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THE NEUROTOXICITY OF ACRYLAMIDE

TIM CHILD

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

March 1996

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THESIS SUMMARY

The experiments described in this thesis compared conventional methods of screening for neurotoxins with potential electrophysiological and pharmacological tests in an attempt to improve the sensitivity of detection of progressive distal neuropathy.

Adult male albino mice were dosed orally with the neurotoxicant acrylamide and subjected to a test of limb strength and co-ordination and a functional observational battery. These methods established a no observable effect level of 100 mg/kg. A dose of 200 mg/kg resulted in abnormalities of gait and reduced limb strength and/or co-ordination.

Analysis of the *in vitro* 'jitter' of the latency of trains of action potentials evoked at a frequency of 30 Hz in the mouse phrenic nerve/hemidiaphragm preparation showed this technique to be unsuitable for detection of the early phases of acrylamide induced peripheral neuropathy (100 mg/kg).

The evoked and spontaneous twitch responses of the hemidiaphragm preparation following *in vitro* exposure to the organophosphorous anticholinesterase compound ecothiopate were altered by *in vivo* pre treatment with acrylamide. Acrylamide caused an increase in the time course of the potentiation of stimulated twitches and a decrease in the maximum potentiation. Spontaneous twitches were reduced in amplitude and frequency. These effects occurred at an acrylamide dose level insufficient to cause clinical signs of neuropathy.

Investigations into the mechanisms underlying these observations yielded the following observations. Analysis of miniature endplate potentials at this dose level indicated prolongation of the life of acetylcholine in the synaptic cleft but the implied decrease in cholinesterase activity could not be demonstrated biochemically or histologically. The electrical excitability of the nerve terminal region of phrenic motor nerves was reduced following acrylamide although a possible compromise of antidromic action potential conduction could not be confirmed. There was no histopathological evidence of neuropathy at this dose level.

Further exploration of this phenomenon is desirable in order to ascertain whether the effect is specific to acrylamide and/or ecothiopate and to elucidate the mechanisms behind these novel observations.

Key Words: Neuropathy, Anticholinesterase, Ecothiopate, Nerve terminal.

This thesis is dedicated to Helen.

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CHAPTER 1

INTRODUCTION

1.1: THE OBJECTIVES OF THE PROJECT:

The major aim of the work presented in this thesis was to compare existing behavioural techniques with more sophisticated electrophysiological techniques in an attempt to identify which procedures were more sensitive in detecting the early stages of neurotoxicity following the administration of a known neurotoxic agent. The detection of neurotoxicity by more specific electrophysiological means would also contribute to the validation of the existing behavioural techniques. Acrylamide was selected as the toxic agent for these investigations since its effects on motor axonopathy are well documented.

The experiments conducted during the course of this investigation covered a wide range of disciplines. To aid the reader, discussion is included at the end of each major section of work relating to a given or closely related discipline(s).

1.2: A BRIEF INTRODUCTION TO ACRYLAMIDE AND ITS USE:

Acrylamide, for which the chemical structure of the monomer is shown in figure one below, is a white crystalline solid with a molecular weight of 71.08 and a melting point of 84.5 °C. It is highly soluble in water (2.15 g/ml at 30 °C) and in polar organic solvents (e.g. 0.86 g/ml in ethanol at 30 °C). It reacts with hydroxy, amino and sulphydryl compounds (Bikales & Kolodny, 1963; Bikales, 1970; Cavins & Friedman, 1967a+b; Druckrey *et al.*, 1953; Hashimoto & Aldridge, 1970). Acrylamide is stable in solution and does not polymerise without the aid of a catalyst.

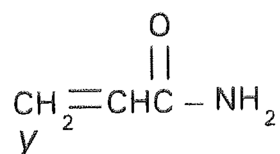


Figure 1.1: The chemical structure of the acrylamide monomer.

The acrylamide molecule consists of two principal functional groups. These are an amine group and a vinyl group conjugated with it. Acrylamide readily undergoes addition reactions at position *y* with compounds having active hydrogen atoms, e.g. molecules containing thiols, hydroxy and amino groups.

Acrylamide became commercially important in the 1950's. Polymerisation of the monomer yields polymers which have many uses. The primary use of acrylamide is as a flocculation agent. However, the major reason for the increase in production of acrylamide

is the increasing application of the polymer to new industrial and commercial operations. Examples of the uses of acrylamide include the following:

1) Flocculators: These are used to separate solids from aqueous solutions and are of importance in mining operations, water purification, metal finishing and the disposal of industrial waste (Spencer & Schaumberg, 1974; Flock & Rausch, 1973).

2) Strengtheners: Acrylamide polymers are used extensively to increase the dry strength of paper and board products (McCollister, Oyen & Rowe, 1964).

