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THE EFFECTS OF PERSISTENT ANTICHOLINESTERASE ACTION AT
THE NEUROMUSCULAR JUNCTION.

JOHN PHILIP BAMFORTH
Doctor of Philosophy

The University of Aston in Birmingham.
September, 1989.

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ASTON UNIVERSITY

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BY

JOHN PHILIP BAMFORTH

A thesis submitted for the degree of : DOCTOR OF PHILOSOPHY - 1989

SUMMARY

The effects of organophosphorus compounds which form a rapidly-ageing complex with acetylcholinesterase (AChE) (e.g. pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate (BOS)) and hence exert a persistent anticholinesterase (anti-ChE) action have been compared with other compounds with a shorter time course of inhibition (e.g. ecothiopate iodide (ECO)). Although the inhibition of AChE produced by BOS lasted longer than that seen with ECO, the time course of the myopathy appeared very similar. BOS also possessed a number of properties which have been seen with other anti-ChEs.

BOS and ECO produced significant increases in neuromuscular "jitter" 5 days after injection, not only in the diaphragm but also in the soleus and extensor digitorum longus muscles. Increases in "jitter" produced by ECO could be prevented by pyridostigmine prophylaxis or rapid treatment with pyridine-2-aldoxime methiodide. Some protection from the BOS-induced increases in "jitter" could be gained by repeated treatment with pyridine-2-aldoxime methiodide, an effect which could not be accounted for simply by enzyme reactivation. From experiments performed in Rej 129 mice it was determined that increases in "jitter", although demonstrated in some dystrophic muscles, could not be used as an early diagnostic tool.

Because sequelae of inhibition were present some time after intoxication, by which time AChE appeared biochemically normal, experiments were performed to investigate inactivation of physiologically important AChE. The time course of extracellular MEPPs was utilised as an indicator of physiologically important AChE and compared with the AChE activity measured by the technique of Ellman *et al.* (1961). It was concluded that the degree of persistence of anti-ChE action was unimportant for the induction of myopathy with a time course of 3-24 hours, but had some importance in events of longer duration.

Keywords: Anticholinesterase. Acetylcholinesterase. Action potentials.

Miniature endplate potentials. Oximes.

Dedicated to the memory of two men, who in the eyes of a young child
appeared great,
Thomas Henry Wightman and Geoffrey Charles Bamforth.

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"IF I CAN SEE SO FAR, IT IS ONLY BECAUSE I STAND ON THE SHOULDERS OF GIANTS."

SIR ISAAC NEWTON.

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ABBREVIATIONS.

ACh	Acetylcholine.
AChE	Acetylcholinesterase.
anti-ChE	Anticholinesterase.
BOS	Pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate.
BuChE	Butyrylcholinesterase.
CANP	Calcium activated neutral protease.
ChE	Cholinesterase.
DFP	Diisopropylfluorophosphate.
DTNB	Dithiobisnitrobenzoate
ECO	Ecothiopate iodide.
EDL	Extensor digitorum longus.
EPC	Endplate current.
EPP	Endplate potential.
HI6	[[[4-(aminocarbonyl)pyridino]methoxy]methyl]- 2-[(hydroxyimino)methyl] -pyridiniumdichloride.
MEPC	Miniature endplate current.
MEPP	Miniature endplate potential.
MCD	Mean consecutive difference.
MIPI	Mean interpotential interval.
MW _t	Molecular weight.
P ₂ S	Pralidoxime Mesylate.
2PAM	Pyridine-2-aldoxime methiodide.
SD	Standard deviation.
SFEMG	Single fibre electromyography.
SR	Sarcoplasmic reticulum.
TEPP	Tetraethyl pyrophosphate.
THA	Time to half amplitude.
TOX	Toxogonin.

1. INTRODUCTION

1.1. THE AIMS OF THIS INVESTIGATION.

Electromyography has become a useful tool in neurophysiological research and clinical diagnosis. With increasing demand for objective criteria, new techniques have been developed and refined, one of which is single fibre electromyography (SFEMG). Extracellular action potentials from single muscle fibres can be recorded by inserting SFEMG electrodes into muscles *in vivo*. These action potentials are generated either voluntarily or by electrical stimulation. Electrical stimulation of muscles is performed by stimulation of the nerve trunk, or for greater selectivity stimulation of intramuscular axon branches. Alternatively, it is possible to activate the muscle fibres directly using an electrical stimulus at one end of the muscle fibre (Stalberg and Trontelj, 1979). Ekstedt (1964) showed that when recording the action potentials from two muscle fibres from the same motor unit there is always a variability in the time intervals between the two muscle fibre potentials at consecutive discharges. This phenomenon is known as jitter. Such variability is increased for long periods following single *in vivo* injections of anticholinesterases (anti-ChEs) (Stalberg and Trontelj, 1979, Kelly and Ferry, 1988).

One of the main purposes of this study was to investigate the effects of various anti-ChEs on jitter in particular with relation to acetylcholinesterase (AChE) inhibition assayed by a biochemical technique. This was done using a technique, developed by Dr. S. S. Kelly, which adapted the concepts of electromyographic jitter to trains of action potentials recorded intracellularly from individual muscle fibres. During this particular part of the study some doubts were cast as to the usefulness of the biochemical assay in accurately measuring physiologically important AChE. An electrophysiological technique, developed as an indicator of such physiologically important AChE activity, was used as a marker to compare with the AChE activity gained by the biochemical method.

Increases in jitter have been reported in patients suffering from muscular dystrophy (Stalberg and Trontelj, 1979). A further aim of the study was to investigate jitter in a murine model of muscular dystrophy, the Rej 129 dy/dy mouse, and to assess whether increases in jitter could be utilised as an early

diagnostic test.

1.2. NEUROMUSCULAR TRANSMISSION.

To review the background to the results which follow, a section is presented on the present knowledge and concepts of neuromuscular transmission. This section firstly summarises the known morphology of the vertebrate neuromuscular junction along with the current view of the passage of events involved in neuromuscular transmission. These present day hypotheses are then expanded to encompass detailed accounts of their derivation.

1.2.1. Synaptic morphology.

The motor endplate of the vertebrate neuromuscular junction generally covers the surface of the muscle cell in a trough or gutter. The vertebrates also have secondary folds of the post-junctional membrane which extend into the muscle cell (figure 1.1). Heuser and Salpeter (1979), using electron microscopy, showed that these secondary clefts contain a network of fibres known as the basal lamina, compared by some to mucopolysaccharide or mucoprotein basement membranes (Zacks and Blumberg, 1961a). The arrangement of these secondary folds varies from muscle to muscle and from one animal to another. However, examples of this type of fast reacting synapse have been found in reptiles (Robertson, 1956), amphibians (Birks *et al.*, 1960) and mammals (Zacks and Blumberg, 1961a). The width of the primary synaptic cleft varies between 20 and 60nM, whilst the secondary folds are 50 to 150nM wide and between 500 and 1000nM deep. The folds are separated from each other by a space of up to 2 μ m. Some electronmicrographs have shown thickening of the pre-junctional membrane opposite the peaks of the secondary folds. It has been suggested that these regions of pre-junctional thickening are the locations from which the vesicles release their quanta (Birks *et al.*, 1960).

1.2.2. The Acetylcholine receptors and associated ion channels.

Investigations into the location of acetylcholine (ACh) receptors have made use of a number of techniques: specific inhibitors, radioactive labels and electronmicroscopy. Porter *et al.* (1973), using tritiated or ^{125}I -labelled α -



Aston University

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Figure 1.1. Diagrammatic representation of a neuromuscular junction. The junctional cleft contains the basal lamina and the nerve terminal is packed with vesicles containing ACh (Bowman, 1980).

bungarotoxin, found 8,500 bungarotoxin binding sites per μm^2 of the post-junctional membrane of the mouse diaphragm. Later work discovered that receptors are not evenly distributed but are found more densely at the peaks of the secondary folds, adjacent to the nerve terminals, in numbers as great as 25,000 per μm^2 . However, very few are located at depths greater than 250nm into the secondary folds (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978).

The endplate channels associated with the ACh receptor are the most well defined channels to date. They were the first ionic channels to be solubilised from their native membrane and have been purified to near molecular homogeneity (Conti-Tronconi and Raftery, 1982). Their complete amino acid and nucleotide sequences have been determined (Noda *et al.*, 1983). This has revealed that the ACh receptor-channel consists of five separate polypeptide chains in one complex, two α chains which bear the ACh binding sites and three other chains assigned β , δ and γ . Their function has been reconstituted by reinserting the purified macromolecule into lipid membranes (Anholt *et al.*, 1984) and they were the first to have the electrical signal from a single open channel recorded (Neher and Sakmann, 1976).

1.2.3. A present day view of the mechanics of neuromuscular transmission.

It is clear that a purpose of ACh at the vertebrate neuromuscular junction is to transmit excitation within the nerve terminal to the muscle, and in so doing generate action potentials in the muscle fibres. But how is this transmission brought about? On the arrival of an action potential within the nerve terminal depolarisation of the pre-junctional membrane occurs. This opens voltage-dependent Ca^{2+} channels, permitting external Ca^{2+} ions to enter the nerve terminal, down its electrochemical gradient, and triggering the release of prepackaged vesicles of transmitter. The ACh crosses the synaptic cleft and combines with the two α sub-units of the ACh receptor-channel complex on the post-synaptic membrane which opens their associated channels allowing small cations to enter the muscle fibres (Takeuchi and Takeuchi, 1959, 1960; Takeuchi, 1963). The depolarisation of the post-junctional membrane which ACh produces, from a resting membrane potential of between 70 and 90mV

negative inside, is caused by an inward flow of Na^+ ions, but the permeability to K^+ is increased at the same time, and the resulting efflux of K^+ prevents the endplate potential (EPP) from moving beyond the zero-potential level. In fact, the null-point of the EPP has been found to be between -10 and -20mV, the liquid-junction potential (del Castillo and Katz, 1954c). Below that level, ACh causes net current to flow inward through the membrane and therefore depolarise the fibre; above that level, the net current flows outward and the potential is in the reverse direction from the normal EPP. Thus the transmitter action tends to displace the membrane potential toward approximately -15mV, beyond the threshold of excitation. This in turn creates local currents in the vicinity, in particular opening Na^+ channels in the perisynaptic region causing an action potential to be propagated along the muscle fibre. In a great deal of electrophysiological work, current is applied as a stimulus and the ensuing changes in potential are measured. Hence the applied current flows locally across the membrane both as ionic current and as capacity current and also spreads laterally to distant patches of the membrane. In voltage clamp, an important technique developed to study ion channels, this process is reversed, that is a voltage is applied and the resultant current measured. In addition, conditions are used to minimise capacity currents and the spread of local circuit currents so that the observed current can be a direct measure of ionic movements across a known membrane area at a known, uniform membrane potential.

Once the channels associated with the ACh receptor close the transmitter is released back into the synaptic cleft where it is rapidly hydrolysed by AChE to choline and acetic acid (see section 1.3). This is rather facile statement to make, but is any enzyme capable of hydrolysing its substrate with the sort of speed necessary for the maintenance of neuromuscular function, particularly during repetitive stimulation? Marnay and Nachmansohn (1937, 1938) attempted to determine whether the cholinesterase (ChE) present in nerve endings was present in sufficient quantities and could hydrolyse ACh at a sufficient rate to maintain neuromuscular transmission. They found that if the rate of hydrolysis of ACh by the nerveless pelvic end of frog sartorius muscle was compared with that of the parts containing nerve endings 100mg of the nerve-free ground muscle split only 0.13mg of ACh, whereas 100mg of the same

muscle containing nerve endings split 0.4-0.8mg, according to the richness in nerve endings. This suggested the presence of a concentration of ChE at the endplates many thousand times higher than that found in muscle tissue, which would enable the hydrolysis of ACh within the refractory period. The rate of activity of AChE is said to among the highest for any enzyme (Silver, 1974). For example, Nachmansohn (1972) calculated that the enzyme associated with 1g of excitable membrane of eel electroplax can hydrolyse more than 30Kg of ACh per hour. It has also been estimated that each enzyme site is capable of hydrolysing 1.6×10^5 to 8.3×10^5 molecules of ACh in one minute (Berry, 1951; Cohen *et al.*, 1955; Wilson and Harrison, 1961). Hobbiger (1976) pointed out that it seems there are at least ten ACh receptors and ten active enzyme sites available for each molecule of ACh released by an action potential. He also estimated that the concentration of ACh in the junctional cleft immediately after it has been released by a nerve impulse, is in the range which will partially inhibit enzyme activity. He nevertheless considers that there would still be sufficient enzyme activity to lower the ACh concentration to inactive levels within one millisecond, well within the refractory period of the muscle membrane (Wilson, 1971). Such rapid enzymatic activity along with diffusion from the synaptic cleft, ensures there is no accumulation of released ACh from one action potential to the next.

The nerve terminal returns to its resting state as the Ca^{2+} that entered is bound and extruded, choline is taken up from the cleft and resynthesised into ACh, and membranes of discharged vesicles are incorporated into the axolemma.

1.2.4. Chemical versus electrical transmission across the neuromuscular junction.

The first speculations on the nature of transmission across junctional regions had long preceded the period of controversy in the 1930's between the exponents of the chemical and electrical hypotheses. DuBois-Reymond (1877) was the first to suggest that junctional transmission could be either chemical or electrical, more probably the former, while Kuhne (1888) thought it more probable that the action current of the nerve impulse excited the muscle fibre at the motor endplate. Elliott (1904) provided the next significant development

when he suggested that sympathetic nerve impulses acted by liberating adrenaline at the junctional regions on smooth muscle, and a little later Dixon (1906) proposed that parasympathetic nerve impulses acted by liberating a muscarine-like substance. Dale (1914) implied that the substance ACh was an ideal candidate for a chemical transmitter at some synapses. This was confirmed a few years later when the heart rate was shown to be slowed when the vagus nerve secreted this substance (Loewi, 1921). It was a later, elegant series of experiments which implied ACh was the transmitter at sympathetic ganglia and the neuromuscular junction (Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1934; Dale *et al.*, 1936; Brown *et al.*, 1936). However, some groups, particularly Eccles (1936, 1937, 1944), postulated that unlike the transmitter actions at post-ganglionic junctions, which had a duration of seconds, the extremely rapid transmission that occurred at the neuromuscular junction was too fast to be achieved by ACh. This gave rise to the hypothesis that the pre-synaptic current was responsible for the brief excitatory action at the neuromuscular junction and that ACh was responsible for the prolonged residual depolarisation (Eccles, 1936, 1937, 1944). A convincing series of experiments by Kuffler (1949), however, finally dismissed the role of electrical transmission at the neuromuscular junction and concluded that ACh was the means by which information was transferred from nerve to muscle. Although dismissed as the mode of transmission at the neuromuscular junction, the concept of electrical transmission has continued, particularly with the discovery of certain synapses whose cell membranes show regions of very close proximity, even fusion in some cases (Hama, 1961, 1962; Bennett *et al.*, 1963). It has been suggested that at these so-called "tight junctions" the mode of transmission is electrical.

Much of the understanding of chemical transmission at synapses has developed from the study of the neuromuscular junction, mainly due to the accessibility and size of the post-junctional membrane along with the availability of modified neuromuscular junctions such as the eel electroplax on which extensive biochemical studies could be performed. The structure is relatively simple consisting of pre- and post-junctional membranes between which is the synaptic cleft, which contains the basement membrane (figure 1.1). A structural arrangement of this kind seems incompatible with a continual

electric cable process across the junction. In fact, if one assumes the electrical resistances and capacities of the synaptic membrane were the same as those which exist along the rest of the fibres, the action current produced by the nerve endings could not depolarise the post-junctional membrane by more than a fraction of a microvolt (Katz, 1966). However, calculations of this sort are fraught with uncertainty, since the electrical properties of these regions are not known, and may well be different at synaptic junctions than elsewhere. Some synapses display much closer proximity of pre- and post-junctional membranes and in some cases of fusion of the two has been reported (Hama, 1961, 1962; Bennett *et al.*, 1963). This may indicate that electrical transmission may occur at certain types of synapse and it could be that membrane fusion of this type is the structural basis for electrical coupling between two cells.

1.2.5. Quantal release of transmitter.

Fatt and Katz (1952) discovered small, spontaneous depolarising potentials in the endplate region of frog muscle fibres whose frequency varied from 0.1-100Hz. These small, spontaneous potentials have the same localisation, time course and pharmacological sensitivities as the much larger endplate potential (EPP) evoked by nerve stimulation, and are thus called miniature EPPs (MEPPs). Direct iontophoretic application of ACh to the post-junctional membrane produces a graded depolarisation, rather than discrete MEPP-sized depolarisations, indicating that the MEPP cannot be produced by a single molecule of ACh, but rather requires the synchronous release of at least a thousand ACh molecules (Fatt and Katz, 1952; Katz and Miledi, 1972). These depolarisations are now known to be due to the opening of ion channels associated with ACh receptors.

When the average EPP amplitude is reduced by bathing the neuromuscular junction in raised Mg^{2+} and reduced Ca^{2+} , the EPPs show stepwise amplitude changes. The size of the EPP amplitude steps appears to correspond to the size of the spontaneously occurring MEPPs, suggesting that the EPP is made up of an integral number of MEPP-sized depolarisations. Del Castillo and Katz (1954a) confirmed this suggestion quantitatively, and demonstrated that the number of MEPPs in the EPP fluctuates in a probabilistic manner described by the Poisson

distribution for random events. This result demonstrates that the MEPP reflects the minimum functional unit of ACh release from the nerve terminal, apart from non-quantal release of ACh. This minimum unit is termed the quantum, and the post-junctional potential produced by a quantum of ACh is called the quantum potential. The number of quantum potentials making up the EPP is called the quantum content of the EPP, and is about 200 in the normal unblocked frog neuromuscular junction. Variations in the average size of the MEPP from one muscle to the next can be accounted for largely by variations in the electrical properties of the muscle fibre rather than in the conductance change underlying the synaptic current (Katz and Thesleff, 1957a), and MEPP amplitudes usually show a unimodal distribution (Fatt and Katz, 1952), suggesting that the size of the released ACh packets is fairly constant.

1.2.6. The vesicular hypothesis.

Following the discovery of the quantal nature of ACh release by Fatt and Katz (1950, 1952) and del Castillo and Katz (1954a), vesicles within the nerve endings were revealed by electron microscopy (de Robertis and Bennett, 1955; de Robertis, 1958), and it was natural to suggest that ACh was packaged within them. The release process involves the temporary fusion of the vesicles with the pre-junctional membrane with the contents being expelled by exocytosis.

Evidence in favour of this hypothesis includes the observations that the nerve terminals are depleted of vesicles by intense, prolonged stimulation, and the surface area of the nerve terminal increases, as one might expect if discharged vesicular membranes were fused with it (Hubbard and Kwanbunbumpen, 1968; Korneliussen, 1972; Ceccarelli *et al.*, 1973; Heuser and Reese, 1973). Electron microscopy has demonstrated that vesicular fusions with the terminal membrane, like MEPPs, are increased by nerve stimulation, and decreased by raised extracellular Mg^{2+} (Heuser *et al.*, 1974).

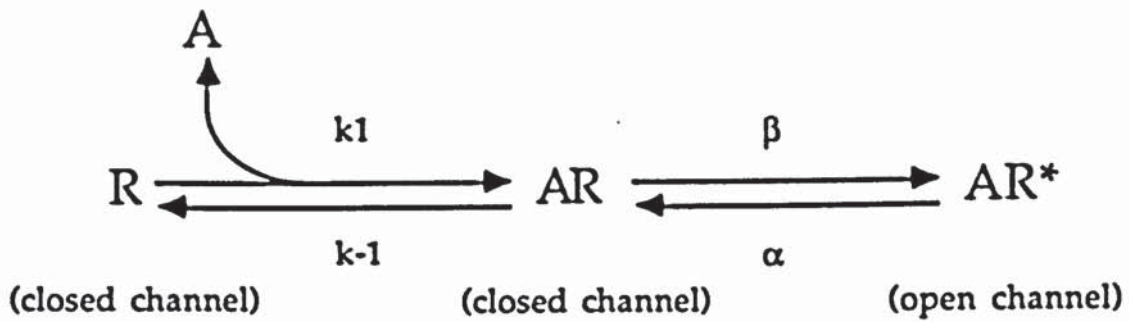
1.2.7. The kinetics of the receptor-channel complex.

The action of ACh released from the synaptic vesicles results in a brief inward postsynaptic ionic current produced by a rapid influx of positively charged ions, in particular Na^+ . This initially results in an endplate potential (EPP), before

the threshold is reached and an action potential generated. These events were described as early as 1951 by Fatt and Katz, and later confirmed by the voltage-clamp studies of other workers (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972a, b). On the arrival of an action potential at the nerve terminal, characterised by an inward flow of current presynaptically, there is a latent period known as the synaptic delay. An investigation of particular interest to the present study is the work of Katz and Miledi (1965) who examined the variability of synaptic delay in the frog. They discovered that, at 20°C, the synaptic delay at a particular endplate had a minimum value of 0.4 to 0.5msec and a modal value of 0.75msec. They also found considerable fluctuation of the intervals during a series of nerve impulses; over 50% occurred within a range of 0.5msec, the rest being spread out in declining fashion over a further 1 to 4msec. The authors discussed the various factors which contributed to the minimum synaptic delay, and concluded that it arose chiefly from the delay in release in transmitter after the arrival of the nerve impulse. This is rapidly followed by the appearance of the EPP post-synaptically. The rate of rise of this EPP is determined by the variation in the release times of the individual quanta and the time taken for released ACh to find unoccupied receptors. The falling phase of the EPC can be described by a single exponential decay with a rate constant α , according to Magleby and Stevens (1972a). In assigning this rate constant they followed the kinetic model for the binding of an agonist (A) to receptor (R) with subsequent opening of the channel as described by del Castillo and Katz (1957) (figure 1.2a). From this they suggested that the binding reaction of ACh to the receptor is so rapid that AR is effectively at equilibrium with free A and R. Then, if the ACh released by the nerve disappeared quickly, the decay of the EPC would be described by the random closing of open channels AR* giving an exponential decay with the rate constant α . The AR complex would then dissociate quickly following channel closure and the ACh would leave the cleft or be hydrolysed by AChE. This view was supported by the noise analysis of Katz and Miledi (1970, 1971) who discovered that the steady application of ACh to the frog neuromuscular junction induced a fluctuating post-junctional response caused by random opening and closing of endplate channels.

A number of observations brought into question the three-state kinetic model

(a)



(b)

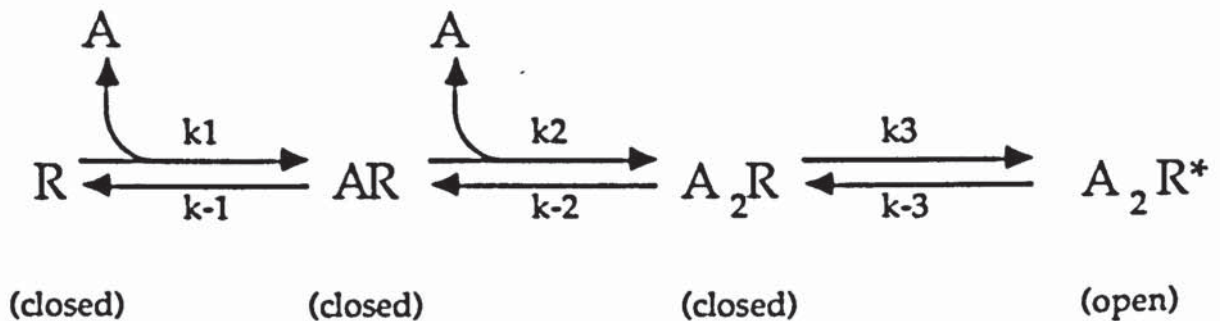


Figure 1.2. (a) The three-state kinetic model of del Castillo and Katz (1957) to describe the activation of the ACh receptor and consequent channel opening. (b) The more accurate four-state kinetic model involving the binding of two molecules of ACh before the channel opens.

described in figure 1.2a. Firstly, if one ACh molecule opens one channel the endplate conductance should increase linearly with ACh concentration at low concentrations, following a curve like the classical Michaelis-Menten equation of enzyme kinetics. This is not the case, the dose-response relationship curves upwards at low concentrations, showing a four-fold conductance increase for a doubling of ACh concentration (Katz and Thesleff, 1957a; Adams, 1975; Dionne *et al.*, 1978; Dreyer *et al.*, 1978). This implies that the opening of the channel requires the binding of two molecules of ACh, a fact confirmed by the isolation of channel macromolecules (Karlin, 1980; Conti-Tronconi and Raftery, 1982). Therefore, the kinetics can be expanded to that seen in figure 1.2b.

Subsequent to the development of the patch clamp technique, Colquhoun and Sakmann (1981) discovered fine structure in what previously published work called "single openings". One opening event was seen to be punctuated by several closings lasting only tens of microseconds, too short to have been detected in the past. It can be said that the channel rapidly passes from the A_2R (closed) state to the A_2R^* (open) before the ACh is lost (figure 1.2b). It was at a similar time that the patch clamp was refined by the use of the "gigaseal", a high resistance seal between the glass of an electrode and a biological membrane, which greatly increased the resolution of channel currents (Hamill *et al.*, 1981). The channel openings have a rectangular appearance expected from a simple open-close interpretation of classical gating kinetics. Also, rather more unexpectedly, the vast majority of single channel openings reach a characteristic "unitary" conductance level, as well as the open events being frequently interrupted by brief closings (flickering).

1.3. CHOLINESTERASES.

1.3.1. Types of cholinesterase.

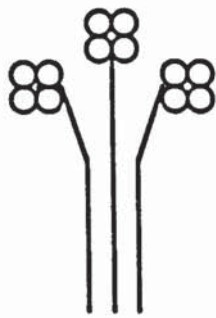
Cholinesterases can be broadly classified into true (or specific) cholinesterases and pseudo (or non-specific) cholinesterases. True cholinesterase is to be found in nervous tissue and striated muscle and is responsible for the destruction of ACh. It is also found in non-cholinergic nerves and other tissues, such as red blood cells, where its function is not clear. Pseudocholinesterase occurs in plasma, intestine, skin and many other tissues, where its role has not altogether been elucidated.

An alternative nomenclature is to describe the enzyme according to the substrate against which they show the greatest activity. Hence, acetylcholinesterase (AChE, acetylcholine acetylhydrolase, which has the enzyme commission classification EC.3.1.1.7) is more active against acetylcholine than against other choline esters and is the main true cholinesterase present in mammals. Butyrylcholinesterase (BuChE, acetylcholine acylhydrolase, EC.3.1.1.8) shows its highest activity against butyrylcholine and is the main pseudocholinesterase found in mammals.

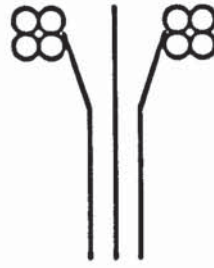
1.3.2. The molecular forms of cholinesterase.

The quest to isolate and determine the structure of cholinesterases was boosted following the development of affinity chromatography. Using this technique it was possible to extract small amounts of cholinesterase and receptor molecules from large volumes of tissue (Rosenberry *et al.*, 1972). It did, however, lead to discrepancies between the molecular weights of extracted material depending on the exact technique used, which was presumably due to the disruption of the integrity of the neuromuscular junctions. Values for the molecular weight of AChE varied from 30 million to as low as 70,000 (Silver, 1974; Rosenberry, 1975). It was suggested that the crudity of the techniques led to such discrepancies and, in the light of the work of Bon *et al.* (1979), this was confirmed. In their studies they were able to identify six different forms of AChE, three of which were globular in nature and three which were designated asymmetric. The three globular forms were aggregates of a number of identical

ASYMMETRIC



A12



A8



A4

GLOBULAR



G4



G2



G1

Figure 1.3. The diagrammatic representation of the globular and asymmetric forms of AChE, as indicated by Bon *et al.* (1979).

chains, each of which had a molecular weight of approximately 71,000 Daltons. Each chain also corresponded to a single enzyme sub-unit with one active site. The forms were assigned G1, G2 or G4 depending on how many globular chains they contained. The three asymmetric forms of the enzyme were called A4, A8 and A12, and consisted of one, two or three of the tetrameric G4 forms of the enzyme respectively, connected to a collagen tail which is 50nm long. The molecular weights of the tails being estimated at approximately 100,000. It is believed that the collagen tails are a component part of the basal lamina, holding the enzyme in place (Massoulié and Bon, 1982). These molecular forms are represented in figure 1.3.

1.3.3. The synthesis and function of AChE.

The asymmetric forms of AChE are thought to be of functional importance in neuromuscular transmission, in particular because of their close proximity to the ACh receptors. Recently, an internal pool of asymmetric AChE has been identified in neuronal and muscle cells in culture by the use of anti-ChEs of differing membrane permeabilities. After irreversible inhibition of all cellular AChE by DFP, the newly synthesised asymmetric AChE appeared in an intracellular compartment within 90 minutes, but did not appear on the cell surface for approximately 2.5 hours (Inestrosa, 1984). Rotundo (1984) also analysed AChE assembly after DFP inactivation using lectins that recognised sugars attached in the rough endoplasmic reticulum and Golgi apparatus, concluding that asymmetric AChE was assembled in the Golgi apparatus. It is believed that the intracellular pathway probably involves the packing of asymmetric AChE into transport vesicles, transportation to the cell surface where it is released and subsequent incorporation into the basal lamina. Moreover, because the AChE and the ACh receptor are transported to the cell surface with similar kinetics, it is possible that they follow a single intracellular transport pathway (Rotundo and Famborough, 1980).

A role of AChE in the neuromuscular junction is to hydrolyse rapidly synaptically released ACh. It is thought that AChE consists of two binding sites within its active centre, an anionic and an esteratic site, as shown in figure 1.4a. The anionic site is believed to contain an ionised carbonyl group which

forms an ionic bond with the cationic head of the ACh molecule. Approximately 0.7nm from this there is the esteratic site which combines with the ester group of the ACh (Wilson and Bergman, 1950).

Once the ACh is reversibly bound to the enzyme the hydrolysis occurs in three steps (figure 1.4b). Firstly the ester bond is cleaved and the choline is lost leaving the acetyl moiety covalently attached to the esteratic site. The acetylated enzyme is rapidly hydrolysed to release acetic acid (Main, 1976). The products of ACh hydrolysis are free to be taken up by the nerve terminal from which new transmitter can be synthesised, a process which will not be detailed here.

1.3.4. Localisation of AChE.

Early work on the localisation of AChE in the synaptic cleft utilised its role as an esterase. A thiol-substituted analogue of ACh was used as a source of sulphur, liberated on the hydrolysis of this false transmitter. This reacted with a reagent which contained lead to produce a precipitate of lead sulphide, a compound which is electron dense. Using this technique, Zacks and Blumberg (1961b) discovered quantities of lead sulphide in the synaptic cleft, however, a more precise idea of AChE location could not be achieved since the stain had a tendency to spread over a large area before being deposited as a precipitate.

Bergman *et al.* (1967) thought AChE was present in the muscle sarcoplasm, close to the post-synaptic membrane, in their studies which used specific substrates of the various types of cholinesterase. In the same year, Teravainen (1967) managed to identify AChE activity associated with the post-synaptic membrane and activity of other non-specific cholinesterases close by in the sarcoplasm.

Rogers *et al.* (1969) attempted to count the number of enzyme binding sites in mouse skeletal muscle using the irreversible organophosphorus anticholinesterase (anti-ChE) diisopropyl fluorophosphate (DFP) labelled with ³²-Phosphorus. There are, however, a number of disadvantages of this technique in localisation of the enzyme. Firstly, the β particles emitted by the ³²-Phosphorus can travel some distance before reacting with the detecting

emulsion. This makes localisation difficult, although an indication of the number of DFP-binding sites can be gained. Within this lies a second problem, binding of DFP is not specific to AChE and thus is likely to give high, inaccurate readings of AChE binding sites. Silver (1974) refined the technique by using Pyridine-2-aldoxime methiodide or pralidoxime (2PAM), a specific reactivator of AChE, to differentiate the AChE from other enzymes. Tritium-labelled DFP is more useful for localisation of enzyme activity since the β particles emitted by tritium are less energetic than those emitted by 32 -Phosphorus and thus do not travel as far before being absorbed (Salpeter, 1969). Using this technique it was discovered that of 8,700 DFP-binding sites per μm^2 of the post-junctional membrane approximately a third could be reactivated by 2PAM. This indicated that there were about 2,500 AChE binding sites per μm^2 of the post-junctional membrane of the sternomastoid and diaphragm muscles of mouse (Salpeter *et al.*, 1972; Barnard *et al.*, 1973).

Hall and Kelly (1971) liberated active endplate AChE from intact rat diaphragm by treating the muscle with collagenase. They also showed that, after 4 hours of such enzymatic treatment, the resting membrane potential was unchanged, action potentials could still be generated, however, the time course of EPPs and miniature endplate potentials (MEPPs) were prolonged, implying that the AChE made been removed without substantial damage to the surface membrane. This suggested that the AChE was situated in a relatively exposed position on the external nerve or muscle cell surface, either on the surface of the membrane or on the external lamina, and was probably not a structural component of the membrane. The final step in the localisation of AChE was the work of McMahan *et al.* (1978), who were able to extract the basal lamina from the synapses of the frog cutaneous pectoris muscle. This basal lamina was then assayed for cholinesterase activity, which was not only found in abundance but also was found to be of the AChE type.

1.4. ANTICHOLINESTERASES.

Anticholinesterase drugs (anti-ChEs) can be divided into two main classes according to their chemical composition and the stability of the enzyme-inhibitor complex. The first class are able to dissociate from the enzyme quickly (reversible anticholinesterases) and are very similar in structure to ACh (e.g. edrophonium). In comparison the second class of compounds display a much more stable and long-lasting complex with the enzyme and are divided into two sub-groups the carbamate (e.g. neostigmine) and organophosphate (e.g. ecothiopate) anticholinesterases.

This study only investigated the actions of the organophosphorus anti-ChEs, in particular three compounds with slightly differing characteristics. Firstly, ecothiopate iodide (ECO), a quaternary compound whose phosphorylated form of AChE ages relatively slowly (for mechanism see section 1.4.1), it is thus open to a certain amount of spontaneous reactivation (figure 1.6) and can be reactivated by oximes (Hobbiger, 1956). Pinacolyl S-(2-trimethylaminoethyl) methylphosphonothioate (BOS) is also a quaternary compound which when attached to AChE rapidly converts to the aged form of the phosphorylated enzyme, this compound shows no reactivation neither spontaneously or following oxime treatment (Harris *et al.*, 1971). The actions of BOS thus tend to last much longer than those of ECO. Finally, sarin is a tertiary compound whose phosphorylated form of AChE ages some what faster than ECO but not as rapidly as BOS. This compound displays little or no spontaneous reactivation, but can be reactivated by oximes (Davies and Green, 1956).

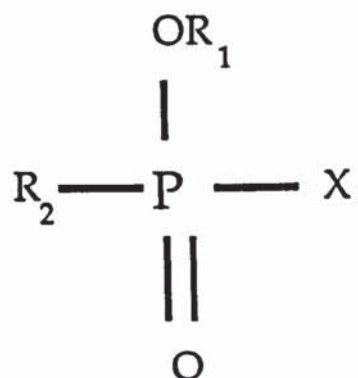
1.4.1. The structure and activity of anti-ChEs.

It is possible to divide the anti-ChEs used in this study into two categories, tertiary and quaternary compounds. Sarin is a tertiary compound possessing the ability to cross the blood-brain barrier and exert its toxic effects centrally. On the other hand quaternary compounds, such as ECO and BOS, restrict their actions to the periphery, because the quaternary nitrogen incorporated into their structures restricts access to the central nervous system.

The mechanisms by which these compounds produce their inhibitory actions occur at the esteratic site of the enzyme (figure 1.4a). Compounds which have a quaternary nitrogen within their structure are believed to be more selective towards AChE as there will be a certain amount of interaction between the enzyme anionic site and positively charged nitrogen of the compound in question.

The first stage of this irreversible inhibition involves the removal of the X group by hydrolysis (figure 1.5). This results in phosphorylation of the esteratic site. Following this stage two processes can occur, reactivation or ageing of the enzyme. Reactivation is the dissociation of the phosphoryl group from the esteratic site by hydrolysis to produce reactivated enzyme capable of cleaving ACh. Ageing involves the hydrolysis of one of the R groups from the phosphorylated enzyme to produce a highly stable form of AChE. Once the AChE-inhibitor complex has aged it can no longer be reactivated either spontaneously or by the use of oximes (see section 1.5). The duration of action of these compounds is thus determined by the relative rates of spontaneous reactivation and ageing of the phosphorylated form of the enzyme. For example, the half time of ageing of the phosphorylated enzyme produced by soman is in the order of 2.4 minutes due to the ease of cleavage of its pinacolyl group (Harris *et al.*, 1971). This process is so rapid that spontaneous reactivation cannot occur. These processes are illustrated in figure 1.6. Of the compounds used in this study BOS can be equated with soman, since their leaving groups are identical (figure 1.5) and one would expect to see no reactivation and a similar rate of ageing. ECO displays some spontaneous reactivation with a half time of between 44 and 64 hours, and a relatively slow rate of ageing with a 41 hour half time (Hobbiger, 1976). Like soman and BOS, sarin shows no reactivation but a much slower rate of ageing with a half time in the order of 3 hours (Hobbiger, 1976). The time course of recovery of the AChE is also dependent on the rate of synthesis and delivery of new enzyme.

General Formula:



Compound	R ₁	R ₂	X
Ecothiopate (ECO)	C ₂ H ₅ -	C ₂ H ₅ O	-SCH ₂ CH ₂ N ⁺ (CH ₃) ₃
Sarin	<i>i</i> -C ₃ H ₇ -	CH ₃ -	-F
Soman	(CH ₃) ₃ C(CH ₃)CH-	CH ₃ -	-F
Pinacolyl S-(2-trimethylaminoethyl) methylphosphonothioate (BOS)	(CH ₃) ₃ C(CH ₃)CH-	CH ₃ -	-SCH ₂ CH ₂ N ⁺ (CH ₃) ₃

Figure 1.5. The chemical structures of some of the organophosphorus compounds of interest to this study. The chemical structures are based on the general formula at the top of the page, and substituted as indicated below.

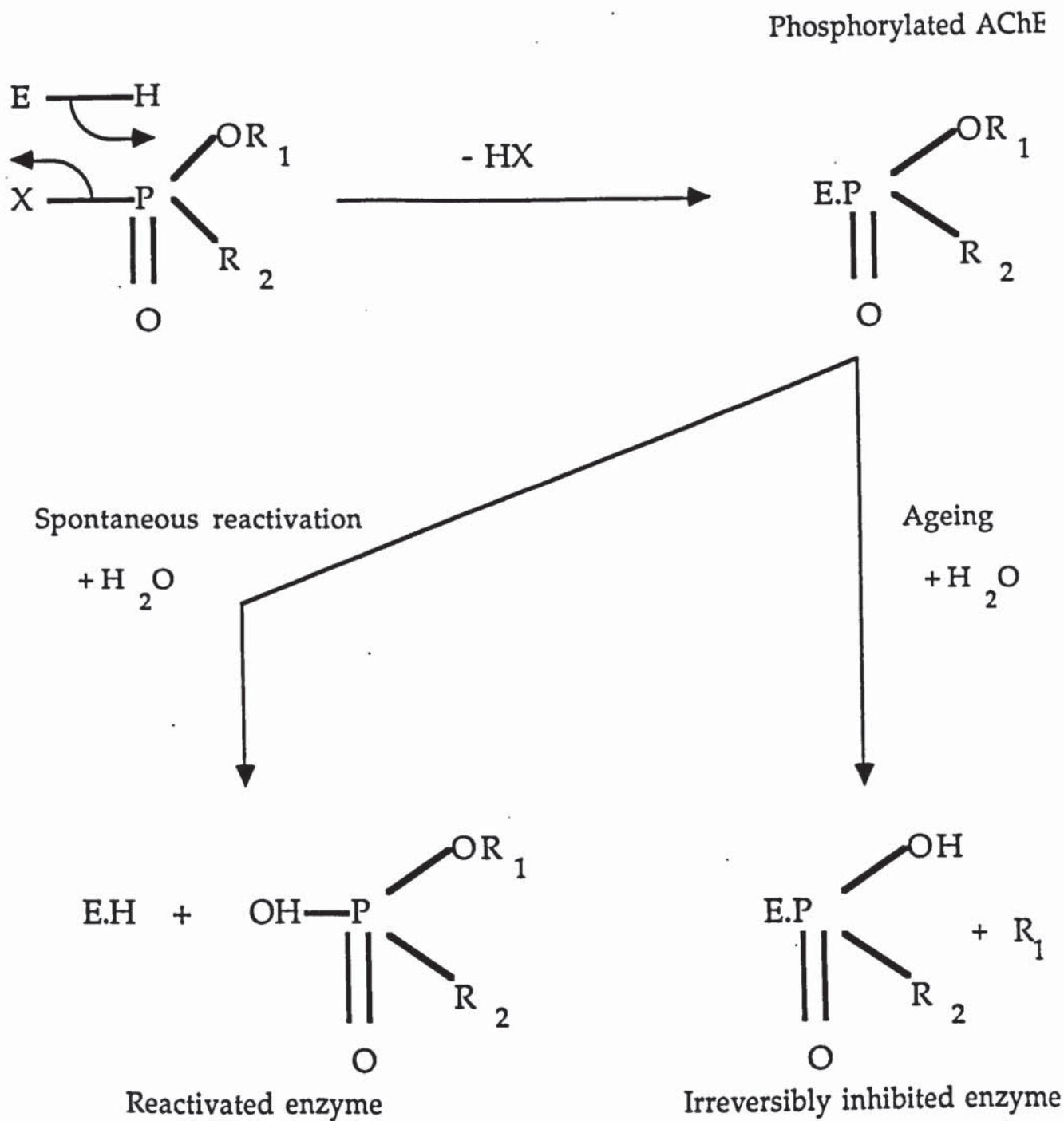


Figure 1.6. The mechanisms of action of anti-ChEs on AChE (E.H), showing the processes of phosphorylation, spontaneous reactivation and ageing.

1.4.2. The effects of exposure to organophosphorus anti-ChEs.

Through the actions on AChE described above, the acute effects of anti-ChEs can be grouped into three areas, nicotinic, muscarinic and central. Although sarin possesses central effects, actions on the central nervous system were not considered of interest in this study. Muscarinic effects such as nausea, vomiting, diarrhoea, sweating, increased bronchial constriction, abdominal cramps, urination, excessive secretion of tears, bradycardia and hypotension, are all symptoms which can be relieved by the prompt injection of atropine (Grob, 1956; Grob and Johns, 1958; Durham and Hayes, 1962). The nicotinic effects, produced by the AChE inhibitory action of these compounds at the neuromuscular junction of skeletal muscle described earlier, are manifested as muscle fasciculations and cramps along with muscle fatigue and muscle weakness (Durham and Hayes, 1962).

These actions at the neuromuscular junction can result in damage of the associated skeletal muscle, particularly in the areas around the endplates (Wecker *et al.*, 1978b; Meshul *et al.*, 1985; Gupta *et al.*, 1987ab; Gupta and Dettbarn, 1987). Studies of this phenomenon and its causes have been performed extensively in our laboratory in recent years (Townsend, 1988; Das, 1989). The purpose of this study is to look at long-term effects of these compounds which occur at times when the myopathy appears to be on the decline.

1.4.3. The effects of anti-ChEs on single twitches.

The response of mammalian skeletal muscle, in the presence of an anti-ChE, to low frequency, supramaximal, indirect stimulation is potentiated because each stimulus triggers repetitive muscle action potentials (Brown *et al.*, 1936). Studies of this so-called twitch potentiation have led authors to a variety of conflicting conclusions. Masland and Wigton (1940) observed that twitch potentiation was associated with repetitive antidromic action potentials in the motor nerve. Since an antidromic impulse originating in an individual nerve terminal will be conducted to other terminals of the same neurone, the production of antidromic firing following a single orthodromic stimulus could explain the potentiation observed. Masland and Wigton (1940) found they

could record antidromic activity in the ventral root of cat hindlimb if they injected ACh intra-arterially. They concluded that anti-ChE induced antidromic firing was due to an action of ACh on nerve terminals as a result of AChE inhibition which would normally limit the diffusion of ACh and thus protect the nerve terminal from this action. These conclusions were challenged by the work of Eccles *et al.* (1942), who were able to record repetitive muscle activity in the absence of antidromic firing, from which they deduced that the repetitive muscle response following a single orthodromic impulse might be initiated by the prolonged endplate potential (EPP) induced by the anti-ChE. Riker and Okamoto (1969) questioned the involvement of ACh in twitch potentiation altogether, suggesting that anti-ChEs triggered antidromic firing by some direct action on the nerve terminals. ECO has been shown to produce twitch potentiation initiated by repetitive muscle action potentials (Morrison, 1977; Ferry, 1988). Such action potentials are thought to be generated ectopically about 0.2mm away from the usual site (Ferry, 1988). Thus it was of interest to compare the actions of BOS with those of ECO in producing such an effect.

1.4.4. Spontaneous twitching of muscle.

In the absence of nerve stimulation organophosphorus anti-ChEs have been shown to produce spontaneous twitching of muscle (Modell *et al.*, 1946, Meer and Meeter, 1956), which can be defined as intermittent synchronised contractions of the muscle fibres constituting a motor unit (Hobbiger, 1976). Spontaneous twitching of muscle, like twitch potentiation, is thought to be caused by antidromic action potentials in nerves originating at a presynaptic site. Since nerve stimulation is not involved, and there is no evidence that the nerve terminals themselves can be depolarised sufficiently to act as stimulus generators in their own right, it has been suggested that antidromic firing is initiated by depolarisation of the first node of Ranvier by spontaneously released ACh, whose lifetime is prolonged by the presence of an AChE inhibitor (Hobbiger, 1976).

1.4.5. The effect of anti-ChEs on post-junctional potentials.

The effects of anti-ChEs in potentiating and lengthening ACh-mediated post-synaptic potentials at neuromuscular junctions have been attributed to the

accumulation of excessive amounts of transmitter within the synapse. Such effects have been demonstrated both in amphibian skeletal muscle (Eccles *et al.*, 1942; Kuba and Tomita, 1971; Kordas, 1972a, 1977; Goldner and Narahashi, 1974; Kuba *et al.*, 1974; Kordas *et al.*, 1975; Fedorov, 1976; Guinan, 1980; Skiliarov, 1980) and mammalian skeletal muscle (Boyd and Martin, 1956a; Blaber and Christ, 1967; Hall and Kelly, 1971).

In particular, the reduced rate of ACh hydrolysis after AChE inhibition results in a prolongation of the time course of spontaneous miniature endplate potentials (MEPPs). Boyd and Martin (1956a) and Blaber and Christ (1967), both showed a 2-3 fold increase in the time course of MEPPs in the cat muscle after the addition of neostigmine. Similarly, neostigmine produced a doubling of the half decay time of MEPPs in the rat diaphragm (Hall and Kelly, 1971).

1.5. PROPHYLAXIS AND TREATMENT OF ORGANOPHOSPHORUS POISONING.

It has been recognised for quite some time that oximes and hydroxamic acids are the most successful therapeutic agents in the treatment of organophosphorus intoxication (Durham and Hayes, 1962). The reversible anti-ChEs, otherwise known as the carbamates, have been utilised as a prophylactic treatment, success being dependent on a prolonged exposure to low doses of the protective agent (Berry and Davies, 1970; Dirnhuber and Green, 1978; French *et al.*, 1979; Dirnhuber *et al.*, 1979).

1.5.1. The mode of action of oximes.

If oximes are administered with suitable rapidity to an animal which has been exposed to an organophosphorus compound they have been shown to successfully reactivate AChE (Lipson *et al.*, 1969; Laskowski and Dettbarn, 1977; Wecker *et al.*, 1978a). The period during which the reactivation is successful is determined by the rate of ageing of the phosphorylated enzyme produced (Figure 1.6). If ageing is extremely rapid, as with BOS and soman, then oximes are unable to exert any effect (Loomis and Salafski, 1963; Heilbronn and Tolegen, 1965; Murtha *et al.*, 1970). Oximes are thought to act by nucleophilic attack at the phosphorus atom of phosphorylated enzyme (figure 1.7), thus the organophosphorus moiety is lost producing reactivated enzyme.

Three oximes were utilised for comparative purposes, pyridine-2-aldoxime methiodide (pralidoxime, 2PAM) and two bispyridinium compounds, toxogonin (obidoxime, TOX) and HI6 (figure 1.8).

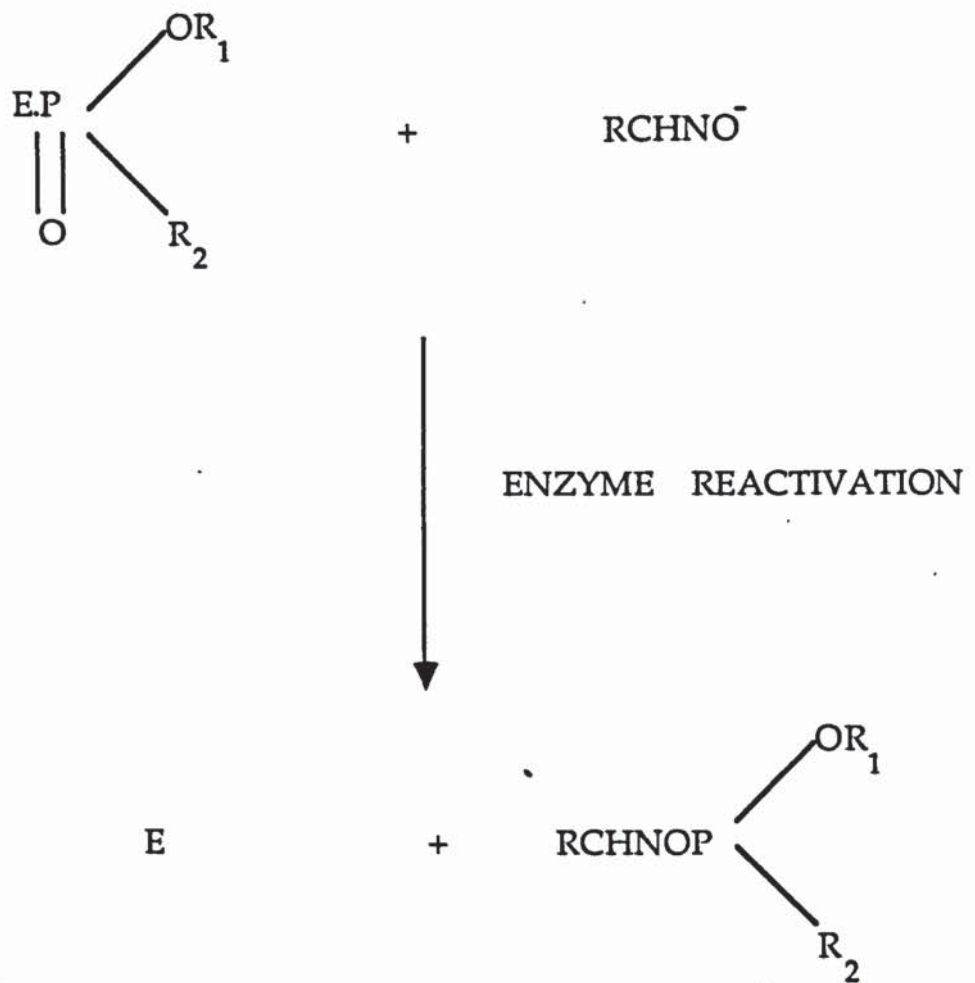
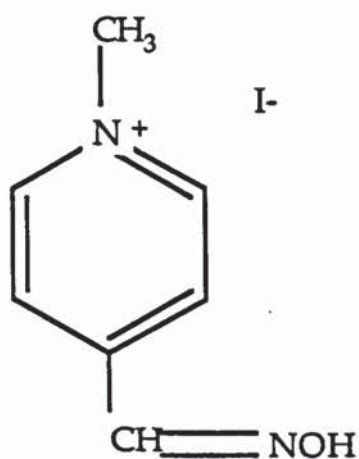
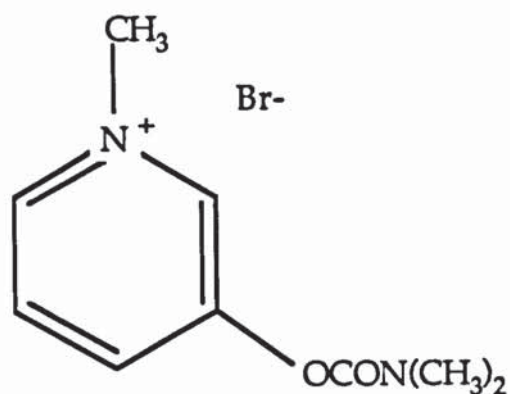


Figure 1.7. The mechanism of action of the oxime anion (RCHNO⁻) on phosphorylated AChE in producing reactivation of the enzyme (E).

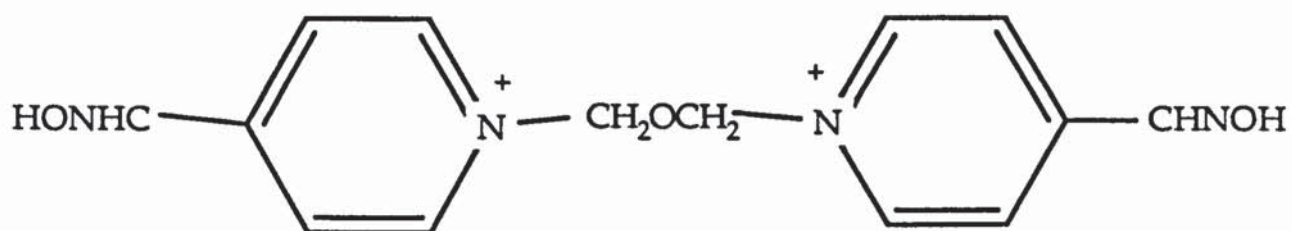
(a)



(b)



(c)



(d)

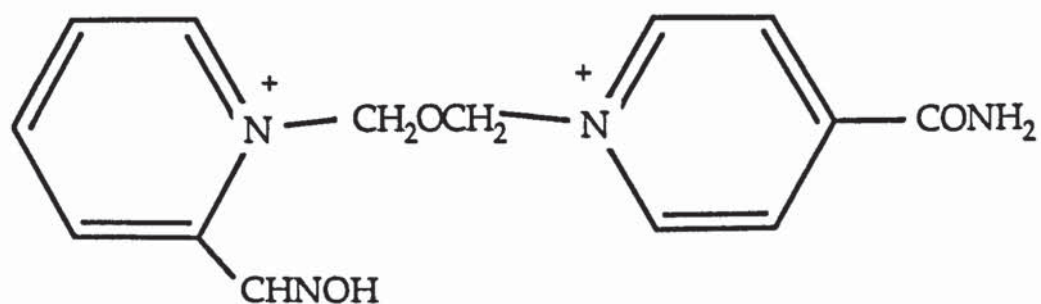


Figure 1.8. The chemical structures of the compounds used in the prophylaxis and treatment of anti-ChE poisoning; (a) 2PAM (b) Pyridostigmine (c) Toxogonin and (d) HI6.

