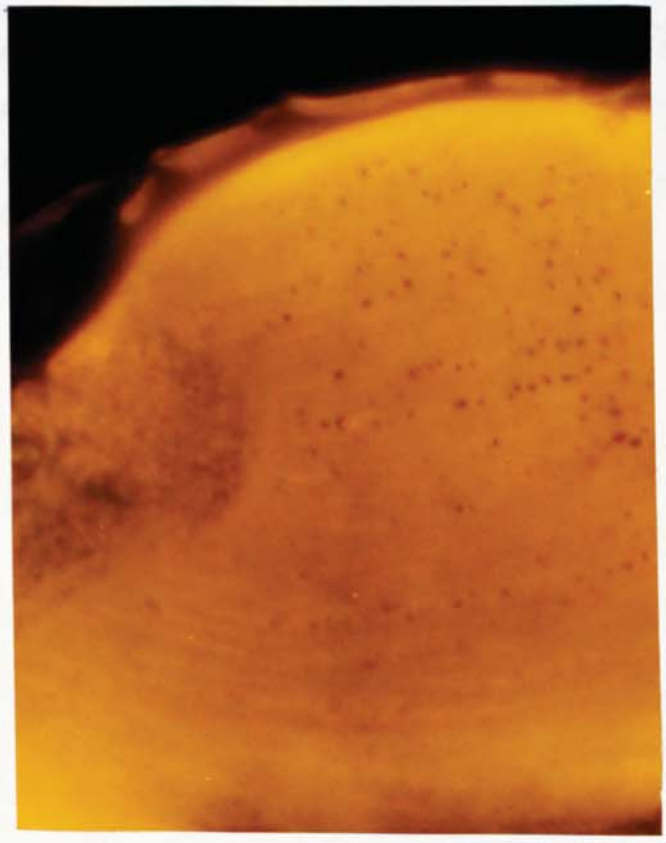


Plate 5.3.10: Same as plate 5.3.9 but longitudinal section. Note formazan granules in procion stained contraction clump and also the location of formazan granules, running parallel to the myofibrils.

consequence of membrane 'leakiness' induced by ECO exposure (see 4.7).

An additional interesting feature to emerge was the observation that some apparently normal fibres and some procioned stained fibres (necrotic fibres) contained formazan (plate 5.3.9). As these preparations were stained first in NBT and later stained with procion, the observations suggest a critical stage in the process of cell necrosis which involves formazan formation and that necrotic cells were not capable of reducing NBT (ie generating O_2^-). This is consistent with the preliminary observations that skeletal damage, induced mechanically by stabbing with a sharp needle, results in intense formazan deposits, providing staining in NBT is done within 15 minutes. These findings are in agreement with Labbe et al (1988) who demonstrated that 48 hours after ischemia, NBT staining could accurately differentiate between viable and necrotic skeletal muscle as determined by electron microscopic evaluation. Furthermore, when biopsies were taken well outside the area of NBT staining all biopsies contained irreversibly injured cells.

The qualitative result discussed above suggests that myopathy induced by a necrotising dose of ECO does involve the generation of O_2^-. Furthermore, because O_2^- was produced by a non-necrotising dose combined with the fact that O_2^- was present early on in the progress of necrotisation suggests that ECO-induced myopathy is partly mediated via oxidative damage.

6.0 TREATMENT OF ECOTHIOPATE-INDUCED MYOPATHY

6.1 Prevention of ECO-induced myopathy by preventing entry of calcium.

6.1.1 Introduction :

The investigation in section 5.1 has clearly established that cellular necrosis induced by ECO at the junctional region of the diaphragm was preceded and accompanied by an influx of Ca^{2+} from the extracellular fluid. Furthermore, evidence was also provided which suggested that the rate of Ca^{2+} entry could govern the extent of cellular necrosis. An elevation in intracellular calcium has been implicated as a causative agent in many experimental and pathological conditions (see 1.5 and 5.1.1), including those induced by OP agents (Leonard and Salpeter, 1979; Toth et al, 1981; Townsend, 1988) and it is suspected that the elevated $[Ca^{2+}]_i$ induced by ECO, is responsible for the subsequent development of myopathy.

The major route for calcium entry into the junctional region is probably through the end plate channels during prolongation and intensification of transmitter action (see 5.1.3) (Evans, 1974; Leonard and Salpeter, 1979) with the possibility that non-quantal release of ACh provides a major contributing source of transmitter action. It has been suggested that Ca^{2+} entry through voltage-dependent Ca^{2+} channels (see 5.1.3) is probably insignificant. However, until recently, it was thought that skeletal muscle Ca^{2+} channels open so slowly upon fibre depolarisation that few channels would even open during an action potential. Now several reports have shown that skeletal muscle actually contains several types of Ca^{2+} channels (see 1.4.1.3) some of which are probably opened by action potentials. Thus in conditions where AChE inhibition causes the half life of transmitter action to be prolonged and intensified, Ca^{2+} entry through Ca^{2+} channels could be significant.

Thus one possible therapeutic intervention for ameliorating ECO-induced myopathy would be to use agents which prevent or reduce the rate of Ca^{2+} entry through biological membranes. Calcium antagonists are commonly used for the treatment of various cardiovascular disorders and there are reports that calcium antagonists reduce the loss of proteins and the ultrastructural damage during the calcium paradox (Baker and Hearse, 1983). In addition these compounds can prevent cardiac muscle necrosis in dystrophic hamsters (Jasmin and Slymoss, 1975) and experimentally induced ultrastructural damage by Bay K 8644.

Furthermore, Dettbarn (1984) reported significant reductions in the number of lesions by prior administration of gentamicin, a receptor blocker, to phospholine (ECO) intoxication.

The main objectives of this section are to evaluate the effectiveness of:

1) various 'receptor blockers' in ECO-induced myopathy?
2) aminoglycosides in ECO-induced myopathy?
3) Ca^{2+} antagonists in ECO-induced myopathy?
4) non-quantal release blockers in ECO-induced myopathy?

6.1.2 Experimental Design :

Mice were divided into 11 groups and received only one of the following drug regime :

Group 1 (control) : mice were injected with ECO (500 n mol kg^{-1}).

Group 2 : mice were injected with mecamylamine (5.5 mg kg^{-1}) a nicotinic ganglion blocker (Bissada et al, 1978) and end plate blockers.

Group 3 : mice were injected with disopyramide phosphate (2.14-4.28 mg kg^{-1}), an antiarrhythmic drug, which has been shown to block open end plate channels (Harvey et al, 1984).

Group 4 : mice were injected with procainamide (3.0 - 6.0 mg kg^{-1}), an antifibrillatory drug, and an open end plate blocker (Galzigna et al, 1972).

Group 5 : mice were injected with netilmicin sulphate (15mg kg^{-1}), an aminoglycoside antibiotic, which has been shown to produce neuromuscular block with a predominant post-synaptic effect (Cuputy et al, 1980).

Group 6 : mice were injected with netilmicin sulphate (30 mg kg^{-1}).

Group 7 : mice were injected with gentamicin sulphate (20 $mgkg^{-1}$), an aminoglycoside antibiotic, which has been shown to produce

neuromuscular block with a predominant pre-synaptic effect (Cuputy et al, 1980) and reported to reduce phospholine (ECO) induced necrosis (Dettbarn, 1984).

Group 8 : mice were injected with verapamil (1.14 - 3.42 mg kg^{-1}), an antiarrhythmic drug, which has been shown to block end plate channels (Wachtel, 1987) and also to reduce cardiac necrosis in dogs (Reimer et al, 1977).

Group 9 : mice were injected with diltiazem (1.71 - 3.42 mg kg^{-1}), used in the treatment of supraventricular arrhythmias and ischemia and also has been shown to block end plate channels (Miledi and Parker, 1981; Wachtel, 1987).

Group 10 : mice were injected with quinacrine (5 mg kg^{-1}), an antimalarial agent, which has been reported to reduce non-quantal release of ACh by 42% (Edwards et al , 1985).

Group 11 : mice were injected with AH1583 (1 mg kg^{-1}) which has been reported to reduce non-quantal release of ACh by 50% (Edwards et al, 1985).

With the exception of group 1, in all the other cases, 15 mins after the administration of the drug in question, each mouse was injected with 500 n mol Kg^{-1} ECO. All injections were administered subcutaneously between the shoulder blades and all drugs were made in saline. The doses administered were equivalent to or 3 x greater than that recommended for use clinically and were corrected for body weight unless otherwise stated.

3 hours after ECO administration mice were anaesthetised and blood samples from the femoral artery were obtained for analyses of serum CK activity (2.4.2). Mice were killed while still anaesthetised and diaphragm removed. One hemidiaphragm was assayed for Ca^{2+} accumulation (2.4.3) and the other was assayed for myopathy using the procion technique (2.3.3).

6.1.3 Results and Discussions :

A single exposure to any one of these drugs alone for 3 hours in vivo, in the doses stated resulted in no obvious behavioural or morphological alterations as compared to atropine controls. Furthermore, prior administration with any of these drugs did not result in modification or prevention of ECO-induced behavioural changes. The time to fasiculation and the extent and severity of behavioural changes were indistinguishable from those treated with ECO alone (results not shown).

6.1.1.3 The effectiveness of various membrane ion channel blockers on the treatment of ECO-induced myopathy :

The effect of various open-ion channel blockers on the prophylactic treatment of ECO-induced myopathy is presented in table 6.1.1.

None of these agents were able to significantly reduce the extent of junctional calcium accumulation and extensive myopathy as indicated by the extent of procion yellow staining nor the elevation in serum CK activity. Although mecamylamine significantly reduced CK activity the value is still indicative of extensive irreversible myopathy (see 4.2.6).

The lack of protection afforded by disopyramide is perhaps the most surprising in view of the fact that in addition to its open-ion channel block (Harvey et al, 1984) at NMJ (Healy et al, 1981) and autonomic ganglia (Byrne et al, 1981), it possesses local anesthetic action, as active as lignocaine (Baines et al, 1976). This additional action was considered to be beneficial to the effectiveness of disopyramide.

The ineffectiveness of these agents possibly suggests the lack of adequate concentration of these agents in the NMJ milieu or alternatively, significant Ca^{2+} entry occurs via routes other than receptor operated channel, perhaps through voltage-dependent Ca^{2+} channels.

Neuromuscular blockade and related respiratory depression are well known side effects of aminoglycoside antibiotics. Most of the antibiotics that block NMT have both pre- and post-synaptic effects (Cuputy et al,1980; Fiekers, 1982; 1983), the relative contributions of these effects to total blocking action varies among the drugs. Dettbarn (1984) reported the protective effect of gentamicin on phospholine (ECO) induced myopathy in rats. The mechanism of protection was attributed to be due to a decrease in evoked transmitter output. The effectiveness of netilmicin and gentamicin were therefore investigated.

Treatment	% Procion staining J	NJ	Serum CK UL-1	Ca^{2+}Accumulation at J (n mol mg-1)
ECO	18.22	2.02	3910	2.07
(500 n mol kg^{-1})	± 8.17	± 2.74	± 1720	± 0.80
N	23	23	32	25
Mecamylamine	15.07	0.86	1870*	2.49
(5.5 mg kg^{-1})+ECO	± 4.58	± 0.82	±535	± 1.22
N	6	6	6	6
Disopyramide	19.59	2.59	3270	1.40
(2.14-4.28mg kg^{-1})	±9.43	± 0.97	±2190	± 0.76
+ ECO, N	6	6	6	6
Procainamide	15.53	2.11	5020	1.82
(3.0-6.0 mg kg^{-1})	±7.39	± 1.11	±4180	± 0.80
+ ECO, N	6	6	6	6

Table 6.1.1: The effectiveness of various end plate channel blockers on the prophylactic treatment of ECO-induced myopathy. Values are mean ± 1SD, where N = number of hemidiaphragms. * denotes significant difference from those of ECO group at 5% level.

It was suggested by E. Harpur (Personal Communication) that the doses for aminoglycosides in mice relate better to surface area than to body weight. Hence, the dose yielding effective therapeutic levels in blood of mice is about 5 times that recommended for a human. Hence, netilmicin at 2 times and 4 times while gentamicin at 4 times the therapeutic dose for man were employed in this study. The effect of these aminoglycoside doses on the treatment of ECO-induced myopathy is shown in table 6.1.2.

In agreement with the previous results, neither of these two agents, even at 4 times the therapeutic dose for man, were able to significantly

reduce either junctional Ca^{2+} accumulation or the extensive myopathy, nor the elevation in serum CK, findings which are directly contradictory to Dettbarn (1984). The reason for this discrepancy is unknown, although there are several differences in the studies that could account for this. Amongst other possibilities, it is possible that the protection at 24 hours post-injection reported by Dettbarn could be due to gentamicin aiding the regeneration process.

Treatment	% Procion staining		Serum CK	Ca^{2+} Accumulation
	J	NJ	UL-1	at J (n mol mg-1)
ECO	18.22	2.02	3910	2.07
(500n mol kg^{-1})	± 8.17	± 2.74	± 1720	± 0.80
N	23	23	32	25
Netilmicin	13.17	1.56	5530	1.48
(15 mg kg^{-1})+ECO	± 6.43	± 1.77	± 3870	± 0.41
N	5	5	5	5
Netilmicin	17.01	0.59	5410	2.53
(30 mg kg^{-1})+ECO	±6.00	± 0.40	± 4340	± 1.13
N	6	6	6	6
Gentamicin	21.27	2.40	5080	2.76
(20 mg kg^{-1})+ECO	±4.73	± 3.47	±2980	± 1.04
N	6	6	6	6

Table 6.1.2: The effectiveness of various aminoglycoside antibiotics on the prophylactic treatment of ECO-induced myopathy. Values are mean ± 1 SD, where N = no. of hemidiaphragms.