3) Grouting agents: One of the most important uses of acrylamide is as a grouting agent in mining and tunnel construction. Liquid acrylamide is pumped into the soil with a cross linking agent and a catalyst and following polymerisation the soil becomes waterproof (Le Quesne, 1979; Auld & Bedwell, 1967; Bikales & Kolodny, 1963).

4) Oil well production: Acrylamide polymers are used to fracture and flood oil bearing strata thus pushing the oil to the surface (MacWilliam *et al.*, 1973).

5) Control of algae: Acrylamide copolymers have been recommended for use in swimming pools and cooling towers to inhibit algae and bacterial growth (Hoover, 1970).

6) Electrophoresis gels: Scientific research frequently utilises acrylamide in the form of polyacrylamide gels for electrophoresis.

Acrylamide has also found applications in adhesives, photography and metal coating technologies. Perhaps the most interesting use of acrylamide, bearing in mind its toxic potential, is in the dissipation of fog where acrylamide monomer and a catalyst are sprayed over affected areas including airfields. The usefulness of the polymers of acrylamide is usually dependent upon the composition and molecular weight of the polymer produced in carefully controlled conditions within large scale reaction vessels with molecular weights of the final polymer ranging from 200,000 to 10,000,000.

Acrylamide has also found a use as a research tool in the neurosciences due to its distinctive mode of neurotoxicity which is described in detail later in this chapter. However, prior to the observation of any neurotoxic effects in test animals, acrylamide must first enter the body and reach the site(s) of action either in the original form and/or as a toxic metabolite. To this end, some understanding of the pharmacokinetics of acrylamide is of great use.

1.3: THE PHARMACOKINETICS OF ACRYLAMIDE:

The absorption,distribution, metabolism and excretion of acrylamide have been studied in some detail (Miller *et al.*, 1982; Hashimoto & Aldridge, 1970; Edwards, 1975). It has been demonstrated that the ability of acrylamide to produce neurotoxicity is independent of the route of administration with intravenous, intraperitoneal, dermal, intramuscular and oral routes all ultimately yielding similar patterns of neurotoxicity (Kuperman, 1958).

Following administration, acrylamide is rapidly distributed to all tissues with little variation associated with the route. This is followed by rapid metabolism and excretion. The work of Miller *et al.* (1982) is of particular interest to the studies presented in this thesis since it utilised a single, non-toxic oral doses of 2,3-^{14}C-acrylamide and concluded that the excretion rate of the radiolabel was independent of the dose. Further work in the same study using a single intravenous dose of 10 mg/kg yielded data on the location and excretion of both the radiolabel associated with metabolites and the parent acrylamide. The concentration of radiolabel was found to be equivalent in all tissues at early time points with there being no concentration in neuronal tissue. Therefore, the relative distribution of acrylamide or its metabolites to nervous tissues does not appear to contribute to its neurotoxicity. However, it is of note that work carried out by Ando & Hashimoto (1972) showed that ^{14}C-acrylamide activity was up to 4 times higher in the distal regions of the sciatic nerve compared to more proximal regions and the brain. The only concentration of acrylamide was found to be associated with erythrocytes. This confirms the findings of Hashimoto & Aldridge (1970) who demonstrated the covalent binding of acrylamide to haemoglobin *in vitro*. This binding accounts for the long elimination half life of radiolabelled acrylamide in the blood which has been reported by Pastoor and Richardson (1981) to be 10.5 days.

Metabolism of acrylamide is rapid. Miller *et al.* reported that the half life of parent acrylamide in all tissues was under 2 hours with there being no trace of acrylamide by 24 hours. This is comparable to the findings of Edwards (1975). Less than 2% of the parent acrylamide dose was excreted in the urine. Seven days after dosing, 71% of the radiolabel was excreted in the urine and 6% in the faeces. Ultimately 90-95% of the dose is excreted in the urine. The main mechanism for the metabolism of acrylamide involves glutathione conjugation. The work of Dixit *et al.* (1980) showed that this route of metabolism is detoxifying since depletion of hepatic glutathione stores leads to earlier onset of toxicity following acrylamide administration. Acrylamide rapidly reacts with hepatic glutathione to form S-β-propionamideglutathione which is eventually excreted as mercapturic acids in the urine. The major urinary metabolite is N-acetyl-S-(3-amino-3-oxypropyl)cysteine (Pastoor *et al.*, 1980). This accounts for approximately half the excreted dose. Miller *et al.* also found three other metabolites which together accounted for 14% of the radiolabel dose. Since it has been demonstrated that acrylamide inhibits its own metabolism via the pathway described above, these may be of considerable interest with regard to the neurotoxic effects of acrylamide, especially in higher dose / longer duration studies. As

mentioned above, a small amount of excretion occurs in the faeces via the bile and there may also be a metabolic pathway leading to a similar amount of excretion evident as expired $^{14}CO_2$ following cleavage of the carbonyl group (Hashimoto & Aldridge, 1970). Miller *et al.* found evidence of enterohepatic circulation of acrylamide and/or its metabolites.