1.5.2. Carbamates and prophylaxis.

The prevention of organophosphorus intoxication has long been provided by carbamates (Dirnhuber and Green, 1978; Gordon *et al.*, 1978; Wecker *et al.*, 1978a,b; Dirnhuber *et al.*, 1979; French *et al.*, 1979). The beneficial action of these compounds relies on their ability to reversibly inhibit AChE. During administration of the carbamate a proportion of the AChE becomes reversibly inhibited and, on exposure to an organophosphorus compound, it is this enzyme which is protected (Berry and Davies, 1970). After a period of time the carbamate dissociates from the AChE, thus enabling it to fulfill its function. It is thought that only a small proportion of the AChE needs to be protected to maintain normal neuromuscular function (Hobbiger, 1976). For example, French *et al.* (1979) demonstrated that pyridostigmine pretreatment reversed the tetanic fade produced by soman in the rat phrenic nerve-hemidiaphragm preparation, despite only a 5% increase in total AChE activity measured by the Ellman *et al.* (1961) technique.

Pyridostigmine bromide was used in this study (figure 1.7). This drug is used extensively in the treatment of myasthenia gravis and is active as a prophylactic against soman poisoning (Berry and Davies, 1970; Gordon *et al.*, 1978).

1.6. MUSCLE FIBRE TYPES.

During the course of this investigation a number of different skeletal muscles were used in an attempt to determine whether any of the increases in jitter seen were dependent on fibre type or differences in functional activity. Hence this section relates the differing properties of the various fibre types found within skeletal muscle, which may aid explanation of the phenomena observed.

1.6.1 White, intermediate and red fibres.

The difference in colour between red and white muscles is due to the presence of a predominance of so-called red fibres which contain a large quantity of the oxygen binding haemoprotein, myoglobin. Conversely pale muscles are composed mainly of white fibres which contain relatively little myoglobin. Most mammalian muscles contain a mixture of red and white fibres, however, there are some examples of muscles which are composed almost entirely of one particular type.

The terms "red" and "white" reflect only a different content of myoglobin and it should not be assumed that the colour is necessarily associated with other characteristics. However, on histological and biochemical examination, a number of generalisations can be made. In the same species, red fibres are usually uniformly thin and contain many mitochondria and fat droplets, while the white fibres are thicker and more variable in diameter with few mitochondria or fat droplets (Close, 1972). It became clear that the classification of fibres into two groups was an oversimplification, and it is now generally recognised that red fibres are divided into two distinct groups, "red" and "intermediate" fibres, as shown in table 1.1a.

White fibres are capable of anaerobic glycolysis, but their low lipid content, low mitochondrial content, and low myoglobin and oxidative enzyme activity generally make them susceptible to fatigue (Table 1.1a). They are therefore adapted for fast, short term, powerful phasic activity. On the other hand, intermediate fibres have poorly developed glycolytic activity and low glycogen stores. They are rich in lipid and are capable of oxidizing it to obtain energy so

(a)

Property	Fibre Classification		
	White	Intermediate	Red
Myoglobin content	low	high	high
Oxidative enzyme activity	low	high	high
Glycolytic activity	high	low	intermediate
Neuromuscular junction	large and complex	intermediate	small and simple
Twitch speed	fast	slow	fast
Susceptibility to fatigue	fatigues easily	resistant to fatigue	fatigues slowly

(b)

Muscle of Rat	Percentage of total number of fibres		
	White	Intermediate	Red
Diaphragm	20	20	60
Extensor digitorum longus	51.2	3.8	45
Soleus	—	80-90	10-20

Table 1.1. (a) The characteristics of the different types of muscle fibres and (b) their distributions in the muscles used in this study. All measurements are taken from the rat (Close, 1972).

that they are resistant to fatigue. This fibre group are thus specialised in low-speed tonic activity, such as in the maintenance of posture. Like intermediate fibres, red fibres are capable of oxidative enzyme activity. Although red fibres are less resistant to fatigue, they fatigue at a slower rate than white fibres. These properties, along with their characteristic nature of fast-twitch, make them ideal for prolonged phasic activity such as that associated with respiration (Close, 1972).

Most muscles studied contain a mixture of the different types of fibres, which is generally compatible with their function. Table 1.1b gives the proportions of the various fibre types in the muscles examined in this study, however the values shown are taken from the rat and it is not clear how close they are to those of murine muscle.

1.6.2. Fast twitch and slow twitch fibres.

Muscle fibres in adult muscles differ in their speeds of contraction and relaxation. White fibres and red fibres are fast contracting whereas fibres of the intermediate type are slow contracting. Thus, the contraction and relaxation speeds of a muscle as a whole will be dependent on the proportions of the fast contracting and slow contracting fibres within it.

Three muscles have been utilised in this study, the soleus, extensor digitorum longus (EDL) and the diaphragm. In the mouse, the EDL and diaphragm muscles are generally classified as fast twitch muscles (Luff, 1981), however, as can be seen from table 1.1b their compositions differ somewhat. The EDL consists of mainly red and white fibres, the large proportion of the latter make it prone to fatigue. The diaphragm on the other hand contains a majority of red fibres and, although generally described as fast twitch, Luff (1981) stated that some of the dynamic properties of the diaphragm are clearly midway between the typical fast twitch EDL and the typical slow twitch soleus. The soleus muscle is composed mainly of intermediate fibres, making it slow twitch in nature.

1.7. MUSCULAR DYSTROPHY.

Muscular dystrophy is a genetically determined disorder which is generally characterised by progressive wasting and weakness of muscles. It covers a broad range of disease states, with a variety of symptoms and severities. A number of species, of which the mouse is one of the most extensively investigated (Michelson *et al.*, 1955), display inherited myopathies analogous to the human state.

1.7.1. Murine models of muscular dystrophy.

Symptoms analogous to those of muscular dystrophy were first observed in an inbred colony of 129 Rej mice at the Jackson Memorial Laboratory, Bar Harbor, Maine (Michelson *et al.*, 1955). Subsequently, an autosomal recessive gene (dy) located on chromosome 10 was found to be responsible for the disease (Stevens *et al.*, 1957; Sidman *et al.*, 1979). Clinical signs of the disease, first detected at about three weeks of age, include: dragging of the hindlimbs; extension of the hindlimbs at an abnormal angle to the body; flexion of the hindlimbs when suspended by the tail and spasmodic nodding of the head. Animals affected by the disease are unable to breed and have a much reduced lifespan (Michelson *et al.*, 1955; Baker *et al.*, 1958). Since the discovery of this model a number of other murine dystrophies have been isolated, among which the most interesting is probably that found in an inbred colony of C57BL/10 mice (Bulfield *et al.*, 1984). The mdx mutation, as it is known, like the human disease state is located on the X chromosome. Mice which are affected are said to display only mild symptoms of the disease and are otherwise described as vigorous and fertile.

1.7.2. The histopathology of dystrophy in 129 Rej mice.

One of the recognised characteristics in skeletal muscle of Rej 129 dy/dy mice affected by the disease is a large variation in fibre size (Harman *et al.*, 1963; Banker, 1967). It was suggested by Rowe and Goldspink (1969) that the very small fibres observed may be atrophying or regenerating, while the very large fibres are hypertrophied to compensate for the reduced number. Another

typical feature is the splitting of muscle fibres, along with affected fibres displaying large numbers of nuclei which are more centrally located than observed in normal fibres (Banker, 1967; Isaacs *et al.*, 1973). Reduced muscle mass also seems to be associated with fat infiltration, both around the fibres and as deposits intracellularly (Michelson *et al.*, 1955; Wechsler, 1966; Makita *et al.*, 1973). It has been shown that in 129 Rej dy/dy mice fast twitch muscles display greater myopathy than slow twitch muscles (Goldspink and Rowe, 1968; Shafiq *et al.*, 1969). Although whether glycolytic or oxidative fast twitch fibres are most affected is not clear (Fahami and Roy, 1966; Susheela *et al.*, 1968, 1969; Cosmos *et al.*, 1973; Butler and Cosmos, 1977).

There is general agreement that there is a decrease in number and diameter of the synaptic vesicles in the nerve terminals of the endplates of dystrophic muscle (Ragab, 1971; Pachter *et al.*, 1973; Banker *et al.*, 1979), however, one team of workers did report increases in the number of synaptic vesicles (Ellisman *et al.*, 1975). Banker *et al.* (1979) described nerve terminal retraction with interposition of Schwann cell cytoplasm or basement membrane proliferation. Alterations of the post-synaptic membrane have also been reported, the number and complexity of the synaptic folds being reduced (Ragab, 1971; Ellisman *et al.*, 1975; Banker *et al.*, 1979; Ellisman, 1981). Another characteristic of such dystrophic animals is axonal demyelination, particularly in areas of the spinal and cranial nerve roots where groups of 50 or more fibres have been found totally unenveloped by Schwann cells, resulting in a reduced rate of conduction (Bradley and Jaros, 1979). Harris and Ribchester (1979a) could find no differences between normal and dystrophic muscles in their characteristics of EPPs or MEPPs, the transmitter null potential, the size of the available store of transmitter, the probability of release of transmitter or the quantal contents of EPPs in response to nerve stimulation of 0.1 to 100Hz. In a later study (Harris and Ribchester, 1979b), they did find that the nerve terminals were much larger in dystrophic muscles than in those of normal animals along with a preponderance of nerve terminal sprouting. It was concluded that this sprouting and expansion of the endplate region was an attempt by the axon to expand the area of synaptic contact in hypertrophied muscle fibres. There is considerable variation in the degree to which the disease affects any one animal (West and Murphy, 1960; Platzer, 1979). These variations from animal to

animal and from muscle to muscle can make the interpretation of results somewhat difficult.

1.7.3. A comparison of murine and Duchenne muscular dystrophies.

A number of qualitative differences between these two states have been noted. In murine dystrophy there are structural and functional changes in the central (Biscoe *et al.*, 1974; Frostholm *et al.*, 1983) and peripheral nervous systems (Bradley and Jaros, 1979; Kuno, 1979; Brimijoin and Schreiber, 1982) which are not seen in Duchenne dystrophy (Dubowitz, 1979). Also Duchenne is a sex-linked disease, the gene being located on the X chromosome, whereas the dystrophy of the 129 Rej mouse is inherited via an autosomal recessive gene (Michelson *et al.*, 1955).

Pathologically, there is a close similarity between Duchenne dystrophy and the dystrophy displayed by the 129 Rej *dy/dy* mouse (Ross *et al.*, 1960). The histopathology, described earlier (section 1.7.2), of the murine dystrophy is closely analogous to that seen in Duchenne dystrophy (Bell and Conen, 1968). Raised serum levels of creatine phosphokinase, diagnostically recognised in Duchenne dystrophy (Bradley *et al.*, 1972), is also seen in murine dystrophy (Watts and Watts, 1980).

These and other similarities have resulted in murine dystrophy being used widely as a model for Duchenne dystrophy, and, although direct comparisons between man and mouse cannot be made, the study of murine dystrophy aids elucidation of basic muscle pathology.

2. MATERIALS AND METHODS

2.1. ANIMALS

2.1.1. Albino mice

Male albino mice, purchased from Bantin and Kingman, Hull and fed *ad libitum* on the company's breeding diet, were ex-breeders, 6-7 months of age and 35-45g in weight. Kelly (1978) and Banker *et al.* (1983) indicate that by six months of age CFHB rats and CBF-1 mice respectively have stable neuromuscular characteristics, it was thus assumed that the mice used could be classed as adult with similar stable properties at the neuromuscular junction.

2.1.2. Dystrophic mice

The dystrophic mice were of the Bar Harbor 129 Rej strain (dy/dy), bred from pairs originally donated by the Muscular Dystrophy Group Research Laboratories, Newcastle upon Tyne General Hospital, Newcastle upon Tyne. The animals affected by the disease could be identified from about three weeks of age by their characteristic posture and dragging of their hindlimbs. It has also been noted that they are lighter in weight and smaller in size than non-dystrophic littermates of the same age (Morgan, 1985).

After weaning, dystrophic mice were placed in a cage with normal littermates as this appeared to increase their chances of survival. Both normal littermates and dystrophic mice were fed *ad libitum*. The dystrophic mice were allowed easier access to food and water by placing the ground food pellets of the breeding diet on the floor of the cage and by fitting the water bottles with longer metal tubes.

No distinction was made between heterozygous and homozygous normal littermates. Muscles from male, clinically normal animals and dystrophic animals aged 8-12 weeks were used depending on which experiment was being performed. In each series of experiments normal and dystrophic mice were matched for age in order to avoid any effects of age on neuromuscular parameters (Kelly, 1978). It is well documented that in these dystrophic mice the hindlimbs are the first muscles affected by the disease (Michelson *et al.*, 1955). The disease progresses from the hindlimbs forward and thus even in the later stages the diaphragm is relatively unaffected (Ross *et al.*, 1960).

2.2 DRUGS

2.2.1. Anticholinesterases Used

All injections of anticholinesterases (anti-ChEs) were made up in a solution of atropine sulphate and 0.9 %w/v sodium chloride in distilled water. The animals were injected subcutaneously between the shoulder blades, with 0.1ml per 20g body weight. The atropine sulphate was given in a dose of 0.7 μ mol/Kg, so as to minimise the muscarinic effects of the anti-ChEs (Grob, 1956; Grob and Johns, 1958). This dose was considered to be the minimum adequate dose to prevent any such effects.

Ecothiopate iodide. Ecothiopate iodide (ECO) was made up from the dry powder portion of Phospholine Iodide BNF eye drops (Ayerst) which contained 12.5mg ecothiopate iodide and 40mg potassium acetate. To this powder 3.26ml of distilled water was added to provide a 10⁻²M solution of ECO. A 100-fold dilution of this solution gave a 10⁻⁴M stock solution. The stock solution contained 6 μ M potassium acetate, which was considered to have a negligible effect on the potassium concentration, in both the *in vivo* injection solution and the physiological saline *in vitro*. *In vivo* doses ranged from 0.025 to 0.5 μ mol/Kg depending on the experiment. *In vitro* a final bath concentration of ECO 0.5 μ M was used. All stocks were stored in a refrigerator.

Pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate. This compound, synthesised Dr.B.Boskovic, will be referred to as BOS. An estimate of the dose required to cause maximal *in vivo* inhibition of muscle acetylcholinesterase (AChE) was taken from Lancaster (1972). Doses ranging from 0.375 to 8 μ mol/Kg were given *in vivo*. For experiments *in vitro* concentrations used were 4 or 8 μ M. In all cases fresh solutions were made up daily to avoid hydrolysis.

Isopropylmethylphosphonofluoridate. Isopropylmethylphosphonofluoridate (Sarin) was used for *in vivo* studies in a range of doses from 0.7 to 1.4 μ mol/Kg, with fresh solutions being made up daily.

Pyridostigmine Bromide. The only carbamate used in this study was

pyridostigmine, which was made up from 1mg/ml ampoules of Mestinon (Roche) injection. This was diluted in 0.9%w/v sodium chloride to give a final injection concentration of 0.38 μ mol/Kg.

2.2.2. Oximes

In all cases the oximes used, whether *in vivo* or *in vitro*, were made up in 0.9%w/v sodium chloride in distilled water. As with the anti-ChEs, in the case of *in vivo* experiments, injections made subcutaneously between the shoulder blades, the injection volume was 0.1ml per 20g body weight.

Pyridine-2-aldoxime methiodide. A dose of 110 μ mol/Kg was used in all experiments *in vivo* involving pyridine-2-aldoxime methiodide (2PAM). Injection volumes were taken from a stock solution of 6mg/ml 2PAM, which was stored below 5°C at all times. For experiments *in vitro* a range of concentrations from 100 μ M to 1mM were used depending on the experiment.

Toxogonin. Toxogonin (TOX) was used *in vitro* in bath concentrations from 50 μ M-1mM.

[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2[(hydroxyimino)methyl] - pyridinium dichloride.

The sample of [[4-(aminocarbonyl)pyridino]methoxy]methyl]-2[(hydroxyimino)methyl]-pyridiniumdichloride (HI-6) was used *in vitro* in bath concentrations from 50-400 μ M.

2.3. MUSCLE PREPARATIONS

2.3.1. Phrenic Nerve-Diaphragm

Mice were killed by cervical dislocation. The skin and muscle overlying the thorax and abdomen were cut away and a triangular window cut in the ribcage to expose the underlying diaphragm and phrenic nerves. Care was taken to dissect as much phrenic nerve as possible, particularly for those experiments requiring indirect stimulation of the diaphragm. The diaphragm was cut out and transferred to a petri dish with a Sylgard (Dow-Corning Ltd) base containing modified Liley's saline (Appendix 2) gassed with 95% O₂, 5% CO₂ at room temperature. It was then separated into left and right halves by cutting down the central tendon and dissecting out the xiphisternum. The ribs were trimmed back to the costal margin and the muscle was freed from blood clots, adipose and connective tissue. The left hemidiaphragm was usually used for contraction studies and electrophysiology experiments since the left phrenic nerve was more accessible than the right. In parameters measured, in particular those of AChE activity (2.5), procion damage (2.4) and electrophysiological studies (2.6), no differences were found between the two halves of the diaphragm.

2.3.2. Extensor digitorum longus (EDL).

Mice were anaesthetised with 2% halothane in 50% nitrous oxide in oxygen. The hindlimbs were shaved and the muscles were dissected out under a dissection microscope. The EDL is composed of four parts held together by a sheath of connective tissue and is connected at its base to digits two to five in the foot. The skin was initially removed from the top of the foot to expose the four tendons which were cut and pulled through the annular ligament at the ankle. Holding these tendons light tension was exerted on the EDL to aid in separation from the surrounding muscle up to its origin just below the knee. As much as possible of the nerve innervating the muscle was also dissected. Muscles were kept moist throughout the dissection with modified Liley's saline. The muscles from both hindlimbs were removed and pinned out on the Sylgard base of a petri dish containing modified Liley's saline gassed with 95% O₂/ 5% CO₂ at room temperature.

2.3.3. Soleus.

The foot of the anaesthetised mouse was pinned at the ankle and through the foot so that it was pointing at right angles away from the animal. The muscles on the surface were separated to reveal the soleus underneath which appeared a deeper red colour than those surrounding it. The central tendon which attached the muscle to the tibia was exposed and cut. Holding this tendon the soleus was gently pulled down and separated from the other muscles until the distal tendon was reached. This was attached to the achilles tendon from where the muscle was cut away. Care was taken to remove as much nerve as possible so as to ensure that stimulation of the muscle was attained easily.

2.4. HISTOLOGICAL METHODS

2.4.1. Procion staining.

Freshly dissected hemidiaphragms, under a small amount of tension, were pinned through the ribs and central tendon on to dental wax and were incubated in a 0.1%w/v solution of Procion Yellow MX4R (ICI) in modified Liley's saline for 60 minutes at room temperature. Modified Liley's saline was then used to wash the stained preparations for 30 minutes, with the saline being changed three times during that period. Throughout the above procedure the preparations were gassed with 95% O₂/ 5% CO₂. The preparations were then fixed for 60 minutes in 4%v/v formaldehyde in 0.1M acetate buffer at pH 4.0. The procion enters cells of which the sarcolemma has been damaged, binding to intracellular proteins (Bradley and Fulthorpe, 1978), and is thus used as a histological indicator of myopathy.

2.4.2. Quantification of procion-staining as an indicator of the extent of myopathy.

The technique described by Townsend (1988) was used to quantify the extent of procion staining in hemidiaphragm preparations. An eyepiece graticule, 0.62mm² in size, comprising 121 points (the intersections of an 11x11 grid), was placed at regular fields of view over each preparation. Fields were selected using a stage micrometer to advance 1mm across the tissue such that the distance between consecutive fields was 0.38mm. In each field the number of points overlying a region stained by procion were counted. This count was expressed as a percentage of the total number of points on the grid. The overall percentage of procion staining was the arithmetic mean of all the fields counted. To enable a comparison of junctional and non-junctional regions, any field containing one or more endplates was classified as part of the junctional region. On average approximately 100 fields of view were counted. Results for both regions were expressed as the percentage of procion staining.

2.4.3. Cholinesterase staining.

Procion-stained and fixed hemidiaphragms were trimmed from the ribs and

stained in the solution described by Karnovsky and Roots (1964) (Appendix 3). The staining was observed under a dissection microscope. In control preparations this would take approximately an hour, however in anti-ChE treated animals a much longer time was required. The stained hemidiaphragms were washed with distilled water and mounted on slides in glycerol jelly. The mounted preparations were viewed using a Zeiss fluorescence microscope, the incident ultra-violet beam being 400-500nm in wavelength, with emitted wavelengths greater than 510nm.

2.5. MEASUREMENT OF ACETYLCHOLINESTERASE IN BLOOD AND DIAPHRAGM.

Acetylcholinesterase (AChE) was measured using the method of Ellman *et al.* (1961). This is a colorimetric technique for determining ChE activity of tissue extracts, homogenates, cell suspensions etc... Enzyme activity can be calculated by measuring the increase in absorbance which occurs when thiocholine reacts with the dithiobisnitrobenzoate ion (DTNB) producing a yellow colour.



The increase in absorbance observed is measured at 412nm and it is known that the thiol group of the DTNB ion reacts with such rapidity that it is not rate limiting (Ellman *et al.*, 1961). One of the major problems with this technique is its lack of specificity; it will measure hydrolysis by AChE, butyrylcholinesterase (BuChE) and any other hydrolysis of acetylthiocholine. Since BuChE is unimportant to neuromuscular transmission the specificity was improved by the use of ethopropazine. In the dose used this compound has been shown to inhibit 100% of non-junctional acetylthiocholine hydrolysing activity whilst only reducing such activity in the junctional region by 20% (S.Das, 1989).

2.5.1. Preparation of the tissue samples.

2.5.1.1. Blood.

Mice were anaesthetised with 2% halothane in a mixture of 50% nitrous oxide in oxygen. The femoral artery was exposed and severed, and 0.1ml of blood was removed from the pool which formed. The blood was mixed with 15.9ml of phosphate buffer, pH 8.0, and 4ml of 2.5×10^{-4} M ethopropazine to give a final concentration of 5×10^{-5} M.

2.5.1.2. Diaphragm.

After the blood sample was removed the mice were killed by cervical dislocation. The diaphragm was dissected out (2.3.1.) and one of the hemidiaphragms prepared for assay. No significant difference was found between the two halves when the AChE activities of left and right hemidiaphragms were compared.

The hemidiaphragm was pinned through the central tendon and costal margin on to Sylgard in the base of a petridish containing modified Liley's saline at room temperature. The fat and connective tissue was cleared from the surface of the hemidiaphragm and a strip of the junctional region, 2-3mm either side of the visible nerve, was cut along its width. This strip was weighed and homogenised with 2ml of ice cold phosphate buffer pH 8.0 in a glass/Teflon homogeniser for one minute. 2ml of the same buffer and 1ml of 2.5×10^{-4} M ethopropazine were added to the homogenate in a specimen tube, and kept on ice. This concentration of ethopropazine has been shown by Das (1989) to completely inhibit non-junctional acetylthiocholine hydrolysing activity. This mixture was sonicated on ice for 30 seconds, using an MSE Ultrasonicator. To remove tissue debris the sample was then centrifuged at 1500g for 15 minutes at 4°C in a Beckmann centrifuge. The supernatant was kept on ice. All solutions used in this assay are specified in Appendix 4.

In some experiments the non-junctional regions of the hemidiaphragm was also assayed. After dissection of the junctional strip, strips either side of this area, that is up to the central tendon and costal margin of the ribs, were cut away and prepared for assay together following the same procedure used with the junctional samples.

2.5.2. The assay procedure.

The assay used followed the procedure set out by Ellman *et al.* (1961) which involves the subtraction of two blanks from the test reaction. The first blank, known as the substrate blank, corrected for the spontaneous hydrolysis of the substrate in the absence of the enzyme sample. This blank was measured for each fresh substrate solution before use. The second blank, the tissue blank,

compensated for the reaction of DTNB with any available thiol groups in the tissue, in the absence of the substrate. The tissue blanks were determined for every sample and run alongside the sample assay. The Pye Unicam spectrophotometer used was set up to assay in duplicate and thus automatically subtracted the tissue blank from the sample assay. The absorbance changes of each sample were measured against its blank at a fixed wavelength of 412nm and 30°C. Thus at one minute intervals the rate of absorbance change (ΔA) with time was followed.

2.5.2.1. The substrate blank.

Four 10ml test tubes were placed in a waterbath at 30°C. 1ml of phosphate buffer pH 8.0 and 1ml of DTNB were added to each tube. 1ml of acetylthiocholine, the substrate, was added to two of the tubes (the test samples) and 1ml of distilled water to the other two tubes (the reference samples). The contents of each tube were mixed and poured into plastic cuvettes. The test and reference samples were placed in the sample and reference beams respectively. Any changes in absorbance was monitored for approximately 8 minutes at 412nm.

The rate of increase in absorbance was determined from the slope of the linear record produced by the spectrophotometer. All measurements were made within the period of constant change on this record which usually involved ignoring the first and last recording. The first was ignored to minimise distortion of the results perhaps by equilibration of the reaction temperature after addition of the substrate, and the last preventing distortion by slowing of the reaction perhaps due to rapid hydrolysis of the substrate. A mean change in absorbance of such substrate blanks in this study was found to be 0.0016 absorbance units per minute.

2.5.2.2. Assay of the sample.

Four test tubes were placed in a waterbath at 30°C and 1ml of test sample and 1ml of DTNB were added to each tube. To two of the tubes 1ml of acetylthiocholine was added (the test samples) and 1ml of distilled water was added to the two remaining tubes (the tissue blank). The contents of each tube

were mixed and poured into cuvettes. Each test sample was read against its corresponding tissue blank, and, as with the substrate blank, any changes in absorbance monitored for approximately 8 minutes.

Again, the rate of increase in absorbance could be gained from the slope of the record produced by the spectrophotometer. The first and last readings were usually ignored, as before.

2.5.2.3. Correction for activity of non-synaptic ChE.

There is a proportion of ChE activity within the muscle fibres which is unimportant to neuromuscular transmission (Massoulie and Bon, 1982) at the neuromuscular junction. When the junctional strip was assayed non-synaptic activity was determined as well as activity within the synaptic cleft. By dissecting out strips of the non-junctional region (figure 2.1) and assaying in the same manner as that used for the junctional samples then a measure of the non-synaptic activity was made. This activity was subtracted from that determined for the junctional region.

2.5.2.4. Calculation of the enzyme units in each sample.

Calculation of the enzyme activity is determined by adaptation of the Beer-Lambert law:-

$$A = \log \frac{I_0}{I_T} = ECL$$

where,

A = the absorbance.

I_0 = 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.

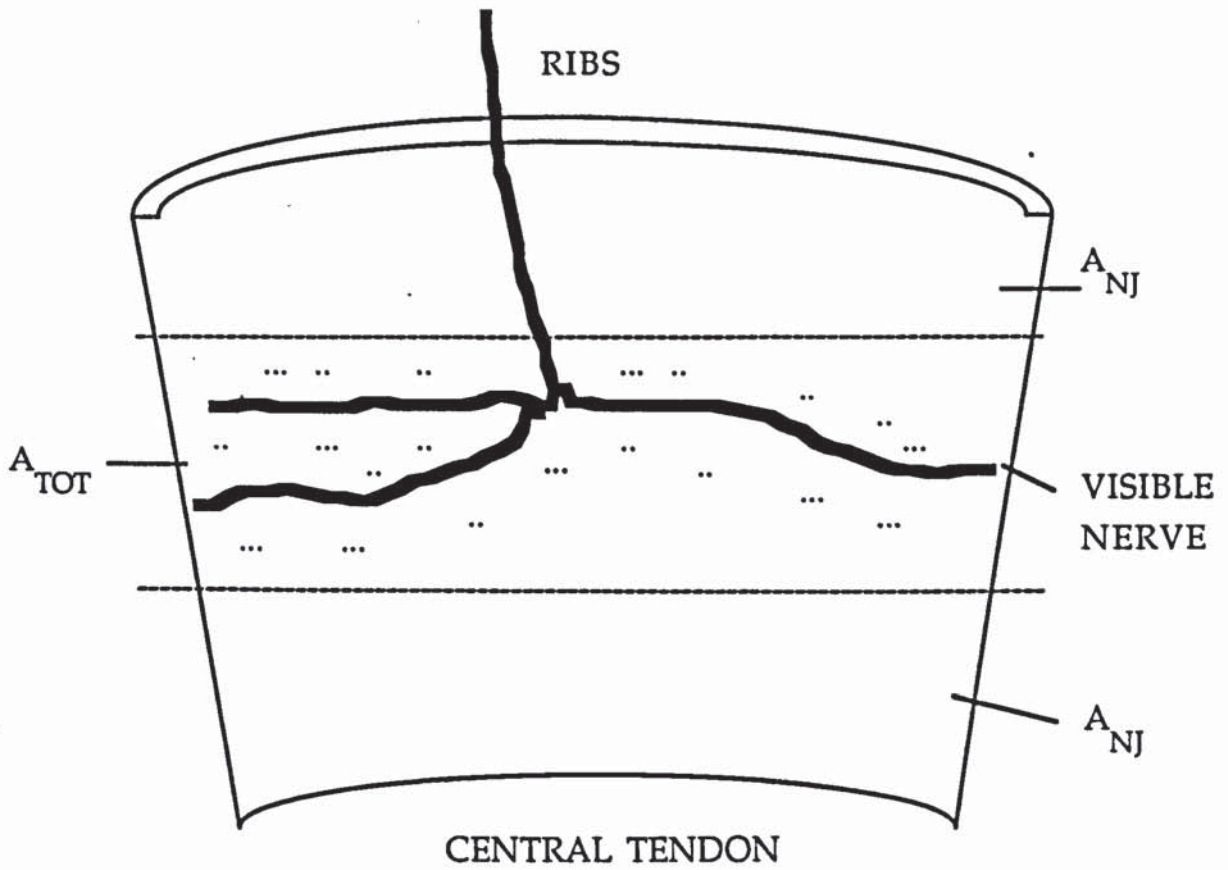


Figure 2.1. A diagrammatic representation of the areas of the hemidiaphragm dissected when determining the non-junctional correction.

2.5.2.5. Determination of the activity in a hemidiaphragm.

The molar extinction coefficient for the DTNB ion is 1.36×10^4 litres/mole/cm, since from Beer-Lambert,

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

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:-

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

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

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

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A}{\text{min} \times \text{cm}} \times \frac{\text{nmol} \times \text{cm}}{\text{ml}} = \Delta A \text{ nmol/min/ml}$$

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 Ymls then the following

applies:-

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

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A \times \text{nmol}}{\text{min} \times \text{mg}} \times \frac{\text{ml}}{\text{ml}} = \Delta A \text{ nmol/min/mg}$$

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

$$\text{Rate of hydrolysis} = \frac{\Delta A \times 220,59}{X} \quad \text{nmol/min/mg}$$

N.B. ΔA is the value following correction for the substrate blank.

2.5.2.6. Whole blood determination.

For this determination $5\mu\text{l}$ of sample was used in each assay. This was a 1 in 200 dilution. Thus in this case $Y=1/200$ and $V=3\text{ml}$, therefore:-

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

the units being calculated as follows:-

$$\text{Rate} = \frac{\Delta A \times \text{nmol}}{\text{min} \times \text{ml}} \times \frac{\text{ml}}{\text{ml}} = \Delta A \text{ nmol/min/ml}$$

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:-

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

N.B. ΔA is the value following correction for the substrate blank.

2.5.2.7. Correction for the non-junctional region.

As described, the absorbance changes of the junctional and non-junctional regions were determined. Using the equation previously described (2.5.2.5.), the enzyme activities of the two regions were calculated :-

A_{TOT} = Total activity of the junctional strip, both functional AChE (ie that involved in neuromuscular transmission) at the neuromuscular junction and non-junctional ChE.

$$= \Delta A/\text{min} \times 220.59 \text{ } \mu\text{mol}/\text{min}/\text{ml}$$

A_{NJ} = Activity of the non-junctional region.

$$= \Delta A/\text{min} \times 220.59 \text{ } \mu\text{mol}/\text{min}/\text{ml}$$

The contribution made by the non-specific ChE activity to that measured in the junctional strip is a function of the ratio of the weights of strip used. Thus the activity of the AChE, functionally useful at the neuromuscular junction (A_J) can be determined from the following equation:-

$$A_J = A_{TOT} - (W_J/W_{NJ} \times A_{NJ}) \quad (1)$$

where W_J = wet weight of the junctional strip

W_{NJ} = wet weight of the non-junctional strip

A_J can be expressed as a function of the weight of the junctional strip taken in the following:-

$$A_J = \frac{A_{TOT} - (W_J/W_{NJ} \times A_{NJ})}{Z} \text{ nmol}/\text{min}/\text{mg} \quad (2)$$

where $Z = \frac{\text{wet weight of junctional strip}}{5} \text{ mg}/\text{ml}$

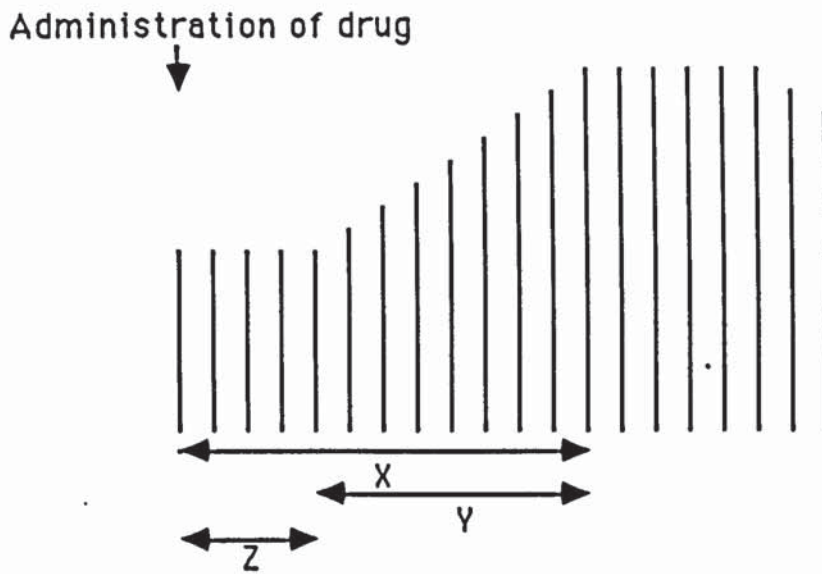
2.6. METHODS USED FOR ORGAN BATH EXPERIMENTS

2.6.1. Single Twitch Experiments

ECO has been shown to increase the contractile response to single supramaximal indirect stimulation (Ferry, 1988). In these experiments the effects of BOS, 4 or 8 μ M final bath concentrations, were compared with the effects of ECO 0.5 μ M.

The phrenic nerve-hemidiaphragm preparation was dissected and maintained at 37 \pm 1 $^{\circ}$ C in an 80ml organ bath containing modified Liley's saline gassed with 95% O₂/5% CO₂. The muscle was attached to two hooks which were passed through the intercostal spaces and the central tendon was gripped by a 0.3g, 0.75cm width, steel bulldog clip which was attached to a Devices UF1 force transducer. The muscle was mounted vertically in the organ bath and the phrenic nerve was stimulated via a Finkleman electrode. The length of the muscle was adjusted until the twitch was maximal. Single, supramaximal stimuli, at 0.2 Hz, were produced by a Devices Digitimer, Pulse Generator and Devices isolated stimulator type 2533 and records of the contractile responses were made on a Washington 400 MD/2 pen recorder. The parameters measured were; time to onset, time to peak amplitude and time from onset to peak amplitude (figure 2.2.(a)).

(a)



(b)

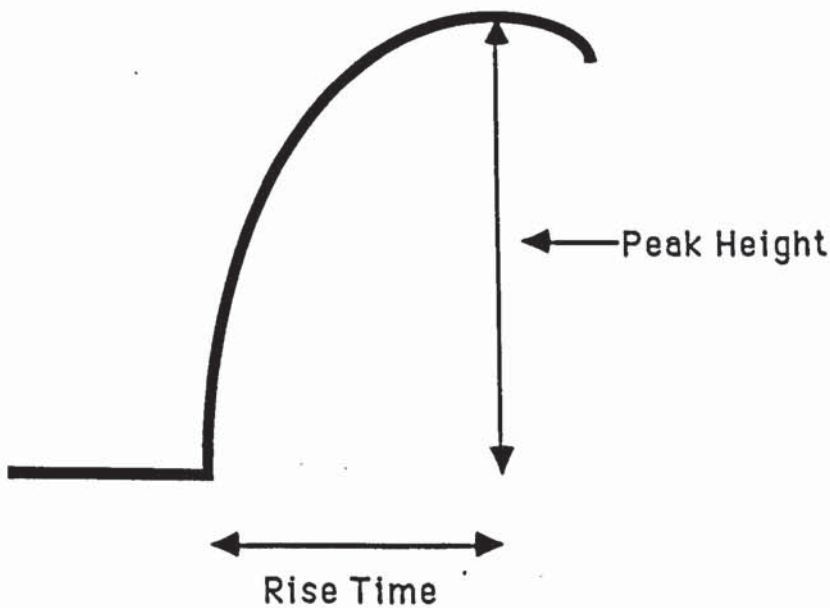


Figure 2.2. Diagrammatic representations of (a) the parameters measured in twitch experiments where,

X= Time to Peak Amplitude (secs)

Y= Time from Onset to Peak Amplitude (secs)

Z= Time to Onset (secs)

(b) The parameters measured on the contractile force traces were the rise time and the peak amplitude height.

2.6.2. Measurement of anti-ChE induced repetitive action potentials and spontaneous twitching.

Mouse phrenic nerve-hemidiaphragm preparations were dissected and a loop of thread was placed through the intercostal spaces. The thread was attached to a steel hook which was directly connected to a Devices UF1 force transducer to determine the muscle's contractile response. The central tendon was then pinned to Sylgard in the base of a 20ml horizontal Perspex organ bath. The muscle length was adjusted to give a maximal twitch response for a given stimulus. This bath incorporated a heater coil through which modified Liley's saline was circulated to enable the temperature to be thermostatically controlled at $37 \pm 1^\circ\text{C}$. The preparations were gassed with 95% O_2 /5% CO_2 , which provided a bubble lift to maintain circulation.

2.6.2.1. Repetitive Action Potentials.

Single supramaximal indirect stimuli were used to stimulate the phrenic nerve via a stimulating electrode. The extracellular field potentials produced by the propagation of action potentials within the muscle were recorded at the cut ends of enamelled Ag wire electrodes, 0.32mm in diameter. AC amplification was used with a bandwidth of 0.16Hz to 10kHz. One electrode was placed close to the muscle and the other remote from the preparation, positioned in such a way as to reduce the stimulus artefact. All recordings were made in the endplate zone where the action current was of minimum latency. The electrical and contractile responses were recorded on a Gould OS4000 digital storage oscilloscope and XY plotter.

After the addition of BOS $8\mu\text{M}$, single stimuli were applied to the nerve at specified time intervals and records of electrical and contractile responses made. The time intervals chosen were 2, 4, 6, 9, 15, 20, 30, 40, 50, 60, 70 and 85 minutes, at which point the BOS was washed out of the bath. Following washout, recordings at 105 and 110 minutes were made. No stimulation of the preparation was made between these time intervals.

The parameters measured were the rise time and the height at peak amplitude, as illustrated in figure 2.2 (b).

2.6.2.2. Spontaneous Twitching.

These experiments were performed in order to determine whether BOS induced spontaneous muscle contractions, a property characteristic of other anti-ChEs. Records of the mechanical events were made. The UF1 force transducer in this case was connected to a Washington 400 MD/2 oscillograph which provided an accurate record of any spontaneous muscle activity.

Preparations were exposed to a final bath concentration of BOS of $8\mu\text{M}$ for a period of 14 minutes. The drug was then washed out of the organ bath with fresh modified Liley's saline and recording continued.

2.6.3. Recording of extracellular miniature endplate potentials (MEPPs).

The current fields which surround a nerve or muscle fibre during propagation of an action potential can be investigated by recording the potential changes which these currents generate between an electrode in the vicinity of the nerve and an indifferent electrode some distance away. The same technique can also be used to measure similar fields which occur around a neuromuscular junction during quantal release of ACh. The basis of extracellular recordings of this type is the work of Lorente de No (1947), who measured field potentials in frog sciatic nerve and explained the changes in shape and size of such potentials in terms of the position of the recording electrode in relation to the current fields.

Recording from the surface of isolated muscle cells in this way, Fatt and Katz (1950a, 1952) discovered spontaneous subthreshold activity. The potentials varied somewhat in size, but had a very consistent time course. They were localised at one region of the fibre and in their shape resembled the EPP, hence the name miniature endplate potentials (MEPPs).

The MEPPs recorded extracellularly have some distinct differences from those sampled intracellularly. Their polarity is reversed, since the microelectrode becomes negative with respect to the bath. The location of the recording electrode is critical, if the microelectrode is moved by mere microns the MEPPs can be lost. Compared to intracellularly recorded MEPPs, extracellular MEPPs

are brief in time course. Similarly the size and frequency distribution of the extracellular MEPPs can be quite different from those recorded internally at the same endplate. Fatt and Katz (1952) believed that this was due to an increased selectivity with the external microelectrode, in that they only recorded potentials generated close to the tip of the electrode.

The potential differences measured by this technique arises from the potential flowing across the endplate membrane and in the external fluid immediately surrounding the electrode tip. The time course of these local currents and the associated external potential which one records is much more rapid than the underlying membrane potential change (Fatt and Katz, 1952).

The left hemidiaphragm was dissected out and pinned onto the Sylgard base of 20ml horizontal Perspex organ bath. The bath contained modified Liley's saline bubbled with 95% O₂/5% CO₂ which was circulated and maintained at 37±1°C. All recordings were made using glass capillary electrodes, with tip 2-5µm, pulled in two stages on a Kopf vertical electrode puller Model 750. They were filled with modified Liley's saline of the same composition as that used in the bath. The recordings were made using a Fenlow AD55 pre-amplifier and a Devices AC amplifier type 3160 (bandwidth 10kHz to 0.16Hz). The extracellular MEPPs were displayed on a Gould 20MHz digital storage oscilloscope type 1425 and a sample of sixteen were averaged at each endplate using a Gould waveform processor type 125. Parameters of the averaged waveform were then determined including the time to half amplitude of the decay phase of the potential (THA). The technique for finding the areas of activity was to place the electrode just on the surface of the tissue in an area likely to contain endplates, then move the tip slowly over the surface using a micro-manipulator until MEPPs appeared on the oscilloscope screen. A minimum of pressure on the surface of the tissue was always used. To prevent any spontaneous twitching of the muscle after anti-ChEs, which is liable to move the electrode from its recording point, 10⁻⁶M tetrodotoxin was added to the preparation 15 minutes before the commencement of recording.

2.6.4. Measurement of "jitter" using intracellular single fibre electromyography.

As stated in the Introduction, Ekstedt (1964) showed that when recording the action potentials from two muscle fibres of the same motor unit there is always a variability in the time intervals between the two muscle fibre potentials at consecutive discharges. This variability is known as jitter. It is due to the variability in transmission time from the point of stimulation to the area where the recording is made. In SFEMG the variability can be expressed mathematically as the standard deviation (SD) around the mean interpotential interval (MIPI). However, there are sometimes slow trends superimposed on the short-term random variation which tend to increase the SD making the value of jitter gained inaccurate. An alternative way of expressing the variability is to use the mean consecutive difference (MCD) over a train of action potentials. This parameter measures short term variability and is relatively unaffected by slow trends, making it much more suitable for the evaluation of jitter (Ekstedt *et al.*, 1974; Stalberg and Trontelj, 1979). Kelly (personal communication) adapted the concepts of jitter measurement in SFEMG to trains of action potentials recorded from individual muscle cells intracellularly. These adaptations to jitter measurement are detailed below.

Three types of preparation were investigated using this technique, hemidiaphragm, EDL or soleus depending on which experiment was being performed. In each case the muscle was dissected out along with as much as possible of the nerve supplying it (2.3.). The muscle was pinned onto Sylgard in a Perspex bath through which modified Liley's saline was passed. This saline was gassed with 95% O₂/ 5% CO₂ and maintained at 37±0.5°C. The nerves were stimulated at 30Hz via a suction electrode with supramaximal pulses of 0.05msec duration. To prevent excessive muscle movement in response to nerve stimulation many pins were used to pin the muscle to the Sylgard, and if necessary some of the fibres were cut.

Intracellular glass capillary microelectrodes filled with 3M KCl were used to record resting membrane potentials (RMPs) and action potentials of uncut fibres. The input device was a World Precision Instruments model M707 preamplifier which was followed subsequently by AC amplification performed

by a Devices type 3160 amplifier (bandwidth 0.16 to greater than 30kHz). The action potentials were displayed on a Tektronix D12 dual beam oscilloscope and recorded on an FM tape recorder (Racal store 4) at a tape speed of 30 inches per second. These recordings were replayed at 15/16 inches per second and analysed using an analogue-to-digital converter and PDP 11/03 minicomputer. With a sampling rate of 20kHz and a record/replay ratio of 32, the effective sampling rate of the initial action potentials was 640kHz. Trains of 30 action potentials were recorded from each muscle fibre and 10 or more fibres sampled from each muscle. Data from a fibre was rejected if, during the train, the RMP fell by more than 5mV, the action potential amplitude fell by more than 10% or the action potential amplitude was less than the RMP.

2.6.4.1. Analysis of "jitter".

Computer programs, devised by Dr.S.S.Kelly, were used to measure the amplitude, time course and latency of each action potential, The latency was the interval between the stimulus and a point on the rising phase at 10% of peak amplitude. This data was used to calculate two parameters in particular, the MCD of action potentials 11-30 and the difference in latency between the 16th action potential and the 1st action potential (Delay). The formula used to calculate the MCD of action potentials 11-30 was,

$$\text{MCD} = \frac{[|L_{11}-L_{12}| + |L_{12}-L_{13}| + \dots + |L_{29}-L_{30}|]}{19}$$

where L_x refers to the latency of action potential x.

It has been shown that in control preparations the initial rundown of latency has reached a plateau by action potential 10, thus measurement of the MCD is made for action potentials 11-30. An accurate pulse generator and Digitimer, used to evoke the action potential trains, were used to estimate the intrinsic error of the processes of record/replay/analysis by providing a train of pulses which were recorded and analysed. These trains gave a value for the MCD of 2.4µsec. Thus the apparatus had a resolution limit of 2.4µsec.

2.7. STATISTICAL TESTS.

Two tests for statistical significance were used within this study, the details of which can be found in Appendix 5. Firstly, a non-parametric test, the Mann-Whitney test, was utilised, except for the data from jitter experiments, since at no point was it shown that the data tested had the characteristics of a normal distribution. Kelly (personal communication) identified a number of different populations of muscle fibres within the data gained from jitter experiments. It was thus decided to use another non-parametric test, the Kolmogorov-Smirnov test, which accounts for such subpopulations, when assessing statistical significance of such jitter data.

3. RESULTS

3.1. THE CHARACTERISATION OF BOS.

Subcutaneous injections of the organophosphorus anti-ChE ecothiopate (ECO) causes myopathy at the endplate region of the mouse diaphragm (Ferry and Townsend, 1986). The progression of this myopathy has been investigated using both histological methods such as procion yellow staining (2.4.1.) and electron microscopy, and biochemical methods such as those which measure AChE (2.5), creatine kinase and intracellular calcium levels. It appears that the necrosis reaches a maximum 6-12 hours after injection and is initiated in the endplate region of the diaphragm, then spreads along the fibre (Townsend, 1988).

On commencement of the jitter studies it was decided to study not only the effects of ECO, but also those of a somewhat longer acting organophosphate. The compound BOS has similarities to the nerve agent soman and forms an identical phosphorylated AChE (see Introduction). This phosphorylated enzyme is prone to rapid ageing with a half life of 2.4 minutes (Harris *et al.*, 1971). Thus the recovery of AChE activity is due only to *de novo* synthesis (Harris *et al.*, 1971). The ECO-inhibited AChE, in contrast, is subject to some spontaneous reactivation (Hobbiger, 1976) and hence one would expect the inhibitory effects of BOS to last for a longer period than those of ECO. A further reason for choosing BOS was that, like ECO, it is a quaternary compound and would thus have very similar distribution characteristics.

Before analysing the effects on jitter of these two compounds it was important to:-

- (a) find a dose of BOS equivalent to a dose of ECO 0.5 μ mol/Kg 3 hours after injection *in vivo*, with regard to extent of myopathy and inhibition of AChE.
- (b) compare the rates of recovery from the myopathy and restoration of AChE after single injections of the two compounds.
- (c) assess whether BOS produced, (i) potentiation of twitch and the associated repetitive muscle action potentials, and (ii) the spontaneous twitching of muscle, often observed with other anti-ChEs.

3.1.1. Myopathy and the associated AChE inhibition 3 hours after a single dose of anti-ChE.

To compare the effects of ECO and BOS on jitter, doses equi-effective in the induction of myopathy, assessed by the Procion technique (2.4.1), were required. Lancaster (1972) studied the distribution of BOS and its tertiary analogue, the maximum inhibitory dose in rodents was estimated to be about 4-8 μ mol/Kg. Therefore, animals were injected with ECO 0.5 μ mol/Kg, or with 4 μ mol/Kg or BOS 8 μ mol/Kg each with 0.7 μ mol/Kg atropine (2.2.1.). After 3 hours blood samples (2.5.1.1.) and diaphragms (2.3.1.) were taken from the mice. The blood and one hemidiaphragm were then assayed for AChE activity (2.5.), the other hemidiaphragm was assessed for myopathy by procion staining (2.4.1.) at the endplate region. Statistical analysis of the difference between animals exposed to an anti-ChE and animals injected with atropine was carried out using a Mann-Whitney test (Appendix 5.1). Throughout the study no differences between untreated mice and those injected with atropine alone were found.

The signs of anti-ChE toxicity, such as fasciculation, appeared significantly earlier at 13.7 \pm 0.5 minutes after ECO 0.5 μ mol/Kg (Das, 1989) compared with 21.3 \pm 3.9 minutes (mean \pm S.D. for 12 animals) after BOS 8 μ mol/Kg ($p\leq 0.05$). Animals exposed to these high doses of anti-ChE initially exhibited increased salivation and lacrimation, simultaneous with rapid fasciculations and body tremor. These fasciculations were often observed as waves of clonic activity, with the animal in a prone position. Two - three hours after ECO 0.5 μ mol/Kg all the animals appeared sedated and gross muscle fasciculations began to decline and by 3 hours appeared normal. After BOS 8 μ mol/Kg, sedation and fasciculation lasted up to 12 hours and in a few cases even longer, however, if signs lasted longer than 12 hours survival was unlikely. Animals injected with doses less than ECO 0.3 μ mol/Kg or BOS 4 μ mol/Kg displayed little or no signs of toxicity.

Table 3.1. shows that, 3 hours after injection, BOS 4 and 8 μ mol/Kg and ECO 0.5 μ mol/Kg each caused a significant reduction of whole blood and diaphragm AChE. The reduction in AChE activity not being significantly different for any of the doses at 3 hours ($p\leq 0.05$). Comparison of the two doses of BOS 30

minutes after injection showed a significant dose related difference in the AChE activity of blood and muscle ($p \leq 0.05$). Thus it appears that, although both doses eventually reached the same maximal degree of inhibition 3 hours after administration ($p \leq 0.05$), the rate of onset of the inhibition produced by the lower dose was slower. This may aid explanation of the data represented in table 3.2, which indicate that ECO 0.5 $\mu\text{mol/Kg}$ and the two doses of BOS produced significant amounts of Procion staining, but BOS 8 $\mu\text{mol/Kg}$ developed a significantly greater amount of staining than did the 4 $\mu\text{mol/Kg}$ dose ($p \leq 0.05$, table 3.2.). It should be noted that the percentage inhibition of diaphragm AChE quoted is derived as follows,

$$\% \text{ Inhibition} = \frac{(\text{Mean activity atropine alone} - \text{Reduced activity after treatment})}{\text{Mean activity atropine alone}} \times 100$$

It may be that the difference in the percentage procion staining produced by the two doses of BOS may be due to the reduction of AChE activity with BOS 4 $\mu\text{mol/Kg}$ being at a much slower rate than that seen with 8 $\mu\text{mol/Kg}$. It appears from this data that BOS 8 $\mu\text{mol/Kg}$ has similar myopathic and AChE inhibitory effects as ECO 0.5 $\mu\text{mol/Kg}$ 3 hours after administration. It was for this reason that the 8 $\mu\text{mol/Kg}$ dose of BOS was used for further comparative investigations of the actions of these two anti-ChEs.

	Time after Injection	Diaphragm AChE activity nmol/min/mg	% Inhibition	Blood AChE activity μmol/min/ml	% Inhibition
Atropine					
alone	3 hours	1.34±0.43 (38)	0	0.90±0.15 (22)	0
ECO	3 hours	0.17±0.04 (8)*	87	0.24±0.06 (8)*	73
0.5μmol/Kg					
BOS	30 mins	0.41±0.14 (4)*	69	0.40±0.05 (4)*	56
4μmol/Kg					
		**		**	
BOS	30 mins	0.15±0.02 (4)*	89	0.18±0.09 (4)*	80
8μmol/Kg					
BOS	3 hours	0.21±0.10 (15)*	84	0.27±0.07 (9)*	70
4μmol/Kg					
BOS	3 hours	0.18±0.05 (11)*	87	0.29±0.07 (7)*	68
8μmol/Kg					

Table 3.1. Levels of AChE activity in homogenates of diaphragm muscle and in whole blood 30 mins and 3 hours after injection of ECO or BOS *in vivo*. Values are given as mean±S.D., with the number of animals in parentheses. * significant difference from atropine alone and ** significant difference between the 4 and 8μmol/Kg doses of BOS (Mann-Whitney; p≤0.05). See text for further details.