6.1.3.2 The effectiveness of various organic calcium antagonists on the treatment of ECO-induced myopathy :

The combined results of the previous two groups of receptor blockers strongly suggest that significant entry of Ca^{2+} occurs via Ca^{2+} channels.

The organic 'Ca^{2+} antagonists' have been found to inhibit a wide variety of membrane related processes, including end plate sensitivity to ACh, K-conductance and blocking of Na^{+} channels (Triggle, 1981). Verapamil and diltiazem, not only block the potential-dependent inward Ca^{2+} current (Triggle, 1981) but also block the 'fast' Na-channel, by decreasing channel lifetime and by reducing the frequency of channel activation, through which Ca^{2+} can also enter the cell (Miledi and Parker, 1981; Wachtel, 1987). The combined properties of these agents were considered beneficial to the effective treatment of ECO-induced myopathy.

The effects of these organic Ca^{2+} antagonists on the treatment of ECO-induced myopathy are shown in table 6.1.3.

The reduction in junctional calcium accumulation by verapamil was just not significant while that of diltiazem was highly significant. In contrast, the extent of myopathy as indicated by procion yellow staining and elevation of creatine kinase activity of serum were similar for the two agents but were not significantly different from ECO (control). The reduction in Ca^{2+} influx appears to be due to antagonism through Ca^{2+} channels rather than end plate channels.

Because Ca^{2+} influx was not prevented, it is possible that this small elevation of $[Ca^{2+}]_i$ is sufficient to initiate the development of a severe myopathy by the end of 3 hour post-injection period. Alternatively, or in addition, there may have been a Ca^{2+} induced release of Ca^{2+} (CROC) (Ford and Podolski, 1970; Endo, 1977; Duncan and Smith, 1980; Fabiato, 1982) and or depolarisation induced release of Ca^{2+} from SR (Endo, 1977) due to the prolongation of ACh half-life at NMJ. Another possibility is the release of Ca^{2+} from mitochondria, in response to an elevation of intracellular Na^{+} as a consequence of prolonged ACh action. Elevation in $[Ca^{2+}]_i$ by any one or a combination of these mechanisms would not be revealed by the Ca^{2+} technique employed in this study.

Treatment	% Procion staining		Serum CK	Ca^{2+} Accumulation
	J	NJ	UL-1	at J (n mol mg-1)
ECO	18.22	2.02	3910	2.07
(500 n mol kg^{-1})	± 8.17	± 2.74	± 1719	± 0.80
N	23	23	32	25
Verapamil	16.65	1.80	4700	0.97
(1.14-3.42 mg kg^{-1})	± 8.82	± 1.03	±2790	± 1.66
+ ECO, N	6	6	6	5
Diltiazem	16.39	1.63	3760	0.59***
(1.71-3.42 mg kg^{-1})	± 9.46	± 1.26	±2570	± 0.22
+ ECO, N	6	6	6	6

Table 6.1.3: The effectiveness of various 'organic' calcium antagonists on the prophylactic treatment of ECO-induced myopathy. Values are mean ± 1 SD, where N = no. of hemidiaphragms. *** denotes significant difference from those of ECO group at 0.2% level.

6.1.3.3 The effectiveness of various putative blockers of non-quantal ACh release on ECO-induced myopathy:

It was suggested in section 5.1.3 that non-quantal release of ACh could provide an important source of agonist action which could play an important role in the genesis or the extent of ECO-induced myopathy. The drug AH5183 and quinacrine were reported to decrease non-quantal ACh release by at least 50% and 42% respectively (Edwards et al,1985). The effects of these putative non-quantal release blockers on the treatment of ECO-induced myopathy are tabulated in 6.1.4.

Treatment	% Procion staining		Serum CK	Ca^{2+} Accumulation
	J	NJ	UL-1	at J (n mol mg-1)
ECO	18.22	2.02	3910	2.07
(500 n mol kg^{-1})	± 8.17	± 2.74	± 1720	± 0.80
N	23	23	32	25
Quinacrine	11.48	1.43	4550	3.60*
(5 mg kg^{-1})+ ECO	± 2.85	± 2.16	±2480	± 0.68
N	5	5	5	5
AH5183	18.86	0.91	1490*	3.30
(1 mg kg^{-1})+ECO	± 8.88	± 1.01	± 376	± 1.23
N	3	3	3	3

Table 6.1.4: The effectiveness of various putative non-quantal ACh release blockers on the prophylactic treatment of ECO-induced myopathy. Values are mean ± 1 SD, where N = no. of hemidiaphragms. * denotes significant difference from those of ECO group at 5% level.

In common with the previous groups of drugs, these two putative agents did not significantly reduce myopathy nor calcium influx at the junctional region, in fact, quinacrine significantly increased Ca^{2+} influx. Furthermore, in the doses used AH5183 was reported to have no effect on either MEPP or EPP amplitudes or quantum content (Ferry and Kelly, 1988).

The apparent decline in procion staining by quinacrine, though not significant, may not be related to inhibition of non-quantal release, but may be due to its free radical scavenging action (see 8.5).

6.2 Prevention of ECO-induced myopathy by quenching free-radicals.

6.2.1 Introduction :

The investigation in section 5.3 has qualitatively shown that cellular necrosis induced by ECO at the junctional region of the diaphragm was preceded and accompanied by the generation of superoxide as detected by vital staining with NBT in combination with SOD.

As a consequence of the potential threat from oxidative damage to the integrity of the cell, aerobic cells have evolved a complex multilevel control system to minimise free-radical related tissue damage. SOD, catalase and glutathione peroxidase are the primary cytosolic enzymatic defence systems whose collective function is to prevent free-radical multiplication and minimise secondary oxidation of lipid hydroperoxides. Vit E is the most efficient natural non-enzymatic, membrane-associated chain-breaking phenolic antioxidant, which functions as a trap for lipid peroxyl (LOO·) and other radicals, effectively inhibiting the peroxidation of cellular membranes (Witting, 1980; Tappel, 1980; Flohe, 1982; Sies, 1985).

Oxidative damage has been implicated in many toxicities, deficiencies and pathological conditions (see 1.8). Furthermore, Vit E deficiency has been shown to enhance the acute toxicity of paraquat in rats (Block, 1979) and endogenous Vit E content of isolated rat hepatocytes exposed to toxic doses of either adriamycin, ethyl methanesulfonate, or the calcium ionophore A23187 has been shown to diminish prior to the expression of the cytotoxicity (Fariss et al, 1985).

Expression of oxidative damage may be as a consequence of overwhelming the antiperoxidation defences by the sheer number of radicals produced. Thus, perhaps a possible therapeutic intervention for alleviating ECO-induced myopathy would be to use agents which prevent or reduce the rate of the radical generation. The ability of Vit E to protect membrane lipids from peroxidative damage has been shown in studies using model membrane systems, subcellular fractions, intact cells, cultured cells and animals (Sandy et al, 1988; Tappel, 1980; Witting, 1980). In recent years considerable evidence has been accumulated suggesting that reperfusion induced injury and arrhythmias are mediated by free-radicals originating primarily during the catalytic cycle of xanthine oxidase. Furthermore, the extent of myocardial necrosis and arrhythmias can be significantly reduced by treatments with various exogenous

antioxidants alone or in combination, which have great medical and clinical implications (Hearse et al, 1986; Gauduel and Duvelleroy, 1984; Chambers et al, 1985; Aoki et al, 1988; Woodward and Zakaria, 1985; Walker et al, 1987; Rao at al, 1983).

The main objectives of this section are to evaluate the effectiveness of :

1) oral pretreatment with Vit E in ECO-induced myopathy?
2) oral pretreatment with N-acetylcysteine (NAC) in ECO induced myopathy?
3) combined oral pretreatment of Vit E and NAC in ECO-induced myopathy?

6.2.2 Experimental design :

7 groups of mice were orally pretreated daily with a specially prepared stainless steel intubation tube for 7 days in the following manner:

Group 1: mice were dosed with water only.

Group 2: mice were dosed with pure corn oil (Mazola) only.

Group 3: mice were dosed with Vit E (dl-α-tocopherol) (500 mg Kg^{-1}) in corn oil. Vit E has been shown to protect hepatocytes against toxic injury (Fariss et al, 1985; Tappel, 1980).

Group 4: mice were dosed with N-acetylcysteine (NAC) (1.0 g Kg^{-1}) (Parvolex). NAC is believed to increase cellular glutathione levels (Lauterburg et al, 1983).

Group 5: mice were dosed with Vit E (500 mg Kg^{-1}) in corn oil and NAC (1.0 g Kg^{-1}) (Parvolex).

Group 6: mice were dosed with acacia gum emulsion alone.

Group 7: mice were dosed with Vit E (500 mg Kg^{-1}) in acacia gum emulsion.

Mice were dosed with 0.1ml of the agent per 20g weight every morning at approximately 09.00 hours and in group 5, an additional dose of NAC was given at 16.00 hours. On the seventh day, 1 hour after the antioxidant, mice were treated with 500 n mol Kg^{-1} ECO and 3 hours later mice were anaesthetised. Blood samples were removed from femoral artery for analysis of blood AChE (see 2.4.1). Mice were killed while still anaesthetised and diaphragms removed. One hemidiaphragm was assayed for diaphragm AChE (see 2.4.1) and the other was assayed for myopathy using the procion technique (see 2.3.3).

6.2.3 Results and Discussions:

Exposure to the various pretreatment regime alone in vivo , in the doses stated, resulted in no obvious behavioural or morphological alterations as compared to atropine controls. Furthermore, pretreatment with various antioxidants for a week did not result in modification or prevention of ECO-induced behavioural changes. The time to fasciculation, that being 15-20 mins and the extent and severity of behavioural changes were indistinguishable from those treated with water + ECO (Results not shown).

The effect of Vit E and NAC alone on ECO-induced myopathy is presented in table 6.2.1 and illustrated in fig 6.2.2.

Pretreatment with these antioxidants alone resulted in significant reduction in the extent of procion yellow staining at the junctional region of the diaphragm. Morphological inspection of these preparations revealed considerable reduction in the number and size of contraction clumps as compared to pretreated water + ECO controls (see plates 6.2.3 and 6.2.4). However, the deformations of EP shape as well as the appearance of hypercontractions were present which were indistinguishable from those of pretreated water + ECO controls.

It is possible that the pretreatment regime in some way affected the mechanism of AChE inhibition and therefore a critical level of AChE inhibition was not attained. This appears unlikely since blood and diaphragm AChE were critically inhibited, in the range associated with a dose of 500 n mol Kg^{-1} ECO (see table 4.10) and extensive myopathy at the junctional region (table 6.2.1). Furthermore, morphological observations of EP shapes together with the behavioural observations are clearly indicative of a critical level of AChE inhibition that can be associated with a dose of 500 n mol Kg^{-1}. Thus, the pretreatment regime did not effect the primary action of ECO as an antiChE.

Treatment	% Procion Staining		AChE Activity	
	J	NJ	Blood	Diaphragm
Water	17.64	0.16	0.23 (75.7)	0.14 (90.4)
+ ECO	± 4.37	± 0.15	± 0.07	± 0.03
N	13	13	9	4
Corn oil	11.86*	0.13	0.27 (72.1)	0.11 (92.2)
+ ECO	± 7.04	± 0.12	± 0.08	± 0.04
N	12	12	5	4
Vit E(Corn oil)	2.60***	0.13	0.23 (75.7)	0.14 (90.1)
+ ECO	± 2.12	± 0.14	± 0.06	± 0.01
N	7	7	5	4
NAC	6.19***	0.15	0.29 (70.3)	0.15 (89.2)
+ ECO	± 3.53	± 0.10	± 0.05	± 0.03
N	10	10	8	8

Table 6.2.1 : Effect of Vit E in corn oil and N-acetylcysteine (NAC) on the prophylactic treatment of ECO-induced myopathy. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively (with % inhibition in parenthesis). Values are mean ± 1SD, where N = no. of hemidiaphragms. *, *** denotes significant difference from those of water + ECO group at 5% and 0.2% levels respectively.