In summary, acrylamide is rapidly dispersed throughout the tissues of the body and is eliminated from them in a monophasic fashion with a half life of approximately 2 hours. However, this is due to the metabolism of acrylamide to yield metabolites which may be neurotoxic themselves and have a terminal half life of approximately 8 days with excretion occurring mainly in the urine.

1.4: THE NEUROTOXICITY OF ACRYLAMIDE:

1.4.1: HUMAN EXPOSURE TO ACRYLAMIDE:

The neurotoxicity of acrylamide was identified both in laboratory animals and in man in the early 1950's following the advent of large scale production of the chemical for the polymer industry. Within a few months of the start of manufacture, several workers at the pilot plant developed a peripheral neuropathy first perceived as a numbness and/or tingling sensation of the fingers. If exposure to acrylamide continued, the workers developed ataxia and weakness of the legs together with uncontrolled sweating of the limbs. They recovered within a week when exposure ceased (Golz; quoted in Fullerton & Barnes, 1966). It has been reported that the signs described above are often preceded by erythema and peeling of the skin even after exposure to solutions of only 1% (Spencer & Schaumberg, 1974). Animal experiments performed at about the same time showed that acrylamide had a cumulative toxic effect and that only the monomer was neurotoxic. The first published report of acrylamide toxicity in animals was by Hamblin in 1956 which was followed by work performed on cats by Kuperman (1958). This work concentrated on the effects of high doses which were found to lead to seizures and ataxia. No pathological signs of toxicity were observed in the central nervous system of these animals. It was not until 1966 that Fullerton & Barnes demonstrated that the main site of the neurotoxic effect of acrylamide was the perihperal nerve fibre in rats. Since the early cases in the pilot plant, human exposure to the monomer has been limited but there have been sporadic reports of human contamination mainly centering on industries where the polymerisation of the monomer is a neccesary stage in the production of commercially useful polymers (Garland & Patterson, 1967). There have been very few non-industrial cases of exposure but one exception was reported by Igisu *et al.* (1975) where the well of a Japanese familly became contaminated during the construction of a nearby sewerage system. These people have probably received the highest dose of anyone exposed to acrylamide. They developed encephalopathy with confusion, disorientation, memory disturbances and hallucinations. They were also ataxic and symptoms of mild peripheral neuropathy only developed 2-3 weeks after the more centrally based effects were seen. This last point is of interest with relation to the findings in most animal studies and other human exposures where the doses are lower and the peripheral signs of neuropathy are the first to develope. Clinical diagnosis of acrylamide poisoning in man is not considered to be problematic since it is most prevalent in workers with known exposure to it and the symptoms are distinct if the practitioner is aware of them. Environmental contamination has so far been avoided with the exception of the case in Japan described above. Many attempts have been made at diagnostic testing for acrylamide neuropathy which have mainly centred on the use of electrophysiological techniques. The reduction in sensory nerve action potential amplitude appears to be the most sensitive of these tests (Takahashi *et al.*, 1971).

1.4.2: ACRYLAMIDE EXPOSURE IN LABORATORY ANIMALS:

The toxicological profile of acrylamide has been well established across a wide range of species including dogs (Thomann *et al.*, 1974), cats (Kuperman, 1958; Leswing & Ribelin, 1969; McCollister *et al.*, 1964; Prineas, 1969), rats (Fullerton & Barnes, 1966; Suzuki & Pfaff, 1973; Tilson & Cabe, 1979), mice (Bradley & Asbury, 1970; Evans & Teal, 1981; Gilbert & Maurissen, 1980), baboons (Hopkins, 1970), monkeys (McCollister *et al.*, 1964), chickens (Edwards, 1975) and quail (Cabe & Colwell, 1981). However, frogs and goldfish appear to show no demonstrable signs of toxicity other than death (Edwards, 1975). Acrylamide toxicity manifests itself clinically in laboratory animals as a range of observations including gait disorders, slight tremors, incoordination, ataxia, muscular weakness, distension of the bladder, loss of body weight and even as convulsions following very high doses (Spencer & Schaumberg, 1974; Cavanagh, 1982). Cessation of exposure often leads to full recovery in all but the most severely affected animals. This pattern of clinical observations is similar to that observed in humans and can be taken as supportive evidence for the use of animal models in the study of the mechanisms which underlie the toxicity of acrylamide. Animals sacrificed following acrylamide exposure show very little macroscopic pathology in the kidneys, spleen, pancreas, adrenals, lungs or testes. Any observations which have been made, e.g. in monkeys (McCollister *et al.*, 1964), have been the result of very high doses which have resulted in the death of the animal. These observations took the form of lung and kidney congestion with necrosis of the liver. More detailed analysis of the kidneys revealed degeneration of the convoluted tubular epithelium and the glomeruli.