Drug	Dose	Time after injection	Percentage procion staining in the endplate region
Atropine alone		3 hours	0.18±0.13 (4)
ECO	0.5µmol/Kg	3 hours	12.1±4.60 (10)* **
BOS	4µmol/Kg	3 hours	3.35±2.26 (4)* **
BOS	8µmol/Kg	3 hours	13.9±3.56 (4)*

Table 3.2. Percentage of surface area stained by procion at the endplate region of the diaphragm 3 hours after injection *in vivo* of ECO or BOS. Values are given as mean±S.D., with the number of animals in parentheses. * significant difference from atropine alone and ** significant difference between BOS 4µmol/Kg and BOS 8µmol/Kg or ECO 0.5µmol/Kg (Mann-Whitney; $p \leq 0.05$). There was no significant difference between ECO 0.5µmol/Kg and BOS 8µmol/Kg 3 hours after injection *in vivo* ($p \leq 0.05$).

3.1.2. The progression of myopathy and reduction of AChE activity after a single injection of anti-ChE.

The effects of ECO 0.5 μ mol/Kg and of BOS 8 μ mol/Kg were observed at various times following their administration *in vivo* (30 mins, 3 hours, 24 hours, 3 days, 7 days, 10 days, 15days).

Each group contained at least four mice which were injected with atropine and either BOS or ECO (2.2.1). Another group, which acted as a control, was injected with atropine alone . At appropriate times blood samples were taken (2.5.1.1.) and diaphragms dissected out (2.3.1.). Samples of whole blood and hemidiaphragm were assayed for AChE activity (2.5.) and the extent of myopathy in terms of percentage procion staining (2.4.1.) at the endplate region was measured. The histology of the stained hemidiaphragms was studied, then photographed in both ultraviolet and tungsten lights using a fluorescence microscope. The myopathic regions were stained with Procion yellow which could be recognised as areas of bright yellow fluorescence. It should be noted that the extent of the procion staining produced by ECO 0.5 μ mol/Kg was measured by and quoted courtesy of Dr. H. E. Townsend.

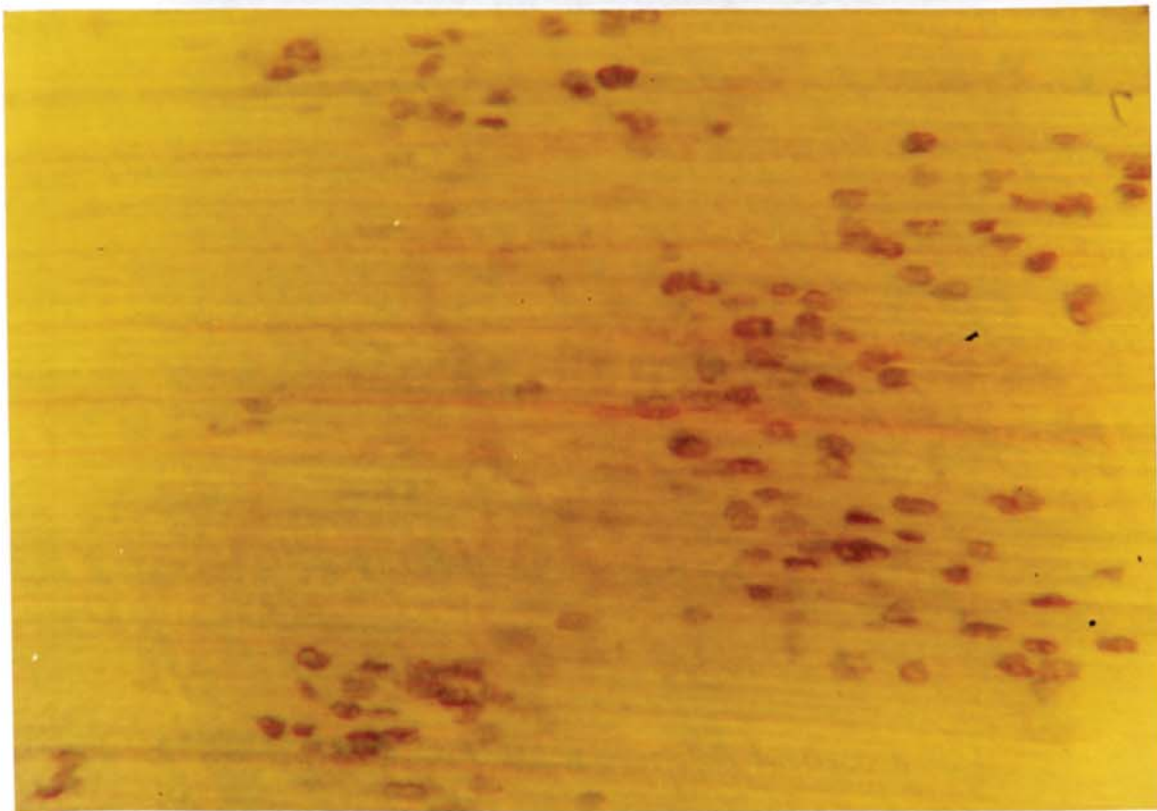
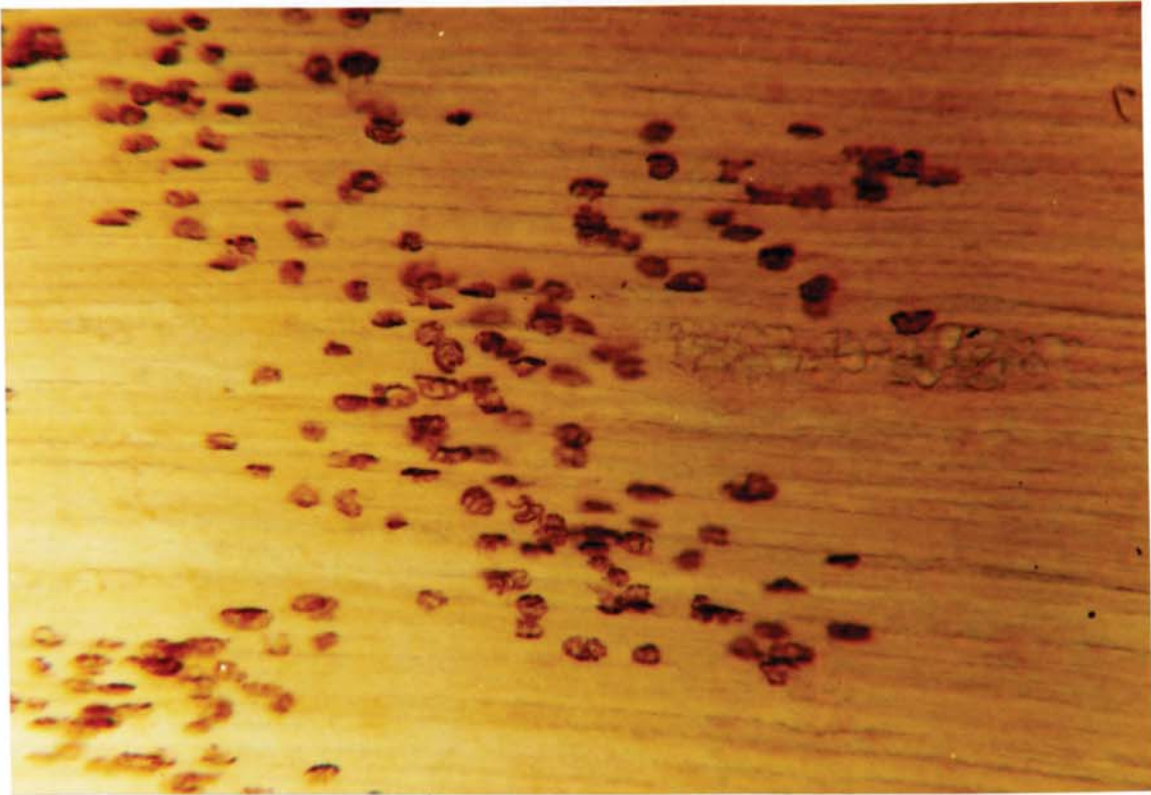


Plate 3.1. Hemidiaphragm of an animal injected with atropine alone, viewed in tungsten (Top) and ultraviolet light (Bottom). The muscle fibres run parallel to each other and show green autofluorescence in ultraviolet light. The endplates are represented by patches of brown stain. The connective tissue has been stained with Procion which has failed to penetrate the fibres themselves. Calibration 100 μ m.

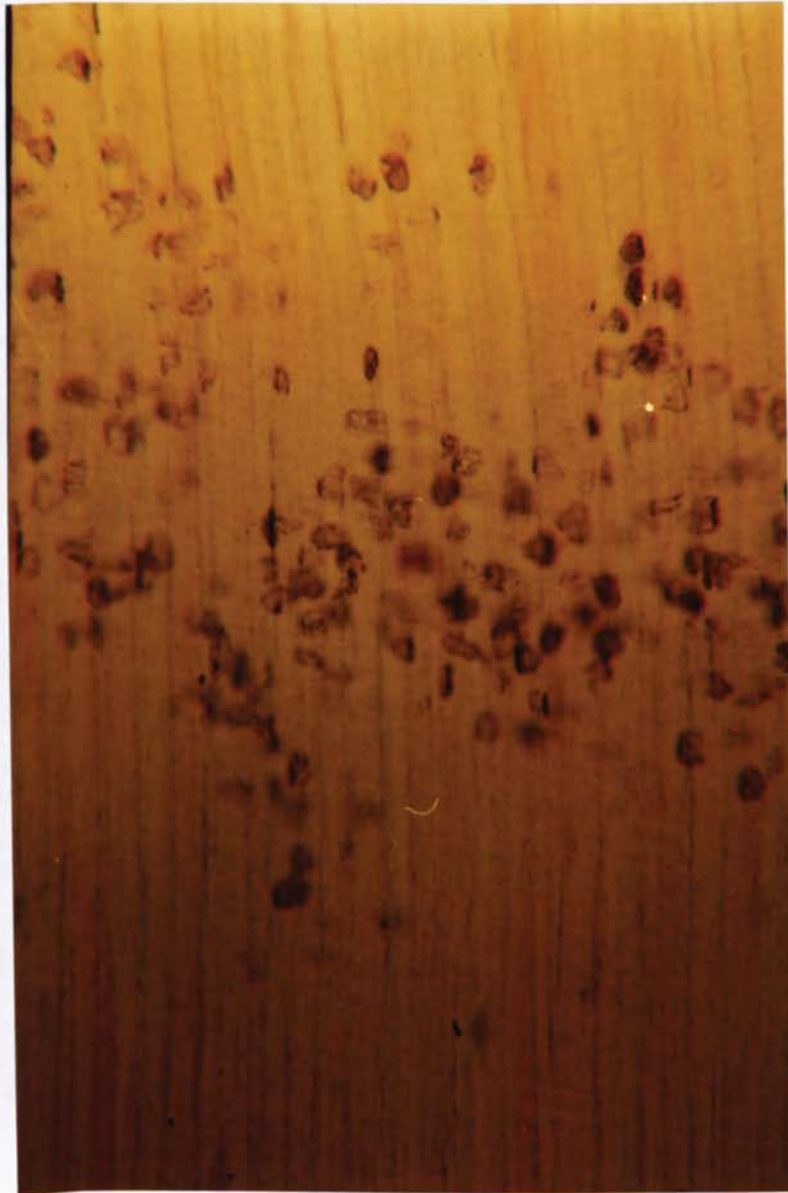


Plate 3.2. Hemidiaphragm preparation 30 minutes after BOS $8\mu\text{mol/Kg}$ *in vivo*, viewed in tungsten light. Procion yellow has not penetrated the fibres but some endplates appear swollen. Calibration $100\mu\text{m}$.

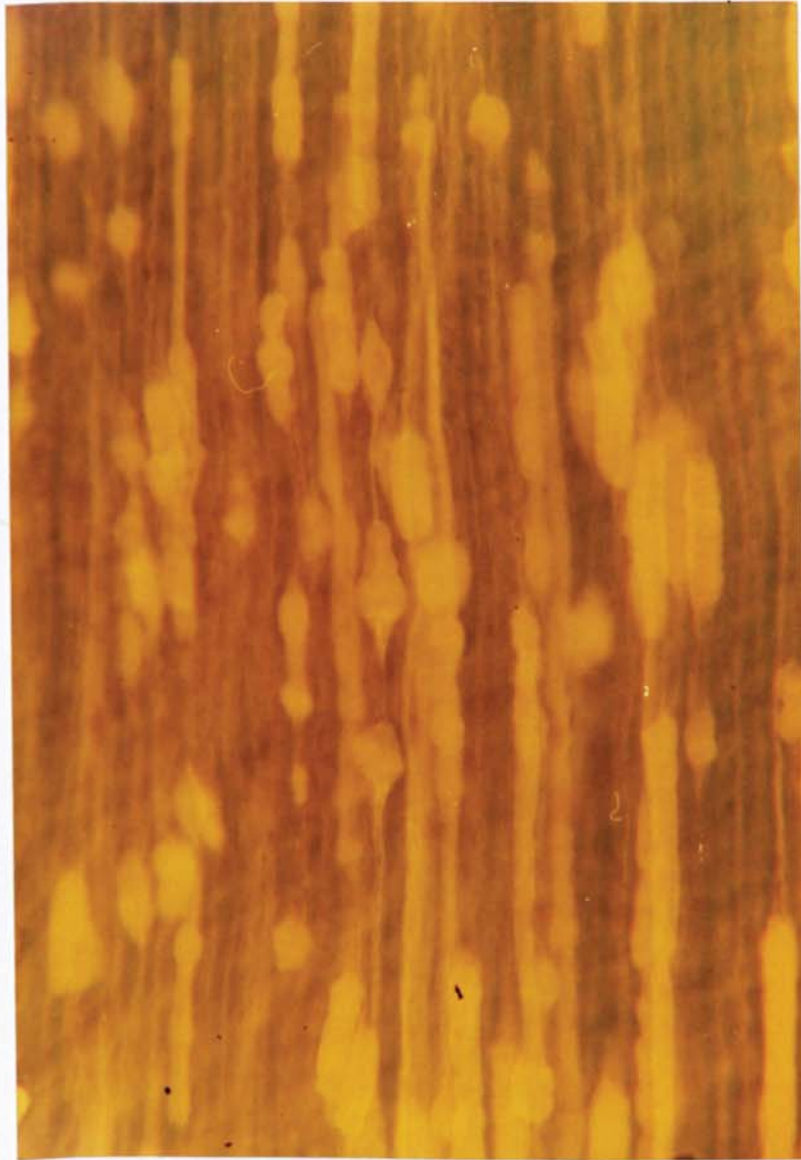


Plate 3.3. Hemidiaphragm preparation 3 hours after BOS $8\mu\text{mol/Kg}$ *in vivo*, viewed in ultraviolet light. Hypercontracted fibres can be observed the majority of which fluoresce yellow, but some are unstained by Procion and fluoresce green. Calibration $100\mu\text{m}$.

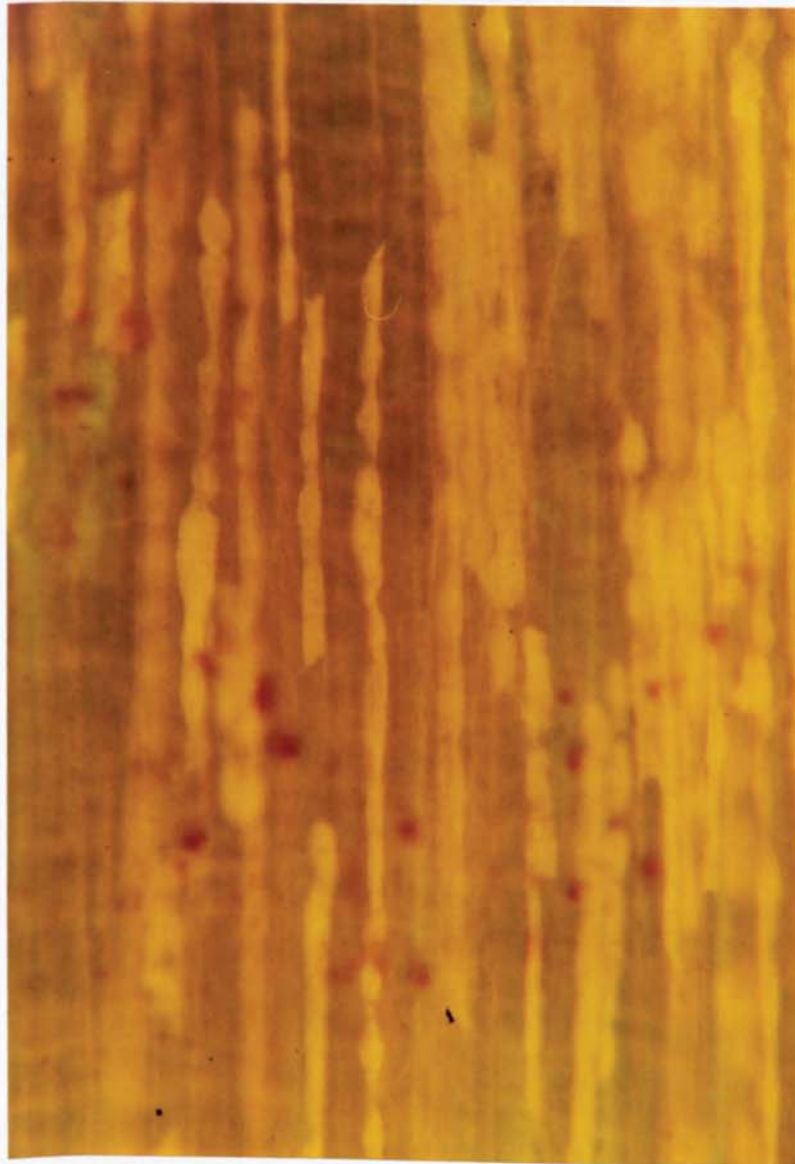


Plate 3.4. Hemidiaphragm preparation 24 hours after BOS $8\mu\text{mol/Kg}$ *in vivo*, viewed in ultraviolet light. The hypercontractions and Procion staining appear similar to the 3 hour level, however they now appear as chains within individual muscle cells. Calibration $100\mu\text{m}$.



Plate 3.5. Hemidiaphragm preparation 3 days after BOS $8\mu\text{mol/Kg}$ *in vivo*, viewed in ultraviolet light. There is no Procion staining and no evidence of hypercontraction, some of the endplates still appear swollen. Calibration $100\mu\text{m}$.

3.1.2.1. Histological examination of the hemidiaphragms.

Hemidiaphragms from mice injected with atropine alone consisted of muscle fibres aligned in parallel with one another along their longitudinal axis (plate 3.1). The fibres displayed the normal transverse striations which represent the regular sarcomere structure characteristic of skeletal muscle (Huxley and Hanson, 1960) together with patches of brown staining found approximately half way along the length of the fibres which were recognised as being endplates (plate 3.1.). If the hemidiaphragm was viewed under ultraviolet light the fibres showed a green autofluorescence (plate 3.1.). None of the fibres showed any yellow fluorescence, suggesting none of the dye had penetrated the fibres, although the Procion did stain the central tendon and connective tissue surrounding the muscle fibres.

The earliest sign of abnormality following the administration of one of the anti-ChEs was swelling of some of the endplates. With ECO 0.5 μ mol/Kg this swelling can be seen within 20 minutes of administration, even though Procion has as yet failed to penetrate the fibres (Townsend, 1988). This effect was also observed with BOS 8 μ mol/Kg 30 minutes after administration (plate 3.2.), again with a lack of penetration of Procion into the fibres. Townsend (1988) found that ECO, produced a significant amount of Procion staining 30 minutes after injection, but BOS does not ($p \leq 0.05$, table 3.3, 3.4), suggesting that the rate of onset of the Procion staining was significantly faster for ECO 0.5 μ mol/Kg than it was for BOS 8 μ mol/Kg ($p \leq 0.05$, table 3.4.). This may be related to the fact that the appearance of the cholinergic symptoms of anti-ChE poisoning seem to occur much earlier with ECO than with BOS.

Three hours after injection of ECO or BOS, hypercontracted regions of the muscle fibre appeared at or close to the endplate area (plate 3.3.). The hypercontraction, usually stained with Procion, was a fairly symmetrical bulging of the fibre, within which the spacing between the cross striations was reduced. The hypercontractions sometimes occurred in chains along the length of the fibre with, in some cases, the contraction so intense as to render the cross striations unrecognisable.

Time after injection	% Procion staining at endplate	Diaphragm AChE		Blood AChE	
		activity nmol/min/mg	% Inhibition	activity μ mol/min/ml	% Inhibition
Atropine					
Control	0.22 \pm 0.36 (14)	1.34 \pm 0.43 (38)	0	0.90 \pm 0.15 (22)	0
	†	†		†	
30 mins	1.38 \pm 1.34 (18)*	0.24 \pm 0.13 (5)*	82	0.42 \pm 0.04 (5)*	53
	†				
3 hours	12.1 \pm 4.60 (10)*	0.17 \pm 0.04 (8)*	87	0.24 \pm 0.06 (8)*	73
		†		†	
24 hours	12.5 \pm 8.26 (11)*	0.54 \pm 0.05 (6)*	60	0.76 \pm 0.19 (6)	16
	†				
3 days	0.20 \pm 0.48 (11)	0.61 \pm 0.07 (4)*	54	0.72 \pm 0.11 (4)*	20
		†			
5 days		1.27 \pm 0.23 (11)	5	0.93 \pm 0.16 (9)	-3
7 days		1.50 \pm 0.26 (5)	-12	0.70 \pm 0.34 (4)	22

Table 3.3. The reduction and recovery of AChE activity in the diaphragm and whole blood samples following a single injection of ECO 0.5 μ mol/Kg *in vivo*. Also the progression of the necrosis indicated by the percentage procion staining in the endplate region of the diaphragm (results courtesy of H.Townsend). The values represent the mean \pm S.D., with the number of animals used in parentheses. * significant difference from atropine alone and † between two values indicates significant difference between them (Mann-Whitney, $p \leq 0.05$).

Time after injection	% Procion staining at endplate	Diaphragm AChE			Blood AChE	
		activity nmol/min/mg	% Inhibition	activity μ mol/min/ml	% Inhibition	
Atropine						
Control	0.18 \pm 0.13 (4)	1.34 \pm 0.43 (38)	0	0.90 \pm 0.15 (22)	0	
		†		†		
30 mins	0.20 \pm 0.19 (4) **†	0.15 \pm 0.02 (4)*	89	0.18 \pm 0.09 (4)*	80	**
3 hours	13.9 \pm 3.56 (4)*	0.18 \pm 0.05 (11)*	87	0.29 \pm 0.07 (7)*	68	
24 hours	12.3 \pm 5.83 (8)* †	0.16 \pm 0.04 (5)*	88	0.37 \pm 0.11 (5)*	59	**†
3 days	0.41 \pm 0.41 (4)	0.19 \pm 0.06 (4)*	86	0.83 \pm 0.05(4)	8	
		**†				
5 days	0.17 \pm 0.17 (4)	0.82 \pm 0.16 (18)*	39	0.71 \pm 0.12 (12)*	21	**
		**		**		
7 days	0.17 \pm 0.14 (4)	0.93 \pm 0.18 (9)*	31	0.80 \pm 0.13 (8)	11	
		**				
10 days		1.10 \pm 0.24 (9)	18	1.06 \pm 0.18 (8)	-18	
				†		
15 days		1.16 \pm 0.19 (8)	13	0.75 \pm 0.11 (4)	17	

Table 3.4. The reduction and recovery of AChE activity in the diaphragm and whole blood samples following single a injection of BOS 8 μ mol/Kg *in vivo*. Also the progression of the necrosis indicated by the % procion staining in the endplate region of the diaphragm. The values represent the mean \pm S.D., with the number of animals used in parentheses. * significant difference from control, ** below a value a significant difference for a ECO 0.5 μ mol/Kg at the same time point and † between two values indicates significant difference between them (Mann-Whitney, $p \leq 0.05$).

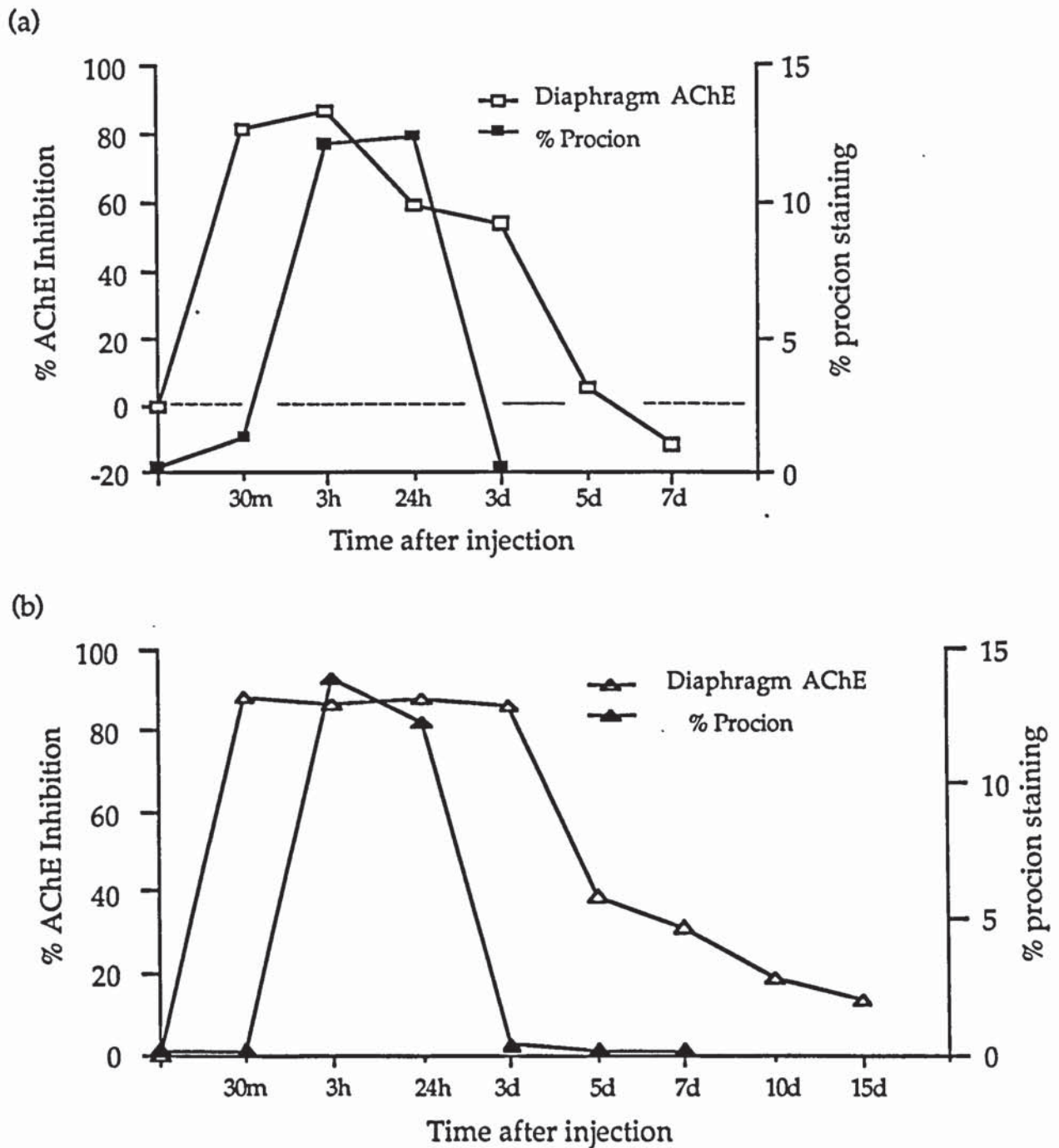


Figure 3.1. Percentage procion staining at the endplate region of diaphragms and % AChE inhibition of diaphragms at various times after (a) ECO 0.5 μmol/Kg and (b) BOS 8 μmol/Kg. The values represent the means of at least 4 experiments. (N.B. m=minutes, h=hours and d=days). All statistical data for this figure can be found in tables 3.3 and 3.4. The procion data after ECO treatment is used courtesy of Dr. H.E. Townsend.

Twenty four hours after administration of the anti-ChE the Procion staining was still extensive, although a large proportion of fibres were unaffected. The amount of staining was very similar to that seen 3 hours after administration (plate 3.4.). After a period of maximal myopathy, 3-24 hours after ECO or BOS, there appeared to be a recovery of the integrity of the muscle fibres. In fact 3 days after injection the muscles looked morphologically normal with no apparent Procion staining (plate 3.5.), although some endplates appeared swollen. From tables 3.3 and 3.4 it can be concluded that, although the rate of appearance of the myopathy was somewhat slower with BOS, at the time points studied there is no significant difference in the myopathy created by the two anti-ChEs.

3.1.2.2. Assessment of the procion technique as a measure of myopathy.

Procion yellow is a water soluble fluorescent dye which is known to penetrate and stain necrotic muscle fibres. It is a small molecule (MWt 500 Daltons: Bennett *et al.*, 1969) which will not penetrate intact, living myocytes (Bradley and Fulthorpe, 1978). Procion stains by binding to intracellular proteins and may be used on living or fixed tissues, *in vivo* or *in vitro* (Bradley and Fulthorpe, 1978).

Both ECO-treated and BOS-treated diaphragms showed myopathy up to 24 hours after administration, indicated by a significant amount of Procion staining (tables 3.3 and 3.4.). However, by 3 days total recovery seemed to have taken place. It has been suggested that most cell membranes have turnover rates in the order of 24-48 hours (Finean *et al.*, 1978; Siekevitz, 1972), thus if the sarcolemma repaired at a similar sort of rate in an anti-ChE treated diaphragm, by 3 days, damaged fibres would be restored and prevent entry of the dye. Alternatively, the debris which procion yellow stains could be removed by the phagocytes attracted to the area of damage.

One drawback of the technique is that it is likely to underestimate extensive myopathy. This is because if two or more damaged, Procion stained fibres overlap only one would be detected by the technique. This will occur less in slightly damaged muscles, but more frequently in extensively myopathic

tissues. Therefore, the greater the myopathy the more likely there will be an underestimation. However, these underestimates are not considered to be a problem in this study it was required only to measure relative not absolute amounts of damage.

3.1.2.3. The recovery of AChE activity.

This series of experiments was designed to compare the rates of recovery of AChE from single injections of ECO 0.5 μ mol/Kg and BOS 8 μ mol/Kg, both of which were known to produce their maximal inhibition of the enzyme by 3 hours after injection (table 3.1.). Both anti-ChEs produced a significant reduction in the blood and tissue AChE activity within 30 minutes of administration. However, a more extensive inhibition of diaphragm AChE than blood AChE occurred (tables 3.3 and 3.4.). In the diaphragm, the percentage inhibition 3 hours after administration was 87% for both ECO and BOS, whilst in the blood it was 73% and 68%, respectively. That is the reduction in AChE activity produced by these doses of ECO and BOS were not significantly different ($p \leq 0.05$).

In the blood, the high level of AChE inhibition seen 3 hours after ECO administration had substantially recovered by 24 hours and was not significantly different from control values (table 3.4.). However, 24 hours after injection of BOS the blood AChE was still 59% inhibited, but by 3 days it had returned to control levels (table 3.4.). By comparison, in the diaphragm, the high level of AChE inhibition seen 3 hours after injection of ECO had begun to recover by 24 hours. By 5 days, the AChE activity showed no significant difference from control values (table 3.3.). In contrast, the high level of AChE inhibition due to administration of BOS 8 μ mol/Kg was maintained for 3 days, from which point recovery commenced. This recovery was completed 10 days after the injection (figure 3.1). It is concluded that ECO 0.5 μ mol/Kg and BOS 8 μ mol/Kg produced a similar degree of inhibition of AChE, and that the recovery is slower after BOS. Germane to this conclusion are the experiments of Newman *et al.* (1984) who attempted to assess the turnover of AChE in rat diaphragms after ECO treatment. They found that in homogenates *in vitro* the phosphorylated enzyme spontaneously reactivated with a half-time of 27

hours. After correcting for such reactivation, the turnover of non-endplate AChE had a half-life of 26 hours whereas the endplate specific AChE was significantly longer but too slow to be quantified. Nevertheless, even if the synthesis of new enzyme is ignored totally the half-time of recovery should still be close to that of reactivation, that is 27 hours. This would suggest that total recovery of diaphragm AChE should occur within 4 to 5 days of exposure to ECO, assuming this would be equivalent to 3 times the time constant. This is consistent with the recovery of diaphragm AChE shown in table 3.3, with recovery complete by 5 days. It is, however, important to realise that the assay used to assess AChE activity involved measurement of the hydrolysis of radiolabelled ACh by homogenised samples of diaphragm. Hence, as indicated later (3.3), the assay, like that of Ellman *et al.* (1961), is fraught with uncertainty when interpreting results. Dettbarn (1984) assessed the half-life of recovery of diaphragm AChE after soman intoxication to be 7.7 days, using the Ellman *et al.* (1961) technique. Using the same calculation, one would expect total recovery by approximately 30 days, somewhat longer than that seen with BOS (table 3.4).

3.1.3. The effects of anti-ChEs on single twitches.

3.1.3.1. Twitch potentiation.

Mouse phrenic nerve-hemidiaphragm preparations were set up in an organ bath as described in 2.6.1. The effects of 4 and of 8 μ M BOS on single twitches at 0.2Hz were recorded and compared to those of ECO 0.5 μ M. The records of contractile responses, from at least 4 experiments, were analysed in a number of ways (fig 2.2a).

Examination of figure 3.3 demonstrates clearly that BOS 8 μ M produced a potentiation of twitch. However, if one compares this to the potentiation exhibited by ECO 0.5 μ M (figure 3.2) its manifestation is somewhat different. ECO caused the twitch height to increase rapidly and then fade to control. This was followed by a gradual return of the potentiation (figure 3.2). With BOS, in the concentrations examined, there was a much slower rise in potentiation from onset to peak, but once this potentiation had occurred it appeared to be maintained for a much longer period than ECO, although over the time period

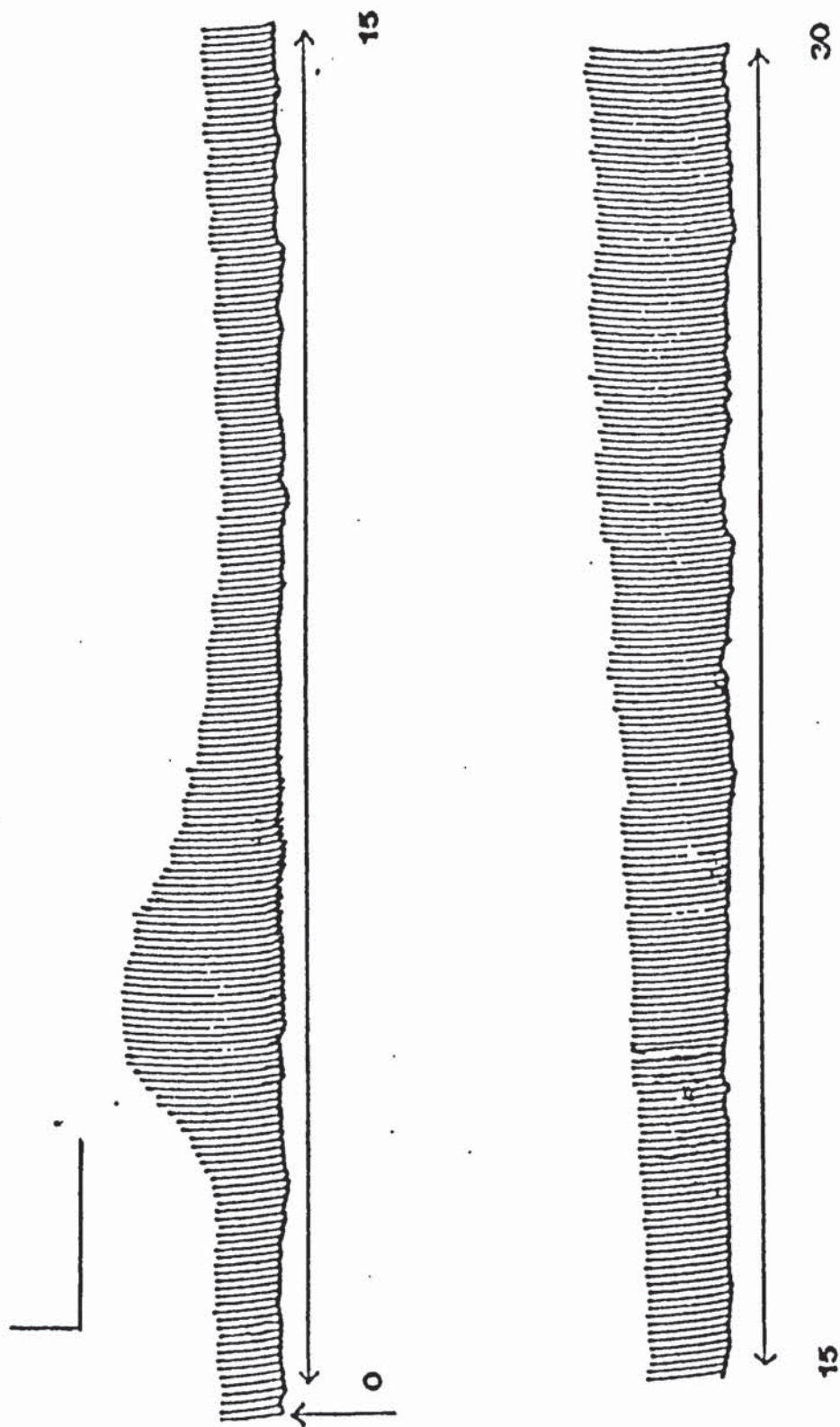


Figure 3.2. Phrenic nerve-hemidiaphragm preparation; records of contractions in response to stimulation of the phrenic nerve with single supramaximal stimuli at 0.2Hz at 37°C. ECO 0.5 μ M was added at the arrow and recordings made for 30 minutes. The horizontal bar represents 2 minutes and the vertical bar 1g force.

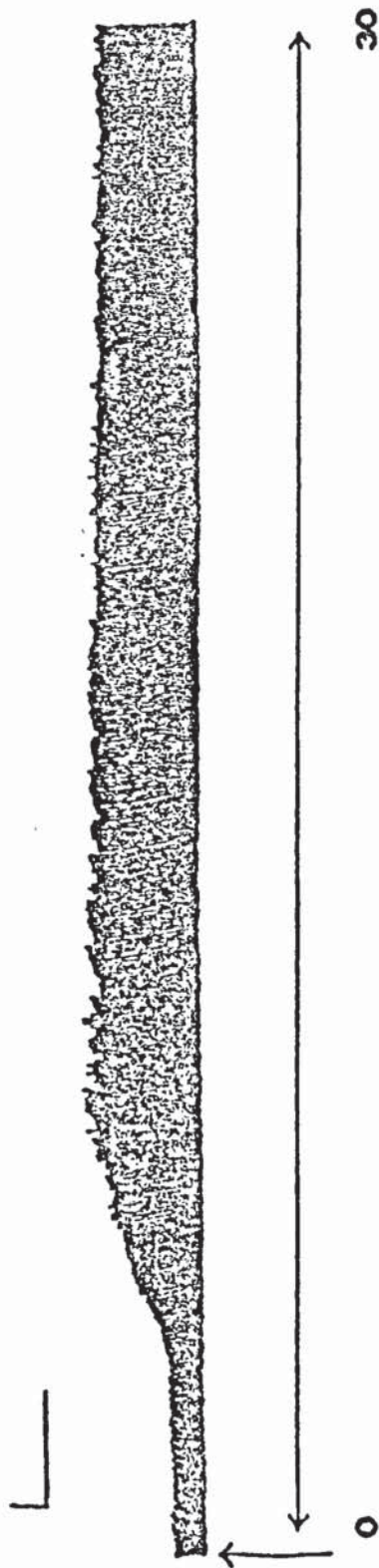


Figure 3.3. Phrenic nerve-hemidiaphragm preparation; records of contractions in response to stimulation of the phrenic nerve with single supramaximal stimuli at 0.2Hz at 37°C. BOS 8µM was added at the arrow and recordings made for 30 minutes. The horizontal bar represents 2 minutes and the vertical bar 1g force.

Drug and Final bath concn	Number of preparations	Onset (secs)	Time to Peak (secs)	Onset to Peak (secs)	% Increase in Twitch Height
ECO 0.5 μ M	6	143 \pm 20	274 \pm 29	131 \pm 16	96 \pm 27
BOS 4 μ M	4	394 \pm 76*	1005 \pm 130*	611 \pm 152*	131 \pm 50
		**	**		
BOS 8 μ M	4	168 \pm 42	543 \pm 227*	374 \pm 189*	206 \pm 31*

Table 3.5. Data for the twitch potentiation experiments to compare the effects of ECO and BOS at stimulation frequency of 0.2 Hz. The measurements were made as in figure 2.2(a) and the values expressed as the mean \pm S.D. from at least four animals. * significant difference from ECO 0.5 μ M and ** significant difference between the two BOS concentrations (Mann-Whitney; $p \leq 0.05$).

studied, there was a gradual fade. The transient reduction in potentiation observed in this 30 minute period with ECO may be due to another effect such as channel block (Tattersall, 1988) which was slower in onset than the potentiation and thus created a period of attenuation of increased twitch height. It does not appear to be a property of BOS.

When the parameters measured for the two anti-ChEs were compared, in particular the BOS 8 μ M and ECO 0.5 μ M concentrations, a number of significant differences were detected. BOS 8 μ M initiated a significantly greater amount of twitch potentiation (206%) than did ECO (96%). No significant difference in the rate of potentiation was found. However, BOS 8 μ M took a significantly longer time to reach peak potentiation from both injection of the drug into the organ bath and from the onset of potentiation than did ECO ($p \leq 0.05$, table 3.5).

If the parameters measured for the two concentrations of BOS were examined, certain concentration-dependent effects came to light. Although both concentrations of BOS produced an increase in twitch height, 206% and 131% increases for the 8 μ M and 4 μ M concentrations respectively, there is no significant difference between them. Similarly, there is no significant difference between the two concentrations in the time taken for the potentiation to reach peak from its onset although for the 8 μ M concentration takes 374 seconds compared with 611 seconds for 4 μ M. However, it did take a significantly longer time to reach the onset and peak of the potentiation from injection of BOS 4 μ M into the bath than for the 8 μ M concentration ($p \leq 0.05$, table 3.5). These values appear consistent with first order kinetics, that is by halving the dose one doubles the time to a critical degree of inhibition of AChE.

3.1.3.2. The association of repetitive muscle action potentials with twitch potentiation.

In order to investigate whether BOS produced twitch potentiation by means of repetitive muscle action potentials, a series of experiments were performed in which the contractile response and associated extracellular field potential were measured together in mouse phrenic nerve-diaphragm preparations after the addition of BOS 8 μ M. At certain intervals (2, 4, 6, 9, 15, 20, 30, 40, 50, 60, 70, 85

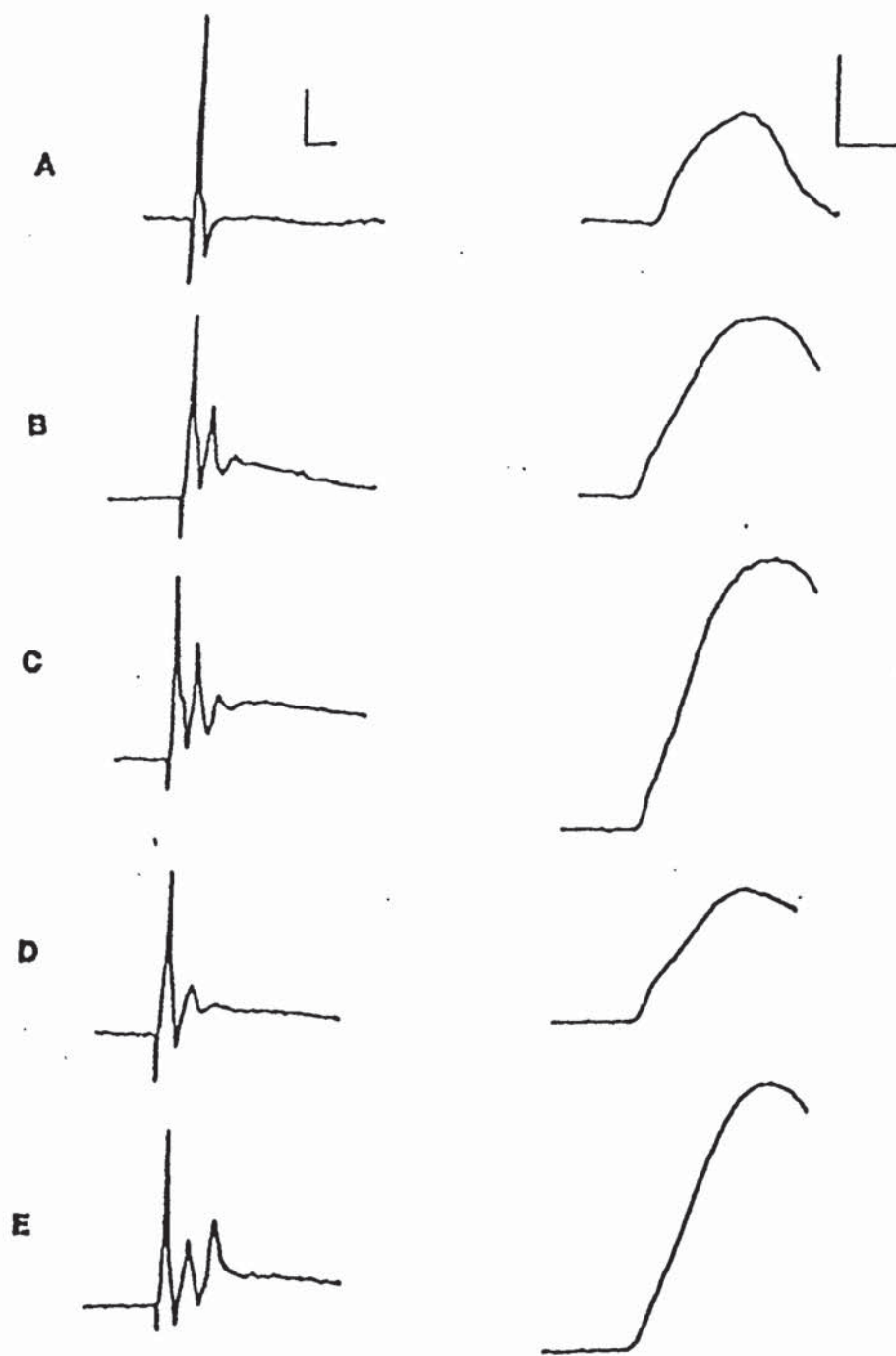


Figure 3.4. Records of indirectly-evoked extracellular field potential at the endplate region and the twitch tension of a hemidiaphragm preparation at 37°C. Left, the potential recorded with silver wire electrodes and A.C. amplification, calibration 2mV, 5msec. Right, the contraction, calibration 1g, 10msec. A- Control responses, B- 6min, C- 9min, D- 85min and E- 105min after BOS 8 μ M, with BOS washed out after 85min.

Time after administration (mins)	Twitch height (mm)	Rise time (msecs)
0	50.8±12.9	14.5±1.2
2	49.7±12.7	14.3±0.6
4	55.8±18.1	14.7±1.3
6	89.3±36.6	19.2±2.2*
9	114.7±32.4*	22.8±2.8*
15	98.0±20.0*	22.4±1.7*
20	93.7±26.5*	23.4±2.1*
30	86.0±29.4*	21.4±1.4*
40	84.5±27.9*	21.8±2.6*
50	70.6±23.9	21.1±1.9*
60	49.3±12.7	18.8±1.5*
70	51.2±14.9	19.1±1.8*
85	47.3±13.2	19.0±1.6*
WASHOUT		
105	92.5±18.3*	23.6±0.8*
110	96.5±13.2*	24.6±1.4*

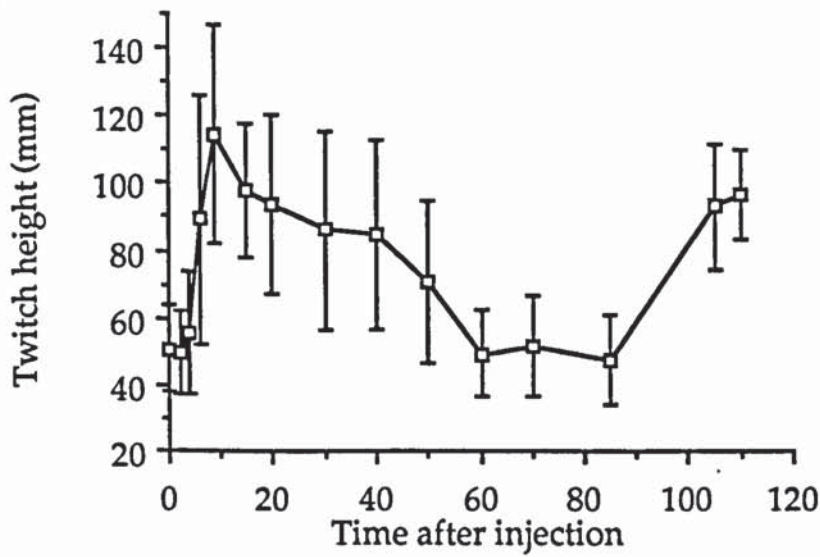
Table 3.6. The changes in twitch height and rise time following the addition of BOS 8µM *in vitro*, the drug was washed out after 85 minutes. The values shown are the means±S.D. from at least four animals. * significant difference from control (Mann-Whitney, $p \leq 0.05$).

minutes) after the addition of the anti-ChE the preparation was stimulated supramaximally via the phrenic nerve and records were made. The BOS was washed out 85 minutes after injection and two further records, at 105 and 110 minutes, were made. From the contractile records two parameters were measured, the twitch height and the rise time.

Figure 3.4 shows sample records of the contractile responses and associated extracellular field potentials for selected time points. It demonstrates that the twitch potentiation was closely connected with repetitive action potentials within the muscle. Figure 3.4 C illustrates the responses at maximum potentiation, 9 minutes after the addition of BOS $8\mu\text{M}$, where the second action potential occurred within 3 msec of the first on the back of a much prolonged endplate potential (EPP). By 85 minutes after the addition of BOS, the contractile response height was not significantly different from the control (Figure 3.4 D), although there was significant prolongation of the time course. The extracellular field potential at the same time showed a much reduced prolongation of the EPP, although there was still some evidence of repetitive activity. Following washout of BOS, a recovery of the potentiation was observed (figure 3.4 E), together with a further increase in the duration of the EPP prolongation and an increase in the amplitude and number of the repetitive action potentials.

Measurement from these experiments are given in table 3.6. A significant potentiation of the contractile response was observed 9 minutes after addition of the anti-ChE, from which point a steady decline occurred until, by 50 minutes, the twitch height was not significantly different from control. However, following removal of the drug after 85 minutes, the two subsequent recordings showed further significant potentiation ($p \leq 0.05$, table 3.6). If the rise time of the contraction is examined (table 3.6), a significant increase was observed 6 minutes after the addition of BOS $8\mu\text{M}$ ($p \leq 0.05$, table 3.6). Although there was a decline in the rise time until the drug was washed out, at 85 minutes, it never returned to control levels. Following the removal of BOS there was again a further increase in rise time ($p \leq 0.05$, table 3.6, figure 3.5). The prolongation of the rise time of the contraction may be due to the production of tetanus within the muscle as a result of its repetitive excitation. Although the potentiation

(a)



(b)

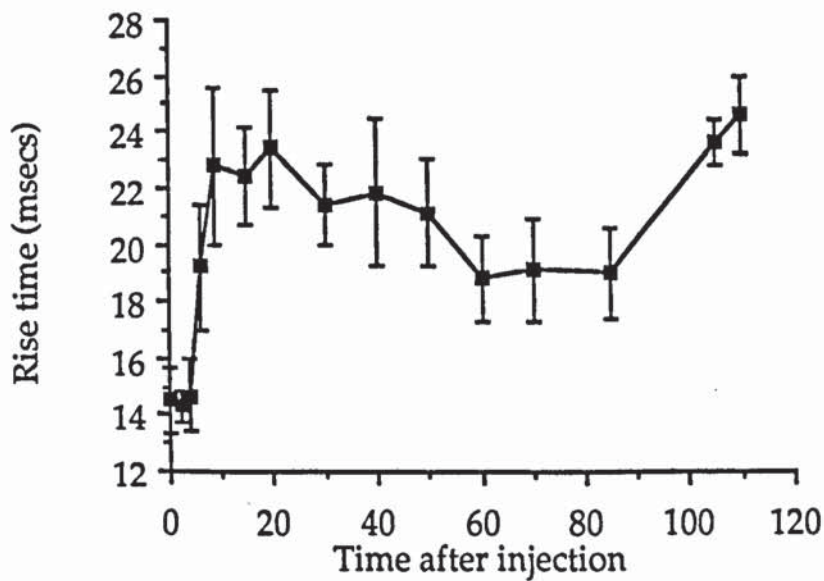


Figure 3.5. The changes in (a) Twitch height and (b) Rise time following the addition of BOS $8\mu\text{M}$ *in vitro*, with the drug being washed out after 85 minutes. The values shown are the means \pm S.D. from at least four animals. Data for this figure is detailed in table 3.6.

wanes, the prolongation may be maintained because of the continued presence of repetitive excitation, evidence of which can be observed in figure 3.4 D.

3.1.4. Spontaneous muscle twitching.

Earlier experiments (3.1.3.2.) had demonstrated that twitch potentiation produced by BOS was related to repetitive muscle action potentials. These experiments were designed to discover whether BOS also possessed the ability to generate spontaneous muscle twitching, as described in 2.6.2.2. Mouse phrenic nerve-hemidiaphragm preparations were set up in an organ bath, BOS was added in a bath concentration of $8\mu\text{M}$, and the contractile activity was recorded. After 14 minutes the BOS was washed out of the bath and recording continued.