The role of Vit E as the most active and effective natural antioxidant in biological systems is no longer questioned (Witting, 1980; Tappel, 1980; Sies, 1985). α-tocopherol, particularly dl-α-tocopherol is the most biologically active and abundant because of superior absorption and retention of the four (α,β,δ and γ) tocopherol compounds collectively referred to as Vit E. Vit E is found almost totally in association with membrane lipids, particularly those of the endoplasmic reticulum (33%)

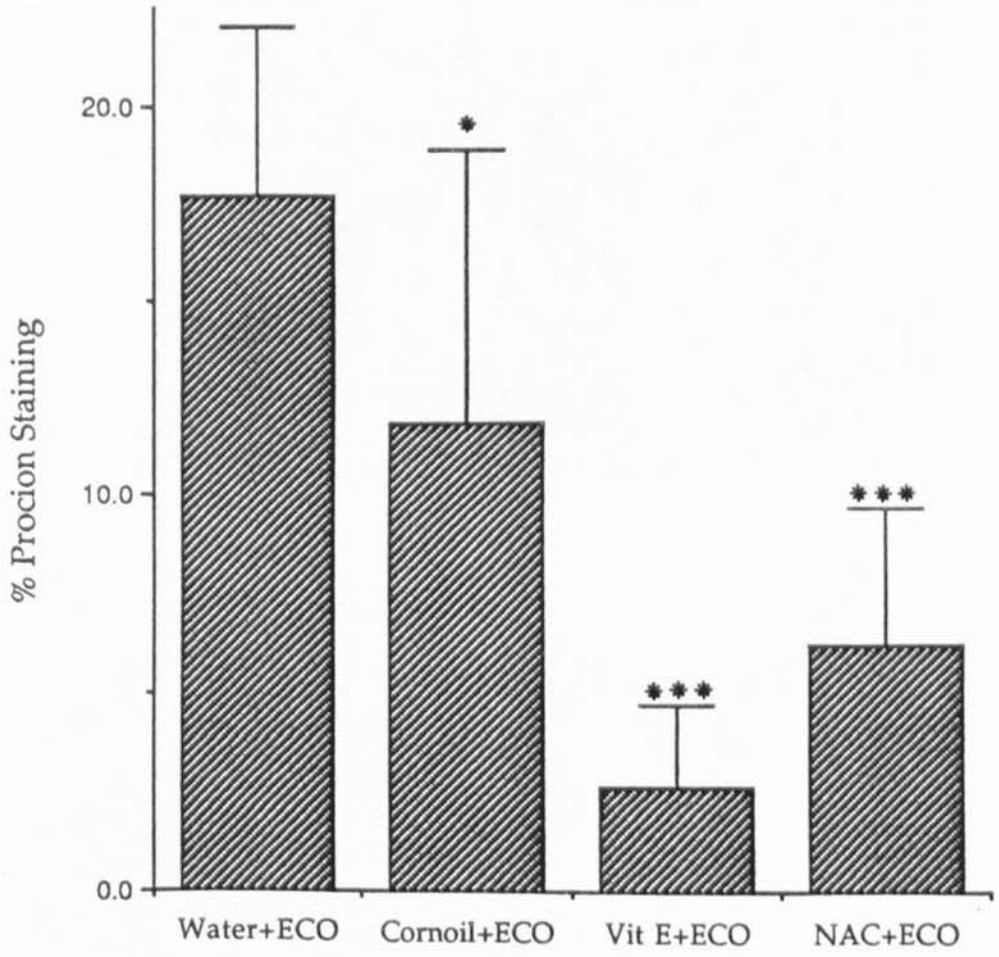


Figure 6.2.2 The effect of Vit E in cornoil and N-acetylcysteine (NAC) on the prophylactic treatment of ECO induced myopathy. Values are mean ± 1SD. *, *** denotes significant difference from those of water+ ECO group at 5% and 0.2% levels respectively.

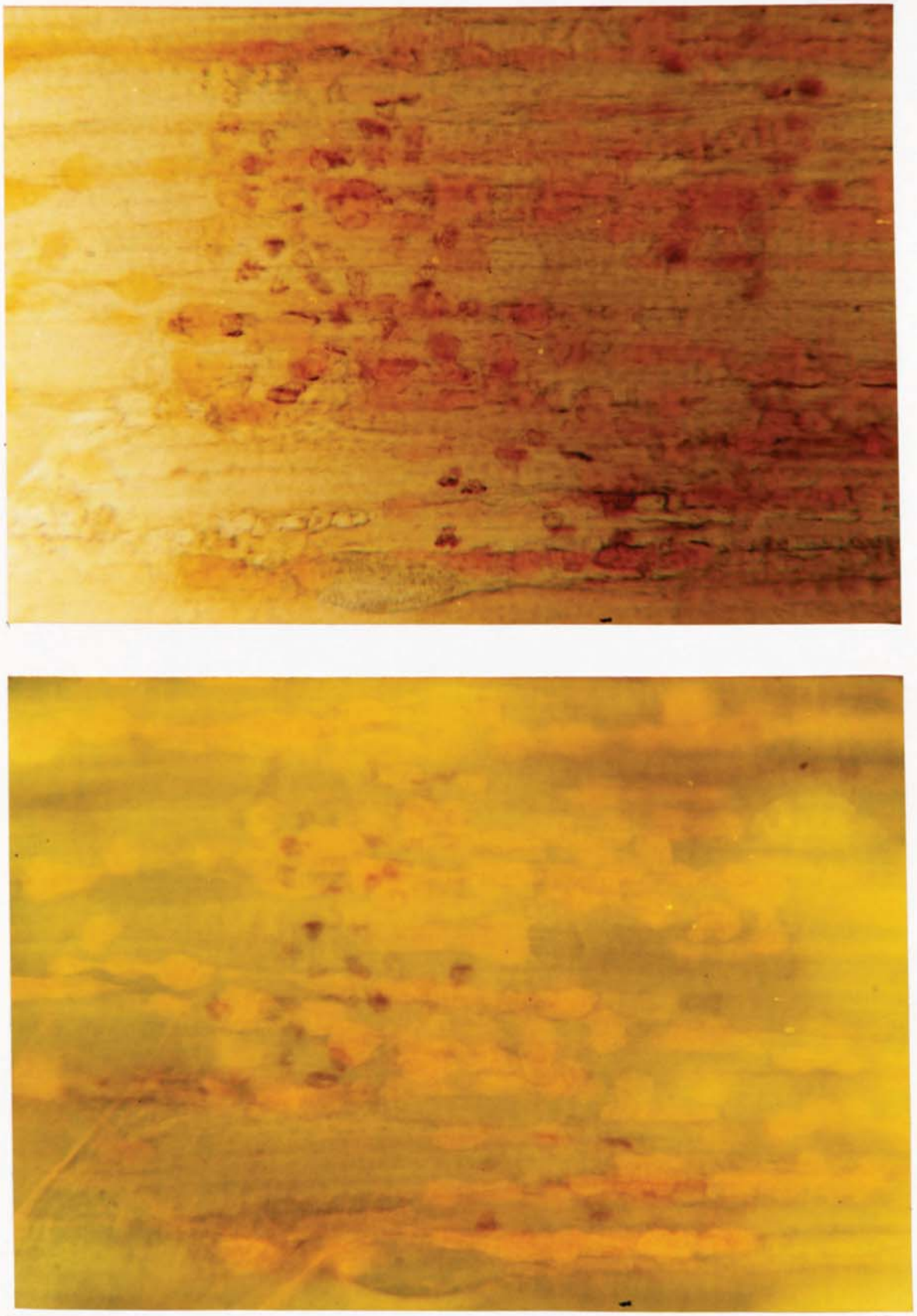


Plate 6.2. 3 : Photograph of whole diaphragm taken from mouse given 500 n mol Kg^{-1} ECO 3 hours previously. Pretreatment for 1 week with water prior to ECO. Vital staining in procion then fixed and stained for ChE (end plates are represented as brown patches). Note extensive procion yellow staining at the junctional region similar to that of plate 4.9. Top: Tungsten illumination. Bottom: UV illumination. Calibration 100 µm.

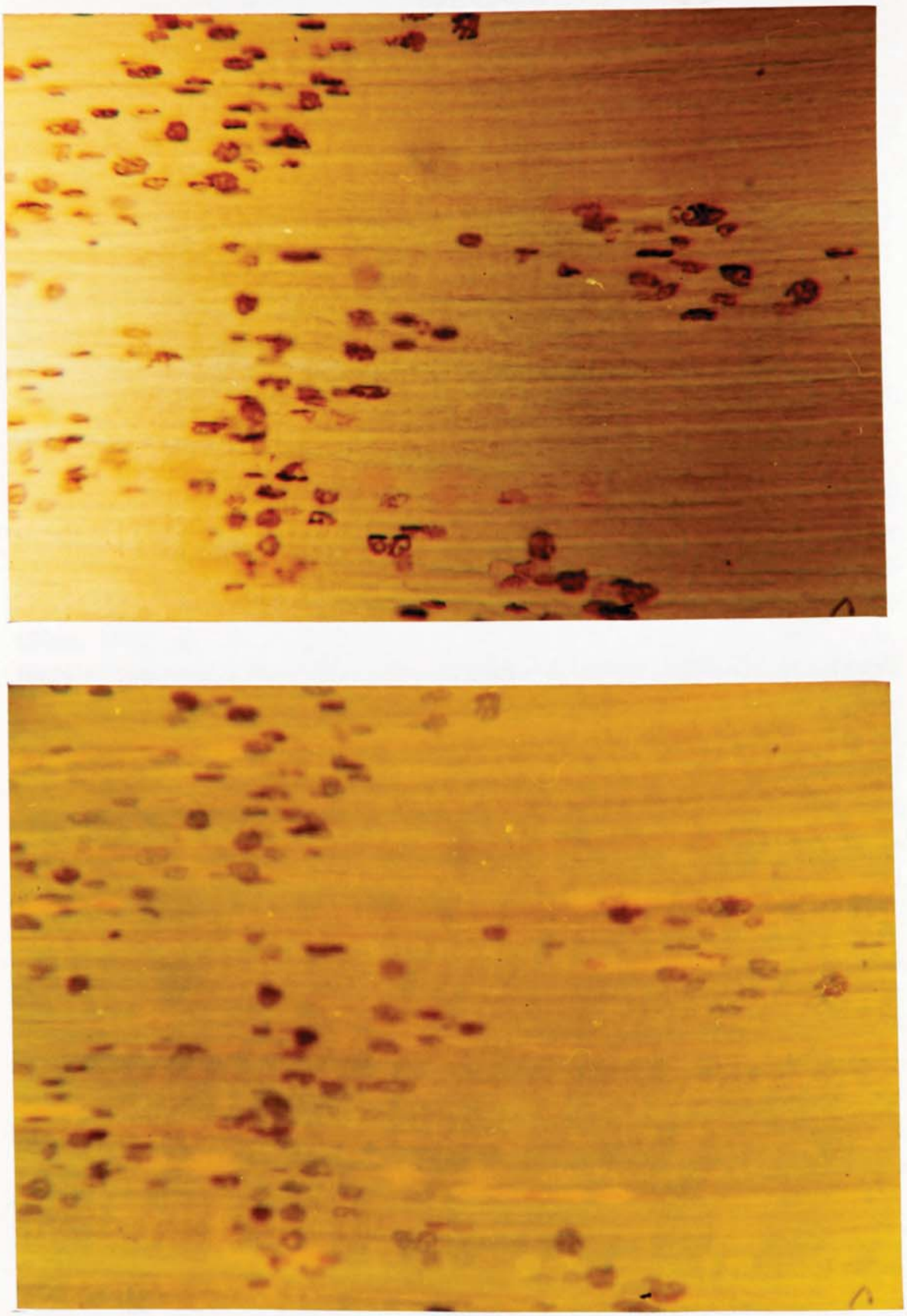


Plate 6.2.4 : Photograph of whole diaphragm taken from mouse given 500 n mol Kg^{-1} ECO 3 hours previously. Pretreatment for 1 week with vitamin E prior to challenge with ECO. Vital staining in procion then fixed and stained for ChE (end plates are represented as brown patches). Note significant reduction in procion yellow staining at the junctional region. Top: Tungsten illumination. Bottom: UV illumination. Calibration 100 μm.

and mitochondria (60%) (Casallany and Draper, 1960). The ratio of Vit E : PUFA within a membrane is an average 1:100 (Kornburst and Mavis, 1980). The reactivity of Vit E with peroxylipid radicals is of the order of 10^4 times that of the reaction of these radicals with PUFA's (Patterson, 1981). Hence Vit E will provide adequate protection against lipid peroxidation until it is consumed, although this could perhaps happen quite rapidly where large numbers of radicals are being produced. Vit E content of hepatocytes exposed to various toxins has been shown to diminish prior to the expression of cytotoxicity (Fariss et al, 1985). Therefore, the protection of ECO-induced myopathy by Vit E is perhaps due to saturation of membrane components such that adequate Vit E is maintained to cope with increased free radical generation. Furthermore, Mason (1942) has shown that Vit E content of many tissues including skeletal muscles (Bieri, 1972) can be varied over a 3 - 5 fold by feeding above minimum daily requirement. It is interesting to note that pretreatment with corn oil alone afforded significant protection (table 6.2.1). The mechanism involved in the protection by corn oil is unknown, however, since 70% of corn oil is PUFA's, then it is possible that these PUFA's react with free-radicals in preference to membrane PUFA, thus membrane PUFA would remain relatively unaffected. Alternatively, some component in the corn oil may behave as a weak antioxidant, in fact corn oil is suggested to contain about 1 mg g^{-1} of Vit E (Willis, 1985).

Vit E functions as a trap for lipid peroxyl (LOO·) and other radicals by acting as a hydrogen donor from the hydroxyl group on the aromatic ring, resulting in the formation of a chromanoxy radical, as suggested by Tappel (1962) and demonstrated some years later (Boguth and Niemann, 1971). A second hydrogen atom, from an unspecified location is also now thought to be available for donation (Simic, 1981). Free radicals are removed from the system by the formation of tocopherol quinone or a dimer (Boguth and Niemann, 1971).

The consequence of dietary Vit E deficiency have been extensively studied (Witting, 1980; Tappel, 1980; Barber and Bernheim,1967). Subcellular fractions from liver , kidney, heart, brain, adrenals, testes and muscle of Vit E deficient animals were all peroxidised in vitro, whereas their control animal counterparts were not (Tappel and Zalkin, 1959; Zalkin and Tappel, 1960). Increased dietary Vit E gave increased protection against in vitro lipid peroxidation (McCay et al, 1971). Furthermore, the activities of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase were significantly increased in the major

adipose tissue and muscle, but not liver, lung and kidney of rats fed on 15.7% tocopherol-stripped corn oil diet (Chow et al, 1973). Tissues that showed increased activities of the protective enzymes are well known as target organs of tocopherol deficiency-induced anomalies, such as ceriod pigment formation in adipose tissue, muscular dystrophy and testicular atrophy. How these enzymes are related to tocopherol deficiency symptoms and whether their increased activities are due to increased synthesis of enzyme or other mechanisms remain to be determined.