Seven preparations were studied in the above manner and all displayed some spontaneous muscle twitching (figure 3.6 A). This activity steadily declined until, by approximately 14 minutes, it was totally abolished. At this point the drug was washed out of the bath and, after a short delay, the spontaneous activity returned. In three of the seven preparations periods of "hyperactivity" were observed following washout, that is bursts of intense contractile activity leading to a tetanus-like effect (figure 3.6 B). It is thought that these periods may be analogous to the sudden surges of body tremor seen *in vivo*. There was also evidence, both before and after washout, of the prolonged localized contraction described by Burd and Ferry (1987). This prolonged contraction, which could last several seconds, followed a spontaneous muscle twitch and is thought to be associated with a prolonged EPP. Burd and Ferry (1987) concluded that this phenomenon was a direct result of AChE inhibition.

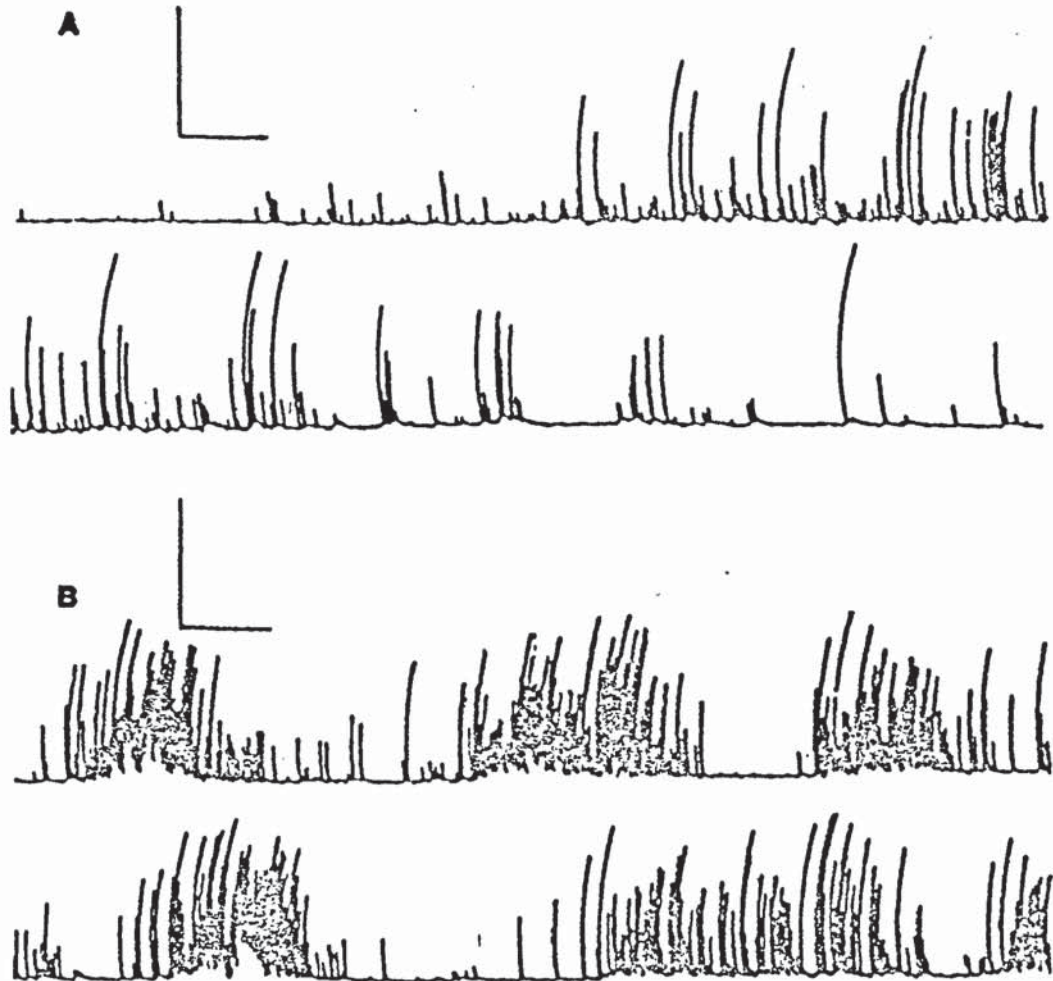


Figure 3.6. Phrenic nerve-hemidiaphragm preparations; record of spontaneous muscle contractions. (A) is a sample of the responses gained on addition of BOS $8\mu\text{M}$ and (B) the responses following washout and giving examples of the phenomenon of "hyperactivity", at 37°C . The horizontal bar represents 30 seconds and the vertical bar 1g.

3.2. THE EFFECTS OF ANTICHOLINESTERASES ON THE VARIABILITY OF THE LATENCIES OF ACTION POTENTIALS.

This technique, developed by Dr.S.S. Kelly, was utilised to examine the effects of anti-ChEs on the variability of the latencies of action potentials (jitter). As described earlier (Introduction), the jitter of extracellularly recorded action potentials from muscle fibres in the same motor unit is considered to be a sensitive indicator of myopathy or neuromuscular disease (Stalberg and Trontelj, 1979). In the laboratory at Aston the technique involved the intracellular recording of trains of thirty action potentials at 30Hz, and analysing their latencies, amplitudes and time courses via computer (2.6.4.). Two parameters were of particular interest:-

- (1) The mean consecutive difference (MCD) of the latencies of action potentials 11-30 (plateau).
- (2) The latency of the sixteenth action potential with respect to the first action potential of the train, known from this point as the "delay".

3.2.1 Summary of the previous work on jitter within the laboratory.

In the laboratory at Aston, prior to the commencement of my studies, Dr. Kelly had determined the time course of increases in jitter following single injections of ECO 0.5 μ mol/Kg (table 3.7) or BOS 8 μ mol/Kg (table 3.8), as well as the dose-dependent effects of these compounds, 5 days after the initial injection (table 3.9). He had established that the maximum increase in MCD and delay at the tendon end of diaphragm muscle cells occurred 1-5 days after administration of ECO 0.5 μ mol/Kg *in vivo*. By 15 days, the delay returned to untreated levels leaving the MCD still significantly raised ($p \leq 0.05$; table 3.7). However, at 27 days both parameters were not significantly different from those of untreated animals. Five days after BOS 8 μ mol/Kg *in vivo* there had been significant increases in MCD and delay ($p \leq 0.05$; table 3.8). By 35 days after injection of BOS, the MCD had returned to untreated levels but the delay had not ($p \leq 0.05$; table 3.8). However, 60 days after administration both MCD and delay were not significantly different from those of untreated animals. In these studies there

Time after injection	MCD (μ sec)	Delay (μ sec)
Untreated	9.9 \pm 3.6 (42)	32 \pm 22 (42)
1 day	23.7 \pm 12.1 (13)*	103 \pm 36 (13)*
3 days	27.9 \pm 32.9 (13)*	107 \pm 50 (14)*
5 days	25.6 \pm 28.0 (39)*	119 \pm 104 (40)*
7 days	17.8 \pm 9.5 (18)*	61 \pm 37 (18)*
15 days	14.7 \pm 7.6 (24)*	48 \pm 38 (24)
27 days	10.1 \pm 3.4 (34)	25 \pm 23 (34)

Table 3.7. The MCD and delay at various times after *in vivo* injection of ECO 0.5 μ mol/Kg, with values given as mean \pm S.D. and the number of muscle fibres from 2 to 5 animals in parentheses. * significant difference from untreated for the parameters of jitter (Kolmogorov-Smirnov; $p \leq 0.05$). Data courtesy of Dr S.S. Kelly.

Time after injection	MCD (μsec)	Delay (μsec)
Untreated	9.9±3.6 (42)	32±22 (42)
5 days	53.3±86.2 (59)*	162±181 (59)*
15 days	12.0±3.8 (28)*	45±28 (28)*
35 days	12.3±4.8 (42)	46±20 (42)*
60 days	11.6±4.0 (14)	37±18 (15)

Table 3.8. The MCD and delay at various times after *in vivo* injection of BOS 8μmol/Kg, with values given as mean±S.D. and the number of muscle fibres from 2 to 5 animals in parentheses. * significant difference from untreated for the parameters of jitter (Kolmogorov-Smirnov; $p \leq 0.05$). Data courtesy of Dr. S.S. Kelly.

Drug and Dose	MCD (μsec)	Delay (μsec)
Untreated	9.9±3.6 (42)	32±22 (42)
ECO 0.5μmol/Kg	25.6±28.0 (39)*	119±104 (40)*
ECO 0.4μmol/Kg	16.8±9.0 (30)*	60±41 (30)*
ECO 0.3μmol/Kg	12.1±5.2 (31)	52±34 (31)*
ECO 0.1μmol/Kg	10.8±5.6 (44)	39±19 (44)
BOS 8μmol/Kg	53.3±86.2 (59)*	162±181 (59)*
BOS 1μmol/Kg	17.0±7.9 (29)*	35±32 (29)

Table 3.9. The MCD and delay, 5 days after various doses of ECO and BOS *in vivo*, with values given as mean±S.D. and the number of muscle fibres from 2 to 5 animals in parentheses. * significant difference from untreated for the parameters of jitter (Kolmogorov-Smirnov; $p \leq 0.05$). Data courtesy of Dr. S.S. Kelly.

was no apparent correlation between MCD and delay, that is fibres with large MCDs did not necessarily have large delays, with the exception that those fibres with a very large MCD tended to also have a very large delay. Thus it appeared that after BOS 8 μ mol/Kg the effects on delay, but not MCD, were longer-lasting than after ECO 0.5 μ mol/Kg. There was no significant difference between the maximum effects of the two drugs on either of the parameters of jitter measured. As maximal increases in the MCD and delay of action potential latency were 5 days after injection of ECO 0.5 μ mol/Kg, the dose-dependency of the increases in jitter were assessed 5 days after administration of the anti-ChE *in vivo*. A dose-dependent effect of ECO on MCD and delay was seen (table 3.9). At 0.1 μ mol/Kg no significant difference from untreated animals was observed and only the 0.3 μ mol/Kg dose showed a significant increase in the delay ($p \leq 0.05$; table 3.9), the MCD was not different from untreated. The 1 μ mol/Kg dose of BOS caused a significant increase in the MCD ($p \leq 0.05$; table 3.9), without affecting the delay. It can also be seen, from the standard deviation of the data, that the greater the dose the greater the variability about the mean. Five days after a high dose of anti-ChE, the fibres sampled encompass a broad cross section of characteristics, from those which were normal to those with extremely large MCD and delays.

In summary, both anti-ChEs increased the MCD and delay of action potentials elicited at 30Hz, the increases being dose related. Although the effects of ECO and BOS on the latencies of action potentials were dose-dependent, lower doses of ECO affect delay more than MCD, but lower doses of BOS affected MCD more than delay. This reinforces the suggestion that MCD was unrelated to delay.

3.2.1.1. The characteristics of the action potentials intracellularly recorded from the tendon end of diaphragm fibres.

The values of resting membrane potential, time to peak (from 10% peak) and amplitude of the action potentials in untreated animals were: 71 \pm 5mV (42), 216 \pm 34 μ sec (35) and 81 \pm 8mV (35), respectively. These values, expressed as the mean \pm S.D., were unaffected by the treatment with an anti-ChE *in vivo*, at any time point. Recordings of typical action potentials are shown in figure 3.7, which illustrates the parameters which were measured. It was shown that, at

30Hz, there was a progressive increase in the latencies of successive action potentials in the early part of the train relative to the first action potential. In untreated preparations, this increase in latency reached a plateau by the tenth action potential of the train (figure 3.8). Approximately 5% of the fibres sampled were excluded from the analysis because action potentials either failed to be generated or failed to be conducted to the recording electrode. Repetitive firing of action potentials was seen in response to single stimuli in about 20% of fibres 5 days after ECO. Of the fibres recorded 5 days after injection of BOS, 50% or more of fibres exhibited repetitive firing in the same manner.

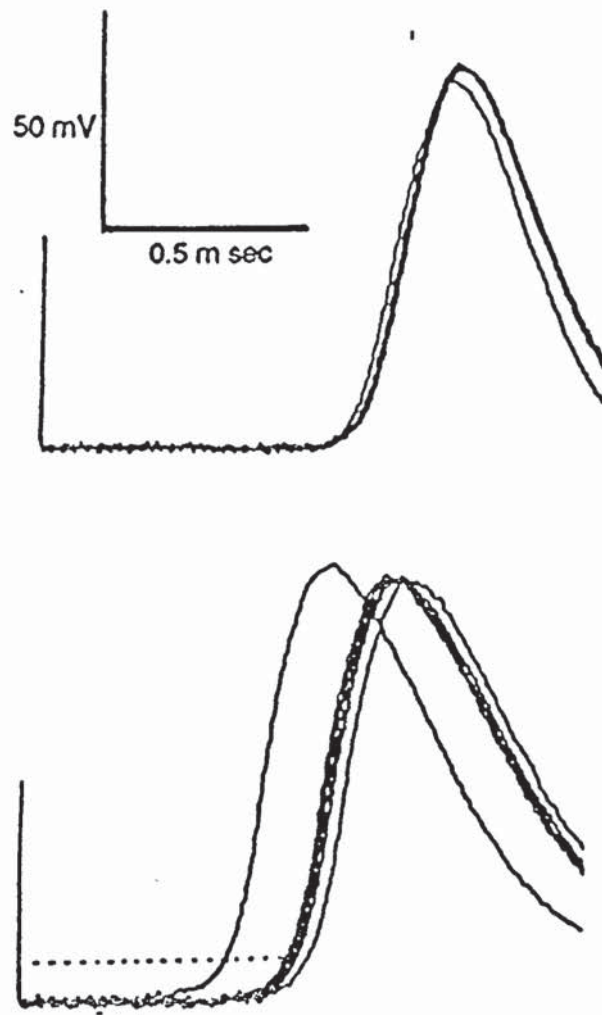


Figure 3.7. Typical intracellular action potentials (30Hz) recorded at the tendinous portion of muscle fibre from untreated muscle (upper) and muscle from an animal 5 days after ECO $0.5\mu\text{mol/Kg}$ (lower). The leftmost trace in each case is the first action potential of the train and the group of six action potentials to the right of the first action potential are action potentials 16 to 21. The broken line on the lower trace intersects with the action potentials at 10% of the peak amplitude and illustrates the point at which latency was calculated. The horizontal and vertical bars represent 0.5msec and 50mV respectively. Figure courtesy of Dr.S.S. Kelly.

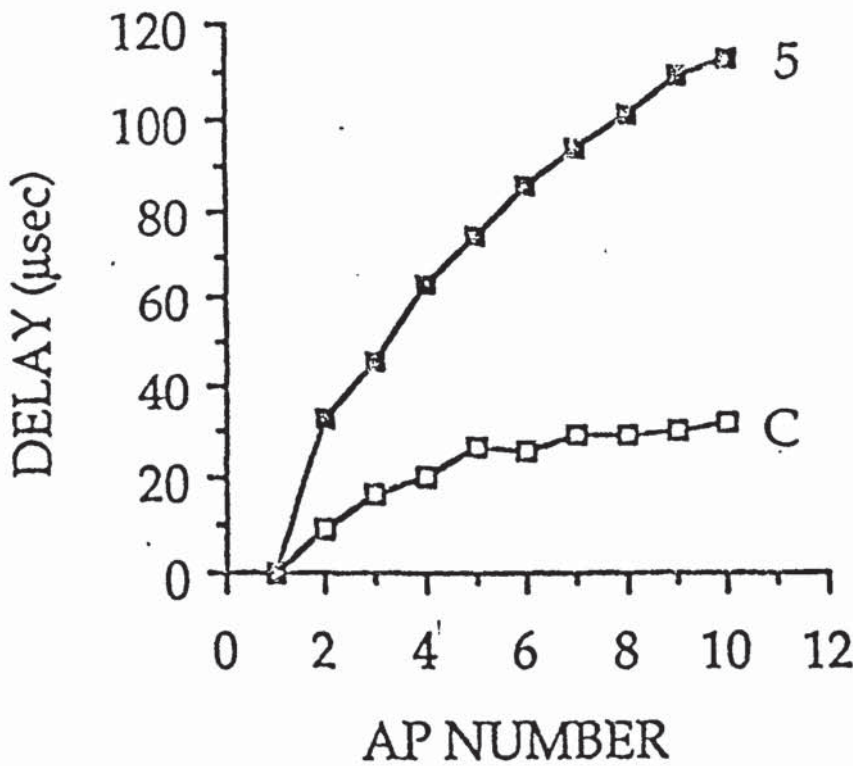
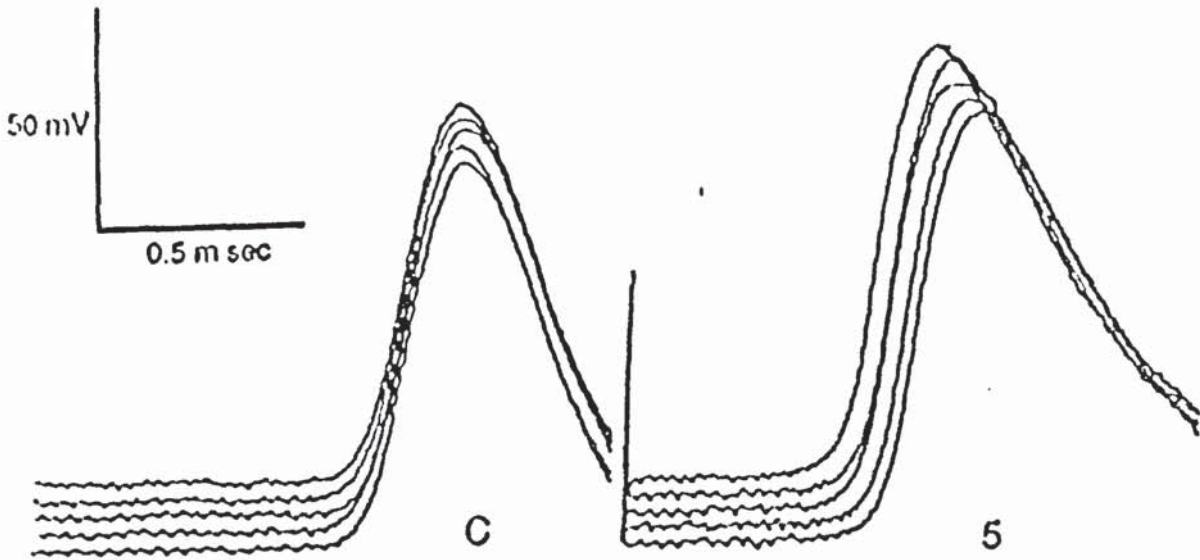


Figure 3.8. Top: Traces show action potentials 1 to 5 elicited at 30Hz from untreated muscle (left) and muscle 5 days after ECO 0.5 μ mol/Kg *in vivo* (right). The delay of action potentials relative to the first action potential (uppermost) is greater in the muscle treated with ECO.

Bottom: Graph showing the mean delay of the first 10 action potentials elicited at 30Hz relative to the first action potential. The open symbols indicate untreated values (n=42) and the closed symbols represent values 5 days after injection of ECO (n=39). Figure courtesy of Dr.S.S. Kelly.

3.2.2. The relationship between inhibition AChE and increases in jitter of diaphragm.

The next section of the jitter project was to investigate whether or not, at times when jitter was measured, there was also inhibition of AChE.

3.2.2.1. The time course of the increases in jitter and of the inhibition of AChE.

Animals were injected with ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg and after various times the animals were killed and their diaphragms assayed for their AChE content (2.5).

(a) Time after injection of ECO 0.5µmol/Kg	Diaphragm AChE activity (nmol/min/mg)	% Inhibition
Atropine Control	1.34±0.43 (38) **	0
3 hours	0.17±0.04 (8)* **	87
1 day	0.54±0.05 (6)*	60
3 days	0.61±0.07 (4)* **	54
5 days	1.27±0.23 (11) **	5
7 days	1.50±0.26 (5)	-12
(b) Time after injection of BOS 8µmol/Kg	Diaphragm AChE activity (nmol/min/mg)	% Inhibition
Atropine Control	1.34±0.43 (38) **	0
3 hours	0.18±0.05 (11)* **	87
5 days	0.82±0.16 (18)* **	39
15 days	1.16±0.19 (8)	13

Table 3.10. The recovery of diaphragm AChE following single injections of (a) ECO 0.5µmol/Kg and (b) BOS 8µmol/Kg *in vivo*, with the values given as the mean±S.D. and the number of muscles assayed in parentheses. * significant difference from atropine control AChE activity and ** between two values indicates significant difference between them (Mann-Whitney; p≤0.05).

Three hours after the injection of ECO 0.5 μ mol/Kg there was an 87% inhibition of diaphragm AChE (table 3.10a). However, 5 days after the injection of ECO AChE levels had returned to control, when the values of MCD and delay were still at a maximum. It was not until 15 days, for the delay, and 27 days, for the MCD, that the values were not different from untreated values. Similarly, 3 hours after the injection of BOS 8 μ mol/Kg there was an 87% inhibition of diaphragm AChE (table 3.10b). As described in section 3.1.2, BOS produces a more prolonged inhibition of AChE than ECO. Five days after injection of BOS 8 μ mol/Kg, when Dr Kelly found the MCD and delay were at a maximum, there was still 39% inhibition of diaphragm AChE (table 3.10b). By 15 days, however, the AChE activity has returned to control levels whereas there the MCD and delay were still significantly increased.

As stated above, the increased delay after BOS, 8 μ mol/Kg, was longer lasting than those after ECO, 0.5 μ mol/Kg. Perhaps this is due to the prolonged inhibition of diaphragm AChE seen with BOS? However, it can be concluded that the increases in jitter do not appear to be related to the inhibition of diaphragm AChE at the time of recording, since these compounds increase jitter for a much longer time than they inhibit AChE.

3.2.2.2. Dose-dependency of the increases in jitter and of the inhibition of AChE.

Animals were injected subcutaneously with a dose of ECO within the range 0.1-0.5 μ mol/Kg or a dose of either BOS 1 or 8 μ mol/Kg. Three hours later they were killed and their diaphragms removed (2.3.1). The diaphragms were then assayed for their AChE content (2.5) in attempt to determine whether the initial AChE inhibition could be related to the increase in jitter measured by Dr Kelly 5 days after injection. The control animals were injected with a 0.7 μ mol/Kg atropine alone and treated in the same manner.

Although the 0.3, 0.4 and 0.5 μ mol/Kg doses of ECO exhibited equivalent amounts of inhibition of diaphragm AChE 3 hours after injection (table 3.11; $p \leq 0.05$), significant dose-dependent differences in the MCD and delay were observed (table 3.9). In fact, the ECO 0.3 μ mol/Kg dose only showed a significant

Drug and Dose	Diaphragm AChE activity (nmol/min/mg)	% Inhibition
Atropine Control	1.34±0.15 (38) **	0
ECO 0.5µmol/Kg	0.17±0.04 (8)*	87
ECO 0.4µmol/Kg	0.17±0.04 (9)*	87
ECO 0.3µmol/Kg	0.16±0.05 (8)* **	88
ECO 0.1µmol/Kg	0.55±0.10 (8)* **	59
BOS 8µmol/Kg	0.18±0.05 (11)* **	87
BOS 1µmol/Kg	0.84±0.07 (4)*	37

Table 3.11. Diaphragm AChE 3 hours after the *in vivo* administration of an anti-ChE, with the values given as the mean±S.D. and the number of muscles assayed in parentheses. * significant difference from control AChE activity and ** between values indicates a significant difference between them (Mann-Whitney; p≤0.05).

increase in delay ($p \leq 0.05$, table 3.9), but the MCD was not different from the atropine control. BOS $1 \mu\text{mol/Kg}$ caused a significant increase in the MCD ($p \leq 0.05$; table 3.9), without affecting delay, though its initial inhibition of diaphragm AChE was only 37%, 3 hours after injection. In comparison, $0.1 \mu\text{mol/Kg}$ ECO inhibited 59% of diaphragm AChE by 3 hours yet had no effect on the MCD and delay. It should be noted that ECO $0.1 \mu\text{mol/Kg}$ caused a significantly greater reduction in the diaphragm AChE activity than did BOS $1 \mu\text{mol/Kg}$, 3 hours after injection ($p \leq 0.05$). However, both 8 and $1 \mu\text{mol/Kg}$ BOS produced significant amounts of AChE inhibition 5 days after injection, 39% and 28% respectively.

In summary, the increase in MCD and delay was related to the dose of the compound, but not directly to the inhibition of AChE 3 hours after administration, when each drug had exerted maximum inhibition of AChE. The duration of the increased MCD and delay were longer than, but may be related to, the duration of AChE inhibition. Although the effects of ECO and of BOS on the latencies of action potentials were dose-dependent, lower doses of ECO affected delay more than MCD, but lower doses of BOS affected MCD more than delay. This reinforces the idea that MCD was unrelated to delay.

3.2.3. The loss of body weight after anti-ChEs and its effect on the variability of the latencies of action potentials.

Over the course of the experiments described above, it was noticed that mice exposed to high doses of anti-ChEs lost weight. To assess whether or not such weight loss may have contributed to the increased jitter observed, a study was made to quantify this reduction in weight, and then to investigate its effect on jitter. All the experiments from this point onwards were performed by myself.

Ten mice, weighing $40 \pm 4\text{g}$, were housed individually and allocated into one of 2 groups. Their weight, food and water intake were monitored over a period of 5 days acclimatisation. On the sixth day, one group was injected with BOS $8 \mu\text{mol/Kg}$ plus $0.7 \mu\text{mol/Kg}$ atropine, and the other group with atropine alone. The values of MCD and delay after an injection of atropine alone were 9.4 ± 2.6 (29) and 32 ± 19 (29) (mean \pm S.D., with the number of fibres in parentheses), neither of which were significantly different from those of untreated animals.

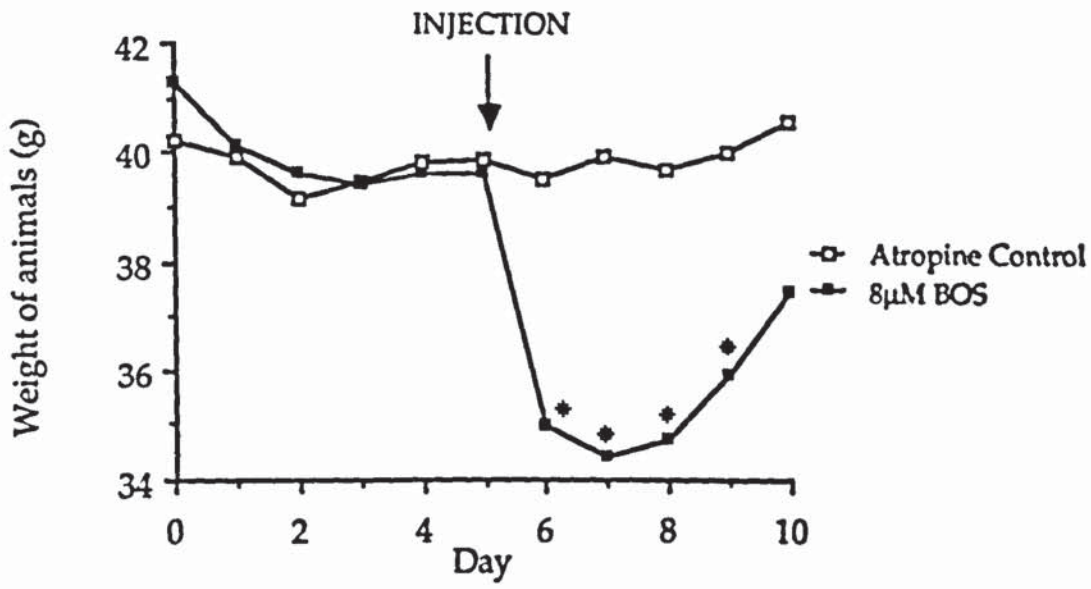
Day	Weight (g)	Food intake (g)	Water intake (ml)
0	40.2±1.6		
1	39.9±1.2	10.7±3.9	8.0±2.2
2	39.1±1.3	5.0±1.1†	6.9±1.3
3	39.4±1.3	5.6±0.8†	7.7±1.6
4	39.8±1.5	7.8±1.5	8.3±1.7
5	39.8±1.8	6.9±1.4	8.0±1.8
INJECTION OF ATROPINE			
6	39.5±1.3*	7.0±2.1*	10.0±2.9*
7	39.9±1.3*	7.0±1.8*	7.5±1.9*
8	39.7±2.0*	6.9±1.0*	7.9±1.8*
9	39.9±2.0*	7.3±2.1	8.0±2.1
10	40.5±1.7	7.0±1.9	6.7±1.6

Table 3.12. The changes in weight, food intake and water intake of control animals over a 10 day period. The animals were dosed at the beginning of the sixth day, with 0.7µmol/Kg atropine. The values represent the mean±S.D. of the data from six animals. † significant difference from the initial value recorded for the group and * significant difference, at the same time point, from the animals injected with BOS 8µmol/Kg plus atropine (Mann-Whitney; p≤0.05).

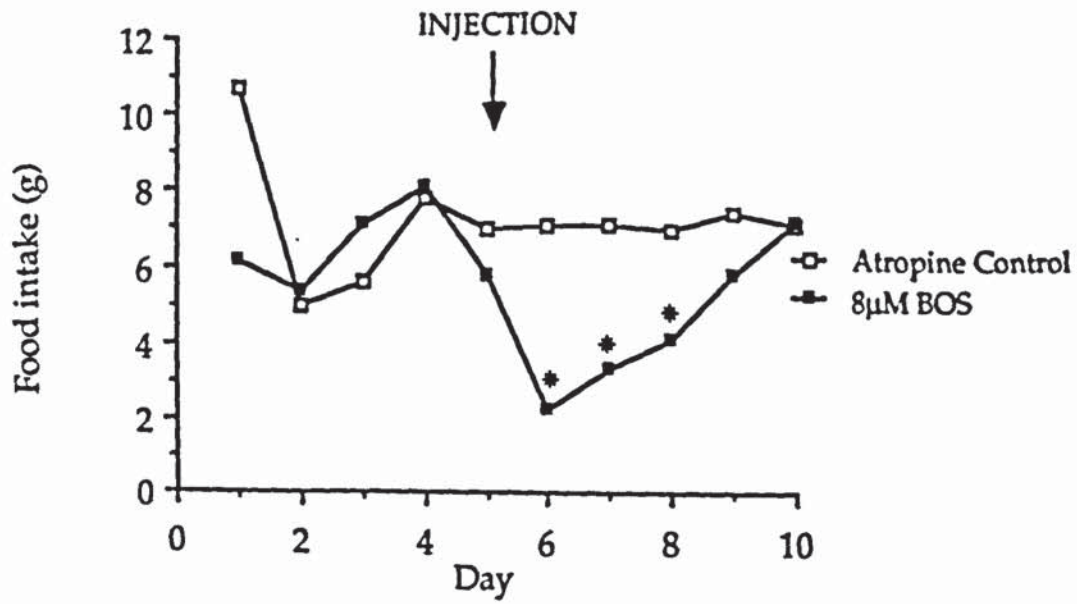
Day	Weight (g)	Food intake (g)	Water intake (ml)
0	41.3±2.4		
1	40.1±1.8	6.1±3.0	6.7±3.1
2	39.6±1.7	5.3±1.5	6.5±2.3
3	39.4±1.5	7.1±4.1	6.7±1.6
4	39.6±1.6	8.1±5.9	7.7±2.8
5	39.6±1.1	5.8±4.0	7.4±2.7
INJECTION OF BOS 8µmol/Kg + ATROPINE			
6	35.0±1.3*†	2.2±0.9*†	3.1±2.2*
7	34.4±3.2*†	3.3±1.9*	3.6±1.9*
8	34.7±3.2*†	4.1±2.4*	4.6±1.3*
9	35.9±3.2*†	5.7±1.5	6.5±0.7
10	37.4±2.7	7.1±0.7	7.6±1.0

Table 3.13. The changes in weight, food intake and water intake of animals injected with BOS 8µmol/Kg plus 0.7µmol/Kg atropine at the beginning of the sixth day of a 10 day period. The values represent the mean±S.D. of the data from five animals. † significant difference from the initial value recorded for the group and * significant difference, at the same time point, from the animals injected with atropine alone (Mann-Whitney; p≤0.05).

(a)



(b)



(c)

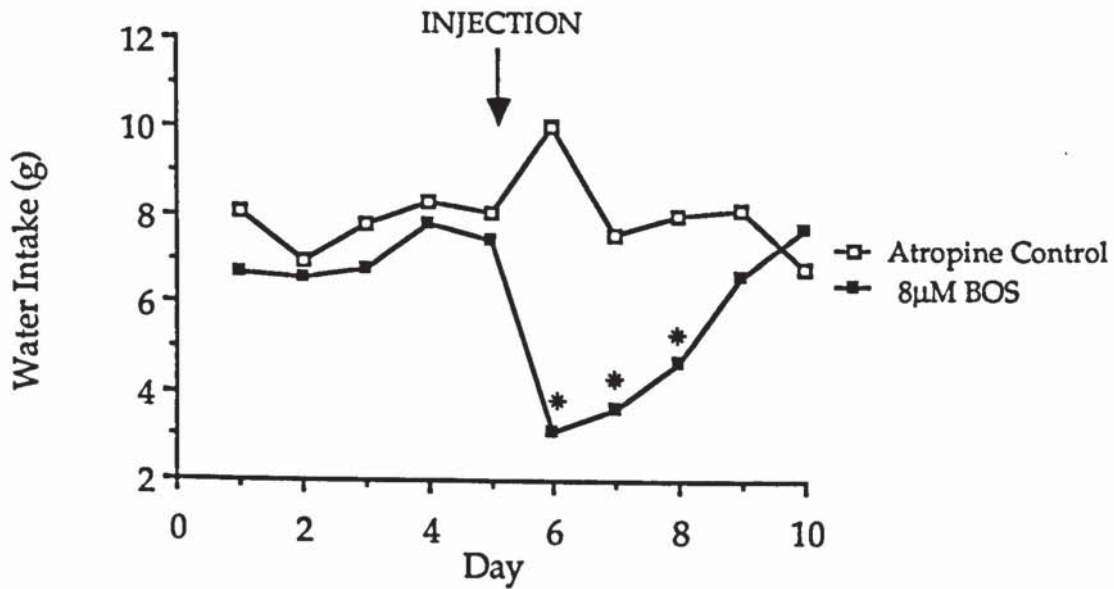


Figure 3.9. The effect on (a) Mean weight, (b) Mean food intake and (c) Mean water intake, of administration of BOS 8µmol/Kg *in vivo* at the beginning of the sixth day of a ten day period. The values represent the means of at least five animals. * significant difference from control animals at the same time point (Mann-Whitney; $p \leq 0.05$).

In the atropine control group no significant reduction in weight, or food and water intake was observed (table 3.12, figure 3.9). However, the group given BOS $8\mu\text{mol/Kg}$ *in vivo* displayed a significant weight loss for a period of 4 days after the injection ($p\leq 0.05$; table 3.13, figure 3.9a). Not only did the group injected with BOS show significant differences from its weight on day 5, but also from the equivalent atropine controls ($p\leq 0.05$; tables 3.12, 3.13, figure 3.9b,c). The fact that this group also displayed significantly less food and water consumption than atropine controls, for the three days subsequent to injection ($p\leq 0.05$; tables 3.12, 3.13), may help to explain the mechanism of such weight loss.

Having quantified the weight loss and reductions in food and water intake, the next experiment was to determine whether such effects would contribute to the increases in jitter observed with BOS. Another group of 3 animals were selected, in the same weight range, and again allowed to acclimatise in individual cages for 5 days. On the sixth day the animals were placed on a dietary regimen equivalent to animals injected with BOS $8\mu\text{mol/Kg}$, the relevant amounts of food and water being given at six hourly periods for the following 5 days (table 3.14). At the end of the regimen the animals were killed and their diaphragms dissected out (2.3.1). The MCD and delay of the latencies of action potentials at 30Hz, recorded from these diaphragms, were calculated as before (2.6.4). To test for significant differences between the groups of jitter data, the Kolmogorov-Smirnov test (Appendix 5.2) was used and the differences were taken to be significant if $p\leq 0.05$ (2-tail).

Day	Mean Weight (g)	Food (g) every 6 hours	Water (ml) every 6 hours
Five days acclimatisation in individual cages with food and water <i>ad lib.</i>			
5	43.2*		
6	38.5	0.6	0.8
7	37.6	0.8	0.9
8	36.8	1.0	1.1
9	37.9	1.4	1.6
10	39.4	1.8	1.9

Table 3.14. The restricted diet to which mice were exposed in an attempt to mimic the reduction in weight experienced following injection of BOS 8 μ mol/Kg *in vivo*. The quantities of food and water were given at six hourly intervals. Also shown is the weight of animals subjected to this diet, the values representing the mean of three mice. * significant difference to the weights of animals injected with BOS 8 μ mol/Kg, at the same time point (Mann-Whitney; $p \leq 0.05$).

Treatment	MCD (μ sec)	Delay (μ sec)
Untreated	9.9 \pm 3.6 (42)	32 \pm 22 (42)
Restricted Diet	7.9 \pm 2.0 (26)*	22 \pm 12 (26)*

Table 3.15. The effect of weight loss, induced by the restricted diet, on MCD and delay. The values represent the mean \pm S.D. of the data from at least 3 animals, with the number of muscle fibres in parentheses. * significant difference from untreated animals (Kolmogorov-Smirnov; $p \leq 0.05$).

Table 3.14 shows the mean weight of 3 animals exposed to the restricted diet. An overall maximum mean weight loss of 6.4g was observed (15% of body weight prior to the restricted diet), compared to 5.2g (13% of body weight prior to injection) produced by the injection of BOS. In fact the weight loss of animals subjected to the diet displayed no significant differences from that of animals given BOS *in vivo*, apart from there being a slight difference initially on the 5th day ($p \leq 0.05$; table 3.14). This suggested that the weight loss initiated by BOS was solely due to the animals reduced dietary intake. Nevertheless, this weight loss did not significantly increase the MCD or delay of the recorded action potential latencies, in fact the reverse was true, that is there was a small, significant decrease in jitter ($p \leq 0.05$; table 3.15). The jitter of animals subjected to the restricted diet was also significantly different from that of animals exposed to BOS $8 \mu\text{mol/Kg}$ for 5 days ($p \leq 0.05$). In conclusion, it can be said that any weight loss produced by BOS did not appear to contribute to any increase in the jitter.

3.2.4. The effect of anti-ChEs on the latencies of action potentials of the soleus and extensor digitorum longus muscles.

This area of the study examined the latencies of action potentials generated in the soleus and extensor digitorum longus (EDL) muscles of the mouse hindlimb, and compared them to those of the diaphragm, in the presence and absence of an anti-ChE. The soleus in the mouse is essentially a slow twitch muscle, whilst the EDL is classified as fast twitch (Close, 1972). The murine diaphragm can also be grouped in the fast twitch category (Davies and Gunn, 1972). Hence, the purpose of this set of experiments was three-fold; firstly, to discover whether the increased jitter after an anti-ChE observed in the diaphragm was also in other muscles, secondly, if such an increase could be related to a particular fibre type and finally, whether or not the 3 untreated muscles displayed the same amount of jitter.

Animals were injected subcutaneously with either a necrotising ($0.4 \mu\text{mol/Kg}$) or a non-necrotising dose ($0.1 \mu\text{mol/Kg}$) of ECO and, after 5 days, their limb muscles removed under anaesthesia (2.3.2 and 2.3.3). Trains of action potentials elicited by nerve stimulation at 30Hz were recorded and values of MCD and

Treatment		MCD (μ sec)	Delay (μ sec)
Untreated	Diaphragm	9.9 \pm 3.6 (42)	32 \pm 22 (42)
	Soleus	9.2 \pm 3.3 (36)	40 \pm 22 (36)
	EDL	10.7 \pm 2.9 (32)	50 \pm 27 (32)
0.4 μ mol/Kg ECO 5 days	Diaphragm	16.8 \pm 9.0 (30)* **	60 \pm 41 (30)* **
	Soleus	26.3 \pm 20.1 (30)*	110 \pm 70 (30)* **
	EDL	19.0 \pm 22.3 (34)*	78 \pm 46 (34)*
0.1 μ mol/Kg ECO 5 days	Diaphragm	10.8 \pm 5.6 (44) **	39 \pm 19 (44) \dagger **
	Soleus	15.3 \pm 10.4 (26)* **	64 \pm 32 (26)*
	EDL	10.6 \pm 6.2 (28)	56 \pm 31 (28) \dagger

Table 3.16. The MCD and delay, after ECO 0.4 or 0.1 μ mol/Kg *in vivo*, with the values given as mean \pm S.D. and the number of muscle fibres used from 2 to 5 animals in parentheses. Action potentials were recorded from the diaphragm, soleus or extensor digitorum longus (EDL), 5 days after injection of the ECO. * significant difference from the appropriate untreated muscle, ** between values indicates a significant difference between them and \dagger significant difference between the EDL and diaphragm after ECO 0.1 μ mol/Kg (Kolmogorov-Smirnov; $p \leq 0.05$). No other differences were found, although tests between the two doses of ECO were not made.

delay of the action potentials latencies calculated. These values were compared with those in untreated animals and with the values calculated from similar experiments in the diaphragm (table 3.9) using the Kolmogorov-Smirnov test.

A dose of ECO 0.4 μ mol/Kg produced a maximal amount of inhibition of diaphragm AChE 3 hours after injection (table 3.11). By 5 days, it is likely these AChE levels would have returned to untreated levels, since in previous experiments the higher 0.5 μ mol/Kg dose had reached untreated levels at the same time point (table 3.10). It is possible, by this time, that any inhibition of AChE which occurred in the soleus and EDL following ECO 0.4 μ mol/Kg would, likewise, no longer be apparent. Nonetheless, as in the diaphragm, the soleus and EDL exhibited a significant increase in MCD and delay, 5 days after injection of this dose of ECO ($p \leq 0.05$; table 3.16). If one examines the effects of ECO 0.1 μ mol/Kg, a dose which in the diaphragm produces 59% inhibition of AChE 3 hours subsequent to administration *in vivo*, no significant increase in the jitter parameters was found in the diaphragm and EDL. A significant increase in the MCD and delay was, however, exhibited in the action potentials recorded in the soleus 5 days after exposure to ECO 0.1 μ mol/Kg *in vivo* ($p \leq 0.05$; table 3.16). This suggests that the soleus, a slow twitch muscle, was more susceptible to ECO than the diaphragm and EDL, fast twitch muscles.

3.2.5. The prevention of anti-ChE induced increases in the variability of latencies of action potentials with carbamates and oximes.

The toxicity of organophosphorus compounds can be prevented by prophylaxis with a carbamate or by treatment with an oxime (see Introduction). The mechanisms by which these compounds exert their action are detailed in the Introduction (1.5). Thus, pyridostigmine and 2PAM were studied in an attempt to prevent the increases in jitter produced by anti-ChEs.

3.2.5.1. The effect of pyridostigmine on ECO-induced increases in jitter.

Mice were injected subcutaneously with pyridostigmine 0.38 μ mol/Kg followed, 30 minutes later, by a dose of ECO 0.5 μ mol/Kg. Five days later the diaphragms were dissected out (2.3.1) and from the diaphragms of some animals trains of action potentials at 30Hz were recorded, and the MCD and delay of their

latencies calculated as described earlier (2.6.4). In other animals diaphragms were assayed for AChE content (2.5).

Table 3.17 shows that AChE in diaphragms from animals pretreated with pyridostigmine and then injected with ECO in the manner described, was approximately 10% less inhibited than AChE in diaphragms 3 hours after injection with ECO alone ($p \leq 0.05$). This $0.38 \mu\text{mol/Kg}$ dose, however, prevented the increases in MCD and delay, the values measured not being significantly different from untreated animals (table 3.18). Although pyridostigmine is itself an anti-ChE, in this relatively low dose, it did not cause any significant increase in the parameters of jitter when used alone (table 3.18).

3.2.5.2. The effect of 2PAM on ECO-induced increases in jitter.

In this series of experiments mice were exposed to ECO $0.5 \mu\text{mol/Kg}$ followed by 2PAM $110 \mu\text{mol/Kg}$ at various times afterwards (on fasciculation, 1 hour, 2 hours, 6 hours and 24 hours). Five days later the animals were killed and their diaphragms removed. The MCD and delay of action potentials were measured from trains of action potentials recorded in some of these diaphragms, whilst some diaphragm samples were assayed for AChE activity.

When 2PAM $110 \mu\text{mol/Kg}$ was injected on the observation of ECO-induced fasciculation, by 3 hours, there was significantly less inhibition of diaphragm AChE (74%) than with ECO alone (87%) ($p \leq 0.05$; table 3.17). However, as with pyridostigmine pretreatment, this dose of 2PAM, given within 15 minutes of ECO, produced a marked reduction in the ECO induced increase in jitter. In fact, in the action potentials recorded after such treatment, there was no significant difference in the MCD and delay from those in untreated animals (table 3.18). As the time between injection of ECO and injection of 2PAM was increased the protection experienced was reduced. The administration of 2PAM within an hour of ECO produced a significant reduction in the MCD ($p \leq 0.05$; table 3.18), however, 2PAM given 2 or more hours after ECO had no significant effect. 2PAM given up to 24 hours after the initial dose of ECO resulted in a significant decrease in the delay from that exhibited by ECO alone. These observations further reinforce the idea that the MCD and delay are not related parameters.

Drug and Treatment	Diaphragm AChE activity 3 hours after injection (nmol/min/mg)	% Inhibition
Untreated	1.34±0.43 (38)†	0
ECO alone	0.17±0.04 (8)*	87
BOS alone	0.18±0.05 (11)*	87
ECO + 2PAM on fasciculation	0.35±0.08 (17)*†	74
BOS + 2PAM on fasciculation	0.20±0.02 (5)*	85
ECO + PYR pretreatment	0.34±0.09 (4)*†	75

Table 3.17. The effects of protective agents on levels of diaphragm AChE 3 hours after *in vivo* injection of ECO 0.5µmol/Kg or BOS 8µmol/Kg. 2PAM (110µmol/Kg) was injected as soon as fasciculations were observed and pyridostigmine (PYR, 0.38µmol/Kg) was injected 30 minutes before injection of ECO. The values represent the mean±S.D., with the number of muscles assayed in parentheses. * significant difference from atropine control AChE levels, † significant difference from the relevant anti-ChE alone (Mann-Whitney; $p \leq 0.05$).

Drug and Treatment	MCD (μsec)	Delay (μsec)
Untreated	9.9±3.6 (42)† **	32±22 (42)† **
ECO alone	25.6±28.0 (39)* **	119±104 (40)* **
ECO + 2PAM on fasciculation.	8.8±3.9 (48)†	35±27 (48)† **
ECO + 2PAM 1 hour	10.5±3.8 (33)† **	62±32 (33)*†
ECO + 2PAM 2 hours	18.7±14.0 (33)*	68±40 (33)*†
ECO + 2PAM 6 hours	19.5±20.5 (33)*	73±58 (33)*†
ECO + 2PAM 24 hours	35.7±28.6 (39)* **	74±87 (39)*† **
ECO + PYR pretreatment	10.9±5.0 (32)†	29±26 (32)†
PYR alone	9.8±4.4 (20)†	44±22 (20)†

Table 3.18. The effects of protective agents on MCD and delay, 5 days after injection of ECO 0.5μmol/Kg *in vivo*. The values represent the mean±S.D. of the data from 2 to 5 animals, with the number of muscle fibres in parentheses. * significant difference from untreated animals, † significant difference from ECO treatment alone and ** between two values indicates significant difference between them (Kolmogorov-Smirnov; p≤0.05).

3.2.5.3. The effect of 2PAM on BOS-induced increases in jitter.

The injection of 2PAM 110 μ mol/Kg on the appearance of fasciculations induced by BOS 8 μ mol/Kg, did not reactivate diaphragm AChE (table 3.17). This was probably due to the rapid ageing of the phosphorylated enzyme produced by BOS (see Introduction). However, Alkondon *et al.* (1988) suggested that oximes could produce their therapeutic effects by mechanisms other than enzyme reactivation and it was thus considered of interest to examine the effects of 2PAM on the increases in jitter after BOS. Mice were given BOS 8 μ mol/Kg *in vivo*, and on fasciculation were injected with 2PAM 110 μ mol/Kg. As detailed later, this single injection of 2PAM produced a reduction in the jitter initiated by BOS 8 μ mol/Kg, however it was not significant. The reduction did, however, suggest that multiple injections may reduce the jitter further. Hence, some animals were given multiple injections of 2PAM following one of two administration protocols. The shorter protocol involved 7 injections over a period of 12 hours; the first injection, being the same as the single dose on fasciculation, was followed by 5 injections of 55 μ mol/Kg at two hourly intervals, with a final injection of 110 μ mol/Kg 12 hours after the first. The longer protocol (12 injections) began as the shorter, but this was followed by five additional 110 μ mol/Kg doses injected 24, 30, 36, 42 and 48 hours after the first. Sets of control animals were also subjected to multiple doses of 2PAM alone. After 5 days, the animals were killed and diaphragms removed (2.3.1). The MCD and delay of the action potentials recorded were calculated. Extracellularly recorded miniature endplate potentials were also sampled from the phrenic nerve-hemidiaphragm preparations of animals exposed to the longer protocol (see section 3.3).

Drug and Treatment	MCD (μsec)	Delay (μsec)
Untreated	9.9±3.6 (42)†	32±22 (42)†
BOS alone	53.3±86.2 (59)*	162±181 (59)*
BOS + 2PAM on fasciculation.	34.9±38.3 (21)*	109±69 (21)*
BOS + 2PAM 7 injections	22.2±22.1 (23)*†	85±86 (23)*†
2PAM alone 7 injections	9.3±3.0 (19)†	32±33 (19)†
BOS + 2PAM 12 injections	41.2±24.2 (33)*	126±79 (33)*
2PAM alone 12 injections	9.0±3.1 (29)†	32±23 (29)†

Table 3.19. The effects of single or multiple doses of 2PAM on the MCD and delay, 5 days after injection of BOS 8μmol/Kg *in vivo*. Details of the experimental protocol and dosage regimens can be found in the text. The values represent the mean±S.D. of the data from 2 to 5 animals, with the number of muscle fibres in parentheses. * significant difference from untreated animals and † significant difference from BOS treatment alone (Kolmogorov-Smirnov; p≤0.05).

Treatment	Time to half amplitude (THA) of MEPPs (msec)
Untreated.	0.42±0.05 (35)†
BOS 8µmol/Kg alone.	0.88±0.18 (37)*
BOS 8µmol/Kg + 2PAM 12 injections.	1.30±0.26 (17)*†
2PAM alone 12 injections.	0.82±0.20 (15)*

Table 3.20. The effect of BOS 8µmol/Kg and the longer protocol of 2PAM dosing (see text for details) on extracellularly recorded MEPPs, 5 days after injection of the BOS *in vivo*. The values represent the mean±S.D. of the data from at least 2 animals, with the number of endplates sampled in parentheses. * significant difference from untreated animals and † significant difference from BOS treatment alone (Mann-Whitney; p≤0.05).

A single dose of 2PAM given on fasciculation did not significantly reduce the MCD and delay produced by BOS alone (table 3.19). If, however, the number of injections of the oxime was increased over the 12 hour period subsequent to administration (7 injections), there was significantly less of an increase in the MCD and delay than was observed with BOS alone ($p \leq 0.05$; table 3.19). Following this discovery it was suggested that administration of 2PAM over an even longer period may produce a greater reduction, hence the employment of a second longer protocol (12 injections) given over the first 48 hours after BOS. Neither single nor multiple injections of 2PAM appeared to have any effect on fasciculations or other signs of toxicity. The MCD and delay in the animals subjected to BOS plus this longer period of 2PAM did not differ from those exposed to the anti-ChE alone (table 3.19). This observation prompted the examination of extracellular MEPPs recorded in the diaphragms of animals exposed to BOS $8 \mu\text{mol/Kg}$, with and without the 12 injection protocol of 2PAM, 5 days after the initial injection, as well as animals to the oxime protocol alone. A dose of BOS $8 \mu\text{mol/Kg}$ created a significant prolongation of the extracellular MEPPs from that of untreated animals ($p \leq 0.05$; table 3.20). However, the addition of 12 injections of 2PAM produced an even greater prolongation ($p \leq 0.05$; table 3.20). Administration of this 12 injection protocol alone also significantly lengthened the time course of the MEPPs ($p \leq 0.05$; table 3.20) suggesting that, at this cumulatively higher dose, another effect of 2PAM was exhibited which was demonstrated instead of any potentially protective action.

3.2.6. The variability of the latencies of action potentials in the Bar Harbor Rej 129 dystrophic mouse, with and without treatment with an anti-ChE.

As stated earlier, increases in jitter are thought to be an accurate indication of myopathy or neuromuscular disease (Stalberg and Trontelj, 1979). The purpose of this work was to investigate jitter in an animal model of muscular dystrophy. This was provided by the Bar Harbor Rej 129 strain of mouse, whose characteristics have been described earlier in the Introduction. The aims were two-fold; firstly to determine whether any increases in jitter could be found in animals which showed few signs of the disease, hence whether such measurements could be utilised as a diagnostic tool, and secondly, to discover if

the dystrophic mice were more susceptible to anti-ChE induced increases in jitter than were their normal littermates.

3.2.6.1. The effects seen in mice aged 12 weeks.

The diaphragms of dystrophic mice and their equivalent normal littermates were dissected out at the age of 12 weeks (2.3.1). At this stage the hindlimbs of the dystrophic animals were atrophied. However, whether the disease had affected the diaphragm was unknown. Action potentials at 30Hz were recorded intracellularly, and the MCD and delay of their latencies calculated. Further groups of dystrophic and normal littermates were exposed to ECO 0.2 or 0.3 μ mol/Kg *in vivo* for 5 days.

In untreated animals, the MCD and delay of dystrophic mice were not different from normal littermates (table 3.21), nor was there any difference between both these groups and untreated 6-7 month old albino mice used in other experiments (table 3.7). When the animals were injected with ECO the dystrophic animals were more susceptible to the toxic effects of anti-ChEs. No dystrophic mice injected with ECO 0.3 μ mol/Kg survived for the required 5 days, whilst of the 4 animals injected with the 0.2 μ mol/Kg dose only 1 survived. The normal littermates, injected with 0.3 μ mol/Kg, showed a significant increase in the MCD and delay compared with the untreated normal littermate 5 days after injection ($p \leq 0.05$; table 3.21). The increase in delay seen in these normal littermates after the 0.3 μ mol/Kg dose of ECO was significantly greater than that seen in albino white mice after the same dose ($p \leq 0.05$), suggesting that the Rej 129 strain of mouse may be more susceptible to the increases in jitter than the albino white mouse strain. At the same timepoint, ECO 0.2 μ mol/Kg created significant increases in both the MCD and delay in dystrophic mice and in their normal littermates ($p \leq 0.05$; table 3.21), when tested against the appropriate untreated animals. However, no significant difference between dystrophic and normal animals could be found.

Animal and Treatment	MCD (μsec)	Delay (μsec)
Normal Untreated	10.4±3.8 (19)	43±17 (19)
Dystrophic Untreated	9.4±5.6 (19)	39±17 (19)
Normal 0.3μmol/Kg ECO	22.4±16.7 (15)*	89±26 (15)*†
Dystrophic 0.3μmol/Kg ECO	No survivors	
Normal 0.2μmol/Kg ECO	18.3±12.8 (24)*	104±49 (24)*
Dystrophic 0.2μmol/Kg (only 1 survivor)	13.4±4.2 (9)*	114±26 (9)*

Table 3.21. The MCD and delay recorded in the diaphragms of 12 week old dystrophic and normal littermate mice (Bar Harbor Rej 129), and the effects of various doses of ECO *in vivo* 5 days after injection. The values represent the mean±S.D. of the data from at least 2 animals (except with dystrophics injected with ECO 0.2μmol/Kg, as stated), with the number of muscle fibres in parentheses. * significant difference from the relevant untreated and † significant difference between normal littermate diaphragm and albino mouse diaphragm after ECO 0.3μmol/Kg (Kolmogorov-Smirnov; $p \leq 0.05$). No differences between the dystrophic animals and the normal littermates were found.

3.2.6.2. The effects seen in mice aged 8 weeks.

In order to overcome the fatal increases in susceptibility to the toxic effects of ECO which was observed in 12 week old dystrophic mice, 8 week old mice were used as an alternative. These mice displayed far fewer signs of the disease and the dose of ECO to which the animals were exposed was reduced to 0.1 μ mol/Kg. Thus these younger animals were exposed to ECO 0.1 μ mol/Kg *in vivo* for a period of 5 days.

In untreated animals, no difference in MCD or delays of dystrophic and normal littermates was observed (table 3.22). Similarly, no differences between these animals, untreated 12 week old animals or untreated 6-7 month old albino white mice were shown (table 3.7, 3.21 and 3.22). The dystrophic mice exposed to ECO 0.1 μ mol/Kg, displayed a significant increase in the MCD compared with the untreated animals, however the delay was unaffected ($p \leq 0.05$; table 3.22). The increases in the MCD and delay seen in the ECO-treated normal littermates were not significantly different from the untreated normal littermates, and they were also not different from the values measured in dystrophic animals of the same age. The delay seen after ECO 0.1 μ mol/Kg in both normal and dystrophic animals was significantly greater than that observed in albino white mice after the same dose ($p \leq 0.05$), reinforcing the idea that the Rej 129 strain may be in some way more susceptible to jitter increases.

Animal and Treatment	MCD (μsec)	Delay (μsec)
Normal Untreated	10.6±6.6 (32)	46±27 (32)
Dystrophic Untreated	9.9±3.9 (35)	44±28 (35)
Normal 0.1μmol/Kg ECO	18.0±15.1 (27)	62±32 (27)†
Dystrophic 0.1μmol/Kg ECO	18.5±18.8 (31)*	84±52 (31)†

Table 3.22. The MCD and delay recorded in the diaphragms of 8 week old dystrophic and normal littermate mice (Bar Harbor Rej 129), and the effects of ECO 0.1μmol/Kg *in vivo* 5 days after injection. The values represent the mean±S.D. of the data from at least 2 animals, with the number of muscle fibres in parentheses. * significant difference from the relevant untreated animal and † significant difference between the Rej 129 mice and albino white mice after the same treatment (Kolmogorov-Smirnov; p≤0.05). No differences between the dystrophic animals and the normal littermates were found.

3.2.6.3. Hindlimb muscles in mice aged 8 weeks.

Having failed to show any significant differences between the jitter displayed in the diaphragms of normal and dystrophic animals, investigations moved to the extensor digitorum longus (EDL) and soleus muscles of the hindlimb. These hindlimb muscles are among the first affected in this model of dystrophy, hence if increases in jitter are brought about by such a disease state then they are more likely to be observed in these muscles.

Dystrophic mice and their equivalent normal littermates, of 8 weeks of age, were anaesthetised and their EDL and soleus muscles dissected out (2.3.2 and 2.3.3). Action potentials at 30Hz were recorded intracellularly, and the MCD and delay calculated. Further groups of dystrophic and normal littermates were given ECO 0.1 μ mol/Kg *in vivo* and 5 days later the EDL and soleus muscles dissected out with the MCD and delay being calculated from the trains of action potentials elicited at 30Hz.

In untreated animals, no significant difference between the dystrophic and normal animals' hindlimb muscle jitter was observed. In normal littermates, the soleus displayed a significantly greater MCD than in the EDL, with the delay being the same ($p \leq 0.05$; table 3.23). There was, however, a significant increase in both parameters in the soleus of the dystrophic mice, when compared to those of the EDL of the same animals ($p \leq 0.05$; table 3.23).

Treatment with 0.1 μ mol/Kg ECO for 5 days *in vivo* did not produce an increase in the MCD or delay in the EDL or soleus of normal littermates, nor were increases observed in the dystrophic animals. There was no significant difference between normal and dystrophic muscles following ECO, apart from there being a significantly greater MCD in the normal EDL than in that of the dystrophic mouse ($p \leq 0.05$; table 3.23) In dystrophic mice, a significant difference in the MCD and delay of the EDL and soleus muscles was again observed ($p \leq 0.05$; table 3.23). Although there was an increase in the soleus' MCD and delay in the normal littermate when compared with the EDL, this was not significant.

(a)	MCD (μ sec)		
	EDL		Soleus
Untreated Normal	8.4 \pm 3.3 (19)	**	14.9 \pm 7.2 (14)*
Untreated Dystrophic	6.8 \pm 2.4 (29)*	**	18.7 \pm 14.7 (21)*
Normal ECO 0.1 μ mol/Kg	12.7 \pm 7.0 (23)†		21.0 \pm 14.5 (15)
Dystrophic ECO 0.1 μ mol/Kg	6.9 \pm 4.3 (16)*†	**	20.3 \pm 14.4 (17)

(b)	Delay (μ sec)		
	EDL		Soleus
Untreated Normal	50 \pm 10 (19)		60 \pm 25 (14)*
Untreated Dystrophic	54 \pm 10 (29)	**	80 \pm 50 (21)*
Normal ECO 0.1 μ mol/Kg	58 \pm 23 (23)		80 \pm 39 (15)
Dystrophic ECO 0.1 μ mol/Kg	51 \pm 14 (16)	**	90 \pm 42 (17)*

Table 3.23. The (a) MCD and (b) delay recorded in the hindlimbs of 8 week old dystrophic and normal littermate mice (Bar Harbor Rej 129), and the effects of ECO 0.1 μ mol/Kg *in vivo* 5 days after injection. The values represent the mean \pm S.D. of the data from at least 2 animals, with the number of fibres in parentheses. Treatment with ECO had no significant effects in either normal littermates or dystrophic animals. * significant difference from the same muscle in the albino mouse under the same conditions, ** significant difference between two values a significant difference between the EDL and soleus in mice treated in the same manner and † a significant difference between the normal and dystrophic animals in the same muscle at the same dose (Kolmogorov-Smirnov; $p \leq 0.05$).

When the EDL and soleus muscles of the Rej 129 strain were compared with the same muscles in the albino white mouse, a number of differences came to light. In untreated animals, both parameters of jitter in the soleus muscles of dystrophic and normal animals were significantly greater than the values observed in the same muscle of albino white mice ($p \leq 0.05$; table 3.16, 3.23). Little difference was seen in the untreated EDL muscles, except that there appeared to be a significantly lower MCD in the dystrophic EDL compared with the albino white mouse EDL ($p \leq 0.05$; table 3.16, 3.23). After ECO $0.1 \mu\text{mol/Kg}$ a number of differences were seen, in particular the delay measured in the soleus of the dystrophic mice was significantly greater than that seen in the albino mouse ($p \leq 0.05$). Also in the dystrophic EDL the MCD was again significantly lower than that observed in the EDL of the albino white mice ($p \leq 0.05$).

In summary, the dystrophic hindlimb muscles did not produce a greater jitter than their normal counterparts, even following treatment with a low dose of ECO. There was, however, a larger jitter in the soleus muscle as compared with the EDL, particularly in the dystrophic hindlimb.

3.3. THE RELATIONSHIP BETWEEN BIOCHEMICALLY MEASURED LEVELS OF AChE AND THE TIME COURSE OF EXTRACELLULARLY RECORDED MINIATURE ENDPLATE POTENTIALS.

During the studies on jitter it was observed that the increases produced by anti-ChEs tended to last much longer than AChE inhibition. This gave rise to the thought that perhaps the biochemical technique of Ellman *et al.* (1961) (2.5) did not produce an accurate measure of AChE active at the neuromuscular junction, especially following anti-ChE treatment.

Biochemical estimates of AChE activity generally measure the rate of hydrolysis of a substrate of AChE. There are those techniques which determine the enzyme activity of tissue homogenates, such as that of Ellman *et al.* (1961) or Siakotos *et al.* (1969), which will not only measure functional AChE (i.e. that responsible for termination of the effects of synaptically released ACh) but also many other constituents which possess the ability to hydrolyse the substrate. There are a number of techniques which claim to determine functional AChE by incubating an intact tissue in a medium containing a quaternary substrate for AChE. The rate of uptake of the substrate by the tissue or the appearance of a metabolite are then assayed and taken as a measure of functional AChE (Fleisher *et al.*, 1960; Mittag *et al.*, 1971; Lancaster, 1972). However, the limitation of such methods is the restricted access of quaternary substances to the synaptic cleft. This problem was highlighted by Lancaster (1973) who showed that, in the isolated rat diaphragm, a quaternary anti-ChE showed less inhibition than the same concentration of its tertiary analogue. A more accurate estimation of functional AChE was thought to be possible using an electrophysiological technique.

The reduced rate of ACh hydrolysis after AChE inhibition results in a prolongation of the time course of both spontaneous miniature endplate potentials (MEPPs) and evoked endplate potentials (EPPs) (see Introduction). However, it was recognised that changes in such potentials could not be quantified in terms of inhibition of functional AChE. Nevertheless, if the technique of Ellman *et al.* (1961) was an accurate assay for functional AChE, after taking the precaution of elimination any interfering reactions with

ethopropazine, then it would be expected that under all conditions there should be a correlation between the biochemical technique and the time course of such potentials. Thus to ascertain whether or not the technique of Ellman *et al.* (1961) accurately measured functional AChE, it was decided to compare the changes in the time course of extracellularly recorded MEPPs with the AChE activity indicated by this biochemical assay under a wide range of conditions and anti-ChEs. A similar study was attempted by Ferry and Marshall (1971) in which the time course of EPPs was used, however, in this case the picture was complicated by the possible alterations in quantal content.

3.3.1. The relationship between biochemically measured levels of AChE and the time course of extracellularly recorded MEPPs 3 hours after injection of an anti-ChE *in vivo*.

Groups of mice were exposed to various doses of Sarin, ECO or BOS for 3 hours *in vivo*, a time at which any AChE inhibition was considered to be at steady state. The animals were then killed and their diaphragms were dissected out (2.3.1). Some hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded from a number of endplates with a patch-type electrode located extracellularly at the endplate (2.6.3). The electrode was manipulated such that the extracellular potentials were as large as possible. The other hemidiaphragms were used for the assay of AChE by the technique of Ellman *et al.* (1961) (2.5).

3.3.1.1. The effect of Sarin on extracellular MEPP parameters.

Sample traces of extracellular MEPPs (figure 3.10) recorded from 5 endplates of animals treated with a range of Sarin doses for 3 hours *in vivo*, as well as 5 from control animals, were selected. Each trace being the average plot of sixteen individual MEPPs. All traces were made on standard 80g A4 paper. Five parameters were then measured:-

(a) Time of the decay of the extracellular MEPP from peak to half amplitude (THA).

(b) The area under the curve as an indicator of the total charge transferred. The

records were accurately cut out and individually weighed, to give an indication of the area under the trace, at the same time the amplitude was also measured. The area under the curve is a product of both intensity and duration of action of ACh both of which are increased by anti-ChEs. Hence it was not surprising when it became apparent that the area was proportional to the amplitude of the record (figure 3.12b), thus relationships between area alone and diaphragm AChE tended to be distorted by amplitude variability. This effect of amplitude was also due in part to the variability in placement of the electrode with respect to the endplate. However, if relative area, a parameter gained by dividing the weight by the amplitude, was plotted against AChE inhibition the relationships were much clearer.

(c) Time taken from 80% to 40% of peak amplitude ($T_{80-40\%}$). Another measure of changes in the decay phase of MEPPs.

(d) Width of the trace at 50% of the peak amplitude ($W_{50\%}$). An indicator of changes in both the rising and decay phases of the MEPP.

(e) Rise time (T_{rise}). An indicator of changes in the rising phase of the MEPP.

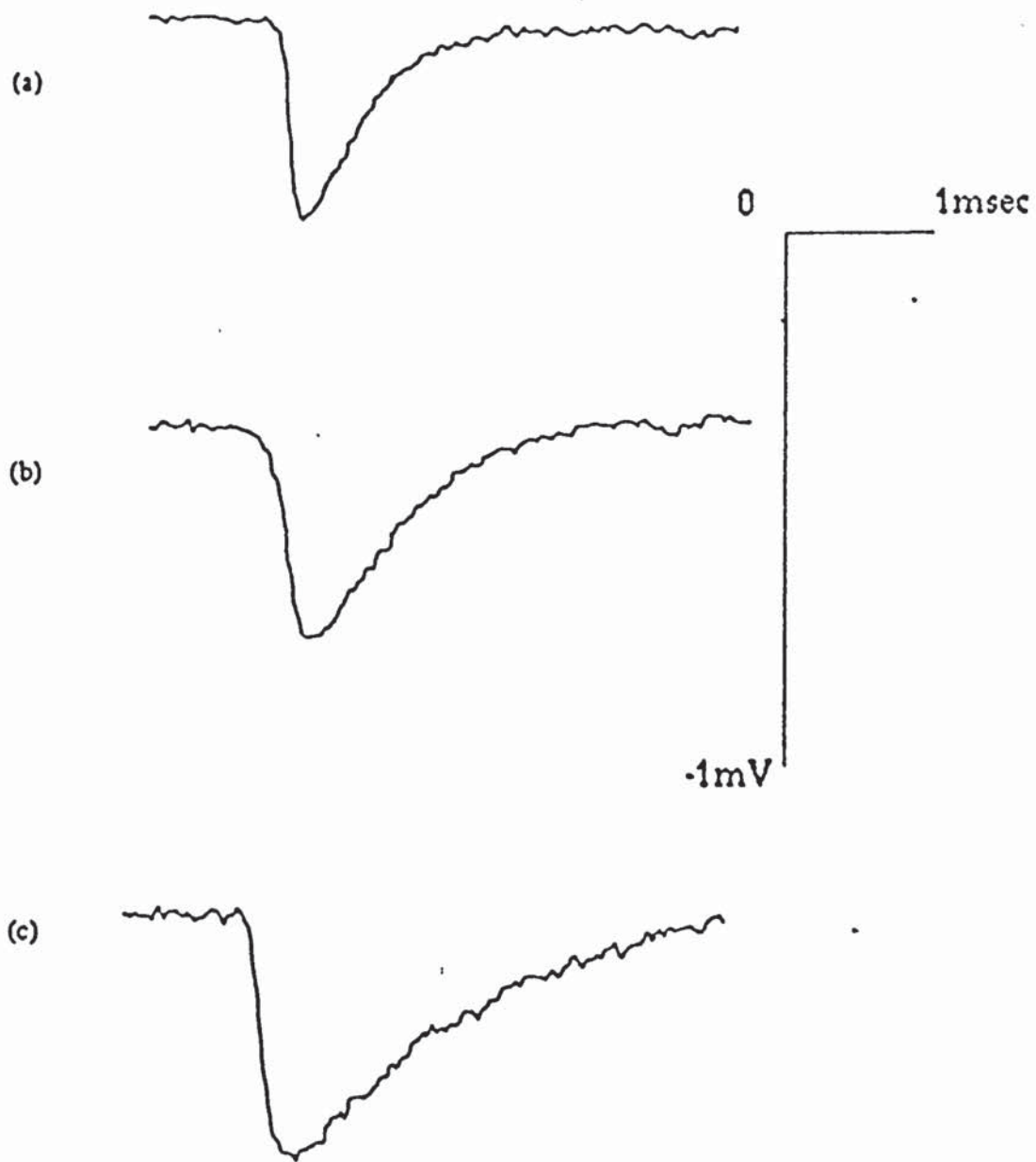
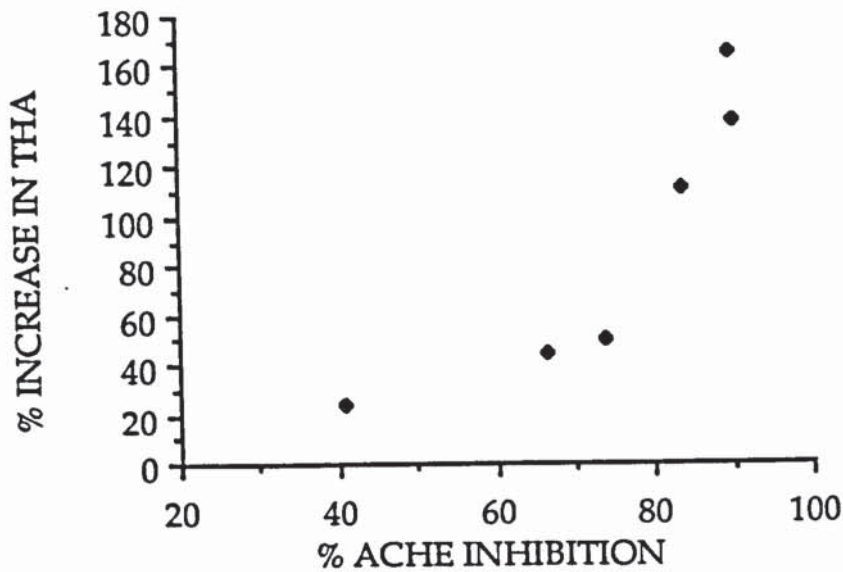


Figure 3.10. Sample traces of negative-going extracellularly recorded MEPPs in mouse hemidiaphragm' *in vitro* at 37°C. (a) Control, (b) 0.77 μ mol/Kg Sarin and (c) Sarin 1.4 μ mol/Kg 3 hours after *in vivo* injection. The horizontal and vertical bars represent 1msec and 1mV respectively.

Dose of Sarin $\mu\text{mol/Kg}$	THA (msec)	Area/Amp (msec)	$T_{80-40\%}$ (msec)	$W_{50\%}$ (msec)	T_{rise} (msec)
Control	0.43±0.04	0.05±0.003	0.32±0.04	0.56±0.05	0.32±0.12
0.700	0.53±0.07*	0.07±0.008*	0.40±0.03*	0.77±0.07*	0.42±0.04*
0.770	0.62±0.11*	0.08±0.011*	0.52±0.08*	0.83±0.13*	0.38±0.11*
0.875	0.64±0.09*	0.08±0.011*	0.53±0.09*	0.86±0.10*	0.42±0.11*
1.050	0.91±0.14*	0.11±0.011*	0.78±0.17*	1.17±0.19*	0.51±0.09*
1.225	1.14±0.16*	0.12±0.016*	0.96±0.14*	1.34±0.18*	0.48±0.13*
1.400	1.02±0.10*	0.12±0.008*	0.87±0.06*	1.28±0.11*	0.58±0.11*

Table 3.24. The effect of various doses of Sarin on 5 measured parameters of the extracellular MEPPs recorded from 5 selected endplates at each dose. The parameters being time to half amplitude (THA), area of MEPP trace divided by its amplitude (W_t/Amp), time from 80% to 40% of peak amplitude on the decay phase ($T_{80-40\%}$), the width of the MEPP at 50% of peak amplitude ($W_{50\%}$) and the rise time (T_{rise}), with the values being given as the mean±S.D. * significant difference from the relevant control (Mann-Whitney; $p \leq 0.05$).

(a)



(b)

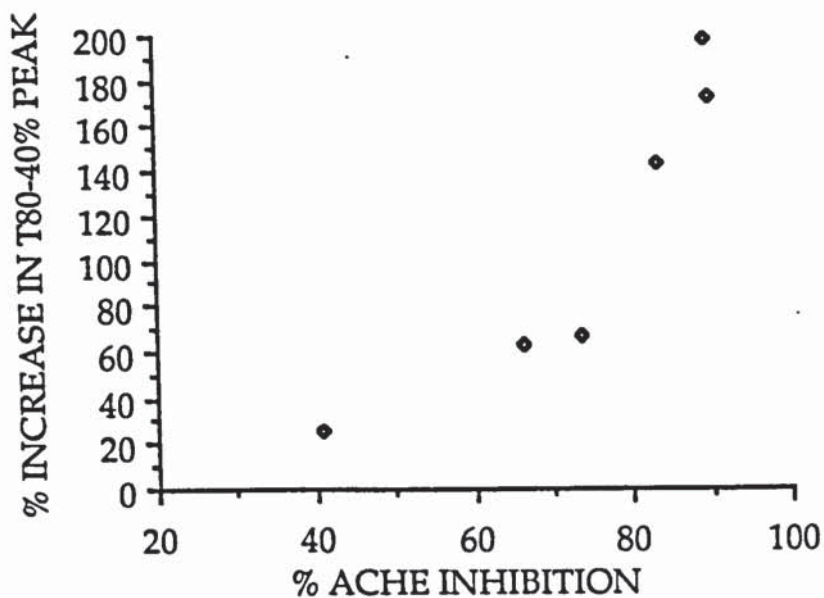
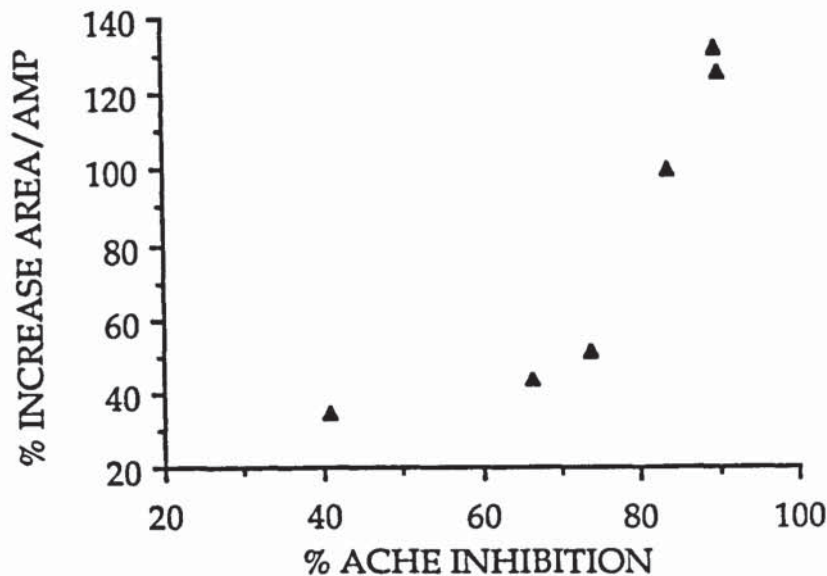


Figure 3.11. (a) The relationship between % increase in time to half amplitude (THA) sampled from 5 endplates at each dose and % inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

(b) The relationship between % increase in time from 80% to 40% peak on the decay phase ($T_{80-40\%}$) sampled from 5 endplates at each dose and % inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

(a)



(b)

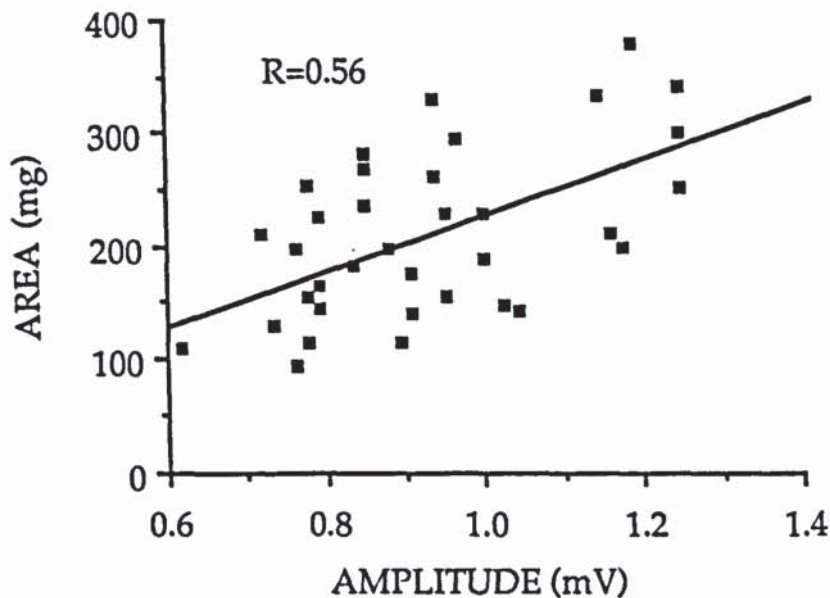
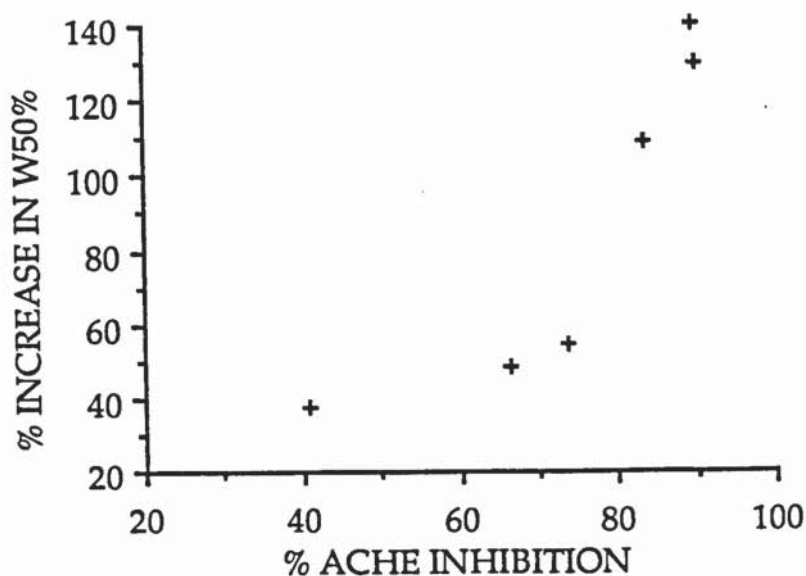


Figure 3.12. (a) The relationship between % increase in area of MEPP trace divided by its amplitude (Area/Amp) sampled from 5 endplates at each dose and % inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

(b) The correlation between weight of MEPP traces and their amplitudes sampled at 35 endplates of animals treated with various doses of Sarin for 3 hours *in vivo*. The correlation coefficient of 0.56 is significant at a 5% level for this number of observations.

(a)



(b)

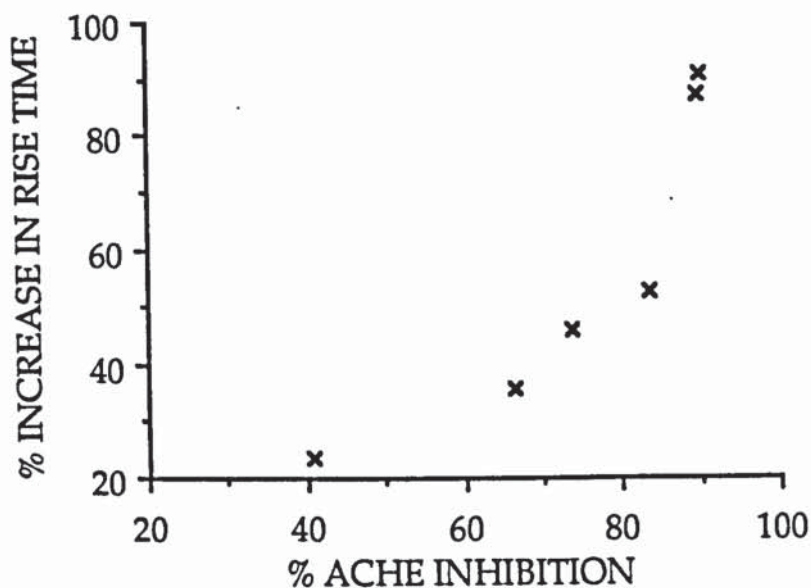


Figure 3.13. (a) The relationship between % increase in width at 50% peak amplitude ($W_{50\%}$) sampled from 5 endplates at each dose and % inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

(b) The relationship between % increase in rise time (T_{rise}) sampled from 5 endplates at each dose and % inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

Table 3.24 shows the effect of Sarin on the parameters measured. Figure 3.11a represents the relationship between the percentage increase in THA and the percentage inhibition of diaphragm AChE, for the 5 sampled endplates at each dose. The other four parameters demonstrated very similar relationships with percentage AChE inhibition (figures 3.11b, 3.12a and 3.13a,b), that is a sudden, sharp increase in effect following doses which caused greater than 70% inhibition. One apparent difference was that the percentage increase in the rising phase, indicated by T_{rise} , appeared to be smaller than with the other parameters, although a similar shaped relationship was observed (figure 3.13b).

It can be seen that all parameters showed very similar relationships with diaphragm AChE inhibition. Thus for a number of reasons from this point onwards the THA was used as the indicator of any prolongation of the time course of extracellularly recorded MEPPs. Firstly, it was easily measured with the Gould waveform processor used to average the extracellular MEPPs. Secondly, for theoretical reasons, since the time constant, τ , of the exponential decay is believed to be equal to the reciprocal of the rate constant for channel closure, α (Magleby and Stevens, 1972a) and one would expect, as stated earlier, that such decay would be affected by anti-ChEs. Finally, any measure which requires the accurate measurement of amplitude using this technique is unreliable, since amplitude is affected by the position of the extracellular electrode. It was shown in preliminary experiments, in which the distance of the electrode from an active site was gradually increased, that the THA was unaffected by changes in amplitude and it was hoped that it would be an accurate indicator of changes in the time course of these extracellular potentials.

3.3.1.2. The effect of Sarin.

The relationship between diaphragm AChE activity assayed by the Ellman *et al.* (1961) technique and the THA is represented in figure 3.14. Also, the effect of doses of Sarin (0.7 to 1.4 $\mu\text{mol/Kg}$) on THA and percentage inhibition of diaphragm AChE can be seen in table 3.25 and figure 3.15, indicating a dose-dependent effect on both parameters. There was a non-linear relationship between the percentage increase in THA and percentage AChE inhibition

(Figure 3.16a). A slow, steady increase in THA occurred up to approximately 66% inhibition of diaphragm AChE. Above this level there was a large increase in the effect on THA, particularly with doses causing greater than 70% AChE inhibition. This plot could be linearised if the reciprocal of the percentage increase in THA was plotted against percentage AChE inhibition. This produced a line with a correlation coefficient of 0.99 (Figure 3.16b) and was particularly useful as it gave a simple representation of the relationship between the two parameters at 3 hours, as well as making deviations from it easily apparent.

Dose of Sarin μmol/Kg	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Untreated	0.43±0.04 (32) **	1.46±0.32 (17) **	0
0.700	0.53±0.06 (24)*	0.86±0.17 (7)* **	41
0.770	0.57±0.06 (18)*	0.49±0.08 (6)*	66
0.875	0.65±0.08 (18)*	0.38±0.05 (7)*	74
0.980	0.74±0.11 (21)*	0.27±0.09 (8)*	82
1.050	0.87±0.15 (18)* **	0.24±0.08 (6)*	84
1.225	1.04±0.14 (20)*	0.15±0.09 (8)*	90
1.400	1.09±0.17 (18)*	0.14±0.03 (8)*	90

Table 3.25. The effect of various doses of Sarin on THA and diaphragm AChE activity 3 hours after *in vivo* injection. The values of the THA represent the mean±S.D. sampled from the number of endplates found in parentheses. The values of AChE activity are the mean±S.D., with the number of animals sampled also in parentheses. * significant difference from the relevant control and ** between two values indicates significant difference between them (Mann-Whitney; p≤0.05).

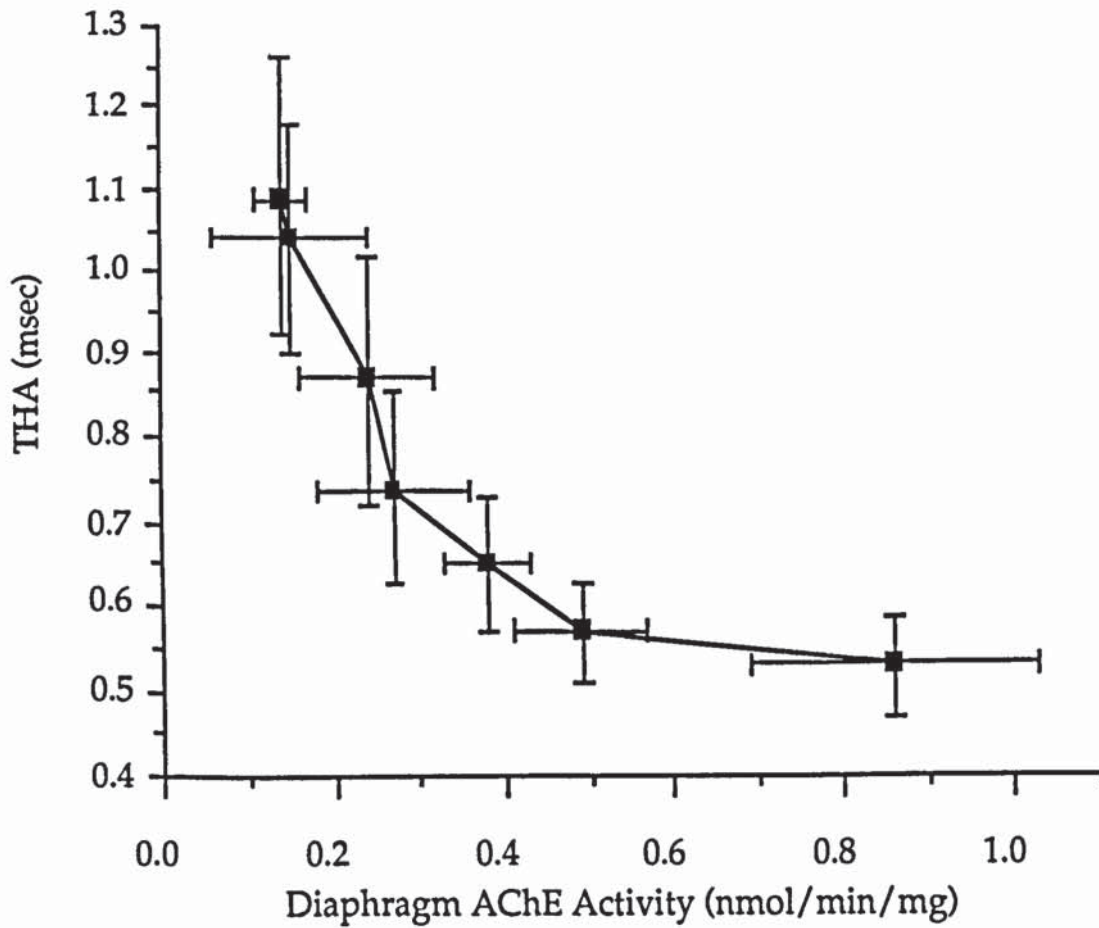
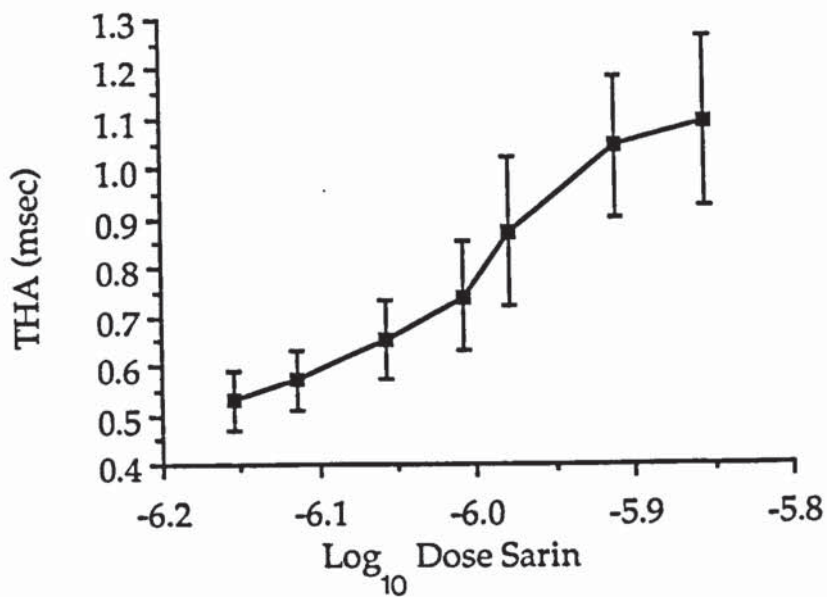


Figure 3.14. The relationship between THA and diaphragm AChE activity measured by the technique of Ellman *et al.* (1961) 3 hours after injection of Sarin *in vivo*, with the values representing the mean \pm S.D.

(a)



(b)

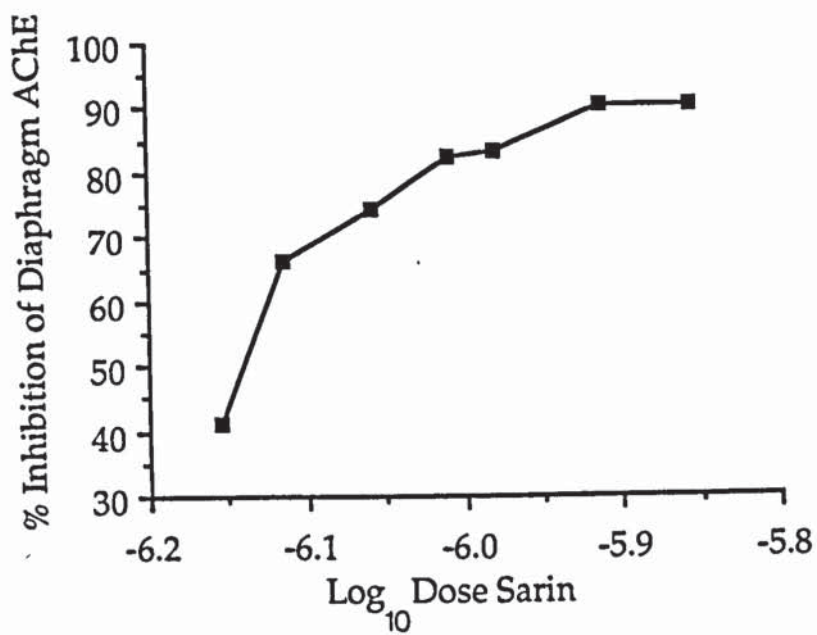
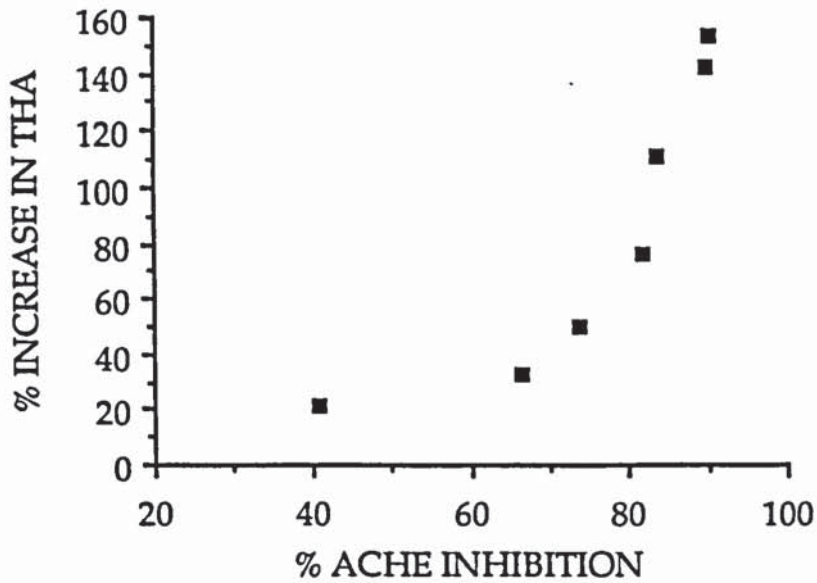


Figure 3.15. The effect of various doses of Sarin on :-
(a) THA (msec), the values represent the mean \pm S.D.
(b) Percentage inhibition of Diaphragm AChE
3 hours after *in vivo* injection. Doses in mol/Kg.

(a)



(b)

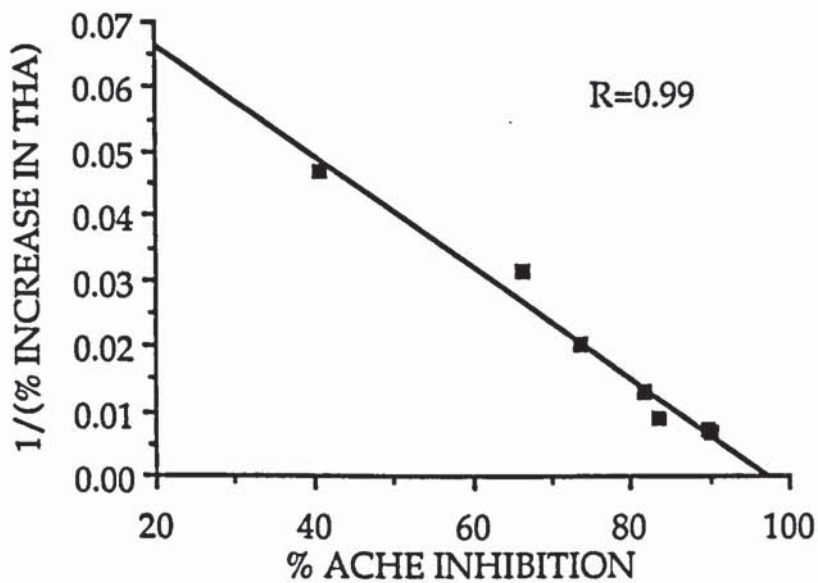


Figure 3.16. (a) The relationship between percentage increase in time to half amplitude (THA) and percentage inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

(b) The relationship between the reciprocal of the percentage increase in THA and percentage inhibition of diaphragm AChE 3 hours after various doses of Sarin *in vivo*.

3.3.1.3. The correction for activity of non-synaptic ChE.

As described in section 2.5.2.3, within the junctional strip (2.5) there is a proportion of the activity which can be assigned to non-synaptic ChE. It was thought that an attempt should be made to compensate for this non-synaptic activity and assess whether such a correction altered any of the relationships between THA and percentage inhibition of diaphragm AChE which had already been determined. It should be noted, however, having used ethopropazine within the assay, which is thought to inhibit much of such non-synaptic activity, one might not expect this correction to have much effect on the relationships observed.

Section 3.3.1.2. describes the determination of the relationship between the THA and percentage inhibition of diaphragm AChE 3 hours after injection of a range of doses of Sarin. This involved the determination of the AChE content of junctional strips of hemidiaphragms (2.5). During the dissection of these junctional strips sections of the non-junctional region were also dissected out and their activities assayed (2.5.2.3). The AChE content of these non-junctional regions gave a direct indication of the non-synaptic ChE element within the junctional regions. Thus the AChE activity of the junctional regions were recalculated by subtracting any activity deemed to be non-synaptic (2.5.2.7).

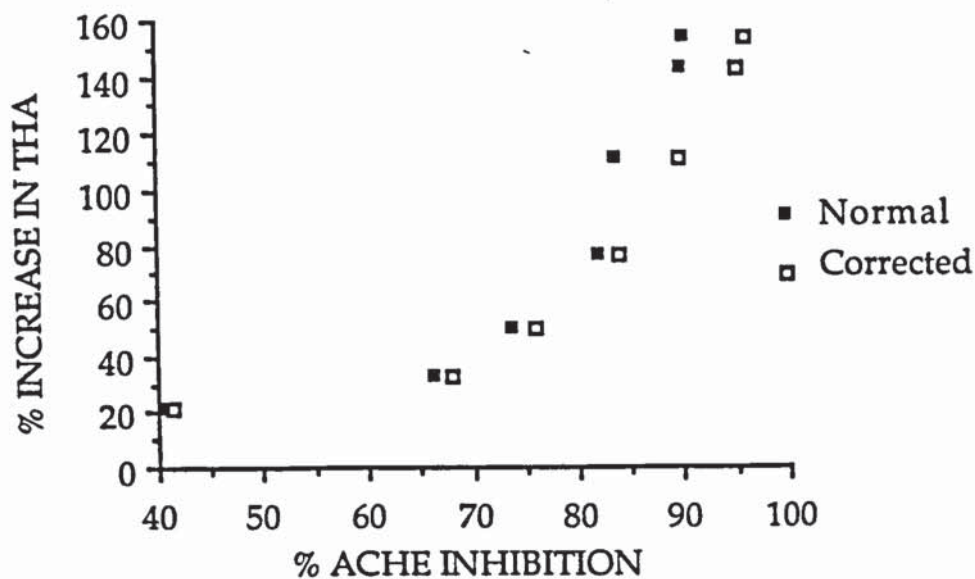
The corrected and uncorrected AChE activities of the diaphragms of animals exposed to doses of Sarin for 3 hours *in vivo* are shown in table 3.26. The corrected values were significantly less than the uncorrected AChE activities after 0.875 and 1.4 μ mol/Kg doses of Sarin ($p \leq 0.05$; table 3.26), other activities were also reduced but not significantly so. When the percentage inhibitions were calculated very few differences between the original and corrected values were observed, the differences appearing to be greater the larger the dose of Sarin used. This difference reached a maximum of 6% with the 1.4 μ mol/Kg dose. Since the percentage inhibitions were relatively unchanged the correction had little effect on the relationship between the percentage increase in THA and the percentage inhibition of diaphragm AChE. As before, there was a steady rise in THA up to approximately 68% inhibition, subsequent to a sudden, rapid increase in effect with the higher doses (Figure 3.17a). Likewise, the reciprocal

of the percentage increase in THA when plotted against percentage inhibition of AChE produced a similar straight line (Figure 3.17b). The slopes of the two straight lines were not significantly different ($p \leq 0.05$).

Dose of Sarin $\mu\text{mol/Kg}$	Diaphragm AChE Activity nmol/min/mg	% Inhibition	Corrected Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	1.46 ± 0.32 (17)	0	1.24 ± 0.34 (17)	0
0.700	0.86 ± 0.17 (7)*	41	0.73 ± 0.15 (7)*	41
0.770	0.49 ± 0.08 (6)*	66	0.40 ± 0.09 (6)*	68
0.875	0.38 ± 0.05 (7)*	74	0.30 ± 0.03 (7)*†	76
0.980	0.27 ± 0.09 (8)*	82	0.20 ± 0.08 (8)*	84
1.050	0.24 ± 0.08 (6)*	84	0.13 ± 0.08 (6)*	90
1.225	0.15 ± 0.09 (8)*	90	0.05 ± 0.10 (8)*	96
1.400	0.14 ± 0.03 (8)*	90	0.05 ± 0.03 (8)*†	96

Table 3.26. The effect of various doses of Sarin on diaphragm AChE activity 3 hours after *in vivo* injection, with the activity being calculated with and without the correction for the non-junctional region (2.5.2.3). The values represent the mean \pm S.D., with the number of animals sampled in parentheses. * significant difference from the relevant control and † significant difference between the original and corrected values at the same dose (Mann-Whitney; $p \leq 0.05$).

(a)



(b)

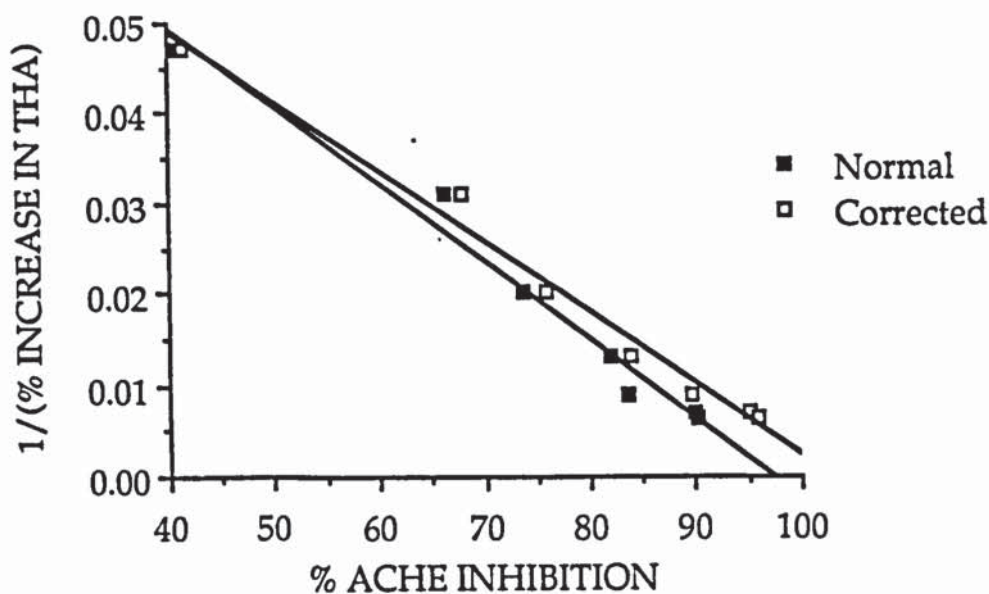


Figure 3.17. (a) The relationship between percentage increase in time to half amplitude (THA) and percentage inhibition of diaphragm AChE, calculated with and without the correction for the non-junctional region (2.5.2.3) and (b) The relationship between the reciprocal of the percentage increase in THA and percentage inhibition of diaphragm AChE, calculated with and without the correction for the non-junctional region (2.5.2.3), after various doses of Sarin.

The use of this correction had merits in that it was a more accurate measure of synaptic AChE. Nevertheless, it has been shown not to affect any of the relationships already highlighted, not only for sarin but also for the quaternary anti-ChE ECO (Das, personal communication). This fact, along with the very time consuming nature of the non-junctional determinations, suggested that its routine use was unpracticable.

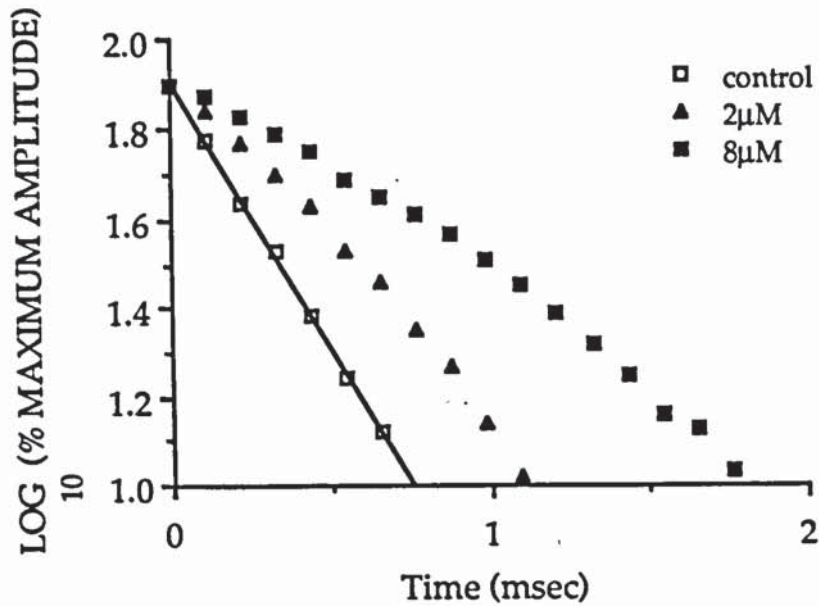
3.3.1.4. The effect of anti-ChEs on the shape of the decay phases of extracellular MEPPs.

As described in the Introduction, the decay phase of both MEPCs and EPCs can be described as a single exponential (Magleby and Stevens, 1972a). Measurements of the decays of control MEPPs were made to discover if they were consistent with such findings. Similar measurements were made on records taken from treated with an anti-ChE, to observe whether such drugs had any effect.

Groups of 5 sample traces from endplates of control animals and animals treated with 2 or BOS 8 μ mol/Kg for 3 hours *in vivo* were selected. The decay phase was analysed in terms of the amplitude at 0.11msec intervals of the time scale, commencing at the point which was 80% of peak amplitude. Measurements were made from the 80% level, rather than peak amplitude, to avoid any distortion of the decay by the rounding of the MEPP which occurs at its peak. These values, measured in millimetres, were expressed as a percentage of the peak amplitude. The mean percentages at each of the 0.11msec intervals were calculated. The logarithms of these percentages were then plotted against the time intervals (NB These intervals represented the time over which the decay phase occurred), as shown in figure 3.18.