While cellular lipids are protected against oxidation by Vit E, to what extent Vit E protects other cellular constituents remains unclear. Oxidative damage in particular to protein sulfhydryl groups, may be more critical to cell viability than damage to cellular lipids.

Glutathione (GSH) is associated with certain aspects of normal structural and functional processes of many cells and in addition plays an indispensable role in the defence against oxidative damage caused by various agents and pathological conditions (Tappel, 1980; Kosower and Kosower, 1976; Witting , 1980; Flohe, 1982). The interior of living cells normally contains a substantial concentration of glutathione (1 - 50 X 10^{-4} M) and a much smaller amount of glutathione disulphide (GSSG) (6 - 200 X 10^{-6} M) (Kosower and Kosower, 1976). There may be substantial quantities of GSH (perhaps 30% of that found free) in the form of protein glutathione mixed disulphides. It is possible that the expression of ECO-induced myopathy is due to depletion of glutathione, consumed by free radical generation (Monte et al, 1984). Like Vit E, GSH is subject to dietary manipulation. The protection afforded by N-acetylcysteine (NAC), a precursor of GSH, is perhaps due to reversal of ECO-induced depletion of GSH, either by promoting synthesis or by maintaining adequate concentration of precursors (Lauterburg et al, 1983). In addition NAC may preferentially react with free radicals, thereby sparing important cellular constituents containing sulfhydryl groups. The results also do not preclude some unknown effect such as direct stabilizing action of sulfhydryl group on cell plasma membrane, an effect that might decrease cell permeability to sodium or calcium ions and hence a potential mechanism of protection.

Glutathione peroxide (GSH-PX) and glutathione-s-transferase (GSH-T) in conjunction with GSH represent part of the primary enzymatic anti-peroxidation defence system in mammalian cells, often referred to collectively as GSH redox cycle. The reaction mechanism for both these enzymes are quite different (Prohaska, 1980). GSH-PX is much more

efficient in removing small amounts of H_2O_2 than is catalase, since Km values for H_2O_2 are 1 μm (Flohe and Brand, 1969) and 1.1 M (Ogura, 1955) respectively. However, both use organic hydroperoxides as substrates (Wendel, 1981), thus reducing lipid hydroperoxides to stable lipids. The removal of hydroperoxides are two fold. Firstly, chain branching is minimised.

$$2\,LOOH + 2GSH \longrightarrow 2LOH + GSSG + H_2O$$

Secondly, branching via secondary initiations is also reduced and decomposition products such as the cytotoxic 4-hydroxyalkenals by secondary reactions, which react with GSH and proteins with essential SH groups, are also minimised (Benedetti, 1980, 1981).

In the course of their activities glutathione peroxides oxidise GSH to GSSG, oxidised glutathione can be reconverted back to its reduced form by the enzyme glutathione reductase.

$$NADPH + H^+ + GSSG \longrightarrow 2GSH + NADP^+$$

This enzyme is present in virtually all mammalian tissues. The activity of glutathione reductase means that for most mammalian cells the ratio of GSH : GSSG is around 100 : 1 (Wendel, 1970; Hazelton and Lang, 1980). The importance of glutathione reductase in maintaining the glutathione redox state has been most clearly demonstrated in cases of eyrthyrocyte glutathione reductase deficiency, which leads to increased membrane fragility and a variety of haematological diseases. Obviously an NADPH supply must be maintained to glutathione reductase activity. One of the major sources of NADPH is the enzyme glucose-6-phosphate dehydrogenase, at the head of the pentose phosphate pathway.

In view of the fact that both Vit E and NAC alone showed considerable protection and since both of these antioxidants seem to have different site(s) and mechanism of action, it was considered that a combined pretreatment could provide a better protection from ECO-induced myopathy. In order to evaluate this, mice were orally pretreated with Vit E and NAC as described in section 6.2.2 (group 5).

The effect of combined pretreatment with NAC and Vit E on ECO-induced myopathy is shown in table 6.2.5 and figure 6.2.6.

Treatment	% Procion Staining		AChE Activity	
	J	NJ	Blood	Diaphragm
Water	17.64	0.16	0.23 (75.7)	0.14 (90.4)
+ ECO	± 4.37	± 0.15	± 0.07	± 0.03
N	13	13	9	4
Vit E(Cornoil)	1.13***	0.11	0.28 (70.6)	0.15 (89.8)
+ NAC +ECO	± 0.66	± 0.10	± 0.07	± 0.05
N	7	7	6	5

Table 6.2.5 : Effect of combined treatment with Vit E in corn oil and N-acetylcysteine (NAC) on prophylactic treatment of ECO-induced myopathy. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively (% inhibition in parenthesis). Values are mean ± 1SD, where N = no. of hemidiaphragms. *** , denotes significant difference from those of water + ECO group at 0.2% level.

In agreement with previous findings, combined pretreatment significantly reduced procion yellow staining at the junctional region, despite critical levels of AChE inhibition in blood and diaphragm.

The synergistic protection was significantly better compared to NAC alone but not compared to Vit E alone. Because of the multilevel defence system (figure 6.2.7), this is perhaps not surprising, since better ultrastructural preservation and/or beneficial effects at cellular membrane by NAC would be masked by the actions of Vit E and will not be detected by the procion technique employed in this study.

Since corn oil on its own showed antioxidant effects and in order to further confirm and validate the protective role of Vit E in ECO-induced myopathy, experiments were done where Vit E was administered in a different vehicle to that of corn oil. In these experiments, Vit E was made up in acacia gum emulsion and orally pretreated as described before.

The effect of acacia alone and acacia + Vit E on ECO-induced myopathy is presented in table 6.2.8 and figure 6.2.9.

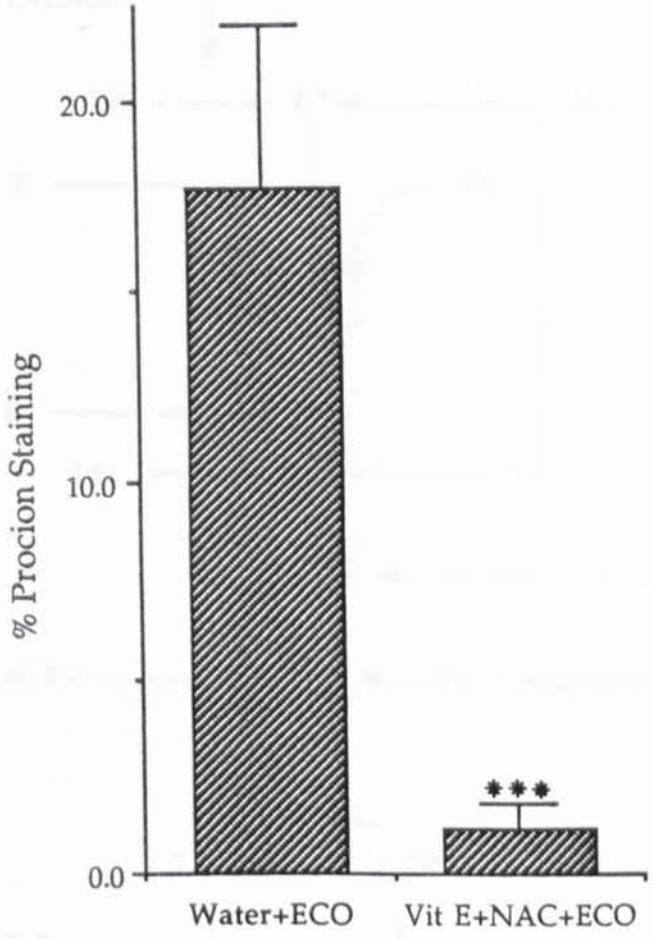


Figure 6.2.6 The effect of combined treatment with Vit E in cornoil and N-acetylctysteine (NAC) on the prophylactic treatment of ECO induced myopathy. Values are mean± 1SD.
*** denotes significant difference from those of water + ECO group at 0.2% level.

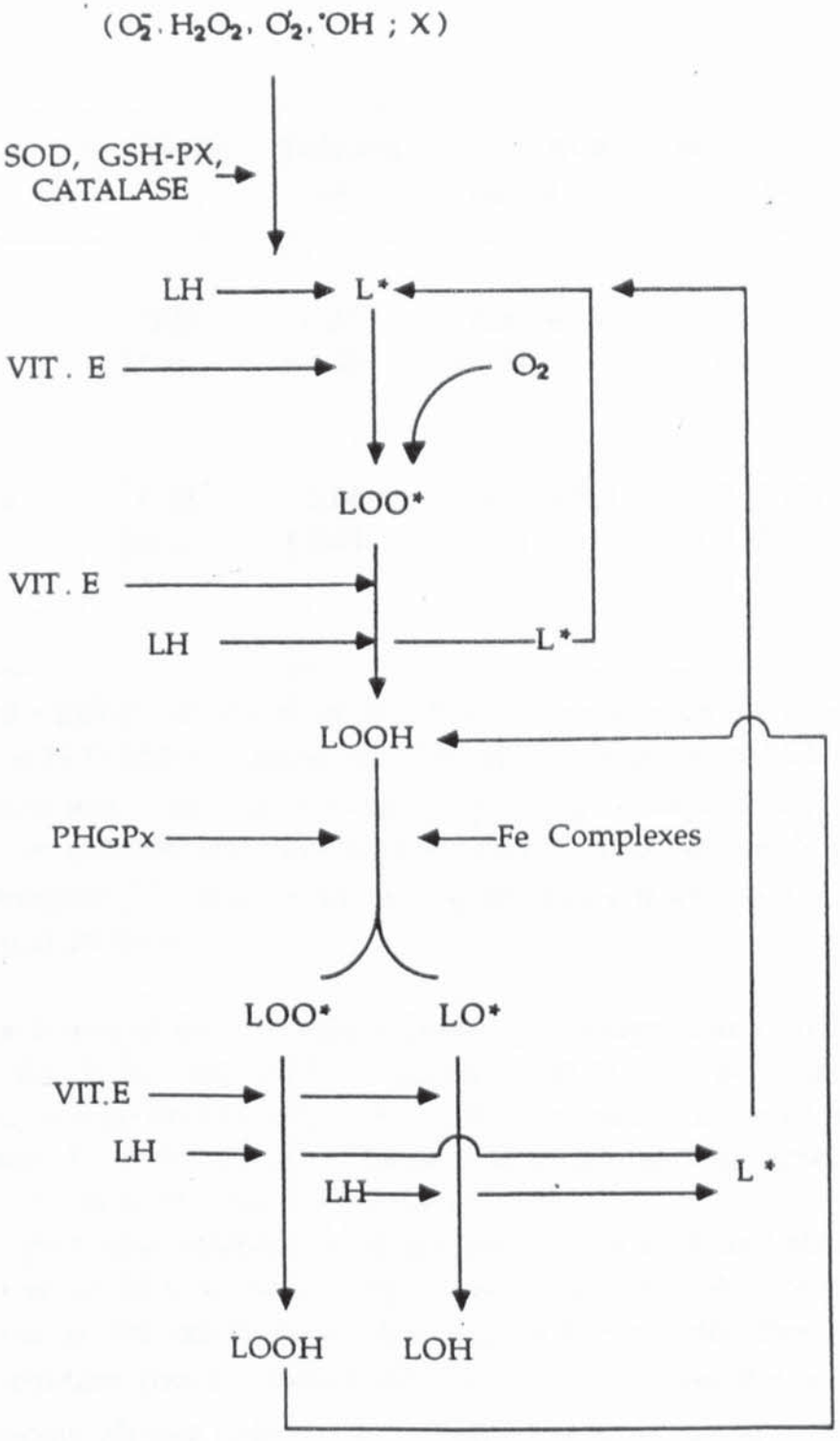


Figure 6.2.7 Scheme of the multilevel system against lipid peroxidation. SOD, superoxide dismutase, GSH-PX, glutathione peroxidase, PHGPx, phospholipid hydroperoxide glutathione peroxidase, L, polyunsaturated phospholipid fatty acid (Novelli and Ursini,1986).

Treatment	% Procion Staining		AChE Activity	
	J	NJ	Blood	Diaphragm
Acacia	17.85	0.03	0.30 (69.0)	0.19 (86.4)
+ ECO	±1.80	±0.04	±0.08	±0.01
N	7	7	7	7
Vit E(acacia)	6.03**	0.04	0.31 (67.5)	0.17 (88.2)
+ ECO	±0.27	±0.03	±0.04	±0.08
N	5	5	5	5

Table 6.2.8 : Effect of Vit E in acacia gum emulsion on the prophylactic treatment of ECO-induced myopathy. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively (with % inhibition in parenthesis). Values are mean ± 1SD, where N = no. of hemidiaphragms. ** , denotes significant difference from those of acacia + ECO group at 2% level.

The extent of procion yellow staining was significantly reduced for acacia + Vit E as compared to acacia + ECO control, thus further confirming the protective effect of Vit E. The extent of procion yellow staining and AChE inhibition of blood and diaphragm for acacia + ECO was similar to those of water + ECO (table 6.2.1).

The protection afforded by pretreatment with Vit E and NAC appear to be as a result of free radical suppression. However, the source of this free radical is yet unresolved. Amongst other possibilities (see 8.5), xanthine oxidase could produce significant O_2^{-}. In healthy tissues, this enzyme exists almost entirely as a dehydrogenase using NAD^+ as an electron acceptor which does not produce O_2^{-}. Under appropriate conditions, such as low O_2 tension, increase in $[Ca^{2+}]_i$ and/or proteolytic action, the enzyme is converted to an oxidase which uses molecular O_2 as the electron acceptor producing $O_2^{-}\cdot$.

Allopurinol a specific blocker of xanthine oxidase in both its dehydrogenase and oxidase form has been reported to reduce free radical

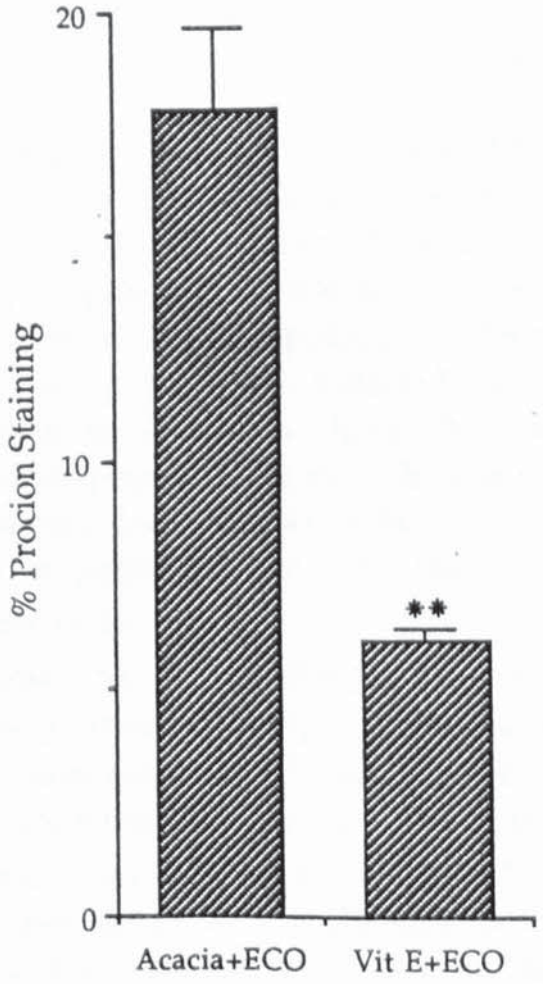


Figure 6.2.9 The effect of Vit E in acacia gum emulsion on the prophylactic treatment of ECO induced myopathy. Values are mean ± 1SD. ** denotes significant difference from those of acacia + ECO group at 2% level.

induced infarcts during myocardial ischemia (Chambers et al, 1985; Hearse et al, 1986). Mice were pretreated with 20 mg Kg^{-1} allopurinol for 5 days, in order to ascertain if xanthine oxidase could be a major source of free radical generation.

The effect of allopurinol on ECO-induced myopathy shown in table 6.2.10 and figure 6.2.11. Pretreatment with allopurinol resulted in significant reduction in the extent of procion yellow staining at the junctional region, despite critical levels of AChE inhibition in blood and diaphragm (see table 6.2.10). Morphological observations were similar to those described earlier for other antioxidants, the size of contraction clumps were much smaller than those encountered with ECO alone, perhaps hinting that spread of free radicals was retarded (see plate 6.2.12). The level of protection were similar to those obtained with NAC alone or Vit E in acacia, suggesting that xanthine oxidase could be a major contributing source of O_2^-.

A prerequisite for the involvement of the xanthine oxidase as a major contributing source of O_2- is the significant accumulation of precursors and substrates eg. inosine, hypoxanthine, xanthine from degradation of adenine nucleotide. The results with allopurinol is conflicting in view of the fact that exposure to 500 nmol Kg^{-1} ECO <u>in vivo</u> for 3 hours did not result in any changes in the levels of high energy phosphate compounds (see 5.2). It is possible that nucleotide breakdown did occur prior to cell death, in only those fibres that stained with procion and perhaps allopurinol exerted its effect in those fibres only. If this is the case, then small changes in adenine metabolites may not have been detectable by the H.P.L.C method employed in this study. Even if the changes in adenine metabolites were large, the failure to detect could be due to the fact that these metabolites are lipid soluble and are therefore freely permeable across biological membrane and hence may have leaked out into the extracellular space (Jennings and Hawkins, 1980; Chambers et al, 1985). Furthermore, in skeletal muscle AMP is deaminated by the action of adenylate deaminase to form inosine monophosphate (IMP), which then is dephosphorylated to form inosine whereas in cardiac muscle the critical reaction is the irreversible dephosphorylation of AMP to adenosine by the 5-nucleotidase of the sarcolemma in which case IMP would not be detected (Jennings and Hawkins, 1980; Jennings et al, 1985). The results of table 5.2.6 support the suggestion that the latter route of adenine breakdown plays the prominent part in skeletal muscle.

Treatment	% Procion Staining		AChE Activity	
	J	NJ	Blood	Diaphragm
Water	17.64	0.16	0.23 (75.7)	0.14 (90.4)
+ ECO	± 4.37	±0.15	± 0.07	± 0.03
N	13	13	9	4
Allopurinol	5.72***	0.17	0.31 (67.5)	0.13 (90.9)
+ ECO	±4.03	± 0.31	± 0.03	± 0.04
N	8	8	8	8

Table 6.2.10 : The prophylactic effectiveness of allopurinol on ECO-induced myopathy. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively (with % inhibition in parenthesis). Values are mean ± 1SD, where N = no. of hemidiaphragms. *** , denotes significant difference from those of water + ECO group at 0.2% level.

Other beneficial effects of allopurinol that are not related to its antioxidant effect might be the facilitation of the 'purine salvage pathway' and/or the direct stimulation of adenylate synthesis, actions which may improve the levels of adenine nucleotides pool.

ECO-induced myopathy was shown to be dependent upon extracellular calcium. Calcium entry via membrane leakiness, perhaps as a consequence of free radicals was suggested as a potential contributing factor in calcium mediated cellular necrosis.

As Vit E was shown to preserve the integrity of cellular membranes, experiments were done to ascertain whether better preservation is associated with a reduction in calcium accumulation and to determine the relative contribution in calcium accumulation from membrane leakiness.

Mice were pretreated with Vit E (500 mg Kg^{-1}) in corn oil as described in 6.2.2. Calcium accumulation at the junctional region was determined as described in section 2.4.3 for those pretreated with Vit E alone and those exposed to 500 n mol Kg^{-1} ECO.

Calcium accumulation at the junctional region was significantly

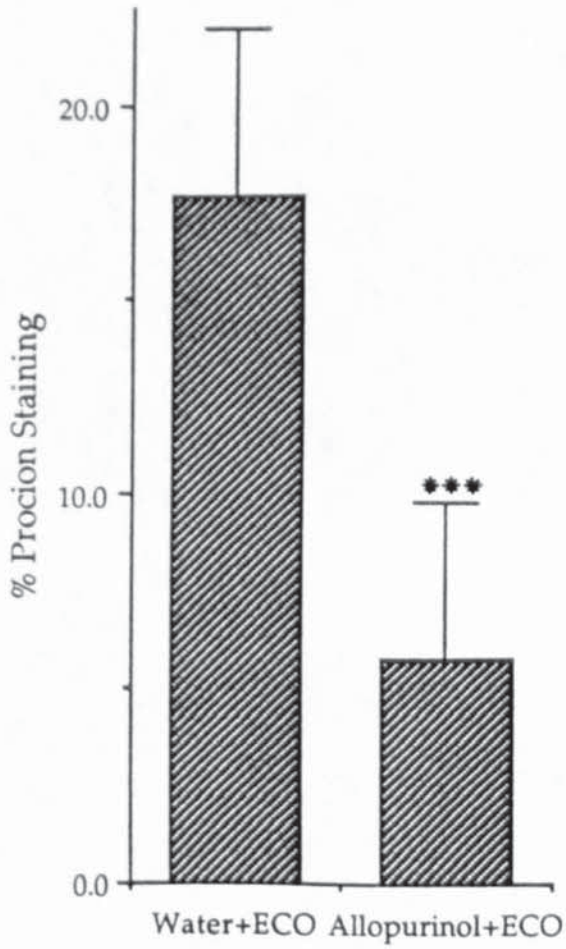


Figure 6.2.11 The prophylactic effectiveness of allopurinol on ECO induced myopathy. Values are mean ± 1SD. *** denotes significant defference from water + ECO control at 0.2% level.

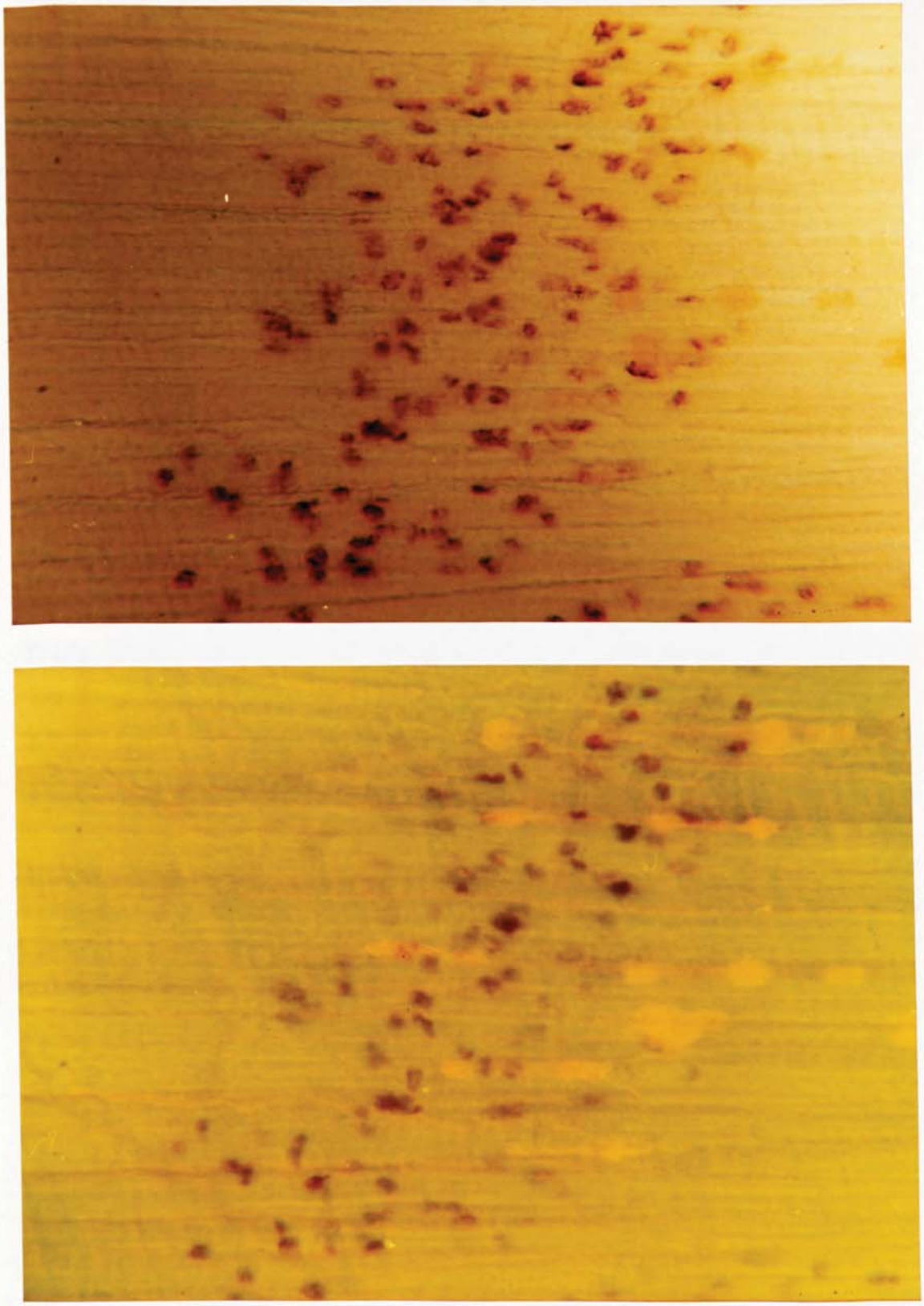


Plate 6.2.12 : Photograph of whole diaphragm taken from mouse given 500 n mol Kg^{-1} ECO 3 hours previously. Pretreatment for 5 days with allopurinol prior to challenge with ECO. Vital staining in procion then fixed and stained for ChE (end plates are represented as brown patches). Note significant reduction in procion yellow staining at the junctional region. Top: Tungsten illumination. Bottom: UV illumination. Calibration 100 μm.

higher for those exposed to Vit E + ECO from those pretreated with Vit E alone but significantly lower by 0.84n mol mg^{-1} from ECO (500n mol Kg^{-1}) (see table 6.2.13 and figure 6.2.14).

	Vit E (corn oil)	Vit E (corn oil) + ECO	ECO
J Ca Accumulation	0.20	1.23^{***}	2.07^{***}
(n mol mg^{-1})	±0.07	$\pm 0.21^{\#\#\#}$	±0.80
N	10	10	25

Table 6.2.13 : Effect of Vit E pretreatment on extent of junctional calcium accumulation (J) after 500 n mol Kg^{-1} ECO, 3 hours <u>in vivo</u> . Values represent mean ± 1SD, where N = no. of hemidiaphragms. *** denotes significant difference from Vit E in corn oil group at 0.2 % level. ### denotes significant difference from ECO group at 0.2% level.