Figure 3.18a represents the decay phases of control MEPPs and those of animals exposed to the two doses of BOS for 3 hours *in vivo*. The control MEPPs followed a single exponential which is said to be an indication of the rate of closing of channels associated with the ACh receptor. BOS produced a dose-dependent prolongation of the decay which could not, in either case, be fitted by a single exponential. It was possible to subtract the control decay, which

(a)



(b)

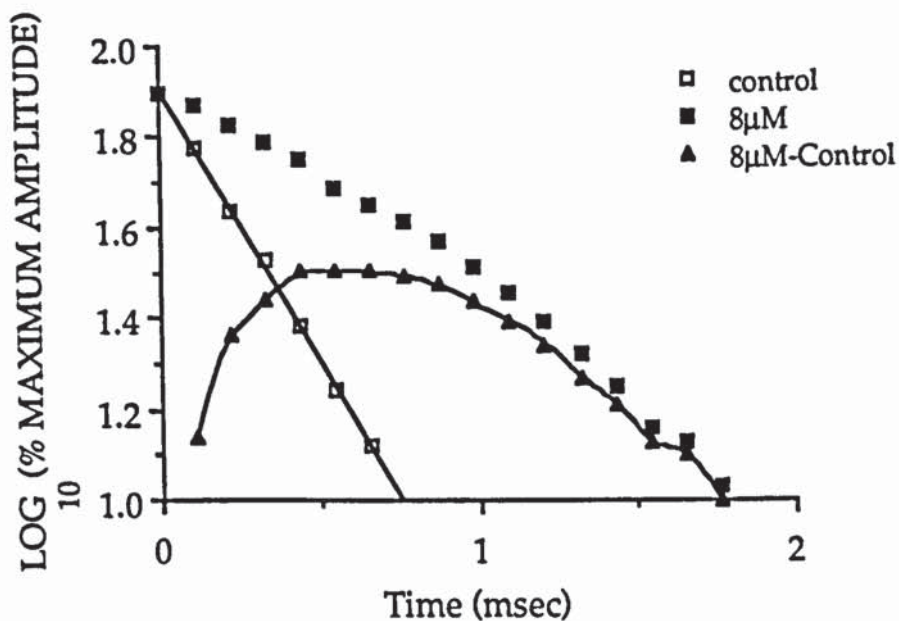


Figure 3.18. (a) Logarithmic plot of the time course of the decay phase of MEPPs of control animals and animals exposed to 2 or BOS 8µmol/Kg for 3 hours *in vivo*. (b) Similar logarithmic plot of the decay phase of MEPPs of control animals and animals exposed to BOS 8µmol/Kg for 3 hours *in vivo*. A further plot, in which the control line was stripped from that treated with BOS, is also represented.

represented closings alone, from the decay produced by *in vivo* exposure to BOS $8\mu\text{mol/Kg}$ for 3 hours. The resultant plot is shown in figure 3.18b. This showed an initial rising phase, presumably an indication of further channel openings as ACh diffused in the synapse in the absence of AChE activity which normally would have rapidly terminated transmitter action. The rising phase was followed by a phase of decay, which again did not appear exponential. Thus it is likely that within this decay there is not only an element produced by channel closings, but also further channel openings. It would be possible to again subtract the control exponential line from this decay to produce a similar plot to the first, but of reduced amplitude. This suggests that the non-exponentiality of the decay phase of MEPPs of treated animals may have been due to re-openings of ACh receptor associated channels as the transmitter diffused out of the synapse, the number of re-openings declining as the time course of the potential progressed, as suggested in the hypothesis of Katz and Miledi (1973).

3.3.1.5. The effect of ECO.

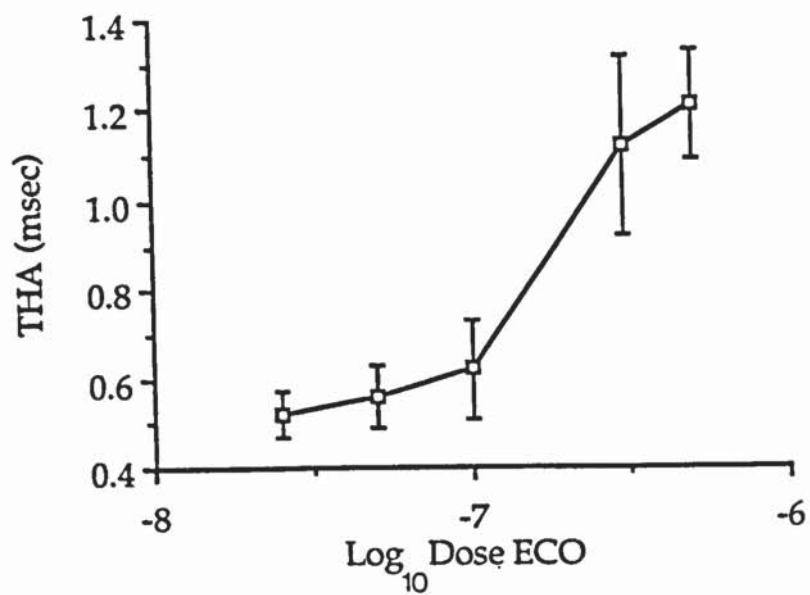
A range of doses, from 0.025 to $0.5\mu\text{mol/Kg}$, of ECO were administered to animals for 3 hours *in vivo*, and the dissected diaphragms treated as indicated in the Introduction to section 3.3.1 (see above).

The effect of ECO (0.025 to $0.5\mu\text{mol/Kg}$) on THA and percentage inhibition of diaphragm AChE can be observed in table 3.27 and figure 3.19, indicating a dose dependent effect on both parameters. There was a non-linear relationship between THA and percentage AChE inhibition (Figure 3.20a). A slow, steady increase in THA occurred up to approximately 60% inhibition of diaphragm AChE. Above this level of inhibition there was a large increase in the effect on THA. If the reciprocal of the percentage increase in THA was plotted against percentage AChE inhibition a linear plot was produced with a correlation coefficient of 0.98 (Figure 3.20b).

Dose of ECO μmol/Kg	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.42±0.05 (35) **	1.34±0.43 (38) **	0
0.025	0.52±0.05 (9)*	0.88±0.09 (5)*	34
0.05	0.56±0.07 (19)*	0.81±0.07 (8)* **	40
0.1	0.62±0.11 (23)* **	0.55±0.10 (8)* **	59
0.3	1.12±0.20 (10)*	0.16±0.05 (8)*	88
0.5	1.21±0.12 (11)*	0.17±0.04 (8)*	87

Table 3.27. The effect of various doses of ECO on THA and diaphragm AChE activity 3 hours after *in vivo* injection. The values of the THA represent the mean±S.D. with the number of endplates in parentheses. The values of AChE activity are the mean±S.D., with the number of animals in parentheses. * a significant difference from the relevant control and ** between values indicates a significant difference between them (Mann-Whitney; $p \leq 0.05$).

(a)



(b)

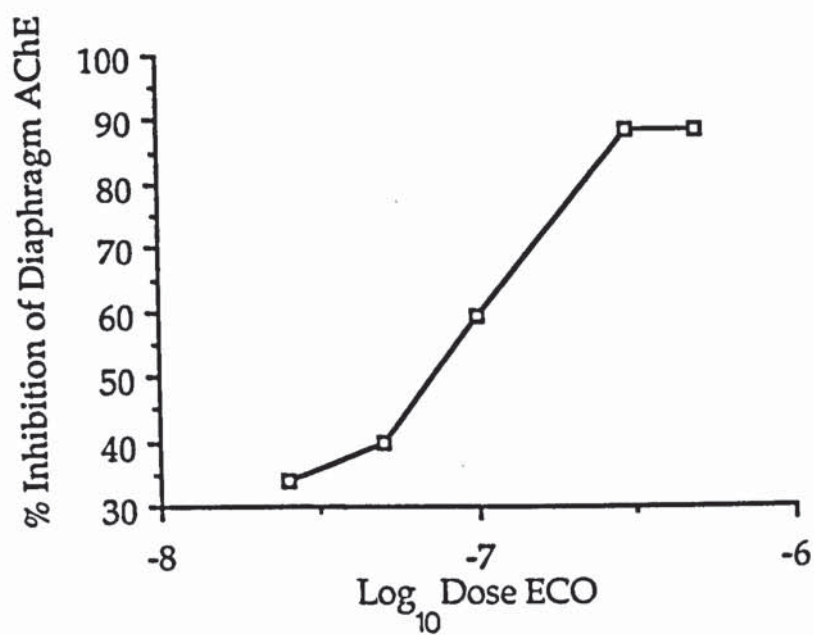
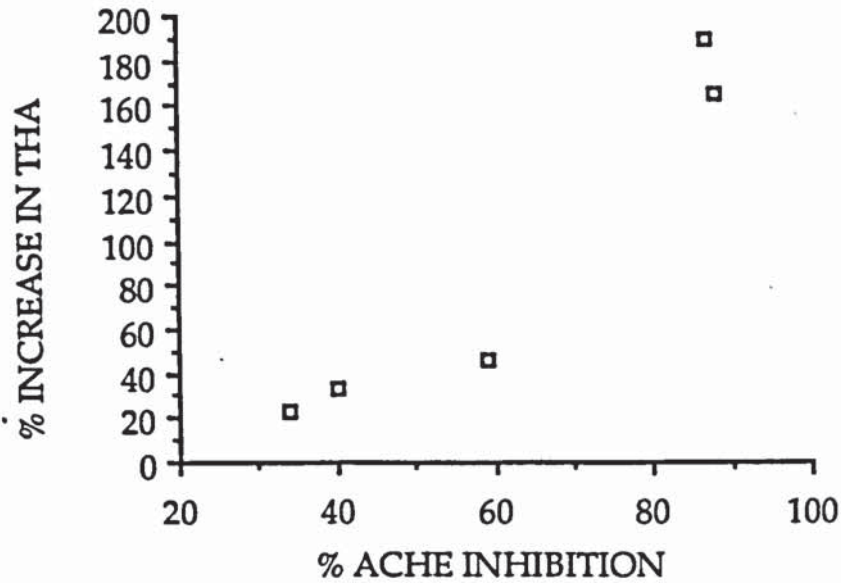


Figure 3.19. The effect of various doses of ECO on :-
(a) THA (msec), the values represent the mean \pm S.D.
(b) Percentage inhibition of Diaphragm AChE
3 hours after *in vivo* injection. Doses in mol/Kg.

(a)



(b)

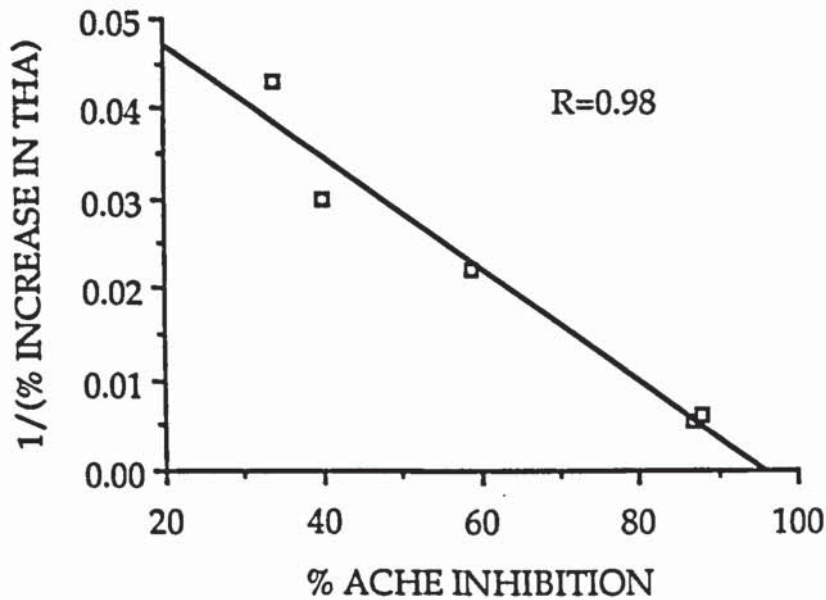


Figure 3.20. (a) The relationship between percentage increase in time to half amplitude (THA) and percentage inhibition of diaphragm AChE 3 hours after various doses of ECO *in vivo*.

(b) The relationship between the reciprocal of the percentage increase in THA and percentage inhibition of diaphragm AChE 3 hours after various doses of ECO *in vivo*.

3.3.1.6. The effect of BOS.

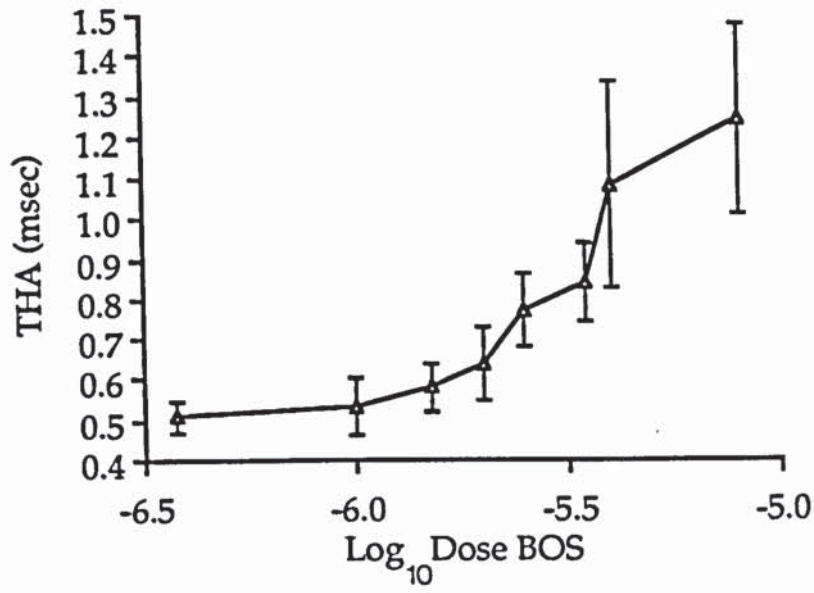
The effect of BOS (0.375 to 8 $\mu\text{mol/Kg}$) on THA and percentage inhibition of diaphragm AChE can be observed in table 3.28 and figure 3.21, indicating a dose dependent effect on both parameters. There was a non-linear relationship between THA and percentage AChE inhibition (Figure 3.22a). A slow, steady increase in THA occurred up to approximately 65% inhibition of diaphragm AChE. Above this level of inhibition there was a large increase in the effect on THA. If the reciprocal of the percentage increase in THA was plotted against percentage AChE inhibition a linear plot was produced with a correlation coefficient of 0.98 (Figure 3.22b).

In summary, it was clear that similar relationships between the time course of extracellular MEPPs and inhibition of diaphragm AChE 3 hours after injection occurred no matter which anti-ChE was used. In fact there were no significant differences between the slopes of the straight line relationships for the three drugs used ($p \leq 0.05$; Student's t-test). It can be said to be independent of the type of anti-ChE, since both tertiary (Sarin) and quaternary (ECO and BOS) compounds were used, as well as reactivatable (Sarin and ECO) and non-reactivatable compounds (BOS).

Dose of BOS μmol/Kg	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.42±0.05 (35) **	1.34±0.43 (38)	0
0.375	0.51±0.04 (11)*	1.13±0.15 (4) **	16
1.0	0.53±0.07 (11)*	0.84±0.07 (4)* **	37
1.5	0.58±0.06 (26)*	0.46±0.08 (4)*	66
2.0	0.64±0.10 (27)* **	0.44±0.06 (4)*	67
2.5	0.77±0.09 (14)*	0.37±0.08 (4)*	72
3.5	0.84±0.10 (19)* **	0.35±0.05 (4)* **	74
4.0	1.08±0.25 (19)* **	0.21±0.10 (15)*	84
8.0	1.24±0.23 (23)*	0.18±0.05 (11)*	87

Table 3.28. The effect of various doses of BOS on THA and diaphragm AChE activity 3 hours after *in vivo* injection. The values of the THA represent the mean±S.D. sampled from the number of endplates found in parentheses. The values of AChE activity are the mean±S.D., with the number of animals sampled also in parentheses. * significant difference from the relevant control and ** between values indicates a significant difference between them (Mann-Whitney; p≤0.05).

(a)



(b)

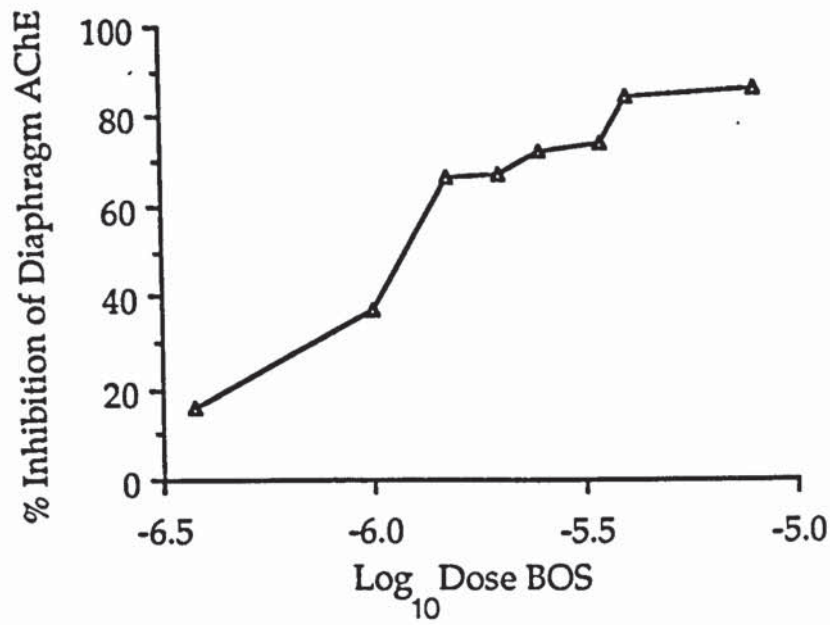
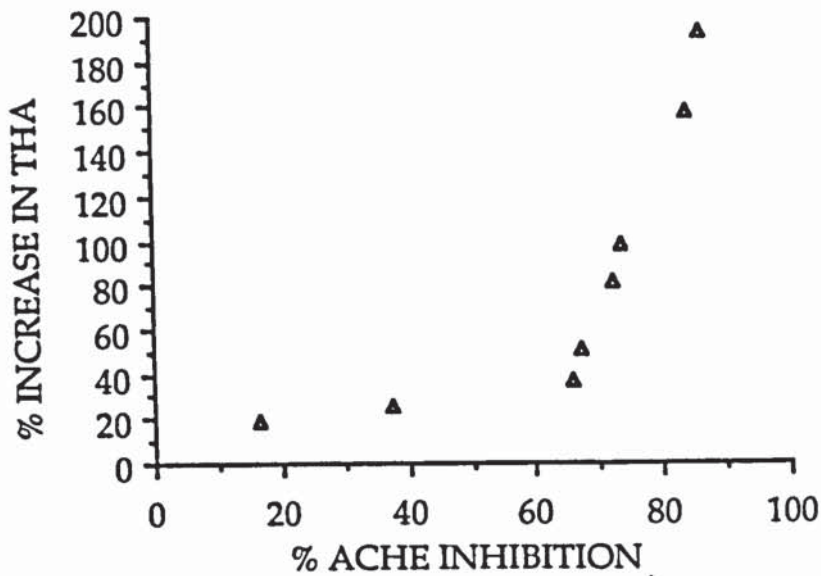


Figure 3.21. The effect of various doses of BOS on :-
(a) THA (msec), the values represent the mean \pm S.D.
(b) Percentage inhibition of Diaphragm AChE
3 hours after *in vivo* injection. Doses in mol/Kg.

(a)



(b)

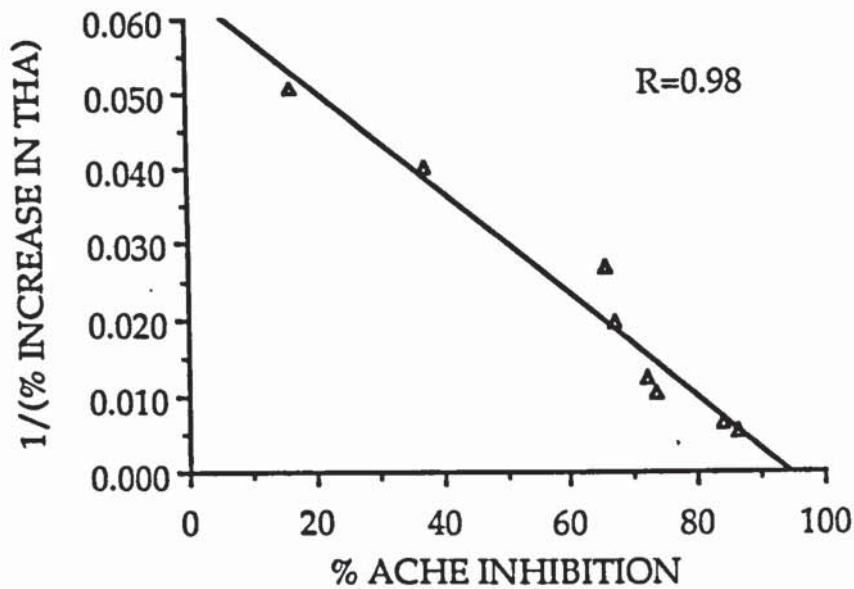


Figure 3.22. (a) The relationship between percentage increase in time to half amplitude (THA) and percentage inhibition of diaphragm AChE 3 hours after various doses of BOS *in vivo*.

(b) The relationship between the reciprocal of the percentage increase in THA and percentage inhibition of diaphragm AChE 3 hours after various doses of BOS *in vivo*.

3.3.2. The recovery of the THA of extracellularly recorded MEPPs following single injections of anti-ChEs.

Having established the relationships between the THA of extracellular MEPPs and the inhibition of diaphragm AChE 3 hours after injection *in vivo* it was decided to discover whether the THA and the AChE activity, measured by the Ellman *et al.* (1961) technique, recovered at the same rate, or whether there was a deviation from this relationship at these longer time points.

Animals were injected with Sarin 1.4 μ mol/Kg, ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg *in vivo* and at various times afterwards the animals were killed and their diaphragms dissected out (2.3.1). Some hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded extracellularly from a number of endplates (2.6.3). Sixteen MEPPs were averaged at each endplate and the THA of the averaged record was measured. The other hemidiaphragms were used for the assay of AChE levels (2.5).

Following the injection of Sarin 1.4 μ mol/Kg *in vivo* there was a significant effect on MEPP THA and AChE activity for up to 10 days, however, by 14 days these parameters had returned to control levels ($p \leq 0.05$; table 3.29). Similarly, following the injection of ECO 0.5 μ mol/Kg *in vivo* there was significant prolongation of the THA of extracellular MEPPs and reduction in diaphragm AChE activity (Table 3.30). By 5 days, although AChE levels had returned to control, a significant prolongation of the THA was observed ($p \leq 0.05$; table 3.30). After injection of BOS 8 μ mol/Kg there was, as with the other anti-ChEs, a significant prolongation of the THA of extracellular MEPPs and inhibition of diaphragm AChE (table 3.31). By 15 days, the AChE levels had returned to control whereas a significant prolongation of the THA was maintained ($p \leq 0.05$; table 3.31)

The relationship between the time course of these extracellular MEPPs and the inhibition of diaphragm AChE at these longer time points can be compared to the relationship at 3 hours after injection by plotting the points on the straight line relationships for the three drugs (see figures 3.16b, 3.20b and 3.22b). These plots, along with those at longer time points, are shown in figure 3.23. It

indicates that at intermediate time points, 7 days, 5 days and 5 days after *in vivo* injection for Sarin, ECO and BOS respectively, the points are outside the 95% confidence limits suggesting the relationship was not the same. However, at even longer times, 10 days and 15 days after injection for Sarin and BOS respectively, the relationship appears to be the same as that at 3 hours. Superimposed upon each graph is the same relationship, but at times longer than 3 hours after injection *in vivo*. The points are represented by crosses and annotated with the time after injection at which the measurements were made. On closer inspection these deviations seem somewhat greater with ECO than BOS, and likewise greater with BOS than sarin. The complex which ECO forms with AChE is known to undergo a certain amount of spontaneous reactivation. Hence if some of this reactivation occurred within the non-functional forms of AChE it would be manifested in an artificially high level of total enzyme when measured by the technique of Ellman *et al.* (1961), without affecting the time course of extracellular MEPPs. BOS and sarin, in contrast, display no such reactivation. Following inhibition, recovery of functional activity is due to the synthesis and assembly of the asymmetrical forms of AChE, much of which is done intracellularly from the enzyme of lower molecular weight. But sarin, unlike ECO and BOS, possesses the capability to inhibit intracellular stores of these components because of its tertiary structure. Thus if the discrepancies between AChE levels measured by the Ellman *et al.* (1961) method and the time course of extracellular MEPPs are due to the rapid appearance of these low molecular weight forms of AChE intracellularly they may be less likely to occur with sarin. Thus it can be concluded that at certain times the Ellman *et al.* (1961) does not appear to give an accurate representation of functional AChE.

Time after Injection	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.43±0.04 (32)	1.46±0.32 (17)	0
3 hours	1.09±0.17 (18)*	0.14±0.03 (8)*	90
3 days	0.94±0.35 (20)*	0.31±0.05 (6)*	79
7 days	0.71±0.16 (20)*	0.61±0.10 (10)*	58
10 days	0.55±0.07 (16)*	0.69±0.19 (9)*	53
14 days	0.46±0.06 (20)	1.43±0.27 (6)	2

Table 3.29. The THA of extracellularly recorded MEPPs and diaphragm AChE activity at various times after a single 1.4µmol/Kg dose of Sarin *in vivo*. Values of THA are represented as the mean±S.D., with the number of endplates sampled in parentheses. The AChE activities are also given as the mean±S.D., with the number of animals sampled in parentheses. * significant difference from the relevant control (Mann-Whitney; p≤0.05).

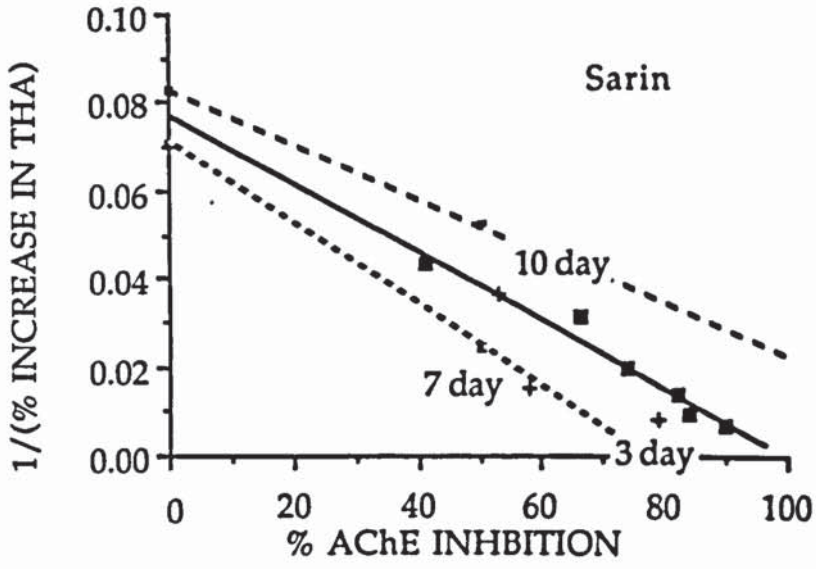
Time after Injection	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.42±0.05 (35)	1.34±0.43 (38)	0
3 hours	1.21±0.12 (11)*	0.18±0.04 (8)*	86
24 hours	0.77±0.10 (20)*	0.54±0.05 (6)*	60
5 days	0.64±0.11 (18)*	1.27±0.23 (11)	5

Table 3.30. The THA of extracellularly recorded MEPPs and diaphragm AChE activity at various times after a single 0.5µmol/Kg dose of ECO *in vivo*. Values of THA are represented as the mean±S.D., with the number of endplates sampled in parentheses. The AChE activities are also given as the mean±S.D., with the number of animals sampled in parentheses. * significant difference from the relevant control (Mann-Whitney; p≤0.05).

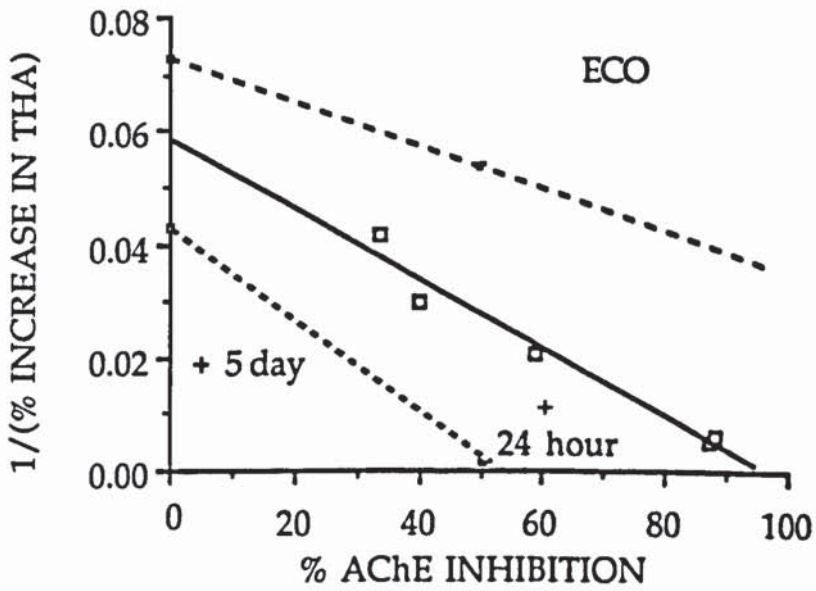
Time after Injection	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.42±0.05 (35)	1.34±0.43 (38)	0
3 hours	1.24±0.23 (23)*	0.18±0.05 (11)*	87
5 days	0.88±0.18 (37)*	0.82±0.16 (18)*	39
15 days	0.49±0.05 (24)*	1.16±0.19 (8)	13

Table 3.31. The THA of extracellularly recorded MEPPs and diaphragm AChE activity at various times after a single 8µmol/Kg dose of BOS *in vivo*. Values of THA are represented as the mean±S.D., with the number of endplates sampled in parentheses. The AChE activities are also given as the mean±S.D., with the number of animals sampled in parentheses. * significant difference from the relevant control (Mann-Whitney; p≤0.05)

(a)



(b)



(c)

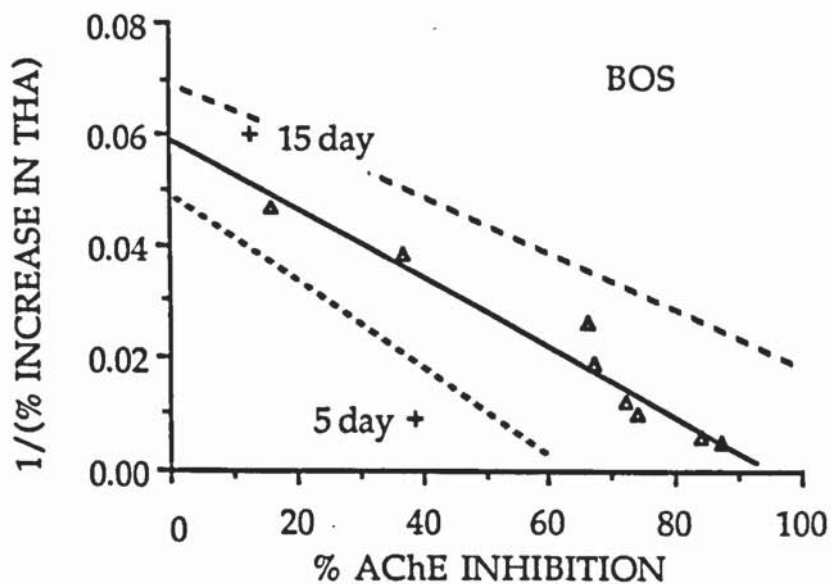


Figure 3.23. The relationship between the reciprocal of percentage increase in THA and percentage inhibition of diaphragm AChE 3 hours after various *in vivo* doses of :-

- (a) Sarin
- (b) ECO
- (c) BOS

Superimposed are the relationships at later time points. The dotted lines represent the 95% confidence limits for the relationships at 3 hours.

3.3.3. An insight into the reasons behind the deviations from the relationships observed at 3 hours.

Having discovered in the previous section (3.3.2) that at intermediate time points there were deviations from the relationships between THA and percentage inhibition of diaphragm AChE found 3 hours after *in vivo* injection. It was suggested that this may be due to a number of possibilities. Firstly, it may be an effect on channel properties due to the presence of higher than normal levels of ACh in the synapse for long periods of time. Alternatively, under the inhibition of AChE there could be a diffusion of transmitter to extrajunctional receptors and it could be such receptors which have different gating characteristics. Finally, during the *de novo* synthesis and transport of AChE, following its irreversible inhibition, there is an initial synthesis and transport of the lower molecular weight constituents of functional AChE. These constituents will be capable of hydrolysing the substrate used in the technique of Ellman *et al.* (1961) and will thus add to the AChE activity measured by this method. They will however have no functional significance at the synapse. The technique Ellman *et al.* (1961) will thus give an artificially high value for functional AChE. A group of mice were injected with ECO 0.5 μ mol/Kg *in vivo*. On the 5th day the animals were each given a further 0.5 μ mol/Kg dose of ECO and after 3 hours were killed and their diaphragms dissected out (2.3.1). Some hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded from a number of endplates extracellularly (2.6.3). Sixteen MEPPs were averaged at each endplate and the THA of the mean record measured. The other hemidiaphragms were used for the assay of AChE levels (2.5).

The effect of this second injection of ECO is shown in table 3.32. The injection returned the THA and the diaphragm AChE to the levels seen 3 hours after the first, no significant difference between the two being observed. This suggests that the deviations may be due to the presence of the low molecular weight forms of AChE which are part of the *de novo* synthesis of functional AChE. The second injection merely re-inhibits that enzyme and any fully synthesised functional AChE, returning the parameters to the levels seen 3 hours after the first injection. If channel properties had changed one might expect a deviation from this standard line.

Time after Injection	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibition
Control	0.42±0.05 (35)†	1.34±0.43 (38)†	0
3 hours	1.21±0.12 (11)*	0.17±0.04 (8)*	87
5 days	0.64±0.11 (18)*†	1.27±0.23 (11)†	5
5 days + 2nd injection ECO 0.5µmol/Kg.	1.29±0.28 (17)*	0.17±0.05 (7)*	87

Table 3.32. The THA of extracellularly recorded MEPPs and diaphragm AChE activity at various times after a single 0.5µmol/Kg dose of ECO *in vivo*. The table also shows the effects on THA and diaphragm AChE of a second dose of ECO 0.5µmol/Kg 5 days after the first, with analysis being made 3 hours subsequent to this second injection. * significant difference from the relevant control and † significant difference from the values gained 3 hours after the initial injection (Mann-Whitney; p≤0.05).

3.4. EFFECTS OF OXIMES OTHER THAN REACTIVATION OF INHIBITED AChE.

Section 3.2.4 described an investigation into the methods of protection against the anti-ChE induced increases in jitter. During these studies it was discovered that injections of 2PAM (the 7 injection protocol) produced significant reductions in both the MCD and the delay of recorded action potentials following administration of BOS *in vivo*, even though no reactivation of diaphragm AChE was evident following a single injection of 2PAM following BOS. This fact and the evidence that 2PAM and other oximes have effects at the nicotinic receptor-channel complex (Alkondon *et al.*, 1988), led to the extension of the MEPP study. BOS and ECO had already been shown to prolong the time course of extracellular MEPPs, thus the effects of oximes on this phenomenon were investigated.

3.4.1. *In vivo* reactivation of AChE.

Four groups of mice were taken. The first two were given either ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg *in vivo*. The other two groups were also given either ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg *in vivo*, followed by a 110 μ mol/Kg dose of 2PAM on commencement of fasciculation. After 3 hours the animals were anaesthetised and blood samples taken (2.5.1.1). The animals were then killed and their diaphragms dissected out (2.3.1). The blood samples, along with some of the hemidiaphragm samples, were assayed for AChE content (2.5). Other hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded extracellularly (2.6.3). Sixteen MEPPs were averaged at each endplate and the THA of the averaged record measured (Figure 3.10).

This dose of 2PAM, after an injection of ECO, produced a significant increase in both blood and diaphragm AChE activity ($p \leq 0.05$; table 3.33), as well as a significant reduction in the MEPP time course, as indicated by the THA. The reductions seen in the inhibition of diaphragm AChE and THA followed the relationships between these two parameters 3 hours after injection of ECO which had been determined earlier in the study, ie the points were on the straight line (Figure 3.20b). However, if BOS 8 μ mol/Kg *in vivo* was followed

Treatment	THA (msec)	Diaphragm AChE Activity nmol/min/mg	% Inhibn	Blood AChE Activity nmol/min/mg	% Inhibn
Control	0.42±0.05 (35)†	1.34±0.43 (38)†	0	0.90±0.15 (22)†	0
ECO	1.21±0.12 (11)*	0.17±0.04 (8)*	87	0.24±0.06 (8)*	73
ECO + 2PAM	0.69±0.09 (22)*†	0.35±0.08 (17)*†	74	0.46±0.09 (9)*†	49
BOS	1.24±0.23 (23)*	0.18±0.09 (11)*	87	0.29±0.07 (7)*	68
BOS + 2PAM	1.33±0.28 (20)*	0.20±0.02 (5)*	85	0.25±0.06 (4)*	72

Table 3.33. The effect of ECO 0.5µmol/Kg and BOS 8µmol/Kg on the THA of extracellularly recorded MEPPs, diaphragm and blood AChE activity after 3 hours *in vivo*. Also shown are the effects of subsequent injection of 110µmol/Kg 2PAM, on the appearance of anti-ChE induced fasciculation, on all three parameters. Values of THA are represented as the mean±S.D., with the number of endplates sampled in parentheses. The AChE activities are also given as the mean±S.D., with the number of animals sampled in parentheses. * significant difference from the relevant control and † significant difference from ECO alone (Mann-Whitney; p≤0.05).

by 110 μ mol/Kg 2PAM on fasciculation no effect on blood AChE, diaphragm AChE or the THA was seen. This was expected since, as stated earlier, the AChE inhibited by BOS ages rapidly and is then no longer open to reactivation by an oxime.

3.4.2. The effect of oximes on the prolongation of the time course of extracellularly recorded MEPPs induced by anti-ChEs.

Mice were injected with either ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg subcutaneously. After three hours the animals were killed and their diaphragms dissected out (2.3.1). The hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded extracellularly from a number of endplates (2.6.3). The flow of fresh saline into the bath was stopped and a 400 μ M dose of 2PAM added. The cessation of the flow of fresh saline was shown not to effect the time course of control MEPPs, 0.43 \pm 0.06 (8) before stopping the flow compared with 0.45 \pm 0.05 (8) after (Mean \pm S.D. with the number of endplates sampled in parentheses). The hemidiaphragms were incubated with the 2PAM for 1 hour, after which MEPPs were again sampled from several endplates (2.6.3). After an adequate number of records were gained the drug was washed out of the bath with fresh saline and the preparation left to equilibrate for a period of 15 minutes. This washout was followed by further recording of extracellular MEPPs and measurement of the THA (2.6.3).

In a similar group of experiments a number of mice were treated with one of the anti-ChEs *in vivo*, as indicted above, for 3 hours. The animals were killed and their diaphragms dissected out (2.3.1). One half of the diaphragm was assayed for AChE activity immediately, while the other half was incubated with 400 μ M 2PAM for an hour *in vitro* as above, then assayed for AChE (2.5). These were performed to assess whether 2PAM was producing any *in vitro* reactivation.

As before, these doses of ECO and BOS produced a prolongation of the THA, to about 1msec. On the addition of 400 μ M 2PAM there was a large, significant reduction in the THA produced by both anti-ChEs ($p\leq 0.05$; table 3.34). On washout there was some return of the prolongation with both drugs, however

Treatment	THA (msec)		
	Anti-ChE alone	+ 400 μ M 2PAM	Washout
ECO 0.5 μ mol/Kg	1.14 \pm 0.21 (17)	0.41 \pm 0.05 (18)*	0.53 \pm 0.08 (22)*†
BOS 8 μ mol/Kg	1.25 \pm 0.25 (18)	0.44 \pm 0.09 (20)*	1.05 \pm 0.19 (17)*

Table 3.34. The effect of 400 μ M 2PAM for 1 hour *in vitro* at 37°C on the THA of extracellularly recorded MEPPs sampled from hemidiaphragms treated with either ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg for 3 hours *in vivo*. The values represent the mean \pm S.D. from at least 2 animals, with the number of endplates sampled in parentheses. * significant difference from the treatment with anti-ChE alone and † significant difference between ECO and BOS at any stage of the experiment (Mann-Whitney; $p \leq 0.05$).

Treatment	Diaphragm AChE activity		Diaphragm AChE activity	
	(nmol/min/mg) Before 2PAM	% Inhibition	(nmol/min/mg) 1 hour 2PAM	% Inhibition
Untreated	1.34±0.43 (38)†	0	1.34±0.43 (38)†	0
ECO 0.5µmol/Kg	0.17±0.06 (4)*	87	0.87±0.07 (4)*†	35
BOS 8µmol/Kg	0.13±0.01 (8)*	90	0.18±0.04 (8)*	87

Table 3.35. The effect of 1 hour incubation with 400µM 2PAM *in vitro* on the AChE activity of hemidiaphragms of animals treated with either ECO 0.5µmol/Kg or BOS 8µmol/Kg for 3 hours *in vivo*. The values represent the mean±S.D. with the number of animals sampled in parentheses. * significant difference from control and † significant difference from hemidiaphragms treated with anti-ChE alone (Mann-Whitney; p≤0.05).

this was significantly greater with BOS ($p \leq 0.05$; table 3.34). The THA with BOS-treated preparations, following the removal of 2PAM, did not quite reach the original levels, the value still being significantly lower than that measured in preparations treated with anti-ChE alone ($p \leq 0.05$; table 3.34). Table 3.35 shows the effect on diaphragm AChE of an hour of incubation with $400 \mu\text{M}$ 2PAM *in vitro*. No significant reactivation was observed with preparations treated with BOS $8 \mu\text{mol/Kg}$ *in vivo*, however, the inhibition of diaphragm AChE following ECO $0.5 \mu\text{mol/Kg}$ for three hours and $400 \mu\text{M}$ 2PAM for an hour *in vitro* was reduced to 35% from 87% seen with the ECO treatment alone ($p \leq 0.05$; table 3.35).

3.4.3. The effect of oximes on BOS-induced prolongation of the time course of extracellularly recorded MEPPs.

The previous experiments demonstrated that $400 \mu\text{M}$ 2PAM produced a reduction in extracellular MEPP THA, without affecting diaphragm AChE activity. The study was broadened to encompass a number of oximes at a range of doses. Disopyramide phosphate has been shown to have blocking properties at the nicotinic receptor-channel complex by Healy *et al.* (1981). This compound was used to determine whether or not the time course of extracellular MEPPs were changed by compounds which were potential blockers.

Mice were injected with BOS $8 \mu\text{mol/Kg}$ and 3 hours later they were killed and their diaphragms dissected out (2.3.1). Hemidiaphragms were placed in an organ bath at 37°C and MEPPs recorded extracellularly from several endplates (2.6.3). A dose of one of the oximes (2PAM, HI6 or TOX) was added to the bath after stopping the flow of fresh saline into the bath. After incubation for an hour *in vitro*, extracellular MEPPs were again recorded from a number of endplates and the THA measured. Once an adequate number of records had been obtained the drug was washed out of the bath with fresh saline and the preparation left to equilibrate for a period of 15 minutes. This washout was followed by yet more recording of extracellular MEPPs and THA measurement (2.6.3).

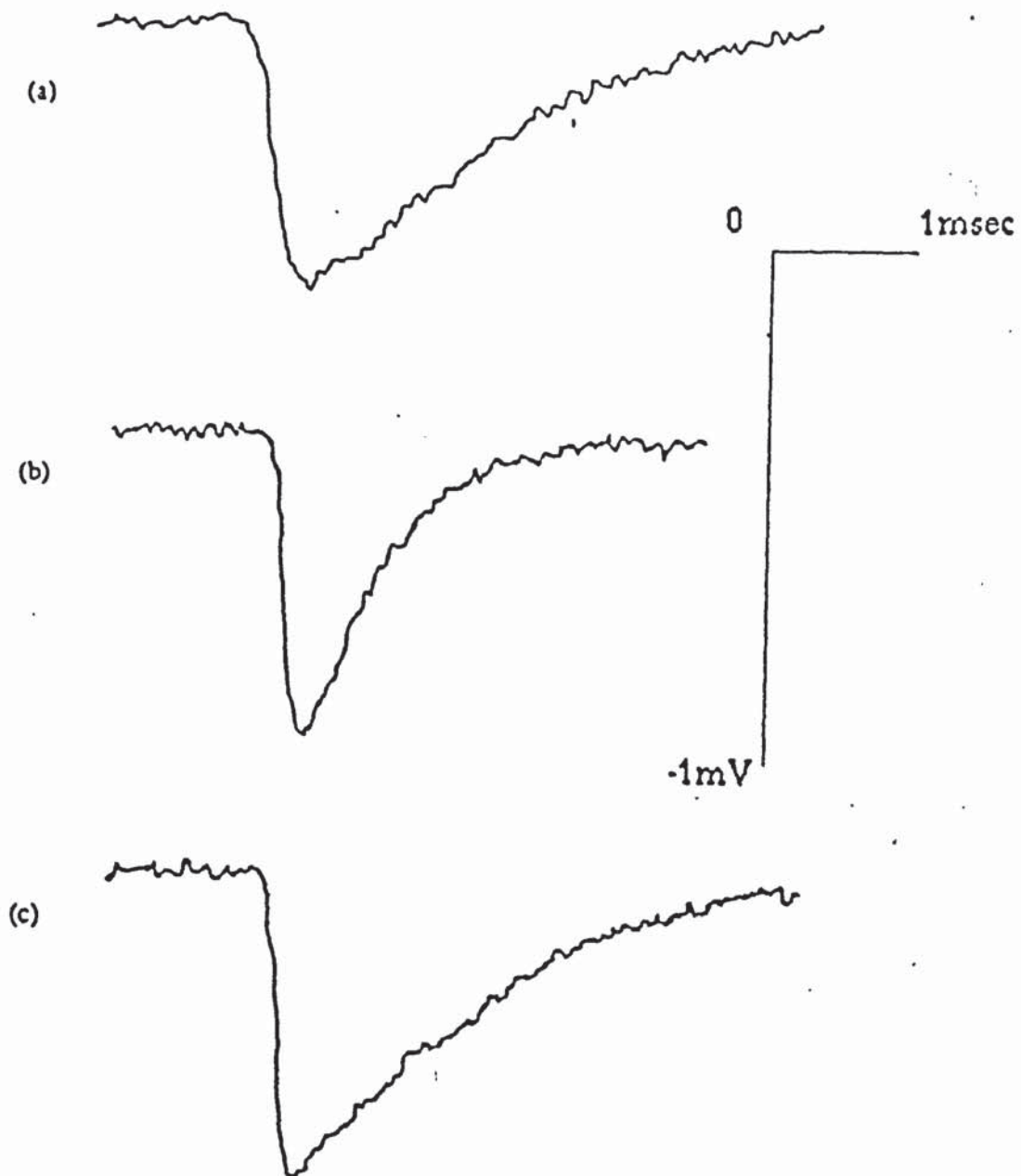


Figure 3.24. Sample traces of negative-going extracellularly recorded MEPPs in mouse hemidiaphragm *in vitro* at 37°C, (a) in a preparation treated with BOS 8 $\mu\text{mol/Kg}$ for 3 hours *in vivo*, (b) the same preparation after a further one hours incubation with 400 μM 2PAM *in vitro* and (c) after washout of the 2PAM. The horizontal and vertical bars represent 1msec and 1mV respectively.

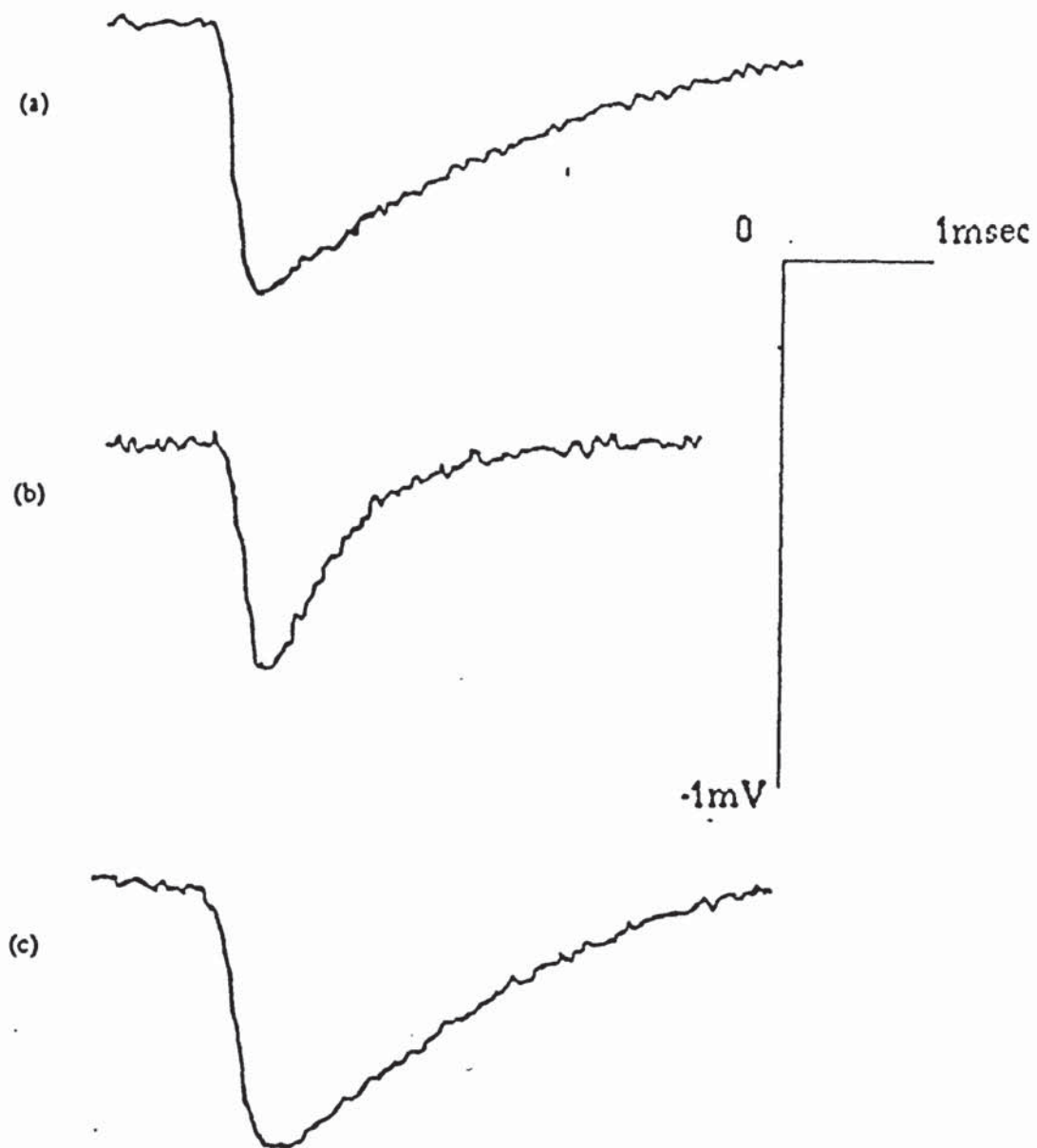
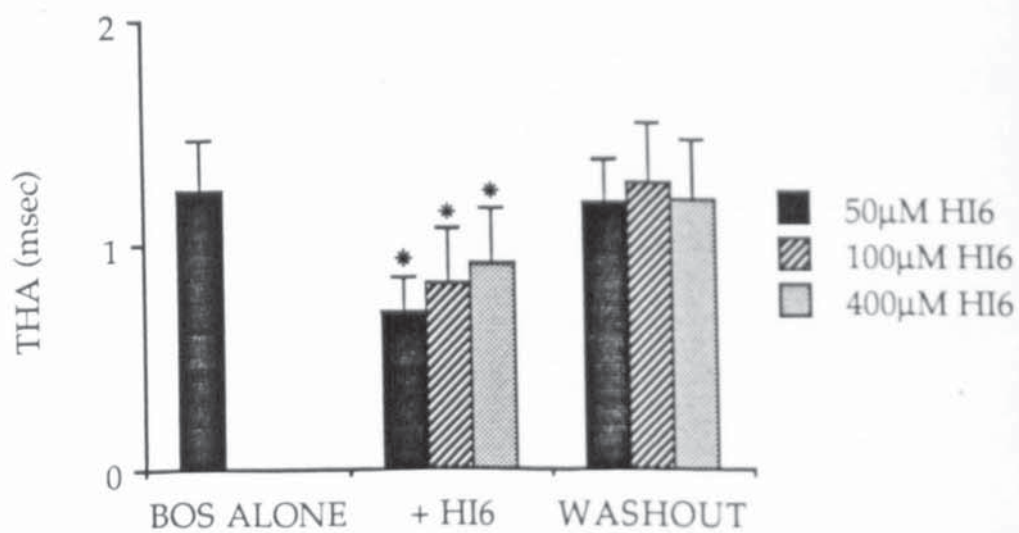
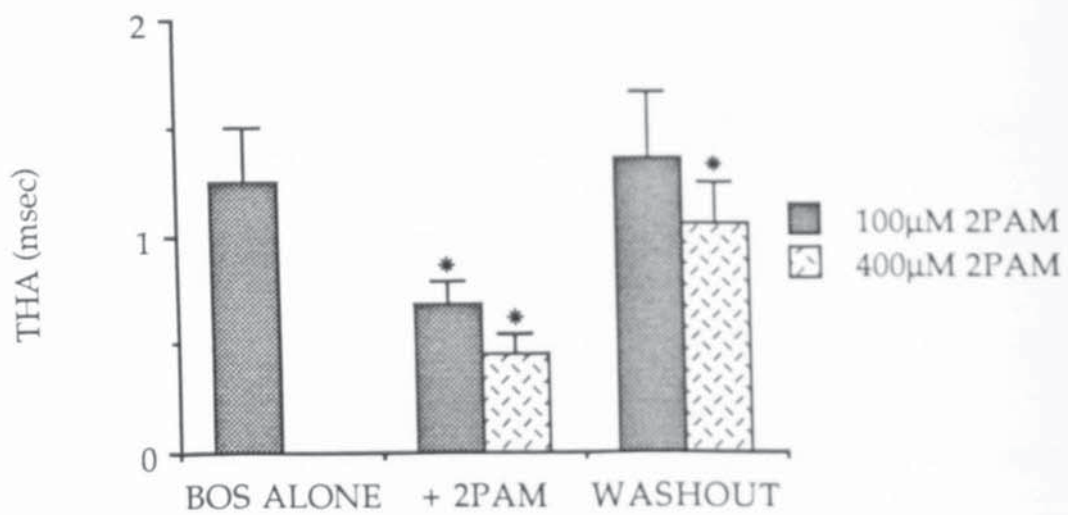


Figure 3.25. Sample traces of negative-going extracellularly recorded MEPPs in mouse hemidiaphragm *in vitro* at 37°C, (a) in a preparation treated with BOS 8 μmol/Kg for 3 hours *in vivo*, (b) the same preparation after a further one hours incubation with 100 μM Disopyramide *in vitro* and (c) after washout of the Disopyramide. The horizontal and vertical bars represent 1msec and 1mV respectively.