The reduction in calcium accumulation at the junctional region by 0.84 nmol mg^{-1} by Vit E may be attributed to 'membrane leakiness'. It was shown in section 6.1 that a range of membrane ion channel blockers were ineffective in preventing ECO-induced myopathy perhaps as a failure of preventing 'membrane leakiness'. If it can be assumed that in the doses used, diltiazem and verapamil block both the Ca^{2+} and the receptor channel, as has been suggested, then the calcium accumulation in table 6.1.3 of 0.59 and 0.97 for diltiazem and verapamil respectively, may be attributable to 'membrane leakiness', assuming other factors are constant, hence the value obtained with Vit E of 0.84 nmol Kg^{-1} are similar in magnitude.

It is interesting to note that the extent of calcium accumulation for those exposed to Vit E + ECO lies between a dose of 200 and 300 nmol Kg^{-1} ECO (see table 5.1.1), further suggesting that the point of irreversibility is dependent upon a critical level of calcium accumulation or alternatively, the prevention of 'membrane leakiness' by Vit E, may have significantly retarded the rate or degree of calcium entry, a factor which has previously been suggested to be critical for determining the extent of procion staining.

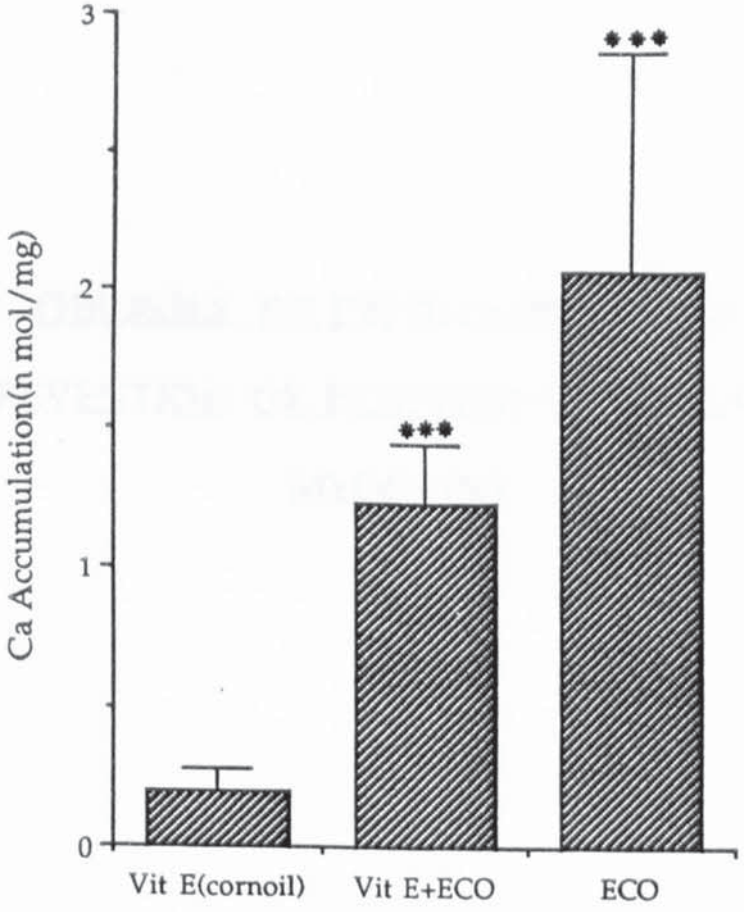


Figure 6.2.14 The effect of Vit E pretreatment on the extent of junctional calcium accumulation after 500nmol Kg^{-1} ECO, 3 hours in vivo. Values are in mean ±1SD. *** denotes significant difference from Vit E in cornoil at 0.2% level.

7.0 THE ROLE OF PYRIDOSTIGMINE IN THE PREVENTION OF ECOTHIOPATE-INDUCED MYOPATHY

7.1 Introduction:

The widespread use of OP, even only as agricultural insecticides imposes a potential threat to the environment and a health hazard to humans. In view of the widespread use, the potential for OP poisoning following absorption of concentrated material during manufacture or handling or following inadvertent medicinal overdose necessitates the availability of effective antidotes.

The most effective therapy for OP poisoning involves either pretreatment with carbamates, pyridostigmine or physostigmine, or the prompt administration of atropine and an oxime, most notably 2-pyridine aldoxime methiodide (2-PAM). The effectiveness of oximes in the treatment of various OP poisoning is believed to be due to the displacement of inhibitor from the enzyme, thereby restoring the AChE function (Durham and Hayes, 1962; Namba et al, 1971). The protective action of carbamates depends primarily upon the ability of the carbamate to reversibly inhibit AChE, by forming a semi-stable carbamylated enzyme (Wilson et al, 1960, 1961) and the subsequent gradual spontaneous decarbamylation releases sufficient AChE to maintain normal function (Gall, 1981; Dirnhuber and Green, 1978; Deshpande et al, 1986; Leadbeater et al, 1985; Deyi et al, 1981).

In contrast to the effective prophylactic effect, pyridostigmine in acute and chronic doses also induces ultrastructural modifications of both pre and postsynaptic structures, similar to those induced by other OP compounds (Hudson et al, 1985; Meshul et al, 1985; Gebbers et al, 1986). In addition subacute doses have been also reported to induce morphological alterations (Hudson et al, 1985).

Such findings raise concern for therapeutic application of pyridostigmine. Furthermore, the question arises whether the 'sign free' dose reported by Dirnhuber et al (1979), ascertained from visual and behavioural observations, is actually 'sign free' at the NMJ and whether repeated exposure to 'sign free' doses of pyridostigmine would still be effective against acute exposure to OP poisoning or would it potentiate the toxicity?

The main objectives of this section are to evaluate whether :

1) a 'sign free' dose of 100 $\mu g\ Kg^{-1}$ pyridostigmine, exposed 3 times daily for 3 days induces any morphological changes? This investigation was initiated by the realisation that those engaged in the manufacture and use of these compounds could be susceptible to multiple exposures to small doses.

2) 100 $\mu g\ Kg^{-1}$ pyridostigmine exposed 3 times daily for 3 days is still effective in preventing ECO-induced myopathy?

7.2 Experimental Design :

Mice were pretreated subcutaneously with 100 μ g Kg^{-1} pyridostigmine 3 times daily at approximately 8 hour intervals for 3 days. One hour after the last dose of pyridostigmine, some of these mice were challenged with 500 n mol Kg^{-1} ECO.

3 hours after the last dose of pyridostigmine or ECO, mice were anesthetised and blood samples were obtained from femoral artery for the determination of blood AChE activity (see 2.4.1) and serum CK activity (see 2.4.2). Mice were killed while still anesthetised and the diaphragm removed and hemidiaphragm produced.

Hemidiaphragms were assayed for AChE activity, calcium accumulation and procion staining for assessment of myopathy. In a selected few of procion stained preparations, determinations of end plate dimensions were made.

7.3 Results :

7.3.1 Behavioural Changes :

A single exposure to 100 μg Kg^{-1} pyridostigmine alone did not induce any noticeable behavioural changes that were distinguishable from atropinised controls. Furthermore, multiple exposure during the first or the subsequent days did not result in behavioural changes. In contrast, multiple pyridostigmine pretreatment was still effective in completely preventing the fasciculations and locomotor inactivity, induced by ECO.

7.3.2 End plate deformations and morphological changes :

Pretreatment with multiple doses of pyridostigmine prevented the subsequent development of severe myopathy associated with acute exposure to 500 n mol Kg^{-1} ECO (see table 7.1). Procion staining was reduced to levels similar in magnitude to those pretreated with pyridostigmine alone and were not significantly different from atropinised control.

Close inspection of pretreated preparations challenged with ECO, revealed numerous end plates with moderately severe distortion that was significantly different from atropine and pyridostigmine alone (see table 7.1). Coupled with this were numerous hypercontractions of moderate severity and the presence of occasional contraction clumps; morphological alterations that were similar to those expected with 200 n

Parameter	Atropine	Pyridostigmine ($100\mu gKg^{-1}$)		ECO
		Alone	+ECO	
Blood AChE	0.96^{***}	0.83^{***}	0.37^{***}	0.25
	±0.18	$\pm 0.13^{\#\#}$	$\pm 0.13^{\dagger\dagger\dagger}$	±0.10
N	42	20 (14.5)	14 ### (61.8)	13 (74.0)
Diaphragm AChE	1.42^{***}	1.09^{***}	0.39^{***}	0.14
	±0.25	$\pm 0.20^{\#\#}$	$\pm 0.12^{\dagger\dagger\dagger}$	±0.06
N	55	8 (23.3)	10 ### (72.4)	18 (90.3)
%Procion Staining(J)	0.16^{***}	0.37^{***}	0.31^{***}	18.2
	±0.20	± 0.62	± 0.17	± 8.2
N	9	13	7	23
%Procion Staining(NJ)	0.28	0.43	0.43	2.02
	±0.30	± 0.62	± 0.54	± 2.74
N	9	13	7	23
CK (U/L)	55^{***}	155^{***}	167^{***}	3910
	±38	$\pm 122^{\#\#}$	$\pm 54^{\#\#}$	±1720
N	20	11	8	32
(J) Calcium Accumulation	0.15^{***}	0.26^{***}	0.87^{***}	2.07
	±0.10	$\pm 0.16^{\#}$	$\pm 0.27^{\dagger\dagger\dagger}$	±0.80
N	47	8	9###	25
EP Ratio	0.61^{*}	$0.73^{***\#}$	$0.86^{***\dagger\#\#}$	1.01

Table 7.1 Effects of multiple doses of pyridostigmine for 3 days on various parameters before and after 500 n mol Kg^{-1} ECO challenge. Blood and diaphragm AChE activity are in μ mol min^{-1} ml^{-1} and n mol min^{-1} mg^{-1} respectively (with % inhibition in parenthesis). Calcium accu,ulation is in n mol mg^{-1}. Values are mean ± 1SD, where N = no. of hemidiaphragms. *, **, *** denotes significant difference from ECO group at 5% and 2% level respectively. †, ††, ††† denotes significant difference from pyridostigmine alone at 5%, 2%, and 0.2% respectively. #, ##, ### denotes significant difference from atropine group at 5%, 2% and 0.2% respectively.

mol Kg^{-1} ECO for 3 hours. In contrast, the morphological alterations induced with pyridostigmine pretreatment alone were much milder, consisting of obvious distortion of EP, infrequent hypercontractions and absence of contraction clumps. The appearance resembling that produced with 100 n mol Kg^{-1} ECO.

7.3.3 Changes in AChE activity :

Multiple exposure to pyridostigmine alone, 3 hours after the last dose, resulted in significant reduction in blood and diaphragm AChE activity by 15% and 23% respectively (see table 7.1). After ECO (500 n mol Kg^{-1}), the AChE activity of both tissues was significantly decreased further, but was significantly lower than the critical level achieved with 500 n mol Kg^{-1} ECO alone (see table 7.1). The levels of AChE activity achieved with pyridostigmine + ECO corresponds to the values obtained with 200 n mol Kg^{-1} ECO (see table 4.10).

7.3.4 Changes in CK :

Pretreatment with multiple doses of pyridostigmine not only prevented the development of severe myopathy but was also effective in preventing the extensive elevation of serum CK activity that accompanies severe myopathy (table 7.1). The CK activity of pyridostigmine pretreatment and pyridostigmine + ECO group although significantly higher than atropine controls (table 7.1) was similar in magnitude to those obtained with non-necrotising doses of ECO (see table 4.24).

7.3.5 Changes in calcium accumulation at junctional region :

Although pretreatment with multiple doses of pyridostigmine successfully prevented the development of myopathy and the extensive CK elevation that accompanies it, it did not completely prevent calcium accumulation at the junctional region of the diaphragm(table 7.1). Pretreatment significantly reduced calcium accumulation but was still significantly elevated compared to either atropine or pyridostigmine alone. Pretreatment with multiple doses of pyridostigmine itself resulted in significant calcium accumulation compared to atropine but was significantly lower compared to either pyridostigmine + ECO or 500 n mol Kg^{-1} ECO exposure, but similar in magnitude to that obtained after 100 n mol Kg^{-1} ECO.

7.4 Discussion :

Pretreatment with certain carbamates, in conjunction with atropine therapy, confers considerable protection against soman poisoning in several species, although there is a marked species variation in the efficacy of the treatment (Dirnhuber et al, 1979; Gordon et al, 1978). Gordon et al (1978) found pyridostigmine to be one of the most effective of the carbamates that were tested in guinea pigs.

Dirnhuber et al (1979) demonstrated that pretreatment of primates with a maximum 'sign free' dose of pyridostigmine iodide (200 μ g Kg^{-1}) offered considerable protection against soman intoxications. Furthermore, in the same study the protection afforded by 50 μ g Kg^{-1} pyridostigmine iodide was suggested to be as effective as pretreatment with 200 μ g Kg^{-1}.

It has been shown by Townsend (1988) that pretreatment with a single dose of 100 μ g Kg^{-1} pyridostigmine, when applied within 2 hours although causing no behavioural changes did successfully prevent ECO-induced myopathy.

In this investigation, pretreatment with multiple doses of pyridostigmine although causing no behavioural changes did consistently produce subtle morphological changes as indicated by a number of different parameters (see table 7.1). These subtle morphological alterations are indicative of subcellular disturbances and therefore suggest that the doses used are not entirely 'sign free' as had been suggested (Dirnhuber et al , 1979).

These changes could be as a result of daily multiple exposure of pyridostigmine over a prolonged period. However, single pretreatment with 100 μ g Kg^{-1} pyridostigmine has been reported by Townsend (1988) to produce slight myopathy. Furthermore, a single non-necrotising doses of ECO has also been shown to produce similar morphological changes indicative of intracellular disturbances. Despite these underlying intracellular disturbances, pretreatment with multiple doses of pyridostigmine was still effective in preventing the development of severe myopathy when challenged with acute dose of ECO. However, the underlying subtle disturbances appear to be slightly exacerbated after ECO challenge.

The findings that pyridostigmine either in a single dose or multiple doses induces subtle morphological alterations is perhaps not surprising since carbamates have been shown to produce ultrastructural damage of pre and post-synaptic structures in skeletal muscles after acute and chronic doses (Hudson et al, 1985; Meshul et al, 1985, Gebbers et al , 1986). Furthermore, subacute doses of pyridostigmine has been

reported to produce ultrastructural changes (Hudson et al, 1986). Perhaps these ultrastructural changes are responsible for the morphological alterations detected by the light microscopy in this study.

The effectiveness of pyridostigmine has been attributed to the carbamoylation of a portion of the tissue AChE, which protects it against irreversible inhibition by OP agents : after poisoning spontaneous decarbamoylation (Wilson et al, 1960, 1961) in parallel with removal or destruction of the OP produces sufficient free AChE to restore normal function (Berry and Davis, 1970; Dirnhuber and Green, 1978; Dirnhuber et al, 1979). The findings that AChE inhibition after ECO is significantly reduced by approximately 15% from the critical level of 90% is certainly consistent with the hypothesis and further supports the view that low levels 1 - 15% of AChE are necessary for the normal neuromuscular function (Hobbiger, 1976).

The inhibition AChE of blood after 100 μ g Kg^{-1} pyridostigmine, 30 - 40 mins after post-injection was 55% as reported by Dirnhuber and Green (1978) and 60% by Hudson et al (1986) and by 4 hours AChE inhibition dropped to 14% (Dirnhuber and Green, 1978). The levels of AChE inhibition 3 hours after pyridostigmine administration was found to be 15% and 23% for blood and diaphragm respectively which are in reasonable agreement with the findings of Dirnhuber et al (1979).

There remains the possibility that pyridostigmine may be exerting protective action by a direct effect unrelated to spontaneous reactivation of AChE. Pyridostigmine has been shown to interact directly with the nicotinic receptor-ionic channel complex (Albuquerque et al, 1984; Akaike et al, 1984). Pyridostigmine showed a weak agonist action with channel openings of low conductance and low frequency characteristics (Albuquerque et al, 1984) and in addition acts as a desensitizing agent (Akaike et al, 1984). Despite these direct effects, French et al (1979) have shown that the reversal by pyridostigmine of the neuromuscular block produced by soman was not attributable to desensitization. Perhaps because the concentration of pyridostigmine employed in the in vitro study or those used in the pretreatment were insufficient to exert any direct effect.

Finally, the findings suggest that the behavioural observations used as a criteria for distinguishing 'sign free' doses are very misleading as regards to the effects occurring at the neuromuscular junction.

8.0 GENERAL DISCUSSION AND CONCLUSIONS

8.1 Role of AChE inhibition in ECO-induced myopathy:

In studying the mechanism(s) involved in antiChE induced myopathy it is not only necessary to accurately measure AChE activity but also convey an accurate picture of the functionally important AChE that is responsible for terminating transmitter action. Although simple and widely used, the biochemical method of Ellman, lacks specificity as regards to AChE and pseudocholinesterases. Hence, as shown in this study, using homogenates as a source of enzymes and acetylthiocholine as the substrate, the ChE activity is derived from many sources whether intracellular,extracellular or from AChE activity or BuChE activity. Consequently, chapter 3.0 was devoted to evaluating ways of accurately estimating AChE and BuChE activity by modifying the Ellman method. The incorporation of ethopropazine and BW284C51 in the assays for estimating AChE and BuChE respectively, enabled the accurate estimation of AChE and BuChE respectively. Although the modified method of Ellman, using homogenates, gives a less distorted picture of synaptic AChE activity than that measured in whole muscles (Miledi et al, 1984), it still assesses AChE which is not involved in the hydrolysis of transmitter. This is indicated by the fact that there are discrepancies between end plate current and the Ellman method at various times after intoxication with organophosphorous compounds (Bamforth et al, 1988).

It has been shown in this study that development of severe myopathy after ECO administration is associated with a critical level and duration of AChE inhibition of approximately 85-90%, similar in magnitude to that reported by others using other OP compounds (Wecker et al, 1978a, b ; Gupta et al, 1985, 1987a,b). It is interesting to note that these authors too have used 'selective' inhibitors in their assays. ECO was found to be an effective inhibitor of BuChE therefore the possibility that myopathy resulting from BuChE inhibition is ruled out since Wecker et al (1978) has shown that treatment in vivo with iso-ompa (25 mg Kg^{-1}) which caused total and irreversible inhibition of BuChE did not show any evidence of either cholinergic hyperactivity or skeletal muscle necrosis.

Exposure to non-necrotising doses of ECO although causing significant reduction in AChE and BuChE did not cause the development of severe myopathy but did result in morphological alterations at the light microscope level with concomitant elevation of serum CK activity. It is possible that the morphological alterations observed at light microscope level represent some ultrastructural damage, since it has been shown that Bay K induced ultrastructural

damage is well advanced at a time when there is very little procion staining (Howl and Publicover, 1987).

Following necrotising doses of ECO, superimposed on these underlying disturbances, was extensive myopathy as indicated by the extent of procion yellow staining at the junctional region concomitant with extensive elevation of serum CK activity.

Since the diaphragm muscle is composed of approximately equal numbers of type I and type II fibres (Gauthier and Padykula, 1966), the possibility existed that exposure to ECO might affect the fibre type differently. However, this is unlikely, since Hudson et al (1985) has shown that the variability in the extent of myopathy is random and not related to a specific fibre type.

The findings that severe myopathy is always found first at the vicinity of the motor end plate, suggest that the nerve terminal plays a decisive role in the development of myopathy. The motor end plate as the primary site of the lesion has been implicated in myopathies produced by other antiChE agents (Gebbers et al, 1986; Meshul et al, 1985; Toth et al, 1981; Wecker et al, 1978a, b; Gupta et al 1985,1987a, b; Laskowski et al, 1975). The importance of the nerve terminal in the production of the myopathy is supported by the following observations: dissection or pretreatment with hemicholinium reduced the number of necrotic fibres during chronic paraxon treatment in soleus, gastrocneumius and quadriceps muscle (Fenichel et al, 1972). Ariens et al (1968) noted a similar effect of phrenic nerve section and were able to induce lesions by chronically stimulating the distal stump.

In the mechanism of acute myopathy caused by OP compounds, accumulation of ACh surplus is a decisive factor (Ariens, 1968; Leonard and Salpeter, 1979), however, the surplus of ACh itself is not sufficient for the development of the myopathy and that a functional interaction between ACh and the ACh receptor is of an even more fundamental importance (Ariens, 1968; Leonard and Salpeter, 1979; Townsend, 1988). Furthermore, it has also been shown that once myopathy is initiated ie. within 60 minutes of ECO administration, the need for nerve stimulation and ACh receptor involvement no longer becomes important and myopathy proceeds regardless (Townsend, 1988).

8.2 Role of calcium in ECO-induced myopathy:

It is well known that extensive subcellular damage in both skeletal and cardiac muscle can be triggered by a variety of agents whose common mode of action appears to be to elevate $[Ca^{2+}]_i$ (Howl and

Publicover, 1987; Rudge and Duncan, 1984; Duncan et al, 1979; Duncan, 1987; Leonard and Salpeter, 1979).

In this study non-necrotising doses of ECO ($\leq$ 200 n mol Kg^{-1}) resulted in significant calcium accumulation at the junctional region concomitant with significant elevation in serum CK activity, indicative of subtle 'membrane leakiness'. The results of the present investigation lend further support to the hypothesis of Salpeter et al (1979) on the role of Ca^{2+} in the development of myopathy. It has been shown that prolonged depolarisation of the sarcolemmal membrane results in an influx of Ca^{2+} into the muscle cell (Evans, 1984; Miledi, 1977; Takeuchi, 1963).

An important feature to emerge throughout this study is the observation that skeletal muscle is capable of buffering extensive fluctuations in calcium. Despite this, several lines of evidence indicated a rise in $[Ca^{2+}]_i$, perhaps due to calcium-induced release of Ca^{2+} (Duncan and Smith, 1980; Fabiato, 1982; Endo, 1977), depolarisation-induced release of Ca^{2+} from SR (Chandler et al, 1976; Schneider, 1986) and Na^{+} induced from mitochondria (Carafoli and Zurini, 1982; Affolter and Carafoli, 1980).

The elevation in $[Ca^{2+}]_i$ may produce ultrastructural damage as has been reported by Hudson (1986) in sub-acute doses of pyridostigmine and perhaps these ultrastructural changes are responsible for the morphological alterations observed at the light microscope level after non-necrotising doses of ECO.

After necrotising doses of ECO, development of severe myopathy is further associated with calcium influx, at doses and time which also show considerable ' membrane leakiness'. It has been shown that the degree of Ca^{2+} entry indeed can determine the extent of procion staining and furthermore Ca^{2+} entry through 'membrane leakiness' could account for up to 29-48% of calcium influx. Hence, it is suggested that the sudden and uncontrolled rate of calcium entry is responsible for the initiation of severe myopathy.

Clearly this investigation suggests two phases to the development of myopathy. The first step represents a disruption of the integrity of the sarcolemmal membrane and is dependent on extracellular Ca^{2+}. Possibly leakage of proteins occurs in this phase and myofilament apparatus and ultrastructural organisation is relatively unaffected (Rudge and Duncan, 1984). Despite the widely differing mechanisms by which this injury may be produced, there is a common functional consequence in what follows. The second step is dependent on extracellular calcium and most likely represents an influx of Ca^{2+} across

the damaged sarcolemmal membrane and down a steep electrochemical gradient. This step at least initiates the pathway by which cellular necrosis is produced. Such schemes have been suggested to occur in calcium paradox (Rudge and Duncan, 1984; Ruigrok, 1985), oxygen paradox (Myers et al, 1985), reperfusion injury (Jennings et al, 1978) and hepatoxicity by toxins (Schanne et al, 1979; Farber, 1981).

8.3 Role of CANP, lysosomes in ECO-induced myopathy:

Calcium plays an important role as a universal modulator of various biochemical processes and a transient rise in cytosolic Ca^{2+} will activate numerous enzymatic systems (Campbell, 1983).

Amongst these CANP, because of the unique localisation (Ishuira et al, 1980) and its capacity to completely remove Z-lines (Busch et al, 1972) together with the lysosomal apparatus with its full complement of acid hydrolases has been implicated to be involved in the initiation of skeletal myopathy (see 1.6 and 1.7). However, the use of CANP inhibitor, leupeptin alone, or in combination with other lysosomal inhibitors has been shown to be ineffective against experimentally induced ultrastructural damage by A23187 or that induced with low levels of Ca^{2+} in chemically skinned cutaneous pectoris preparations (Duncan et al, 1979; Duncan, 1987). In further support of these observations is the demonstration by Townsend (1988) that leupeptin failed to prevent ECO-induced myopathy. In view of these results and the fact that skeletal muscles have low lysosomal content (Bird and Carter, 1980), suggest that at least in the case for ECO, CANP and the lysosomal system do not play a major role in the initiation of ECO-induced myopathy and at best plays an ancillary role in 'mopping up'.

8.4 Role of high energy phosphates in ECO-induced myopathy:

Increased elevation of intracellular calcium forces respiring mitochondria to maintain Ca^{2+} homeostasis by sequestering the excessive amounts of this ion. This may be partly responsible for the early swelling and vacuolation, demonstrated on numerous occasions (Laskowski et al, 1975,1977; Meshul et al, 1985; Hudson et al, 1985, 1986) but more importantly prevents oxidative phosphorylation. Consequently, cellular necrosis has been suggested to be due to depletion of cellular high energy phosphate. This study has shown that ECO-induced myopathy is not due to the depletion of high energy phosphate

and supports the findings of Duncan (1987) that experimentally induced damage is not attributable to loss of cellular high energy phosphate. In contrast, the findings of Gupta and Dettbarn (1987) showed significant depletion of high energy phosphate at a time coincident with the appearance of necrotic lesions, perhaps suggesting that cellular depletion of high energy phosphate is a consequence of myopathy, a view which can be partly supported by the findings of this study.

8.5 Role of free radicals in ECO-induced myopathy:

The results of the histochemical staining experiments combined with the results of the antioxidant experiments strongly suggest that ECO-induced myopathy at least in part is mediated via free radicals. The potential sources of free radical generation which may be associated with ECO-induced myopathy are numerous (see 1.8).

Allopurinol, a specific inhibitor of both the dehydrogenase and oxidase form, has been demonstrated to be effective against the reduction of infarct size and the genesis of arrhythmias in reperfusion induced damage (Hearse et al, 1985; Chambers et al, 1985; Akizuki et al, 1984) and in addition has been consistently reported to be more effective in rat than canine myocardium presumably because rat myocardium contains higher xanthine oxidase activity (Woodward and Zakaria, 1985; Aoki et al, 1988). In agreement with these findings, allopurinol has been shown in this study to significantly protect against ECO-induced myopathy, presumably by suppressing the production of O_2^-. Thus xanthine oxidase could be a major contributing source of free radical in ECO-induced myopathy, however, the result also indicates that the xanthine oxidase is not wholly responsible for ECO-induced myopathy.