Treatment	THA (msec)		
	BOS 8 μ mol/Kg	+ treatment	Washout
100 μ M 2PAM	1.25 \pm 0.25 (18)	0.67 \pm 0.11 (14)*	1.35 \pm 0.31 (15)
400 μ M 2PAM	1.25 \pm 0.25 (18)	0.44 \pm 0.09 (20)*	1.05 \pm 0.19 (17)*
50 μ M HI6	1.24 \pm 0.22 (19)	0.70 \pm 0.15 (15)*†	1.19 \pm 0.18 (16)
100 μ M HI6	1.24 \pm 0.22 (19)	0.83 \pm 0.23 (14)*	1.28 \pm 0.20 (12)
400 μ M HI6	1.24 \pm 0.22 (19)	0.92 \pm 0.24 (15)*†	1.20 \pm 0.26 (13)
100 μ M TOX	1.24 \pm 0.24 (24)	0.82 \pm 0.20 (21)*	1.19 \pm 0.15 (16)
400 μ M TOX	1.24 \pm 0.24 (24)	0.69 \pm 0.15 (16)*	1.19 \pm 0.16 (8)
100 μ M Disopyramide	1.30 \pm 0.23 (4)	0.41 \pm 0.08 (16)*	1.16 \pm 0.22 (16)

Table 3.36. The effect of various treatments, *in vitro* at 37°C, on the THA of extracellularly recorded MEPPs in hemidiaphragms dissected from animals which had been treated with BOS 8 μ mol/Kg for 3 hours *in vivo*. The values represent the mean \pm S.D. from at least 2 animals with the number of endplates sampled in parentheses. * significant difference from the animals treated with BOS alone and † significant difference between the 50 μ M and 400 μ M doses of HI6 (Mann-Whitney; $p \leq 0.05$). There were no other significant differences.



(c)

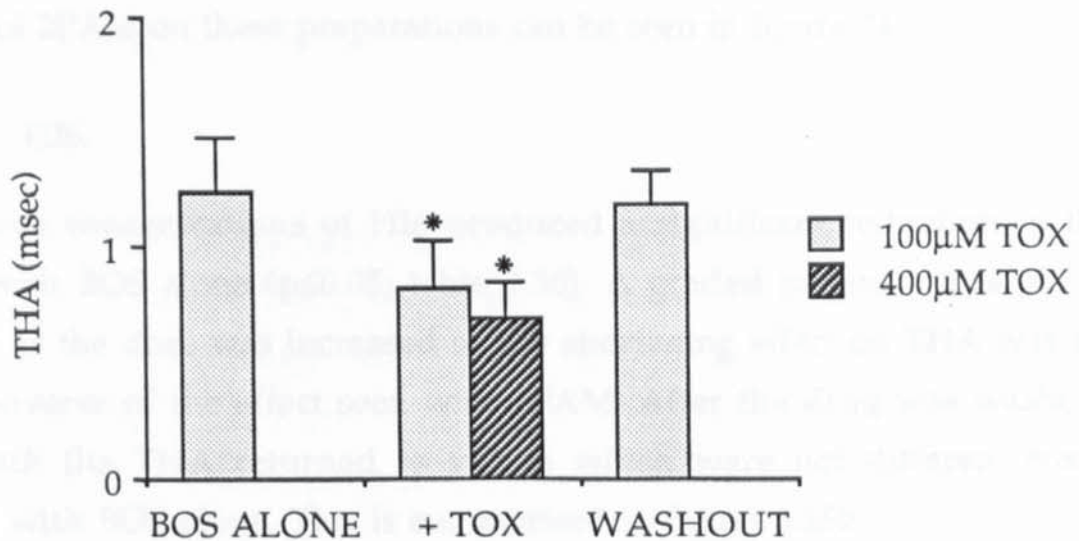


Figure 3.26. The effect on the THA of extracellular MEPPs at 37°C *in vitro*, recorded in hemidiaphragms exposed to BOS 8µmol/Kg for 3 hours *in vivo*, of various concentrations of:-

- (a) 2PAM
- (b) HI6
- (c) Toxogonin

with the values representing the mean±S.D.

* significant difference from BOS alone (Mann-Whitney; $p \leq 0.05$).

3.4.3.1. 2PAM.

Both concentrations of 2PAM produced a significant reduction in the THA seen with BOS alone ($p \leq 0.05$; table 3.36). The reduction produced by the 400 μ M dose appeared greater than that shown by the 100 μ M dose, but the values were not significantly different. On washout a degree of recovery of the prolonged THA was observed, total recovery occurring after the 100 μ M dose. After washing out 400 μ M 2PAM the THA maintained a level which was significantly lower than that seen with BOS alone ($p \leq 0.05$; table 3.36, figure 3.26a). Sample traces of the effect of 2PAM on these preparations can be seen in figure 24.

3.4.3.2. HI6.

All three concentrations of HI6 produced a significant reduction in the THA seen with BOS alone ($p \leq 0.05$; table 3.36). A graded response was observed in which as the dose was increased so the shortening effect on THA was reduced, the converse of the effect seen with 2PAM. After the drug was washed out of the bath the THA returned to values which were not different from those found with BOS alone. This is summarised in figure 3.26b.

3.4.3.3. Toxogonin (TOX)

Both concentrations of TOX produced a significant reduction in the THA seen with BOS alone ($p \leq 0.05$; table 3.36). As with 2PAM the reduction seen with the 400 μ M dose appeared greater than that seen with the 100 μ M dose, but the difference was not significant. On washout the THA returned to values which were not different from those found with BOS alone. This is summarised in figure 3.26c.

3.4.3.4. Disopyramide.

The channel blocker disopyramide, in a dose of 100 μ M, produced a significant reduction in the THA produced by BOS alone ($p \leq 0.05$; table 3.36). Following the removal of the disopyramide by washing there was a return to the prolongation of the THA, which was not significantly different from the THA measured with BOS alone. Sample traces of the effect of disopyramide on these preparations can be seen in figure 25.

3.4.4. The effect of oximes on untreated preparations.

The experiments described in sections 3.4.2 and 3.4.3 established that oximes possessed properties other than reactivation. They produced reductions in the prolonged THA produced by BOS alone, yet 2PAM in particular does not affect diaphragm AChE levels. The fact that disopyramide was capable of producing a similar effect indicated that oximes might be having an effect at the nicotinic receptor-channel complex. The following experiments were performed to determine whether the effects could be demonstrated on untreated preparations, as well as those treated with an anti-ChE.

3.4.4.1. 2PAM.

400 μ M and 1mM concentrations of 2PAM produced a significant reduction in untreated THA ($p \leq 0.05$; table 3.37), but although the reductions appeared concentration-dependent the differences between concentrations were not significant. The effects are summarised in figure 3.28a. Sample traces of the effect of 2PAM on these preparations can be seen in figure 27.

3.4.4.2. HI6.

The 100, 200 and 400 μ M concentrations of HI6 created significant increases in the THA from that of untreated hemidiaphragms ($p \leq 0.05$; table 3.38). 50 μ M HI6, however, displayed no significant effect on untreated THA, but was different from the other three in concentrations used ($p \leq 0.05$; table 3.38). There were no differences between the 100, 200 and 400 μ M concentrations, even though the effect appears concentration-dependent. This prolongation of the THA is the converse of the effects seen with 2PAM. The effects are summarised in figure 3.28b.

3.4.4.3. Toxogonin (TOX).

A significant reduction of untreated THA was produced by 50 μ M TOX, a concentration which also displayed significantly different properties from those of the 400 μ M and 1mM concentrations ($p \leq 0.05$; table 3.39). The 400 μ M and 1mM concentrations resulted in a significant increase in the THA, in a concentration-dependent manner ($p \leq 0.05$; table 3.39, figure 3.28c).

Treatment	THA (msec)
Untreated	0.43±0.04 (32)
100µM 2PAM	0.40±0.06 (18)
400µM 2PAM	0.38±0.07 (32)*
1mM 2PAM	0.37±0.04 (15)*

Table 3.37. The effect of various concentrations of 2PAM on extracellularly recorded MEPPs of untreated hemidiaphragms *in vitro* at 37°C. The values represent mean±S.D. from at least two animals, with the number of endplates sampled in parentheses. * significant difference from the untreated control (Mann-Whitney; $p \leq 0.05$). No significant difference between the concentrations was found.

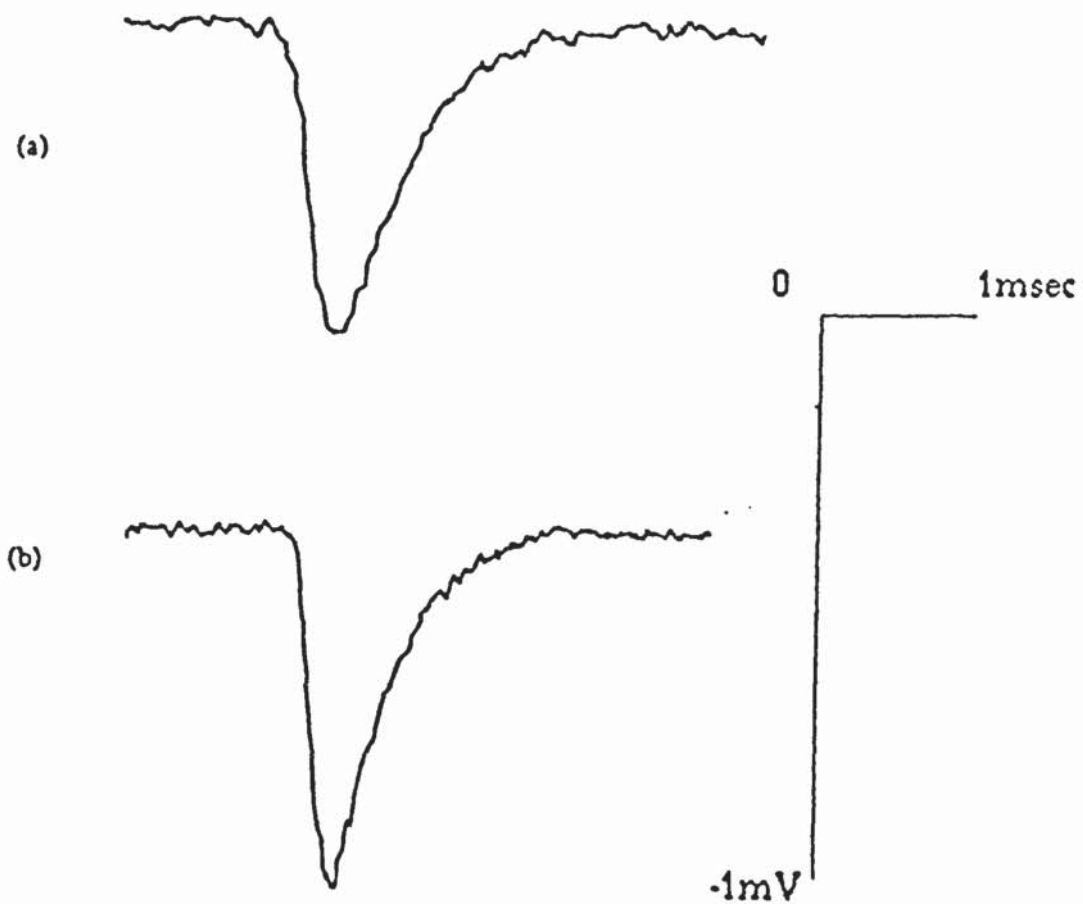


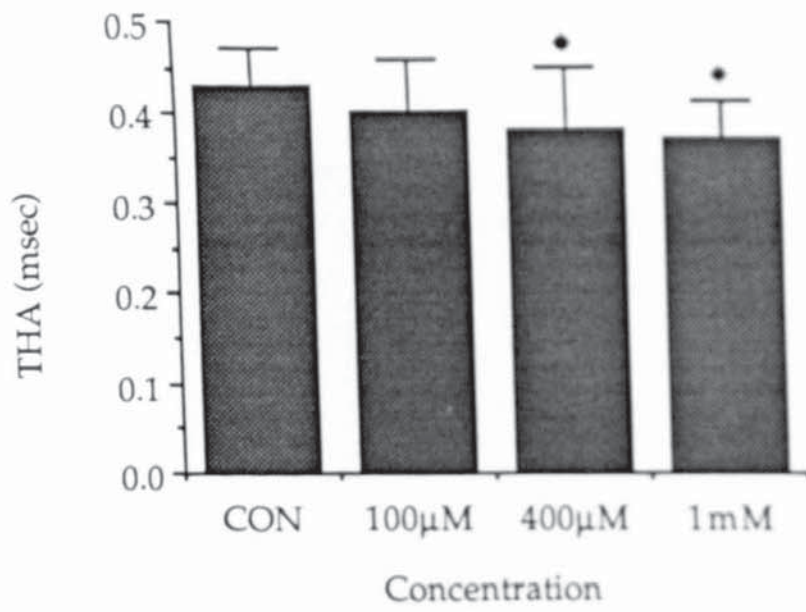
Figure 3.27. Sample traces of negative-going extracellularly recorded MEPPs in mouse hemidiaphragm, *in vitro* at 37°C, (a) in an untreated preparation and (b) the same preparation after a further one hours incubation with 400 μ M 2PAM *in vitro*. The horizontal and vertical bars represent 1msec and 1mV respectively.

Treatment	THA (msec)
Untreated	0.43±0.04 (32)
50µM HI6	0.42±0.06 (16)
100µM HI6	0.49±0.07 (19)*†
200µM HI6	0.53±0.09 (16)*†
400µM HI6	0.54±0.14 (15)*†

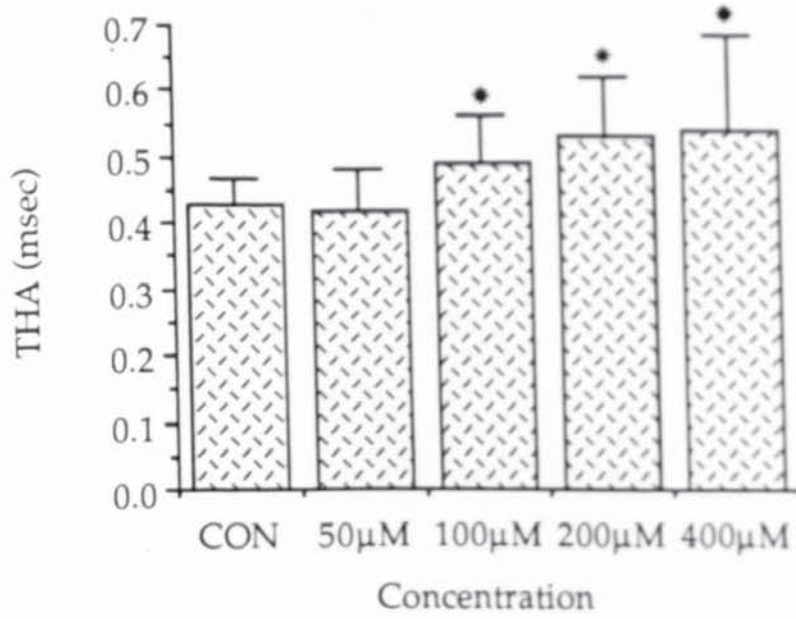
Table 3.38. The effect of various concentrations of HI6 on extracellularly recorded MEPPs of untreated hemidiaphragms *in vitro* at 37°C. The values represent mean±S.D. from at least two animals, with the number of endplates sampled in parentheses. * significant difference from the untreated control and † significant difference from the 50µM concentration (Mann-Whitney; $p \leq 0.05$). No other significant differences between the concentrations were found.

Treatment	THA (msec)
Untreated	0.43±0.04 (32)†
50µM TOX	0.40±0.05 (15)*
100µM TOX	0.41±0.03 (16) **
400µM TOX	0.48±0.04 (17)*† **
1mM TOX	0.57±0.10 (20)*†

Table 3.39. The effect of various concentrations of TOX on extracellularly recorded MEPPs of untreated hemidiaphragms *in vitro* at 37°C. The values represent mean±S.D. from at least two animals, with the number of endplates sampled in parentheses. * significant difference from the untreated control, ** between values a significant difference between those two concentrations and † significant difference from the 50µM concentration (Mann-Whitney; $p \leq 0.05$). No other significant differences between the concentrations were found.



b)



(c)

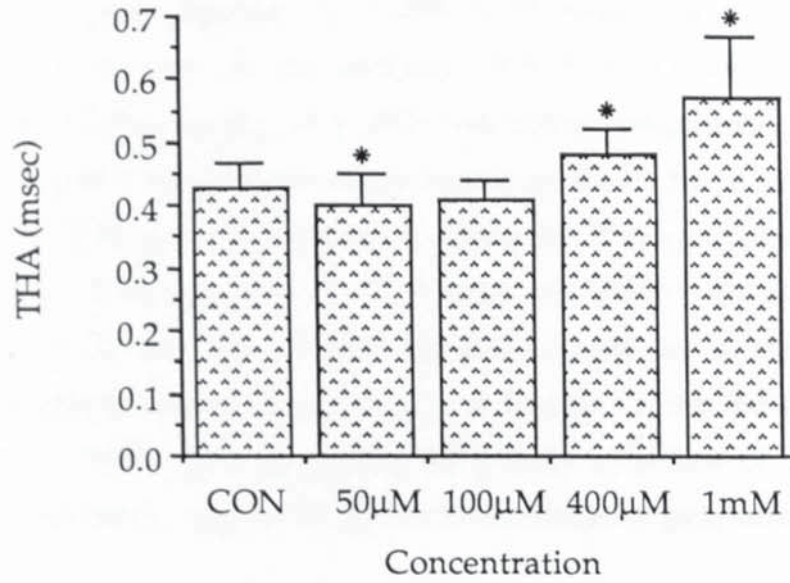


Figure 3.28. The effect on the THA of extracellular MEPPs, recorded in untreated hemidiaphragms *in vitro* at 37°C, of various concentrations of:-

(a) 2PAM

(b) HI6

(c) Toxogonin

with the values representing the mean±S.D., from at least 2 animals.

* significant difference from untreated control (Mann-Whitney; $p \leq 0.05$).

In summary, these experiments suggest that the oximes used possess properties other than reactivation of AChE. All of the oximes used displayed the ability to reduce the prolongation of MEPP THA resulting from BOS treatment *in vivo*, however only one of the oximes, 2PAM, has been used to demonstrate the unreactivable nature of a BOS-inhibited preparation. In the concentrations used, 2PAM was able to reduce not only the THA in animals treated with BOS, but also that seen in untreated animals. The converse was true with HI6 in untreated animals, which displayed an ability to prolong untreated THA. Finally, TOX was able to prolong and reduce the untreated THA, prolongation at high doses and reduction at the lower. It can be concluded that only the effects of 2PAM can be explained purely in terms of channel block, the other two compounds appear to have more complex properties.

3.5. EXTRACELLULARLY RECORDED MEPPs, DIAPHRAGM AChE AND THE EFFECT OF TEMPERATURE.

This short study was performed for two reasons. Firstly, all the *in vitro* work in this thesis was undertaken at 37°C, whereas the standard temperature for the Ellman *et al.* (1961) technique, used for AChE assay, was 30°C. This led to the question: if the temperature at which the assay was performed was increased to 37°C would it affect the AChE activity measured?

Secondly, all patch clamp investigations are performed at room temperature (Hamill *et al.*, 1981), the technique previously used to determine the action of oximes at the nicotinic receptor-channel complex (Alkonidon *et al.*, 1988). The questions to be answered were; how did this reduction to room temperature affect AChE activity and the time course of extracellular MEPPs and could any prolongation of the extracellular MEPP time course be explained merely in terms of a reduction in AChE activity, or was there any alteration in the properties of the channels?

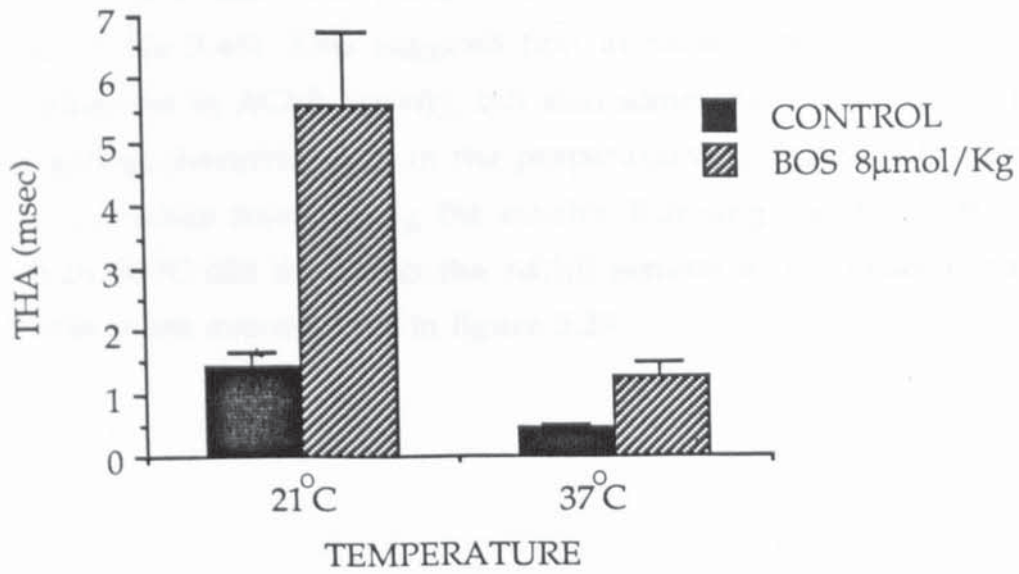
Two groups of animals were taken, one of which was injected with BOS 8µmol/Kg. Three hours later, both groups of animals were then killed and their diaphragms removed (2.3.1). Samples of hemidiaphragm were assayed for AChE (2.5) at room temperature (21°C), 30°C and 37°C. Some hemidiaphragms were placed into an organ bath at room temperature or 37°C and extracellular MEPPs recorded from a number of endplates from which the THA was measured (2.6.3).

The reduction of the organ bath temperature from 37°C to room temperature produced a large, significant increase in the THA of both untreated animals and those treated with BOS 8µmol/Kg ($p \leq 0.05$; table 3.40). Similarly, a reduction in the temperature at which the AChE assay was performed from 37°C to room temperature produced a marked, significant drop in the AChE activity in untreated animals and those which underwent BOS treatment ($p \leq 0.05$; table 3.40). However, these inhibitions of AChE were not great enough to explain the prolongations of THA experienced. For example, the value for the THA of untreated extracellular MEPPs measured at room temperature was 1.4msec, a

Treatment	Temperature (°C)	THA (msec)	Diaphragm AChE activity nmol/min/mg
Untreated	21°C	1.44±0.21 (15)*	0.77±0.12 (8)*†
Untreated	30°C		1.34±0.43 (32)†
Untreated	37°C	0.43±0.04 (32)†	1.37±0.17 (6)†
BOS 8µmol/Kg	21°C	5.58±1.14 (14)*†	0.11±0.04 (7)*†
BOS 8µmol/Kg	30°C		0.18±0.05 (11)*
BOS 8µmol/Kg	37°C	1.24±0.23 (23)*	0.18±0.07 (7)*

Table 3.40. The effect of temperature (21°C, 30°C and 37°C) on the THA of extracellularly recorded MEPPs and diaphragm AChE activity of untreated animals and animals exposed to BOS 8µmol/Kg for 3 hours *in vivo*. The values of THA are the mean±S.D. from at least 2 animals, with the number of endplates sampled in parentheses. The values for AChE activity also represent the mean±S.D. with the number of animals used in parentheses. * significant difference, for either parameter, from untreated for the parameter at 37°C and † significant difference from animals treated with BOS 8µmol/Kg at 37°C (Mann-Whitney; p≤0.05).

(a)



(b)

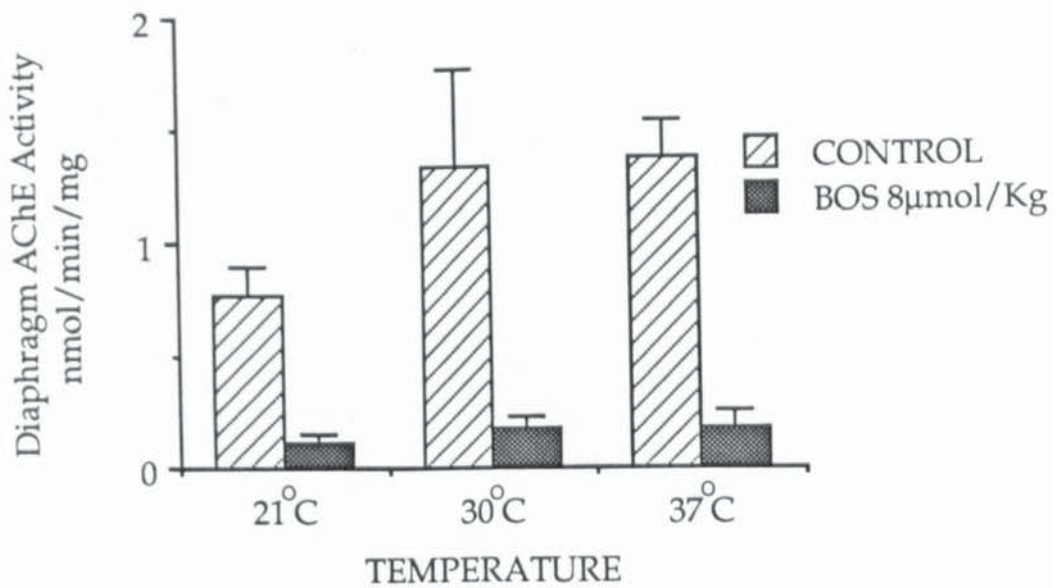


Figure 3.29. The effect of temperature on (a) THA of extracellular MEPPs recorded *in vitro* and (b) Diaphragm AChE activity in untreated animals and animals exposed to BOS 8µmol/Kg for 3 hours *in vivo*. The values in each case represent the mean±S.D.

value produced by approximately 90% AChE inhibition at 37°C (see section 2.4), however, in this case the AChE inhibition was approximately 40% when measured (table 3.40). This suggests that at room temperature there was not only a reduction in AChE activity, but also some change in channel properties, such as gating characteristics, in the preparations, a fact which must be taken into account when interpreting the results. Running the AChE assay at 37°C rather than 30°C did not affect the AChE activity at the doses examined. The overall effects are summarised in figure 3.29.

4. DISCUSSION.

4.1. THE CHARACTERISATION OF BOS.

4.1.1. Anticholinesterase-induced myopathy.

The present study established that the myopathy produced in mice exposed to BOS 8 μ mol/Kg followed a very similar time profile to that produced by ECO 0.5 μ mol/Kg. This was despite the fact that the inhibition of AChE produced by BOS 8 μ mol/Kg appeared to be much more prolonged than that seen with ECO 0.5 μ mol/Kg. As explained earlier (1.4.1), the observed prolongation of action was probably due to the capability of the phosphorylated AChE, which BOS produces, to age rapidly. The myopathy observed with BOS began, as with ECO (Townsend, 1988), at the junctional region of the diaphragm but spread along the fibre with increasing time after administration (3.1.2). The early manifestations of anti-ChE-induced myopathy included hypercontraction of the junctional region, indicative that free Ca²⁺ was elevated in the sarcoplasm (Huxley, 1973). This hypercontraction could be seen with both ECO (Townsend, 1988) and BOS, and corresponded to the changes Hudson *et al.* (1985) observed at the endplate 30 minutes following injection of pyridostigmine to rats.

Discrepancies exist in the literature regarding the time course of anti-ChE-induced myopathy and its subsequent recovery. The time course of soman-induced necrosis was determined by Gupta *et al.* (1987b) in the rat. Using the method developed by Fenichel *et al.* (1972) they estimated the number of necrotic fibres in a sample of hemidiaphragm, finding that necrosis steadily increased, reaching a maximum 24 hours after a subcutaneous injection of soman, and then declined, displaying full recovery by 7 days. Similarly, Dettbarn (1984) followed the recovery of diaphragm muscle after single subcutaneous injections of soman and systox. Again the necrosis appeared to last for 1 to 3 days but had recovered by 7 days. Ariens *et al.* (1968) examined regeneration following DFP/paraoxon administration to rats. Such regeneration had begun 2 days after the injection, however, some necrosis was still apparent 10 days later. All of these studies reported a longer recovery period than seen in this investigation with BOS and that seen by Townsend (1988) with ECO. The reason for this discrepancy may lie in the technique used to assess the myopathy; the procion technique will show no myopathy if the integrity of the cell membranes alone has recovered. This may occur before the ultrastructural parameters used

to assess necrosis in the studies described earlier. To determine beyond doubt when recovery from necrosis is complete further ultrastructural work, particularly by electron microscopy, is required. This has been performed quantitatively with ECO, at an electron microscopic level, by Townsend (1988). The integrity of the cells examined recovered at times long after procion staining had ceased. Alternatively, the discrepancy may be due to a species difference between rat and mouse.

It is also clear that, as with ECO, extensive inhibition of diaphragm (86%) and blood AChE (68%) preceded the development of any myopathy. This critical level of inhibition, in the region of 85-90%, is comparable to that seen in other studies (Wecker *et al.*; 1978a,b, Gupta *et al.*; 1985, 1987a,b). Thus it is suggested that the myopathy is a catastrophic event produced by a rapid inhibition of AChE over a relatively short period of time, a criterion satisfied by both ECO and BOS. Once this event has occurred the necrosis will progress and recover over a predetermined time period, independent of further AChE inhibition.

The consequence of rapid AChE inhibition is the accumulation of ACh within the neuromuscular junction (Katz and Miledi, 1973) which results in an influx of Ca^{2+} into the cell (Ashley and Ridgeway, 1968; Evans, 1974; Miledi *et al.*, 1977) and thus an increase in the levels of intracellular Ca^{2+} . This effect has been shown to be one of the earliest biochemical effects of such inhibition and is thought to be a cause, rather than an effect, of myopathy (Townsend, 1988). Significant elevations of intracellular Ca^{2+} are even seen in low, non-necrotising doses of ECO (Das, 1989). However, influx of Ca^{2+} from extracellular fluid may not be the only source of elevated intracellular Ca^{2+} . Intracellular Ca^{2+} -buffers may release Ca^{2+} , most notably in the mitochondria and sarcoplasmic reticulum (SR) (Oberc and Engel, 1977). The prolonged action of ACh within the synaptic cleft during AChE inhibition will be reflected in a much prolonged depolarisation of the post-synaptic membrane, which may in turn promote release of Ca^{2+} from the SR (Endo, 1977). Alternatively, elevated intracellular Ca^{2+} can itself induce release of Ca^{2+} from the SR (Ford and Podolski, 1970; Endo, 1977; Fabiato, 1982). Another possibility is that the prolonged depolarisation produces an increase in intracellular Na^{+} which, in

turn, initiates release of Ca^{2+} from mitochondria via the $\text{Na}^+/\text{Ca}^{2+}$ transporter of the inner mitochondrial membrane (Affolter and Carafoli, 1980; Carafoli and Zurini, 1982). The fact that the mitochondria and the SR become swollen following treatment with an anti-ChE suggests that at least some of the intracellular Ca^{2+} is bound. The appearance of large, electron-opaque deposits, thought to be due to precipitation of excess Ca^{2+} with inorganic phosphate within mitochondria, (Greenawalt *et al.*; 1964, Townsend, 1988) implies that the loading of mitochondria with Ca^{2+} may play an important role in the development of anti-ChE-induced myopathy. Although Hudson *et al.* (1985) suggested that the myopathy observed is a random process, independent of fibre type, ultrastructural studies have identified that cells which appear resistant to necrosis following high dose anti-ChE have a high volume density of mitochondria (Townsend, 1988). Such cells may thus possess an ability to buffer higher concentrations of intracellular Ca^{2+} and hence necrosis would not be initiated. Such elevations in intracellular Ca^{2+} may initiate a number of potentially harmful processes which could lead directly to cellular necrosis. These include activation of CANP (Toth *et al.*, 1983) or the proteases of the lysosomal system (Kar and Pearson, 1978), superoxide production and the associated lipid peroxidation (Das, 1989), depletion of the glycogen and adenine nucleotide stores (Gupta and Dettbarn, 1987) and activation of phospholipases (Weglicki, 1980). As well as these biochemical changes, it is thought that there may be initiation of certain mechanical events within the contractile machinery of the affected cell. Portions of the cells may undergo contractions of far greater intensity than normally observed, as demonstrated by the areas of hypercontraction seen microscopically, which may directly result in rupture of the sarcolemma (Ferry, personal communication).

A link between the extent of myopathy and the rate of onset of AChE inhibition was indicated by the differing amounts of procion staining produced by the $4\mu\text{mol/Kg}$ and $8\mu\text{mol/Kg}$ doses of BOS 3 hours after a subcutaneous injection (3.1.1), a time at which the AChE inhibitions in the diaphragm were identical. However, further examination of the inhibition 30 minutes after injection revealed the inhibitory action of the $8\mu\text{mol/Kg}$ dose to be complete, whereas the $4\mu\text{mol/Kg}$ dose had yet to reach its maximal effect. This suggests that if the rate of onset of the extensive AChE inhibition was relatively slow then cells coped

with any associated trauma perhaps by Ca^{2+} sequestration or even its extrusion from the cell. Whereas, if the rate was much more rapid then the buffering capacities of such cells become overloaded resulting in a cascade of harmful events that could eventually lead to necrosis. Wecker *et al.* (1978a) reported that the faster the rate of inhibition produced by paraoxon in the rat, the greater the number of necrotic lesions. A significant amount of procion staining had occurred 30 minutes after ECO 0.5 $\mu\text{mol/Kg}$ (Townsend, 1988), whereas BOS 8 $\mu\text{mol/Kg}$ displayed no significant effect. This may have been a pharmacokinetic effect, that is, ECO appeared to be taken into the peripheral circulation at a faster rate than BOS, thus exerting its inhibitory effects more rapidly. Such an effect would also explain why signs of anti-ChE intoxication, in particular fasciculations, were seen much sooner after injection of ECO than after BOS.

4.1.2. BOS and muscle twitch.

BOS, like ECO (Morrison, 1977; Ferry, 1988), possessed the ability to potentiate twitch responses to low frequency, supramaximal, indirect stimulation (3.1.3). This effect was, as with other anti-ChEs, associated with repetitive action potentials within the muscle fibres (Morrison, 1977; Ferry, 1979; 1988, Clark *et al.*, 1983, 1984). Werner (1960) and Riker and Okamoto (1969) thought that the repetitive muscle firing was due to antidromic firing along the axon reflex, resulting in the recruitment of an entire motor unit. The origin of such antidromic activity being at the nerve terminals (Masland and Wigton, 1940).

It has also been shown that antidromic activity can be initiated after muscles are cut and prevented from discharging (Barstad, 1962), which indicates that the firing is at least in part due to a direct action at the nerve terminals, whether that be by the anti-ChE (Riker, 1975) or the transmitter itself. The work of Aizenman *et al.* (1986) revealed that transmitter release is essential for such antidromic activity, which tends to argue against a presynaptic action of the anti-ChE.

An alternative hypothesis expounded by a number of workers is that the action potentials within the muscle possess the capability to excite the nerve terminals

(Eccles *et al.*, 1942; Lloyd, 1942; Paton 1963; Ferry, 1988). It is also thought that it may in fact be the repetitive action potentials in the muscle which initiate antidromic activity, since during the first action potential, the nerve terminals will be refractory (Eccles *et al.*, 1942; Ferry, 1988).

It has also been suggested that K⁺ efflux, following prolonged endplate depolarisation in the presence of anti-ChEs, may be the initiator of antidromic activity (Diamond, 1959; Katz, 1962; Hohlfeld *et al.*, 1981). Two pieces of evidence, however, appear to contradict this hypothesis. Firstly, if K⁺ is able to elicit antidromic activity, then injection of KCl should elicit discharges, but none have been found (Feng and Li, 1941). Secondly, if ACh or anti-ChEs cause a significant efflux of K⁺, there should be an increase in spontaneous transmitter release, and there are reports which have failed to observe this (Fatt and Katz, 1952; Liley, 1956; Hubbard *et al.*, 1965). In contrast, however, Wecker *et al.* (1978b) found that an *in vivo* injection of paraoxon greatly increased the MEPP frequency in the phrenic nerve-hemidiaphragm preparation, as well as increasing quantum size and the amplitude of the first EPP of a train.

Over the 30 minute period examined in the present investigation, BOS displayed the biphasic effects observed by van der Meer and Meeter (1956) with DFP, that is an initial potentiation followed by a gradual fade towards control levels (3.1.3.2). This can be explained by an anti-ChE action, whereby cumulated ACh causes depolarisation (potentiation) followed by desensitisation (return to control). With BOS this desensitisation could be reversed by washout, resulting in a return of the potentiation (3.1.3.2). When Morrison (1977) examined the effects of ECO in the rat diaphragm he described a similar return of potentiation on washout. He also found a dose-dependent onset of the antidromic activity similar to the dose-dependent onset of twitch potentiation seen with BOS. This in turn was related to the differing rates of onset of AChE inhibition which were demonstrated *in vivo* (3.1.1). From this evidence he concluded that a direct action of ECO on nerve terminals was unlikely and proposed that ECO worked via an anti-ChE action to prolong the EPP, thus initiating repetitive muscle action potentials, and enhancing the action of ACh at the nerve terminals. An aspect of the potentiation seen with ECO, but not BOS, is a transient period of reduction in twitch height (figure 3.2). This may be due to another effect of ECO,

such as channel block (Tattersall, 1988), which reduces twitch height. This appears to be slower in onset than the potentiation and results in a short period of twitch height attenuation.

BOS also demonstrated the ability to initiate spontaneous muscle action potentials (3.1.4), an effect also displayed by many anti-ChEs (Masland and Wigton, 1940; Eccles *et al.*, 1942; Riker and Wescoe, 1946; van der Meer and Meeter, 1956; Blaber and Goode, 1968; Webb and Bowman, 1974). Two mechanisms for such activity have been suggested. Firstly, a direct depolarising action of the drug itself, since anti-ChEs such as ECO have been shown to possess agonist actions at the nicotinic ACh receptor (Tattersall, 1988). Secondly, an anti-ChE action, which allows the endogenous ACh to depolarise a pre-synaptic site, whether that be the pre-synaptic membrane or the first node of Ranvier. This pre-synaptic activation would lead to antidromic activity and thus contraction within the motor unit.

One piece of evidence against a direct agonist effect is the observation that DFP possesses the capability to produce spontaneous activity (van der Meer and Meeter, 1956). It is unlikely that DFP displays any direct effects since it cannot bind to the anionic site of the ACh receptor (Karlin, 1973; Rang, 1975). Similarly, DFP binds to the esteratic, rather than the anionic, site of the AChE molecule. Thus the simplest explanation of such activity in this case is that DFP acts by an anti-ChE action. However, it is quite possible that anti-ChE drugs such as ECO and BOS, which contain thiocholine groups within their structures, suggesting an ability to combine with the anionic sites on the ACh receptor and the AChE molecule, will also display agonist actions.

An observation of particular interest in the present investigation was the appearance of "hyperactivity" in some preparations following removal of BOS from the bath (figure 3.6). This was characterised by periods of intense spontaneous activity of high frequency leading to a tetanus-like effect, followed by periods of contractile silence. It is thought that these periods of silence were due to desensitisation induced by the magnitude of activity at pre-synaptic sites. The periods of desensitisation lengthened until contractile activity ceased. It is possible that the periods of "hyperactivity" observed are analogous to the bursts

of fasciculations seen *in vivo*.

Wecker *et al.* (1978a) suggested that the decisive factor was the duration of "cholinergic hyperactivity", be that spontaneous or impulse-evoked antidromic activity, triggered by a critical degree of AChE. The longer the enzyme remained inhibited (up to 2 hours), the longer the period of overactivity lasted, which in turn led to a proportionate increase in muscle fibre necrosis.

4.2. THE EFFECTS OF ANTICHOLINESTERASES ON THE VARIABILITY OF LATENCIES OF ACTION POTENTIALS.

4.2.1. The relationship between inhibition of diaphragm AChE and increases in jitter.

The main purpose of this aspect of the study was to determine whether the increases in jitter seen after anti-ChE treatment could be related to the reduction in AChE activity measured by the Ellman *et al.* (1961) technique at the time of the experiment. Thus, the most interesting observation brought to light by these studies was that, at times when the MCD and delay were at a maximum, the AChE activity, measured by the technique of Ellman *et al.* (1961), was at or near untreated levels for both ECO and BOS (table 4.1). In fact, with both compounds the increases in the MCD and delay returned to untreated levels at times long after AChE activity in the diaphragm had returned to normal. This observation is corroborated by similar work performed with sarin (Smith, personal communication). As discussed later (4.3), the time course of extracellular MEPPs, or more specifically the THA, was used in an attempt to find a more accurate indicator of physiologically important AChE. As can be seen in table 4.1, there appeared to be some relationship between the increases in jitter and the prolongation of the THA in that there were increases in jitter at times when the time course of extracellular MEPPs was significantly prolonged. Further work is required to discover whether such a relationship is genuine. However, it should be noted that the effects on jitter displayed no correlation with the AChE inhibition measured by the technique of Ellman *et al.* (1961) at the time of recording.

The effect on delay of BOS lasted longer than that of ECO and it may be that this was related to the prolonged inhibitory properties of BOS which were discussed earlier (1.4.1). Hence, it appears that the duration of the effects of ECO and BOS on jitter may be related to the duration of the reduction in AChE activity. It is possible that inhibition of AChE triggers a process which is maintained for some time after the initiating event has subsided.

The increases in jitter also displayed dose-dependency (3.2.1), however, these again appear to be unrelated to AChE inhibition, assayed by the technique of

(a)

Time after injection	MCD (μ sec)	Delay (μ sec)	AChE activity (nmol/min/mg)	THA (msec)
Control	9.9 \pm 3.6	32 \pm 22	1.34 \pm 0.43	0.42 \pm 0.05
1 day	23.7 \pm 12.1*	103 \pm 36*	0.54 \pm 0.05*	0.77 \pm 0.10*
3 days	27.9 \pm 32.9*	107 \pm 50*	0.61 \pm 0.07*	
5 days	25.6 \pm 28.0*	119 \pm 104*	1.27 \pm 0.23	0.64 \pm 0.11*
7 days	17.8 \pm 9.5*	61 \pm 37*	1.50 \pm 0.26	
15 days	14.7 \pm 7.6*	48 \pm 38		
27 days	10.1 \pm 3.4	25 \pm 23		

(b)

Time after injection	MCD (μ sec)	Delay (μ sec)	AChE activity (nmol/min/mg)	THA (msec)
Control	9.9 \pm 3.6	32 \pm 22	1.34 \pm 0.43	0.42 \pm 0.05
5 days	53.3 \pm 86.2*	162 \pm 181*	0.82 \pm 0.16*	0.88 \pm 0.18*
15 days	12.0 \pm 3.8*	45 \pm 28*	1.16 \pm 0.19*	0.49 \pm 0.05*
35 days	12.3 \pm 4.8	46 \pm 20*		
60 days	11.6 \pm 4.0	37 \pm 18		

Table 4.1. A summary of the recovery of MCD, delay, diaphragm AChE activity and the THA of extracellular MEPPs following single injections of (a) ECO 0.5 μ mol/Kg and (b) BOS 8 μ mol/Kg, with the values given as the mean \pm S.D. * significant difference from the relevant control ($p \leq 0.05$).

Ellman *et al.* (1961), 3 hours after injection. 0.3, 0.4 and 0.5 μ mol/Kg doses of ECO produced equivalent amounts of AChE inhibition at 3 hours, yet when the MCD and delay were measured 5 days after injection, a dose-dependent effect was observed. In fact the 0.3 μ mol/Kg dose displayed no significant effect on the MCD. It may be that it is the rate of onset of AChE inhibition that determines the extent of the jitter increases rather than the final level of inhibition. For example, it has been demonstrated that 4 μ mol/Kg and 8 μ mol/Kg doses of BOS produced an equivalent reduction in the AChE activity 3 hours after injection, yet the onset of this inhibition was slower with the lower dose (3.1.1). It has been suggested earlier (4.1) that this phenomenon may be the reason for the 8 μ mol/Kg dose producing a significantly greater amount of myopathy, as indicated by the procion technique. Thus, although there is no direct evidence for this hypothesis, the above may be a parallel in the studies of anti-ChE induced myopathy. A relatively simple experiment to confirm this idea would be to give the same dose of anti-ChE by one of two routes; firstly, as before, by subcutaneous injection, and secondly, by some slow-release mechanism. If the hypothesis was to hold, then when one examined the jitter 5 days later, higher values would be gained from animals injected with the anti-ChE by the subcutaneous route.

Kelly (personal communication) highlighted a number of reasons why the MCD and delay appear not to be related phenomena. 0.5 μ M ECO *in vitro* had a greater effect on delay than on MCD and also delay recovered before MCD after *in vivo* injection of ECO 0.5 μ mol/Kg. In addition, 1 μ mol/Kg BOS increased MCD but not delay, whereas 0.3 μ mol/Kg ECO increased delay without affecting the MCD. Furthermore, there was no correlation between the two parameters in the same muscle fibres, with or without the injection of ECO.

Three hours after injection, 1 μ mol/Kg BOS increased the MCD despite producing only 37% inhibition of diaphragm AChE, yet 0.3 μ mol/Kg ECO inhibited 88% of diaphragm AChE at the same time point but had no effect on the MCD. By 5 days, however, diaphragm AChE showed complete recovery from injections of ECO whereas animals injected with 1 μ mol/Kg BOS maintained 28% inhibition. This suggests that increases in the MCD may be related to prolonged inhibition of AChE over several days rather than acute

effects. Increases in the delay appear to require a high, acute level of inhibition. In the doses examined only those which produced inhibition in the region of 87% or greater 3 hours after injection had a significant effect on delay by 5 days. In summary, from the evidence gained thus far from the diaphragm it appears that the MCD may be related in some way to prolonged inhibition of muscle AChE whereas the delay seems to require its rapid, maximal inhibition. These hypotheses require further in depth investigation before they can be confirmed, for example, if the theory about the MCD were to hold then repetitive low doses of ECO might be expected to increase the MCD without affecting delay.

4.2.2. The weight loss experienced with anti-ChEs and its effect on jitter.

Mice injected with BOS 8 μ mol/Kg displayed a significant reduction in their mean weight for 4 days after the injection, due to a reduced intake of food and water (3.2.3). The weight loss occurred during the first 2 days of exposure, a period during which signs of intoxication were at a maximum, followed by a steady recovery to untreated levels by the fifth day. This 13% weight loss can be compared to the 25% weight loss observed by Gupta *et al.* (1986) in rats exposed to multiple low doses of DFP. They stated that maximal weight loss occurred when the severity of signs was greatest. Mice exposed to an equivalent amount of dietary restriction (3.2.3) displayed weight loss which was not significantly different to that observed in animals injected with BOS 8 μ mol/Kg. This suggested that the weight loss was due to dietary restriction alone rather than to any metabolic effect.

Such dietary restriction did not contribute to the increases in jitter observed with BOS. In fact, it produced a small but significant reduction in the jitter. A possible explanation for this reduction in jitter is that, after dietary restriction, there might have been an increased quantum content of EPPs or a reduced threshold for excitation of the muscle fibre, which would increase the safety factor for neuromuscular transmission. With an increased safety factor, the rundown of EPP amplitudes at the beginning of a train of stimuli could mean that action potentials would be generated lower down the rising phase of the EPP, a point at which the rise is steeper and less likely to increase variability. This would reduce the plateau MCD and initial delay of action potentials.

There is at least some evidence that this may be possible. Kelly (1979) showed that 30 day old rats exposed to one weeks dietary restriction displayed an increase in the mean quantum content of the first EPP of a train of EPPs, without having any effect on the quantum contents of plateau EPPs elicited at 10Hz. He concluded that this increase could have been due either to an increase in the store of quanta for release, or to an increased probability of release or a combination of the two. An increase in these parameters would increase the quantum content of the first EPP without affecting the quantum content of the plateau EPPs, since this is determined by the rate of mobilisation. Kelly (1979) also showed that there was a decrease in the safety factor in these animals after dietary restriction, but after the rats were returned to food *ad libitum* there was a rapid increase which "overshot" untreated values. In the present investigation the food and water available was returned to untreated levels gradually over the 5 day period in an attempt to mimic the effects of BOS. In fact, weight gain had commenced by the third day. It is possible that such an "overshoot" of the safety factor had occurred by the fifth day when electrophysiological recordings were made. It is, however, important to note that the observations of Kelly (1979) were made in young animals; the same study showed that older animals were more resistant to dietary effects.

4.2.3. The effects of anti-ChEs on the jitter of the soleus and EDL muscles.

Five days after albino mice were injected with 0.4 μ mol/Kg ECO the diaphragm, soleus and EDL muscles all displayed a significant increase in the MCD and delay. In fact, the soleus showed a significantly greater increase in the delay than the other two muscles. Five days after 0.1 μ mol/Kg ECO only the soleus exhibited significant increases in the MCD and delay. These results suggest that the soleus, a slow twitch muscle, was more susceptible to ECO than the diaphragm and EDL, which are fast twitch muscles (3.2.4).

It is possible to explain this observation in terms of differential AChE inhibition in the various skeletal muscles due to the anti-ChE having different pharmacokinetics. Soleus and diaphragm have been shown to display a greater proportion of AChE inhibition than that seen in the EDL of the rat, after soman (Gupta *et al.*, 1987a) or tabun (Gupta *et al.*, 1987b). These varying degrees of

AChE inhibition correlate well with the observed differences in the number of necrotic lesions within the muscle (Wecker *et al.*, 1978a; Gupta *et al.*, 1987b). To be more precise, it has been determined that inhibition of the 16S form of AChE is the trigger mechanism which leads to necrosis. The level of the 16S form of AChE can be assessed by its sedimentation coefficient and electrophoretic mobility, and is equivalent to the asymmetric form of the enzyme located extracellularly as part of the basal lamina and which was designated A12 by Bon *et al.* (1979). It is this 16S form which is thought to be the functionally important endplate enzyme (Hall, 1973). Gupta *et al.* (1987a,b) demonstrated an absence of inhibition of the 16S form of AChE in the EDL coincident with a lack of lesions in the same muscle after soman and tabun treatment. In contrast, the soleus and diaphragm both developed necrosis following significant inhibition of the 16S form of AChE.

It is important to note that there is evidence that the recordings made in this study were not made in necrotic fibres. Firstly, because a greater proportion of muscle fibres had increased MCD and delay than showed histological damage after an injection of ECO, and secondly, because visibly damaged fibres seemed to be electrically silent 24 hours after injection. Finally, doses of anti-ChE which did not produce necrosis in the diaphragm increased MCD or delay (Kelly, personal communication). Nevertheless, as stated earlier, the increases in jitter were thought to be at least initiated by AChE inhibition, thus knowledge of this differential inhibition from muscle to muscle is important.

In contrast to the effects seen with soman and tabun, other inhibitors such as paraoxon (Wecker *et al.*, 1978a) and DFP (Gupta *et al.*, 1985, 1986) showed the slowest rate of AChE inhibition and the lowest number of necrotic lesions in the soleus. Several possible explanations for this differential necrotic activity between inhibitors were put forward by Meshul *et al.* (1985). They include pharmacokinetic variables which influence the delivery of a particular organophosphate, variations in the location of AChE in soleus and EDL (Dettbarn, 1981, 1984; Groswald and Dettbarn, 1983), and changes in ACh release due to different firing patterns (Misulis *et al.*, 1987).

Alternatively, the reason for the increased susceptibility of the soleus could be

due to the nature of its fibre type, which is predominantly intermediate. It could be that the buffering capacity of the sarcoplasm, which is responsible for containing any potentially lethal rises in intracellular ions such as Ca^{2+} , is lower in the soleus fibres compared with the EDL and diaphragm. This would make such fibres more susceptible not only to necrosis but also to subtle alterations in neuromuscular transmission. However, there is a preponderance of mitochondria in the fibres of the soleus, the organelles which perform much of this cellular buffering, suggesting that this may not be the case (Close, 1972). Nevertheless, this does not preclude other mechanisms by which such fibres express increased susceptibility to anti-ChEs. There is evidence that the sarcolemma of slow twitch muscle fibres possess a $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism which those of fast twitch muscles do not (Leoty, 1984), and this property in itself may provide a likely mechanism for the soleus' increased susceptibility. Following anti-ChE treatment the action of ACh is extended within the cleft, which is manifested in the prolongation of the time course of endplate currents (3.3). This may result in an increased Na^+ concentration within the cell which in itself may be detrimental. However, if there is active $\text{Na}^+/\text{Ca}^{2+}$ exchange across the membrane this could lead to an overload of Ca^{2+} within the fibre, along with the cascade of detrimental actions which such an event stimulates.

In conclusion, it is clear that the observations in this section of the study may be due to a number of factors which may in themselves be interactive. Firstly, it may be due to the pattern of activity experienced within the different muscles, that is fast, phasic activity as opposed to those which are slow and tonic. In addition, there may be other criteria which involve the fibre type characteristics, such as Ca^{2+} buffering or $\text{Na}^+/\text{Ca}^{2+}$ exchange. Finally, the pharmacokinetics of the anti-ChE chosen may play an important role.

4.2.4. The prevention of anti-ChE induced increases in jitter with carbamates and oximes.

4.2.4.1. The effect of pretreatment with a single dose of pyridostigmine on ECO-induced increases in jitter.

Certain carbamates, pyridostigmine in particular, have been shown to protect against organophosphorus poisoning in a number of species when used

prophylactically together with atropine (Dirnhuber and Green, 1978; Gordon *et al.*, 1978; Wecker *et al.*, 1978a; Dirnhuber *et al.*, 1979; French *et al.*, 1979). The work of Dirnhuber *et al.* (1979) demonstrated that a maximum "sign free" dose of pyridostigmine iodide (200 μ g/Kg) offered considerable protection against intoxication with soman. In the same study a lower, 50 μ g/Kg, dose was shown to be as effective. Townsend (1988) was able to prevent ECO-induced myopathy in the mouse by pyridostigmine pretreatment, in a dose equivalent to that used in this study, 2 hours or less prior to the anti-ChE. Despite Dirnhuber *et al.* (1979) assigning such doses as "sign free", there is evidence that they cause subtle morphological changes indicative of subcellular disturbances (Hudson *et al.*, 1985; Meshul *et al.*, 1985; Townsend, 1988; Das, 1989).