Since mitochondria have a large capacity for Ca^{2+}, consequently the intramitochondrial transport system will be dissociated which besides preventing ATP production, will generate H_2O_2 and O_2^- (Boveris and Cadenas, 1982; Loschen et al, 1974). Mitochondria from a variety of sources has been shown to produce H_2O_2 (Boveris and Cadenas, 1982) and Loschen (1974) has shown that O_2^- can be considered as a stoichiometric precursor for mitochondrial H_2O_2. Much evidence points to the reduced forms of ubiquinone as a source of mitochondrial O_2^- and H_2O_2. The rate of H_2O_2 production by isolated mitochondria depends on the mitochondrial metabolic state and H_2O_2 generation is highest in state 4. Furthermore, under conditions of low e^- flow, as in antimycin supplemented mitochondria, it has been observed that

addition of uncouplers (eg. ionophores, Ca^{2+}) stimulates H_2O_2 formation up to 13 fold (Boveris and Chance, 1973; Cadenas and Boveris, 1980) and this has been explained in terms of a collapse in membrane potential. Interestingly, this phenomena may occur also <u>in vivo</u> : rats treated with 2,4-dinitrophenol show a 50 fold increase in H_2O_2 production by isolated liver mitochondria which is accompanied by a 5 fold increase in mitochondrial SOD activity (Dryer et al, 1980). It is likely that the mitochondrial production of O_2^- and the H_2O_2 contributes significantly to the generation of other free radicals since up to 20% of the O_2^- and H_2O_2 escape from intact mitochondria despite the presence of mitochondrial antioxidants (Boveris and Cadenas, 1982).

It is possible that lipid peroxidation resulting from free radical activation either from mitochondria or xanthine oxidase or as a direct consequence of Ca^{2+} activated phospholipase A_2 results in the disruption of membrane cytoarchitecture (see 1.10). The involvement of free radicals is two fold: firstly the free radical-induced release of arachidonic acid (AA) and subsequent formation of prostaglandins and endoperoxides may further aggravate the injury; secondly there is evidence that the AA cascade pathway generates free radicals. It has been demonstrated by Duncan and Jackson (1987) that nordihydroguaiaretic acid (NDGA) and chloropromazine both protected against DNP and A23187 induced intracellular enzyme efflux suggesting that both lipoxygenase and cycloxygenase pathways are involved in sarcolemmal breakdown. In support of these findings is the observation that pretreatment with quinacrine (10 mg Kg^{-1}), a potent phospholipase A_2 inhibitor (Blackwell and Flower, 1983), significantly reduced the extent of procion yellow staining after ECO administration (6.85 ± 3.38, $n = 5$, $P<0.002$). The protection afforded by quinacrine or the prevention of enzyme efflux with chloropromazine and NDGA, as reported by Duncan and Jackson (1987), could also be due to their antioxidant actions. The protection afforded by quinacrine, is similar in magnitude to those obtained with allopurinol or NAC alone. However, it is possible that better preservation of sarcolemmal membrane does not necessarily mean better protection against ultrastructural damage (Duncan and Jackson,1987; Howl and Publicover, 1987).

Activated leukocytes, which may begin to infiltrate the diaphragm within 3 hours of ECO administration (Ariens, 1968), release among other things, oxygen radicals and products of the AA cascade. Although the theory that leukocytes release free radicals and kill otherwise viable cells cannot be denied, however, it is very unlikely that the injury seen in the present study is predominantly leukocyte-related for two reasons.

Firstly, the earliest sign of leukocyte infiltration was reported to be between 12 - 24 hours after ECO administration (Townsend, 1988) and secondly, even if some leukocytes were present, the amount of superoxide produced would probably be insufficient to produce such an extensive myopathy.

8.6 Summary:

In summary, all the results suggest that the transition from reversible to irreversible injury is of sudden onset and a multitude of factors probably acting in concert may be responsible. The exact mechanism(s) for the critical transition from reversible to irreversible injury still remains unknown but the involvement of Ca^{2+} and free radicals appears to be crucial.

In non-necrotising doses of ECO, the influx of calcium forces the SR and mitochondria to buffer this ion. As a consequence of mitochondrial Ca uptake, the production of O_2^-, H_2O_2 and other free radicals will inevitably lead to lipid peroxidation. Lipid peroxidation or products of lipid peroxidation may initiate the slow release of Ca^{2+}, since hydroperoxides have been shown to induce Ca^{2+} release from loaded liver mitochondria (see Richter and Frei, 1985), even though the mitochondria are structurally and functionally intact. Furthermore, free radical generation may also reduce SR Ca uptake velocity as well as Ca^{2+} ATPase activity (Hess et al, 1981).The consequent rise in $[Ca^{2+}]_i$ is responsible for activation of xanthine oxidase, phospholipase A_2, as well as the subtle morphological alterations observed at these doses. However, as a result of a slow rate of O_2^- generation in combination with the effective multilevel antioxidant system, the onset of irreversible damage is prevented.

After necrotising doses of ECO, the rapid entry of calcium not only enhances mitochondrial free radical generation but perhaps simultaneously activates cytosolic xanthine oxidase, membrane phospholipase A_2. Consequently, by overwhelming the multilevel antioxidant systems by the sheer number of free radicals produced, rapid cellular damage and intracellular havoc is produced. Mitochondria and SR release their Ca^{2+} which together with 'membrane leakiness' aggravate the rise in $[Ca^{2+}]_i$ which induces a massive contraction of myofibrils and perhaps the generation of stressful forces upon an already weakened myofibril resulting in the formation of contraction

clumps and cell death.

Finally, by unravelling and understanding the complexity of some of the mechanisms involved in ECO-induced myopathy it has been possible by appropriate therapeutic intervention to prevent or reduce the extent of ECO-induced myopathy.

APPENDIX

A1. Source of reagent

Reagent	Source
Acetic Acid	BDH
N-acetylcysteine (Parvolex)	Duncan Flockhart Ltd.
Acetylthicholine Iodide	BDH
Adenosine 5'-monophosphate(AMP)	Sigma III
Adenosine 5'-diphosphate (ADP)	Sigma IV
Adenosine 5'-triphosphate (ATP)	Sigma
AH5183	Gift from Glaxo
Allopurinol	Sigma
Atropine Sulphate	Sigma
BOS	CDE
Calcium Chloride ($CaCl_2$)	BDH-Aristar
Catalase	Sigma (46,200 units)
Copper Sulphate ($CuSO_4$)	Hopkin + William Ltd
Creatine hydrate	Sigma
Creatine kinase assay kit	Sigma
Dextrose	Fisons-AR grade
Diltiazem	Sigma
Disodium Hydrogen Orthophosphate	BDH-Analar
Disopyramide Phosphate	Sigma
Dithiobisnitrobenzoate	BDH
Ecothiopate Iodide	Ayerst
Ethopropazine hydrochloride	Sigma
Formaldehyde	BDH-Analar
Gentamicin Sulphate	Sigma
Glutaraldehyde	BDH-Aristar
Glycerol Jelly	BDH
Hydrochloric acid (concentrated)	BDH-Aristar
Inosine 5'-monophosphate (IMP)	Sigma
Lanthanum Chloride ($LaCl_3$)	Fisons
Magnesium Chloride (MgCl2)	BDH
Mannitol	Sigma
Mecamylamine	Sigma
Methanol H.P.L.C. grade	Fisons
Netilmicin Sulphate	Sigma
Nitroblue tetrazolium (NBT)	Sigma III

Phosphocreatine	Sigma
Potassium Chloride (KCl)	BDH-Analar
Potassium Ferricyanide	Hopkin + Williams Ltd
Procainamide	Sigma
Procion Yellow MX4R	ICI
Pyridostigmine bromide (Mestinon)	Roche
Quinacrine	Sigma
Sodium Acetate	Fisons-SLR grade
Sodium Hydrogen Carbonate ($NaHCO_3$)	BDH-Analar
Sodium Citrate	BDH-Analar
Sodium Chloride	BDH-AR
Sodium Dihydrogen Orthophosphate	BDH-Analar
Sodium Hydroxide	BDH-Analar
Superoxide Dismutase (SOD)	Sigma (30,000 units)
Tetrabutylammonium hydroxide	Sigma
dl-α-Tocopherol (Vit E)	Sigma
Tricholoroacetic acid (20%)	BDH
Trisodium Citrate	BDH-Analar
Tween 80	Sigma
Verapamil	Sigma

A2. Composition of the physiological saline solution (pH 7.4).

Table A2.1. Composition of this physiological saline was identical with that described by Liley (1956) except for a greater dextrose content as recommended by Krnjevic and Miledi (1958).

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

NB. $CaCl_2$ added was as 1.0M $CaCl_2$ volumetric solution (2ml per litre) and gassed with 95% O_2/ 5% CO_2. This solution is refered to as physiological saline.

A3. Composition of the Cholinesterase stain.

Table A3.1. Preparation of cholinesterase stain pH 6.0 (Karnovsky 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	12.5mg	acetylthiocholine iodide and dissolve.	
3. Add:-	30mM	Copper Sulphate	2.5ml
	5mM	Potassium Ferricyanide	2.5ml

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

A4. Composition of the solutions used in the AChE assays.

A4.1. 5,5-Dithiobis(2-nitrobenzoic) acid (DTNB).

(a) Stock solution.- a 0.01M DTNB solution was used which, kept below 4°C, had a shelf life of approximately a week.

Table A4.1. Composition of the 0.01M DTNB stock solution.

Substance	
DTNB	39.6mg
NaHCO3	15.0mg
0.1M Phosphate Buffer, pH 7.0.	10.0ml

(b) Assay solution - a 1:40 dilution of the stock solution in distilled water was used in the assay.

A4.2. Composition of the buffers used.

Table A4.2. Composition of phosphate buffer, pH 7.0.

0.2M $Na_2HPO_4.12H_2O$	30.5ml
0.2M $NaH_2PO_4.2H_2O$	19.5ml
Distilled Water	50ml

Table A4.3. Composition of phosphate buffer, pH 8.0.

0.2M $Na_2HPO_4.12H_2O$	47.4ml
0.2M $NaH_2PO_4.2H_2O$	2.6ml
Distilled Water	50.0ml

A4.3. Composition of the substrate, acetylthiocholine iodide.

The substrate was made up fresh on the day of the assay and was in a final cuvette concentration of 0.5mM.

Table A4.4. Composition of the acetylthiocholine iodide solution.

Acetylthiocholine iodide	21.67mg
Distilled Water	50.0ml

A4.4. Composition of the inhibitor Ethopropazine.

A final sample concentration of $5x10^{-5}M$ ethopropazine was used in all samples.

Table A4.5. Composition of the ethopropazine stock solution.

Ethopropazine	21.8mg
Distilled Water	25.0ml

This stock solution was diluted 1:10 in phosphate buffer, pH 8.0, to give a concentration of $2.5x10^{-4}M$, before adding it to the tissue samples.

A5. Statistical tests.

A5.1. Mann-Whitney test.

This test is used in situations where the data is assumed to be non-parametric in nature, that is it has not been shown to have a normal distribution. It assumes that the two samples to be tested are independent, the scores in each group are drawn at random and they are rankable. The null hypothesis that the two samples are drawn from two populations with the same distribution characteristics is tested by using equation 1 :-

$$R' = n1\,(n1 + n2 + 1) - R \qquad (1)$$

where n1 is the number of scores in group 1, n2 is the number of scores in group 2 and R is the sum of the ranks in the smaller group (always group 1). To determine the level of significance of the difference under consideration the smaller value of R or R' can be compared with the values of R given in the tables for the appropriate number of scores in each group. If the calculated value of R or R' is equal or less than the value of R given in the 5% confidence level column of the statistical tables, then the null hypothesis would be unlikely to be true, i.e. the two populations from which the samples were drawn have significantly different distribution characteristics. These could be differences in the mean, variance or skew of the populations or a combination of these. However, a significant result indicates a difference in the medians of the populations sampled.

For large samples equation 2 has to be used:-

$$z = \frac{n1\,(n1 + n2 + 1) - 2R}{\sqrt{\frac{n1n2\,(n1 + n2 + 1)}{3}}} \qquad (2)$$

The significance of z can be assessed with reference to the appropriate table. A value of z equal to or greater than that given in the 5% confidence level column suggests that the null hypothesis is untrue.

A5.2. Linear regression.

When a linear regression line is fitted to a set of data points, the following criteria must be fulfilled before the regression of one variable on another may be said to be statistically significant. The slope of the regression must be significantly different from zero and Pearson's correlation coefficient (r) must have a value large enough to indicate that the calculated regression line is a significantly close fit to the data points. r is calculated using equation 4:-

$$r = \frac{N\Sigma XY - \Sigma X \Sigma Y}{\sqrt{[(N\Sigma X^2 - (\Sigma X)^2)(N\Sigma Y^2 - (\Sigma Y)^2)]}} \qquad (4)$$

where X and Y are two variables and N is the number of paired data scores. The value of r so obtained can be compared to the values of r for the number of pairs of variables in the tables in order to determine the level of significance for the correlation.

The slope of the line of best fit (b) is then calculated using equation 5:-

$$b = \frac{N\Sigma XY - \Sigma X \Sigma Y}{N\Sigma X^2 - (\Sigma X)^2} \qquad (5)$$

The intercept of this line (c) is calculated using equation 6:-

$$c = \frac{\Sigma Y - b\Sigma X}{N} \qquad (6)$$

Thus we can describe the association of X and Y in terms of the following equation of a straight line:-

$$Y = bX + c \qquad (7)$$

All calculations were performed using the "MDCSTAT" program on Northstar microcomputer.

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