As stated earlier (1.5), the mode of action of pyridostigmine is thought to be by reversible inhibition of a portion of the functionally important AChE, resulting in its protection from irreversible inhibition by organophosphorus agents. After poisoning, decarbamylation (Wilson *et al.*, 1960, 1961) occurs along with removal or destruction of the carbamate, producing sufficient free AChE to restore normal function (Berry and Davies, 1970; Dirnhuber and Green, 1978; Dirnhuber *et al.*, 1979). This study has shown that 0.38 μ mol/Kg (100 μ g/Kg) pyridostigmine given 30 minutes prior to a dose of ECO 0.5 μ mol/Kg produced a 12% reduction in the AChE inhibition, from the 87% inhibition seen with ECO alone. This observation is consistent with, and supports the view that only low levels of AChE protection are necessary for relatively normal neuromuscular function (Hobbiger, 1976). French *et al.* (1979) found that maintenance of neuromuscular function was attained by protection of only 5% of total AChE by pyridostigmine. However, it should be remembered that the AChE levels in the present study and that of French *et al.* (1979) were measured by the technique of Ellman *et al.* (1961), which has been shown to be inadequate in accurately measuring physiologically important AChE. It may be that although the enzyme protected is only a small percentage of total AChE within the preparation it may all be of the asymmetrical form and thus functionally important. Thus if a method was devised to accurately measure this population of AChE one may find that pyridostigmine, in the doses utilised, protects quite a large proportion.

Other actions of carbamates have been suggested. Eccles and MacFarlane (1949) thought that the curare-like depression of ACh sensitivity in the presence of a carbamate may be due to competitive inhibition (Siefert and Eldefrawi, 1974) or binding to other sites on the the nicotinic ACh receptor (Carpenter *et al.*, 1976). These hypotheses have been confirmed by recent patch clamp studies with pyridostigmine. Its ability to prolong both endplate currents (EPCs) and miniature endplate currents (MEPCs), as well as its action as a weak agonist capable of inducing desensitisation, have been demonstrated in the frog (Albuquerque *et al.*, 1984). Pyridostigmine (50-100 μ M) decreased the frequency of channel opening events activated by ACh and induced a modified form of the ACh channel currents. In the presence of pyridostigmine channel conductance was lower, though channel lifetime remained unaltered or only slightly prolonged. In addition, channel openings were frequently interrupted by fast flickers (Akaike *et al.*, 1984). It is, however, important to related these reports in the context of the concentration of pyridostigmine used in this study. If one assumes a total volume distribution of 10ml in a mouse of 40g then the maximum concentration of the carbamate at the neuromuscular junction is likely to be 1.5 μ M, suggesting channel block, in this situation, may have a negligible effect.

Whether the prophylaxis produced by pyridostigmine is by protection of a portion of the AChE, by some direct action or by a combination of the two, is unclear. Nevertheless, 0.38 μ mol/Kg pyridostigmine given 30 minutes before a 0.5 μ mol/Kg dose of anti-ChE prevented the increases in MCD and delay when measured 5 days later (3.2.5.1).

4.2.4.2. The effect of single doses of 2PAM on ECO-induced increases in jitter.

Administration of 110 μ mol/Kg 2PAM upon commencement of ECO-induced fasciculation, like pyridostigmine, prevented the increases in jitter seen with ECO alone 5 days after injection (3.2.5.2). If the 2PAM was administered 2 or more hours after the dose of ECO, no reduction in the MCD was observed. In contrast, 2PAM could be given up to 24 hours after ECO, and still produced a significant reduction in the delay, further suggesting that the MCD and delay are unrelated phenomena (Table 3.18).

2PAM has been found to prevent the development of myopathies induced by paraoxon (Laskowski *et al.*, 1977; Wecker *et al.*, 1978a,b), parathion, phosdrin (Brachfeld and Zavron, 1965) and DFP (Ariens *et al.*, 1968, 1969), but was relatively ineffective against soman (Loomis and Salafski, 1963; Heilbronn and Tolegan, 1965; Murtha *et al.*, 1970). The effectiveness of the oxime treatment was however dependent on the delay with which it was administered after the organophosphorus intoxication (Townsend, 1988).

Many workers have reported successful reactivation of AChE by oximes, if applied soon after its inhibition by organophosphorus compounds (Lipson *et al.*, 1969; Murtha *et al.*, 1970; Laskowski and Dettbarn, 1977; Wecker *et al.*, 1978a). They have been thought for many years to act by nucleophilic attack of the phosphorus atom at the enzyme inhibitor complex. The organophosphorus moiety is thus removed and hydrolysed, and the oxime residue then undergoes a further reaction to regenerate the active enzyme (Durham and Hayes, 1962). The diminished effectiveness with time of 2PAM against organophosphorus-induced myopathies is believed to be due to ageing of the inhibited enzyme (Lipson *et al.*, 1969; Durham and Hayes, 1962). After ageing has occurred the inhibited enzyme will no longer yield to nucleophilic attack, which is the basis of oxime reactivation (Lipson *et al.*, 1969). The lack of effectiveness of 2PAM against soman intoxication is thought to be due to rapid ageing of the soman-AChE complex (Loomis and Salafski, 1963; Murtha *et al.*, 1970).

Even though 2PAM, injected on the commencement of fasciculation, prevented the increases in jitter usually observed after ECO, there was only a 13% reactivation of diaphragm AChE 3 hours after injection of ECO. It has been suggested that such reactivation is not sufficient to explain recovery of neuromuscular transmission. It is possible that at least some of this recovery may be due to one of the range of recently reported additional properties of oximes at frog (Alkondon *et al.*, 1988) and mouse (Tattersall, personal communication) endplates. Alternatively, it could be that the amount of enzyme reactivation, indicated 3 hours after injection, does not give a true representation of the reactivation occurring at earlier times. It has been demonstrated earlier *in vitro* (table 3.35) that 2PAM possesses the ability to

reactivate substantial quantities of AChE if it is reached in large enough concentrations. Hence, there is the possibility that, at times earlier than 3 hours, there is a significantly greater amount of AChE reactivation which, at the time of assay, has been re-inhibited by the ECO still present. The discovery that pyridostigmine and 2PAM prevented the increases in jitter confirmed the centrality of the inhibition of AChE in the induction of such increases.

4.2.4.3. The effect of 2PAM on BOS-induced increases in jitter.

Single doses of 2PAM given on fasciculation produced no reactivation of diaphragm AChE 3 hours after injection of BOS. This is believed to be due, as with soman, to the rapid ageing of the BOS-AChE complex (Loomis and Salafski, 1963; Murtha *et al.*, 1970; Harris *et al.*, 1971). Likewise, 2PAM, given on commencement of BOS-induced fasciculation, had no effect on the increases in jitter when recorded 5 days later (3.2.5.3). However, if 7 injections of 2PAM were given over a 12 hour period, as described in section 3.2.5.3, a significant reduction in the BOS-induced jitter increases was observed. This effect is similar to that seen by Smith and Muir (1977) and Smith *et al.* (1981) who were able to produce recovery of neuromuscular function even when the inhibited AChE had undergone ageing and was no longer open to reactivation. This may again be due to the other effects of oximes which have been identified (Alkondon *et al.*, 1988; Tattersall, personal communication). These results stimulated an investigation into these actions at a macroscopic level (sections 3.4 and 4.4).

The number of injections of 2PAM was increased to 12 over a 48 hour period (3.2.5.3) in the hope that prolonged, repetitive dosing would maintain the levels of 2PAM within the synaptic cleft, thus increasing the probability of protection by some direct action being observed. In fact, following this 12 injection protocol, no reduction in the MCD and delay was seen. At 5 days, animals treated with BOS 8 μ mol/Kg followed by the 12 injection protocol displayed a significantly greater prolongation of the THA of extracellular MEPPs than animals injected with BOS alone. Furthermore, if animals were administered the 12 injection protocol alone, recorded extracellular MEPPs also displayed significant prolongation of the THA (Table 3.20). A possible explanation for this phenomenon is that during the prolonged administration of 2PAM, whose half-life for elimination is 1 to 1.5 hours (Sidell and Groff, 1971; Josselson and

Groff, 1978), there was a steady rise in the plasma concentration and hence the concentration within the synaptic cleft. At these high concentrations, 2PAM may have other actions which have a detrimental, rather than a protective, effect.

The picture was further complicated by the observation that the 12 injections protocol alone produced significant prolongation of the THA of extracellular MEPPs 5 days after the first injection. This prolongation was not significantly different from that observed with BOS alone. The same protocol, however, had no effect on jitter whereas BOS 8 μ mol/Kg produced significant increases in both the MCD and delay at the same time point.

Thus, it appears that 2PAM is a molecule possessing a range of properties which are displayed to varying degrees at different concentrations. A simple hypothesis would be that 2PAM has at least two properties besides that of enzyme reactivation. Firstly, an action which is protective and secondly one which compromises neuromuscular transmission. Although the 12 injection protocol of 2PAM appeared to display a similar prolongation of THA to that of BOS 8 μ mol/Kg at 5 days, the other actions of the oxime may have been sufficient to prevent triggering of the mechanism which produces jitter increases. However, when the inhibitory actions of BOS are combined with the detrimental effect of the 12 injections of 2PAM any protective effect may not be great enough to prevent substantial increases in the MCD and delay.

4.2.5. Jitter in the Bar Harbor Rej 129 dystrophic mouse, with and without anti-ChE treatment.

At no point in this section of the study was any difference found in the jitter recorded in normal littermates and dystrophic mice, apart from the EDL of the normal animals having a slightly higher MCD than that displayed by their dystrophic counterparts after exposure to 0.1 μ mol/Kg ECO for 5 days (3.2.6). This suggests that the EDL of the dystrophic mouse was more resistant to the effects of ECO than that of the normal littermate. Alternatively, there may be a population of fibres within the EDL of the dystrophic mouse which may be more susceptible and necrotise. A similar population of fibres may occur in the

EDL of the normal littermate which although compromised have less of a necrotic tendency. Since action potentials cannot be recorded from necrotic fibres it would appear in this situation that the EDL of the normal littermate was affected far more than the same muscle in the dystrophic animal.

The 12 week old dystrophic mice exhibited a greater mortality rate than their normal equivalents following anti-ChE treatment. An insight into this increased susceptibility may come from the observation that some dystrophic muscles display a decreased sensitivity to curare. In particular, certain hind-limb muscles have been shown to possess this property (Baker and Sabawala, 1963; Kelly *et al.*, 1986). However, there appeared to be no difference in the isolated mouse diaphragm (Harris and Ribchester, 1979a). A number of explanations for this phenomenon have been put forward. One is that there is an increase in ACh receptors on the postsynaptic membrane of dystrophic EDL muscles (Howe *et al.*, 1976, 1977), however, this is not consistent with certain binding studies (Marusyk and Monckton, 1976; Matthews-Bellinger, 1980; Kelly *et al.*, 1986).

Another explanation for such curare resistance is that in murine muscular dystrophy there is an abnormality at some presynaptic site which increases evoked ACh release (Beaulnes *et al.*, 1966). This view is supported by the work of Kelly *et al.* (1986) who were able to show that the dystrophic EDL displayed a greater EPP quantum content and a greater initial rundown of EPPs. However, in contrast, some studies failed to find any differences between the quantum contents of dystrophic and normal mouse muscle (Carbonetto, 1977; Harris and Ribchester, 1979b), although these discrepancies may be due to the type of statistics used to estimate such quantum contents.

Quantum content is believed to be the product of the number of quanta available for release (n) and the probability (p) that a quantum will be released by a particular stimulus (del Castillo and Katz, 1954b). Hence, an increase in quantum content must be due to an increase in n and/or p . Kelly *et al.* (1986) could find no difference in the mean value for n between normal and dystrophic EDL muscles, but the mean value for p was significantly greater. They suggested that such increases in p were unlikely to be the result of increased intraterminal Ca^{2+} ion concentration and was more likely due to a

difference in the neuronal membrane at these dystrophic nerve terminals.

Ferry (1988) examined the effect of reduced Mg^{2+} , which itself increases quantum content, on ECO-induced potentiation of single twitches. He found that the neuromuscular block, which followed the initial potentiation, was far greater in the environment of reduced Mg^{2+} . If this situation is paralleled to dystrophic mice injected with an anti-ChE, it is possible to suggest that the greater quantum content of their muscles produces a rapid neuromuscular block. This may be fatal if it occurs in respiratory muscles, leading to death by respiratory failure.

Table 3.23 shows that there appears to be an inherently greater jitter in the soleus muscles of the hindlimb than in the EDL, particularly for the dystrophic animals. No differences were found between soleus muscles of the dystrophic and normal mice and, unlike albino mice, $0.1\mu\text{mol/Kg}$ did not increase jitter. These observations may at first appear difficult to reconcile since fast twitch muscles have been shown to exhibit greater myopathy than slow twitch muscles (Goldspink and Rowe, 1968; Shafiq *et al.*, 1969). But, as stated earlier, there is evidence to suggest that, when measuring jitter, one is not recording from necrotic fibres (Kelly, personal communication). Thus, the extent of myopathy may not be directly related to the increases in jitter. One possible explanation is that the soleus muscle displays a greater resistance to necrosis than the EDL. In the passage of time there is a natural turnover of cells in a muscle, involving cell death and regeneration. The EDL may display a greater susceptibility to this process and would thus contain more myopathic cells from which action potentials cannot be recorded. The soleus muscle on the other hand may exhibit a resistance to these processes, possibly from the increased buffering capacity of its predominant fibre type, thus displaying less cell mortality and hence less myopathy. However, there may be a substantial challenge to the cell sufficient to cause disturbance of neuromuscular transmission apparent in the jitter increases.

Alternatively, the soleus muscles could have a reduced safety factor when compared with the EDL, whether that be via a reduction in the quantum content of EPPs or via an increased threshold. Action potentials would thus be

generated near the peak of the EPP, introducing extra variability in a train of stimulation. This, however, seems unlikely, since some dystrophic muscles actually display an increase in safety factor (Harris and Ribchester, 1979b).

SFEMG studies have been performed in many forms of muscular dystrophies. However, Stalberg and Trontelj (1979) reported that jitter increases were only observed in the more affected muscles of patients in which the disease was progressive. The origin of these jitter increases in dystrophy are unclear although several possible reasons have been cited. Firstly, it may be due to irregularities in impulse propagation in degenerating muscle fibres before complete transmission failure takes place. Secondly, there could be disturbed motor endplate function. Motor endplate abnormalities such as focal atrophy of the synaptic folds have been reported, with preservation of the ACh receptors (Engel *et al.*, 1977). A third possibility is that impulse transmission disturbances may be the result of uncertain transmission in regenerating fibres, which are formed from satellite cells. Finally, such disturbances are often seen in cells undergoing re-innervation, particularly in newly formed nerve sprouts and immature motor endplates (Stalberg and Trontelj, 1979).

Whatever the reason for increased jitter in patients suffering from muscular dystrophy it is clear from this and other studies that such increases, although indicative of neuromuscular disturbance, cannot be used as an early clinical sign of such disease states.

4.2.6. The origin of anti-ChE-induced increases in jitter.

Although an investigation into the origin of the anti-ChE induced increases in jitter has not been within the brief of this study, it is interesting to relate the experiments of Kelly (unpublished) which addressed this problem.

It had been suggested that the increases in jitter were the result of an increased variability of EPP rise times (Stalberg and Trontelj, 1979). Kelly (unpublished) has examined the aetiology of this problem in much more detail. In untreated preparations, there seemed to be little if any extra latency variability or delay introduced by the generation of the action potential or its conduction along the

muscle fibre. This was indicated by the MCD and delay of the EPPs being sufficient to account for the MCD and delay of action potentials recorded near the tendon. After ECO treatment, the increases in jitter could not be accounted for by any increases in jitter of the EPPs, suggesting that the actions of the ECO were postsynaptic, be that some sort of compromise of action potential propagation along the muscle fibre or muscle action potential generation.

When muscle fibres were stimulated directly near one tendon and the action potential generated was recorded near the other tendon, no difference between the MCD and delay in untreated or ECO-treated preparations was discovered even though the action potentials were recorded after passing through the endplate region. These results suggested a necessity for neuronal generation of action potentials before such jitter increases could be observed and that a variability in propagation along the fibre did not seem to be involved.

The alternative hypothesis was that the generation of the action potential near the endplate was the likely origin of anti-ChE induced increases in jitter. For example, after anti-ChE treatment, there could be a decrease in the quantum content of EPPs or an increase in the threshold necessary for the excitation of the muscle fibre, which would reduce the safety factor of neuromuscular transmission. With a reduced safety factor, the rundown of the EPP amplitudes at the beginning of a train of stimuli could mean that action potentials would be generated closer to the peak of the EPP during the train. This could increase the plateau MCD and initial delay of action potentials. Kelly (unpublished) found that there was a decrease in MEPP amplitude 5 days after ECO treatment, which was thought to be due to a reduction in the input resistance, suggesting a possible decrease in the safety factor. However, when the safety factor was calculated no significant alteration was discovered.

Kelly (unpublished) found that, 5 days after ECO treatment, there was an increased variability of EPP amplitude, which suggests that there may be increased variability of the take-off point of the action potentials from the EPPs.

The final hypothesis which as yet has not been investigated, is that after the ECO treatment, there is an alteration in the membrane properties around the

endplate which increases the area over which the muscle action potential can be generated. This larger area will cause action potentials to be generated at slightly different points. Thus, jitter increases would be due to an increased variability in conduction times.

4.3. THE RELATIONSHIP BETWEEN BIOCHEMICALLY MEASURED LEVELS OF AChE AND THE TIME COURSE OF EXTRACELLULARLY RECORDED MEPPs.

4.3.1. The relationship 3 hours after injection of an anti-ChE *in vivo*.

The aim of these experiments was to relate the extent of the prolongation of the decay phase of extracellularly recorded MEPPs after exposure to an anti-ChE to biochemically measured AChE, in the belief that the time course of extracellular MEPPs may be indicative of physiologically important AChE. A similar study was performed by Ferry and Marshall (1971) using prolongation of EPPs as an index of the increased transmitter action after anti-ChEs in the rat. They produced a calibration curve relating the experimentally determined prolongation of the EPPs and calculated inhibition of the physiologically important AChE. The AChE inhibition was calculated using pseudo first-order kinetics whereas, in this study, AChE activity was measured by the technique of Ellman *et al.* (1961), since the main purpose was to determine how the biochemical assay was related to physiologically important AChE.

The relationship between the prolongation of the THA of extracellularly recorded MEPPs and the percentage inhibition of AChE, measured by Ellman *et al.* (1961), was non-linear (3.3.1). At low levels of inhibition there was a small, but significant, increase in the THA, followed, at inhibitions greater than approximately 60%, by a sudden, rapid increase. This effect was shown to be independent of the type of organophosphorus compound used, whether it be tertiary, quaternary, rapidly aged or open to reactivation. These observations implied that the two methods do not measure the same populations of AChE. For example, the Ellman *et al.* (1961) technique may determine the AChE activity within tissue homogenates which may contain intracellular forms of AChE, that also possess the ability to hydrolyse acetylthiocholine. However, these forms of the enzyme perform no role in the termination of transmitter actions, indicated by the THA.

An alternative hypothesis is that the relationship could be due to a diffusion effect. Low doses may only inhibit AChE at the periphery of the synaptic cleft,

and it may be that this enzyme is not important in terminating the effects of spontaneously released quanta. With higher doses, the physiologically important enzyme deep within the cleft may then be inhibited, creating the sudden, rapid increase in THA which was seen. Lancaster (1973) demonstrated that the quaternary nature of some compounds restricted their access into the neuromuscular junction when compared with their tertiary analogues. The relationship 3 hours after injection was consistent for both quaternary and tertiary compounds which may suggest that the reason for its non-linearity was not diffusion related. A variation on this idea is that the decay of extracellular MEPPs depends upon 3 parameters, AChE activity around the peaks of the post-junctional folds in the proximity of the ACh receptors, AChE activity deep within the junctional folds and diffusion of the transmitter out of the synaptic cleft. It is possible that after low doses of anti-ChEs the AChE around the receptors is inhibited, whereas the inaccessible enzyme found within the folds is relatively unaffected. Thus, when a quanta of transmitter is released the time course of its action is almost unchanged, since it is either rapidly hydrolysed by the vast amounts of AChE still present within the folds or it diffuses out of the cleft. However, on treatment with a higher dose of anti-ChE virtually all the enzyme is inhibited, thus termination of transmitter actions is solely determined by diffusion, during which further receptor interactions can occur.

Finally, a third possibility is that the relationship is due to the excess of AChE within the synaptic cleft, the function of this enzyme being to maintain the integrity of neuromuscular transmission. Studies have shown that in mouse diaphragm the number of AChE binding sites, determined by the binding of [³²P]- or [³H]- DFP, is 3×10^7 (Waser and Reller, 1965; Barnard *et al.*, 1971b), whereas it is 4.6×10^7 in the rat (Barnard *et al.*, 1971a). From this Hobbiger (1976) estimated that there were ten active enzyme sites for each molecule of ACh released by a nerve impulse. Thus, substantial inhibition of AChE could occur in the cleft before a pronounced effect on the THA would be seen.

4.3.2. The effect of anti-ChEs on the shape of the decay phases of extracellular MEPPs.

During his analysis of the effects of prostigmine on the time course of EPPs in the frog, Kordas (1972a) found that the falling phase of the potential could be

divided into a non-exponential phase close to the peak, followed by an exponential portion, the duration of both phases varying under different experimental conditions. The exponential portion is believed to be due to the random closure of the ion channels associated with the ACh receptor, prior to the dissociation of the transmitter-receptor complex (Colquhoun, 1971). Kordas (1972a) reported a prolongation of the non-exponential phase of the EPP decay in the presence of prostigmine which is very similar to observations made in this study. The present investigation showed that, as the dose of anti-ChE was increased, the proportion of the decay which could be fitted by a single exponential was reduced, until, by maximal inhibition there was little if any exponential fit (3.3.1.4). These findings are also consistent with those reported by other workers (Goldner and Narabashi, 1974; Linder *et al.*, 1984; Madsen *et al.*, 1985). It has been tentatively suggested (Magleby and Terrar, 1975; Linder *et al.*, 1984) that this curvature reflects the steady diminution with time of the likelihood that any one ACh molecule, when it is released by an ACh-receptor complex, again acts to open a channel. This decline in probability arises from the requirement of two ACh molecules for channel opening, which makes the rate of channel opening proportional to the square of the local ACh concentration (Katz and Thesleff, 1957b; Dreyer *et al.*, 1978; Colquhoun, 1978; Adams, 1981).

It was thought by some groups that free diffusion from the synaptic cleft was too rapid to account for the prolonged time course of such potentials after anti-ChE treatment (Eccles and Jaeger, 1958; Kuba and Tomita, 1971; Magleby and Stevens, 1972a). This led to the suggestion that the prolongation might be a consequence of an increase in the mean lifetimes of channels activated by ACh (Kuba and Tomita, 1971; Magleby and Stevens, 1972a). However, this was dismissed by the subsequent experiments of Katz and Miledi (1973) who, based on a spectral analysis of voltage-noise accompanying the action of ACh, reported that anti-ChEs did not greatly affect the mean lifetime of these channels. They hypothesised that the prolongation which occurs in the presence of an anti-ChE is due to the diffusion of the excessive amounts of ACh out of the synaptic cleft. During this diffusion the molecules repeatedly bind to receptors in their path, thus prolonging the potential. In contrast, when AChE is fully active such repetitive binding does not occur, since as soon as the transmitter-receptor complex dissociates, the ACh is rapidly hydrolysed. Figure 4.1 represents a



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Figure 4.1. Hypothetical distribution of activated ACh receptors after release of three quanta onto neighbouring sites on the subsynaptic membrane. Density of activated receptors (open ion channels) is shown on the z-axis; the x,y-plane represents the post-synaptic membrane. The progressive change in the number of open channels is drawn at three different times: T_1 at the peak of the synaptic current, T_2 and T_3 during its falling phase. (A) When AChE is fully active, there is little opportunity for lateral diffusion from the point of release, and within 0.3 msec the ACh is bound to the receptors. The declining phase of the current is due to the closing time of the ionic channels. (B) When AChE is inhibited, ACh is able to diffuse along the synaptic membrane so that at T_2 and T_3 it covers overlapping areas, leading to interaction and prolongation of the synaptic current (Hartzell *et al.*, 1975).

similar situation in which three quanta have been released in the presence and absence of AChE. There are a number of other reports which support these ideas (Hartzell *et al.*, 1975; Magleby and Terrar, 1975; Linder *et al.*, 1984).

4.3.3. The recovery of the THA of extracellularly recorded MEPPs following single injections of anti-ChEs.

It has been established in this study (3.3.4) that at intermediate time points, 7, 5 and 5 days after *in vivo* injection of Sarin, ECO and BOS respectively, the relationship between the THA of extracellular MEPPs and the inhibition of diaphragm AChE does not obey the relationship at 3 hours. That is, at these time points the prolongation of the time course of the extracellular MEPPs appears to last longer than the AChE inhibition.

There are two possible explanations for these discrepancies at intermediate time points. Firstly, it may be that the high levels of ACh, experienced within the cleft for long periods after AChE inhibition, cause an alteration in channel properties. There are a number of ways in which this could be achieved, it could in some way prolong the mean lifetime of the channel or diffuse to extrajunctional receptors with different gating characteristics. On the other hand, there could be a shift in the population of the channels at the endplate.

Alternatively, during *de novo* synthesis of new diaphragm AChE, following its irreversible inhibition, there could be production of large amounts of the low molecular weight forms of AChE prior to its biosynthesis into physiologically important AChE. This low molecular weight AChE would be measured by the Ellman *et al.* (1961) technique since it possesses the ability to hydrolyse acetylthiocholine, the substrate used in the assay. However, it would not aid destruction of quantally released ACh, hence the potentials would remain prolonged.

Relevant to the first hypothesis are the experiments of Tattersall (personal communication) who examined the effects of ECO on single channels associated with the nicotinic ACh receptor using the patch clamp technique. He compared the properties of channels in the endplate region of the flexor digitorum brevis of mice exposed to a single injection of ECO 0.5 μ mol/Kg for 3 days with those of

untreated animals. No significant difference between the two groups was discovered, suggesting that an alteration in channel properties did not occur. Further evidence against the first hypothesis was given by the experiments in section 3.3.5. Here a second injection of ECO 0.5 μ mol/Kg given 5 days after the first merely re-inhibited any new enzyme which had been synthesised and returned the time course of the extracellular MEPPs to that seen 3 hours after the initial injection. In other words the 3 hour relationship again held, implying no change in channel properties. Also the fact that 5 days after ECO one can still record spontaneous contractions and repetitive action potentials suggests that the AChE levels have not returned to those of untreated preparations. These experiments also tend to suggest that the second of the 2 hypotheses is the more likely.

There is some evidence to suggest that recovery of low molecular weight AChE occurs much more rapidly than AChE which is physiologically important, supporting the second hypothesis. Grubic *et al.* (1981) examined the recovery of the different forms of AChE in the rat diaphragm after complete irreversible inhibition with soman. They reported an initial slow recovery during the first 2 days, with only 4S and 10S present at the endplate region. Asymmetric molecular forms of AChE, those which are physiologically important, only appeared later and, even one week after poisoning, their activity was only 50% of the normal value. They also reported that the synthesised AChE displayed a limited ability to attach to the subcellular structures and were retained in the muscle, suggesting that this was another reason for its delayed recovery. Similarly, Goudou and Reiger (1983) reported that 16S and 10S forms of AChE were the most sensitive to anti-ChE treatment. They used a methylphosphorothiolate derivative (MPT) to irreversibly inhibit the AChE of mouse diaphragm, finding that the molecular form which showed most rapid recovery was 4S, and thus these authors hypothesised that it may perform a precursor role in the biosynthesis of the asymmetric forms of AChE. Thus, from the above reports and those of this study there is strong evidence that the discrepancies found between the recovery of AChE activity, assayed by the Ellman *et al.* (1961) method, and the THA of extracellular MEPPs after anti-ChE intoxication, may be due to the rapid appearance of low molecular weight AChE which is unimportant in termination of quantal transmitter release.

4.4. EFFECTS OF OXIMES OTHER THAN REACTIVATION.

4.4.1. In vivo reactivation of AChE.

If the mono-pyridinium oxime 2PAM, in a dose of 110 μ mol/Kg, was given to mice on commencement of fasciculation induced by ECO 0.5 μ mol/Kg *in vivo*, 13 and 24% reactivation of diaphragm and whole blood AChE respectively was achieved 3 hours after injection of the anti-ChE. However, no reactivation was observed in animals injected with BOS 8 μ mol/Kg followed by the identical oxime treatment (3.4.1). As has been stated on several occasions, this is thought to be due to the rapid ageing of the AChE-BOS complex, which renders it unsusceptible to oxime reactivation.

4.4.2. The effects of oximes on the prolongation of the time course of extracellularly recorded MEPPs induced by anti-ChEs.

The *in vitro* actions of 2PAM could not be explained as simply as those seen *in vivo*. When mice were exposed to 400 μ M 2PAM for an hour *in vitro* after 3 hours exposure to ECO 0.5 μ mol/Kg or BOS 8 μ mol/Kg *in vivo*, there was a significant reduction in the THA of extracellular MEPPs. After the oxime was washed out there was some return of the prolongation of THA, however, a significantly greater return was seen in preparations exposed to BOS (3.4.2). The AChE activity of diaphragms exposed to one of the anti-ChEs, followed by 2PAM in the same manner, was also assayed. Diaphragms exposed to ECO 0.5 μ mol/Kg *in vivo* followed by 400 μ M 2PAM *in vitro* displayed 52% reactivation of their AChE, whereas those exposed to BOS 8 μ mol/Kg followed by the same dose of 2PAM did not display any reactivation (3.4.2). Hence, the reduction in the THA of extracellular MEPPs produced by 400 μ M 2PAM in diaphragms exposed to BOS could not be explained in terms of enzyme reactivation and must have been due to some other effect. Recent studies have also indicated that the reactivation mechanism alone is not sufficient to explain the antidotal effects of oximes against organophosphorus poisoning in experimental animals (Smith and Muir, 1977; Smith *et al.*, 1981; Su *et al.*, 1986).

In the light of these results the study was broadened to encompass two bis-pyridinium oximes, HI6 and TOX. The effects of the three compounds on the

prolongation of THA of extracellular MEPPs produced by BOS *in vivo* was investigated (3.4.3). The effect of a recognised blocker of channels associated with the nicotinic ACh receptor, disopyramide, was also examined.

Disopyramide has been shown to be useful in the treatment of atrial and ventricular arrhythmias (Dean, 1975) and can be used in the management of cardiac arrhythmias occurring during anaesthesia. It has not only been shown to have atropine-like actions (Baines *et al.*, 1976), but also blocks transmission at sympathetic ganglia (Byrne *et al.*, 1981). Similarly, Healy *et al.* (1981) identified disopyramide's ability to block neuromuscular transmission in rats, suggesting the mechanism by which this occurred was either postjunctional anticholinergic, local anaesthetic or an alteration in the control of Ca²⁺ movement. Harvey *et al.* (1984) reported that disopyramide produced a concentration and voltage dependent reduction in the amplitude and the time constant of decay of neurally evoked endplate currents (EPCs) in the cut costocutaneous muscle of the garter snake. They concluded that disopyramide possessed a non-competitive blocking action at the neuromuscular junction, which was irreversible by anti-ChEs. The voltage dependent nature of this block suggested that it was mediated via blockade of the open form of the ACh receptor-channel complex. A similar result was observed in the present study in which incubation of diaphragms, exposed to BOS 8µmol/Kg for 3 hours *in vivo*, with 100µM disopyramide for 1 hour *in vitro*, produced a reduction in the observed THA of extracellular MEPPs. Following removal of the disopyramide, the prolonged THA returned and was not significantly different from that seen in mice exposed to BOS alone. This not only re-inforced the idea that disopyramide could block open channels associated with the ACh receptor, but confirmed that any such activity could be identified by this technique.

All three oximes examined possessed the ability to reduce the prolonged THA seen after BOS intoxication *in vivo*, although their effects appeared slightly different. In the doses used (100 and 400µM) 2PAM and TOX both produced a reduction in the THA in what appeared to be a dose dependent manner. In fact, although the reduction appeared greater with the higher doses the differences between doses were not significant. In contrast, HI6 displayed dose dependency but in the opposite direction. As the dose was increased so the reduction in THA

was diminished.

Local anaesthetics, such as procaine, display an ability to block nicotinic ACh receptor-channels in the open state (Adams, 1977, Neher and Steinbach, 1978). Classically, this effect alters the shape of endplate currents so that the decay takes the form of an initial sharp phase followed by a period of more prolonged, gentle decline. Evidently, endplate channels become blocked soon after they open, but the drug prevents them from closing. Later they may conduct briefly again after the blocking drug leaves, a process which may be repeated several times. The kinetics of such block are illustrated in figure 4.2., following the model of Neher and Steinbach (1978). This type of block was the first to be examined at the single channel level and revealed the flickering conductance of one channel as single drug molecules enter and leave the channel stochastically (Neher and Steinbach, 1978). Although the oximes and disopyramide, under the conditions utilised in these studies, reduced the THA of extracellular MEPPs, the classical change in endplate current shape representative of open channel block was never observed (figures 3.24 and 3.25).

Alkondon *et al.* (1988) recently published evidence of an extensive range of properties of oximes which may aid explanation of any antidotal actions which cannot be attributed to reactivation. They identified three major, additional actions; firstly, an ability to hydrolyse ACh, analogous to the functions of AChE, secondly, an excitatory action which produced an increase in the open probability of the channels associated with the ACh receptor and finally, an inhibitory action at receptors manifested as a decrease in the mean open time and burst time of ACh-activated channel openings. The majority of the results of Alkondon *et al.* (1988) were gleaned from the frog *Rana pipiens* at room temperature or below, thus care should be taken in comparing them with this study which was performed at approximately 37°C in the mouse. It is believed, however, that many of the properties identified by Alkondon *et al.* (1988) will also be applicable to the mouse.

Alkondon *et al.* (1988) demonstrated that 2PAM and HI6 (50-500µM) could cleave acetylthiocholine, which was analogous to the hydrolysis of transmitter

by the natural enzyme AChE. However, 2PAM appeared approximately twice as potent as HI6 in this respect. This AChE-like action may play an important role in organophosphorus intoxication, were it could account for the hydrolysis of excessive amounts of ACh present within the synaptic cleft. These authors also identified a weak anti-ChE effect within the same concentration range, which was thought to be responsible for the increases in EPC amplitude and twitch tension observed. This effect will, however, be insignificant under conditions of organophosphorus intoxication, where maximum inhibition of the enzyme is already prevailing.

The excitatory effects of these compounds were demonstrated by Alkondon *et al.* (1988) at a macroscopic level in the form of an increase in the twitch tension and an increase in the amplitude and decay time constants of EPCs, these effects being similar to those seen with 2PAM seen by Karczmar *et al.* (1968). This could be due to presynaptic effects, postsynaptic effects such as AChE inhibition (Magleby and Stevens, 1972a; Kordas *et al.*, 1975), or to direct membrane effects. Increased quantal content and ACh output has been demonstrated in the presence of 2PAM (Edwards and Ikeda, 1962; Goyer, 1970), however, Alkondon *et al.* (1988) were unable to confirm such observations. Similarly, they were unable to demonstrate any direct membrane effects. Alkondon *et al.* (1988) thought it unlikely that such effects were due to an anti-ChE effect, since the increases in EPC amplitude and time course occurred at concentrations which did not show any such activity. Agonist properties of the oximes themselves at the ACh receptor were also ruled out. They suggested that the increase in the frequency of channel openings caused by oximes was in fact due to their ability to counteract desensitisation, thus increasing the number of receptors available for activation.

The increase in channel opening probability observed in the presence of oximes could be potentially useful in the recovery of endplates which have undergone organophosphorus intoxication. Organophosphorus compounds impair neuromuscular transmission via desensitisation of ACh receptors produced either by the excessive amounts of ACh, direct effects of the organophosphates themselves or by a combination of the two (Karczmar and Ohta, 1981). Thus, any reduction in such desensitisation may restore normal neuromuscular

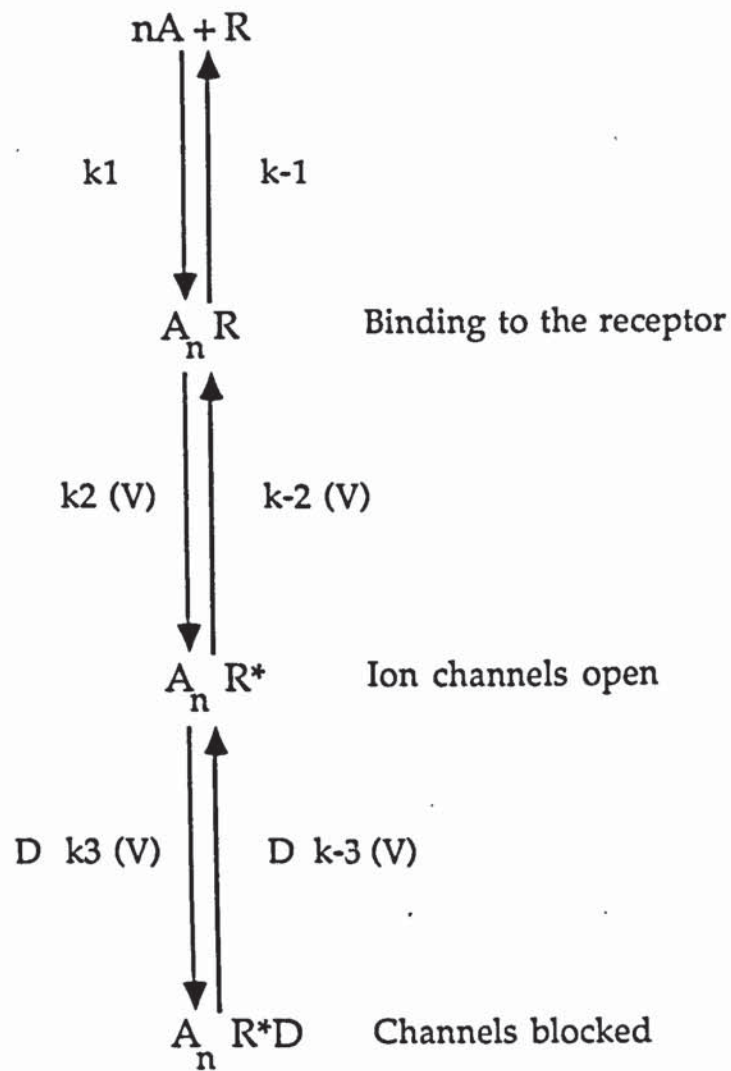


Figure 4.2. The simple sequential model of ion channel blockade (Neher and Steinbach, 1978). Where R is the receptor, n is the number of molecules of the agonist, A, and D is the blocking drug.

function.

The inhibitory effects of oximes were manifested in the experiments of Alkondon *et al.* (1988) in a reduction in twitch tension, a decreased amplitude and decay constant of EPCs and in a decrease in mean channel open and burst times. They concluded that these effects were most likely to be due to ion channel blockade, possibly by the mechanism represented by the simple sequential model which has been used to explain the blocking kinetics of a number of other drugs (Alder *et al.*, 1978; Neher and Steinbach, 1978), represented in figure 4.2. In this model, R, the receptor, interacts with n molecules of ACh (A) to form an agonist bound but non-conducting species, A_nR . This undergoes a conformational change to form a conductive state, A_nR^* . A_nR^*D is assumed to have no conductance because it is blocked by the drug, D. The forward and backward rate constants for the blocking reaction are k_3 and k_{-3} respectively, and V indicates the steps which are voltage sensitive. Alkondon *et al.* (1988) calculated that for HI6 the forward rate constant for blocking was 4 to 5 times faster than for 2PAM.

Using the same patch clamp technique, Tattersall (personal communication) reported HI6, TOX and pralidoxime mesylate (P_2S) (identical active moiety as 2PAM) had similar voltage-dependent blocking actions on open ACh channels at the endplates of the flexor digitorum brevis muscle of the mouse. Again using the simple sequential model of open channel block (Neher and Steinbach, 1978), Tattersall found that the unblocking rate for P_2S was much slower than that seen with HI6 and TOX.

This open channel blocking action could again be related to the compounds' antidotal properties. By virtue of such blockade, oximes may modulate receptor function by shortening channel duration, which may protect receptors from excessive stimulation by accumulated ACh, while at the same time maintaining enough channel currents to sustain physiological function.

It is clear that, of the mechanisms mentioned above, the AChE-like action, the open ion channel blocking properties or a combination of the two could be responsible for the reduction in the THA of extracellular MEPPs in diaphragms

exposed to BOS following *in vitro* oxime treatment. As has been stated above, as the concentration of HI6 was increased so the reduction in the THA was not as great, suggesting that, at these higher doses, the excitatory effects of HI6 were active, resulting in some additional prolongation of the MEPP time course. This effect was not apparent with 2PAM and TOX.

4.4.3. The effect of oximes on untreated preparations.

2PAM (400 μ M and 1mM), as in preparations exposed to BOS *in vivo*, produced significant reductions in the THA of extracellular MEPPs in untreated animals. In the concentrations examined, this oxime did not appear to exhibit any of the excitatory properties described by Alkondon *et al.* (1988). Again the reduction in the THA could be due to either an AChE-like effect or the blocking of open ion channels, yet, since excessive amounts of ACh are not present in untreated preparations, the former mechanism may be the less likely.

HI6 (100-400 μ M) produced prolongation of the THA of extracellular MEPPs recorded in untreated preparations. This may be due to one of two mechanisms; the excitatory effects of oximes, which Alkondon *et al.* (1988) thought was due to a prevention of desensitisation; or an effect of the weak anti-ChE potential of these compounds (Alkondon *et al.*, 1988). The latter mechanism appears unlikely since Alkondon *et al.* (1988) showed in their system that 2PAM was a more potent anti-ChE than HI6, yet 2PAM demonstrated no prolongation of the untreated THA. This suggests that HI6, in higher doses, is a potent excitatory agent, a fact which appears to be confirmed by its effect on BOS intoxicated preparations.

The low dose (50 μ M) of TOX produced a significant reduction in the THA of extracellular MEPPs of untreated preparations. As the dose was increased (400 μ M and 1mM), however, significant prolongation of the THA was seen. It appears that with low concentrations of TOX the AChE-like or ion channel blocking effects prevail, but as the dose increases these effects are overcome by some excitatory mechanism, be that via inhibition of AChE or mechanisms such as the prevention of receptor desensitisation.

4.4.4. Antidotal actions of oximes *in vivo* other than reactivation.

This and many other studies have identified antidotal actions that could not be attributed to enzyme reactivation (Smith and Muir, 1977; Smith *et al.*, 1981; Su *et al.*, 1986). As described earlier, prolonged administration of 2PAM (the 7 injection protocol) decreased BOS-induced jitter, 5 days after injection of the anti-ChE (3.2.5.3). This effect may be due to one or a combination of the mechanisms identified by Alkondon *et al.* (1988), that is, AChE-like activity or the excitatory or inhibitory mechanisms. When the period of 2PAM administration and number of 2PAM injections (the 12 injection protocol) were increased, protection from the effects of BOS was no longer achieved. An insight into the reasons behind this lack of protection may be given by the examination of the THA of extracellular MEPPs of animals subjected to the 12 injection protocol alone, which was significantly prolonged at 5 days. Similarly, the THA of animals exposed to BOS 8µmol/Kg followed by the 12 injection protocol of 2PAM displayed a significantly greater THA of extracellular MEPPs than that seen with BOS alone at the same time point. It may be that during this chronic exposure to 2PAM there was a distortion of the channel associated with the ACh receptor which increased the mean open channel lifetime. This would prolong the time course of extracellular MEPPs, as well as countering any protective effects which 2PAM might possess. This theory could be tested with ease using the patch clamp technique.

These results indicate that there may be a role for oximes in the treatment of intoxication with organophosphates which are not prone to enzyme reactivation. This treatment would involve repeated dosing of the oxime, which would have to be accurately titrated to gain the maximal antidotal effect. However, it seems from these results that care would have to be taken with such repetitive dosing, since too many injections of oxime may, in fact, be detrimental.

4.5. EXTRACELLULARLY RECORDED MEPPs, DIAPHRAGM AChE AND THE EFFECT OF TEMPERATURE.

Two fundamental problems were addressed by this section of the investigation. Firstly, the effect of temperature on diaphragm AChE and secondly, the effect of such changes on the time course of extracellular MEPPs (3.5).

All biochemical assays were performed at 30°C using the technique of Ellman *et al.* (1961), from which direct inferences were made about the enzyme activity in other experiments undertaken at 37°C. This raised the obvious question as to whether such assays gave an accurate measure of AChE activity, despite the temperature difference. The AChE activity of control and fully inhibited mouse diaphragms were assayed at 30°C and 37°C. No significant differences were found between activities measured at the two temperatures. This implied that the levels of diaphragm AChE found at 30°C were representative of the enzyme activity in experiments performed at 37°C.

Extracellular MEPPs of untreated animals and animals exposed to BOS 8µmol/Kg for 3 hours *in vivo* were recorded at room temperature (21°C). The diaphragm AChE activity of animals treated in the same manner was also assayed at room temperature by the technique of Ellman *et al.* (1961). The decay phase of the extracellular MEPPs recorded in both untreated and treated mice displayed temperature dependency, as indicated by significant prolongation of the THA. This prolongation was far in excess of that which could be explained simply in terms of AChE inhibition. For example, in control preparations there was approximately a 40% reduction in enzyme activity from which one would expect, from earlier results (3.3.1), a 30-40% increase in the THA. In fact, the THA, when measured, showed approximately a 3-fold increase. In other words, it appeared that the reduction in enzyme activity was not the rate-determining factor.

A number of studies have shown the decay phase of EPPs and MEPPs to be highly temperature sensitive, with a Q_{10} of approximately 3 (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972b; Kordas, 1972b; Anderson and Stevens, 1973; Gage and McBurney, 1975; Head, 1983; Kordas and Zorec, 1984).

Such decay is also affected by membrane potential (Takeuchi and Takeuchi, 1959; Gage and Armstrong, 1968; Kordas, 1969; Gage and McBurney, 1972; Magleby and Stevens, 1972a; Anderson and Stevens, 1973). Similar temperature sensitivity is presented in this investigation, yet the question arises as to which step in the decay of the ACh-induced conductance increase is rate-limiting.

The high Q_{10} of the decay of these post-synaptic events has been used as an argument against diffusional dilution of ACh being rate-limiting (Magleby and Stevens, 1972b). Also, it is difficult to explain how diffusional loss of ACh should be affected by post-synaptic membrane potential (Gage and McBurney, 1975). AChE activity could conceivably be affected by membrane potential, but the voltage-sensitivity remains unchanged if the AChE is inhibited (Gage and McBurney, 1975). This evidence and the results presented in this investigation suggest that the the rate-limiting effect in the changes in MEPP decay is not AChE activity. The sensitivity of the rate-limiting reaction to membrane potential implies that the reaction occurs within the post-synaptic membrane (Magleby and Stevens, 1972a,b; Kordas, 1972a,b; Gage and McBurney, 1975). Kordas (1972a,b) and this suggests that the rate-limiting step is the dissociation of ACh from the receptors. However, Magleby and Stevens (1972a,b) presented the hypothesis that the rate-limiting reaction involves a conformational change in the transmitter-receptor complex which has a dipole moment.

Whatever the mechanism, the ion channels associated with the ACh receptor appear to remain open for longer periods at lower temperatures, reinforcing the care which should be taken in interpreting data from patch clamp studies. Similarly, making direct inferences between such data and that gained from experiments performed at the usual body temperature of the experimental animal should be made tentatively. For example, can a compound be assumed to be a blocker of open ion channels at 37°C from data produced by the patch clamp technique at room temperature when the channels are clearly open for far longer periods?

4.6 CONCLUSIONS.

1. BOS 8 μ mol/Kg produced an equivalent amount of procion staining as ECO 0.5 μ mol/Kg 3 hours after subcutaneous injection.
2. Despite the inhibition of AChE lasting longer after an injection of BOS than after ECO, the time course of the myopathy, as indicated by the procion technique, was very similar for both compounds.
3. BOS produced twitch potentiation associated with repetitive action potentials within the muscle and spontaneous fasciculations.
4. Both anti-ChEs produced dose-dependent increases in jitter which did not appear to be related to the inhibition of diaphragm AChE, measured by the technique of Ellman *et al.* (1961), at the time of recording. The increased MCD and delay may be related to the duration of AChE inhibition or to the rate of onset of such inhibition.
5. The weight loss experienced in mice exposed to BOS 8 μ mol/Kg did not contribute to the increases in jitter experienced 5 days after the initial injection.
6. Increases in jitter were found in the soleus and EDL 5 days after subcutaneous injections of ECO.
7. Single doses of pyridostigmine, as prophylaxis, or 2PAM, as a treatment, prevented the ECO-induced increases in jitter 5 days after injection. Multiple doses of 2PAM given over the 12 hours subsequent to the anti-ChE displayed an ability to reduce the BOS-induced increases in jitter 5 days after injection, an effect which could not be accounted for by enzyme reactivation.
8. Increases in jitter could not be utilised as an early diagnostic indicator of muscular dystrophy in the Rej 129 mouse.
9. The relationship between the time course of extracellular MEPPs and

biochemically measured AChE was assessed 3 hours after an *in vivo* injection. The time course of these extracellular MEPPs was only greatly affected at higher than 60% inhibition of diaphragm AChE. When longer time points were examined it appeared that the biochemically assayed AChE recovered at a faster rate than did the MEPPs. This was thought to be due to the early recovery of the low molecular weight forms of AChE which would be detected by the biochemical assay but, since these forms are not functionally important, not by the electrophysiological technique.

10. 2PAM, TOX and HI6 were shown to have effects, other than enzyme reactivation, on the time course of extracellular MEPPs, one of which may be open channel blocking.

11. The effects of temperature on the time course of extracellular MEPPs could not be explained merely in terms of reduced enzyme activity.

APPENDICES

A1. Source of reagent

<u>Reagent</u>	<u>Source</u>
Acetic Acid	BDH
Acetylthiocholine Iodide	BDH
Atropine Sulphate	Sigma
BOS	CDE
Calcium Chloride (CaCl ₂)	BDH-Aristar
Copper Sulphate (CuSO ₄)	Hopkin + Williams Ltd
Dextrose	Fisons-AR grade
Disodium Hydrogen Orthophosphate	BDH-Analar
Disopyramide Phosphate	Sigma
Dithiobisnitrobenzoate	BDH
Ecothiopate Iodide	Ayerst
Ethopropazine	Sigma
Formaldehyde	BDH-Analar
Glycerol Jelly	BDH
HI6	CDE
Magnesium Chloride (MgCl ₂)	BDH
Potassium Chloride (KCl)	BDH-Analar
Potassium Ferricyanide	Hopkin + Williams Ltd
Procion Yellow MX4R	ICI
Pyridine-2-aldoxime Methiodide	Sigma
Pyridostigmine Bromide (Mestinon)	Roche
Sarin	CDE
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
Tetrodotoxin	Sigma
Toxogonin	CDE

A2. Composition of the Lileys solution.

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

<u>Substance</u>	<u>Concentration (mM)</u>
NaCl	137
KCl	5
CaCl ₂	2
MgCl ₂	1
NaH ₂ PO ₄	1
NaHCO ₃	12
Dextrose	25

NB. CaCl₂ was added as 1.0M CaCl₂ solution (2ml per litre). This, referred to as Lileys saline in this thesis, was gassed with 95% O₂/ 5% CO₂.

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.

<u>Substance</u>	
DTNB	39.6mg
NaHCO ₃	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 ₂ HPO ₄ ·12H ₂ O	30.5ml
0.2M NaH ₂ PO ₄ ·2H ₂ O	19.5ml
Distilled Water	50ml

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

0.2M Na ₂ HPO ₄ ·12H ₂ O	47.4ml
0.2M NaH ₂ PO ₄ ·2H ₂ O	2.6ml
Distilled Water	50.0ml

A4.3. 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. Ethopropazine.

A final sample concentration of $5 \times 10^{-5} \text{M}$ 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 $2.5 \times 10^{-4} \text{M}$, 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 (ie 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 :-

$$R' = n_1 (n_1 + n_2 + 1) - R \quad (1)$$

where n_1 is the number of scores in group 1, n_2 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. A significant result indicates a difference in the medians of the populations sampled.

For large samples equation 2 has to be used:-

$$z = \frac{n_1 (n_1 + n_2 + 1) - 2R}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{3}}} \quad (2)$$

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. Kolmogorov-Smirnov (K-S) two sample test.

This test is particularly useful for data within which there appear to be a

number of distinct populations from which a particular sample can be drawn. It assumes that the two samples are independent, the scores within each sample are drawn at random and that the ordering of the categories is meaningful and not arbitrary. The null hypothesis, as with the Mann-Whitney test, is that the two samples have the same distribution characteristics.

A number of ranked categories are assigned within which a particular score may fall and the two samples are then summarised in terms of the cumulative frequencies (C) in each of these categories. Each C is converted to a cumulative proportion (CP) by dividing by the appropriate sample size, n_1 or n_2 . If this is done correctly the sum of the CPs for both samples should be 1.0. The difference between the CPs for both samples in each category is calculated and the category with the largest difference, irrespective of sign, is assigned the letter D. K is computed using equation 3:-

$$K = D \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \quad (3)$$

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

A5.3. 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:-

$$R = \frac{N\sum XY - \sum X \sum Y}{\sqrt{[(N\sum X^2 - (\sum X)^2)(N\sum Y^2 - (\sum Y)^2)]}} \quad (4)$$

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:-

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

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

$$c = \frac{\sum Y - b\sum X}{N} \quad (6)$$

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

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

All calculations were performed using the "MDCSTAT" program on an Amstrad PCW 8512 microcomputer. The Mann-Whitney and Kolmogorov-Smirnov tests were taken from Meddis (1975).

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