Whilst acrylamide toxicity has been demonstrated to be relatively insensitive to the route of administration, there is some inter-species variation in the susceptibility of animals to acrylamide reported in the literature. However, it is important to bear in mind the criteria used to determine the presence or absence of a toxic effect. For example, if one were to solely rely upon bodyweight changes as an index of the toxicity of acrylamide one would require a larger dose in order to observe an effect than if the landing foot splay of the animal was recorded since a slight abnormality of gait would probably not affect the feeding of a laboratory animal. The ever increasing array of experimental techniques and the toxicological findings which result from them, make the accurate estimation of a minimum dose at which some form of toxicity can be observed akin to trying to hit a moving target. This having been said, the calculation of the classical acute LD_{50} across a large range of species yields a figure of 180-250 mg/kg (McCollister *et al.*, 1964; Tilson & Cabe, 1979; Pryor *et al.*, 1983; Fullerton & Barnes, 1966; Hashimoto *et al.*, 1981; Cabe & Colwell, 1981). This indicates some commonalty between species with regard to their succeptability to acrylamide.

It is now known that the clinical observations made following the exposure of humans or laboratory animals to acrylamide are most directly related to effects of acrylamide on

nervous tissue. The site(s) of this neurotoxicity together with the mechanisms which have been proposed to underlie it are introduced in the following section.

1.5: MORPHOLOGICAL AND PHYSIOLOGICAL EFFECTS OF ACRYLAMIDE ON NERVOUS TISSUE:

The main characteristic of acrylamide neuropathy is the distal multifocal degeneration of the axon focussing primarily on the nerve terminal and adjacent Nodes of Ranvier (DeGrandchamp & Lowndes, 1990; DeGrandchamp *et al.*, 1990; Fullerton & Barnes, 1966; Schaumberg *et al.*, 1974). This degeneration then progresses proximally (Cavanagh, 1982; Miller & Spencer, 1985). Susceptable axons in both the central and peripheral nervous systems are affected (Spencer & Schaumberg, 1977). In general, it is accepted that the pattern of neuropathy observed following acrylamide administration is similar to that seen after treatment with neurotoxicants such as n-hexane, 2,5-hexanedione and methyl n-butyl ketone. This pattern of degeneration has been termed 'central-peripheral distal axonopathy'. Not all nerves are equally susceptable. In general the longer and larger diameter nerves are more sensitive to acrylamide induced damage and there is evidence that sensory nerves are more likely to be the first to succumb to it (Sumner, 1980; Cavanagh, 1982). Exactly why this should be the case is not clear since as stated earlier in section 1.1, acrylamide is rapidly distributed throughout the total body water. Studies by Jennekens *et al.* (1979) have contradicted this viewpoint by concluding that initial motor terminal degeneration is widespread and not limited to only the longest and largest diameter fibres. Possible mechanisms to explain acrylamide neuropathy are discussed at length in section 1.6 below. The remainder of this section will concentrate on the morphological and physiological characteristics of the neuropathy.

1.5.1: MORPHOLOGICAL CHANGES ASSOCIATED WITH ACRYLAMIDE NEUROPATHY:

In the first studies of experimental acrylamide intoxication, no pathological changes were observed. However, these studies by Kuperman (1958) and McCollister *et al.* (1964) failed to examine peripheral nerves since at that time it was assumed that the behavioural changes seen following the admimistration of large doses of acrylamide had their origin in the central nervous system, a view which still has some recent support (Gold *et al.*, 1992). It was in 1966 that Fullerton & Barnes identified that the primary morphologically detectable change in the nerves of sub-chronically poisoned animals was to be seen in the distal regions of peripheral nerves. Prineas (1969) conducted more detailed electron microscope surveys in the cat and found that the first signs of any ultrastructural change was the increase in 10 nM neurofilaments, mitochondria and other organelles in the axoplasm of nerve terminals and preterminal Nodes of Ranvier thus demonstrating the multifocal nature of acrylamide neuropathy. This increase in neurofilament concentration

was most marked in the distal regions of peripheral nerves. Whether these findings precede the clinically observable neurological symptoms associated with acrylamide is open to debate with some workers detecting pathological changes only after the onset of clinical abnormality. This may be the result of the experimental paradigm employed in these studies since others have returned the opposite finding (DeGrandchamp & Lowndes, 1990; DeGrandchamp *et al.*, 1990). Subsequent work confirmed the observations of Prineas and also demonstrated the presence of membranous materials, possibly smooth endoplasmic reticulum, in terminal regions (Suzuki & Pfaff, 1973; Cavanagh, 1982, Jennekens *et al.*, 1979). If exposure continues, the number of neurofilaments increases resulting in marginalisation of synaptic vesicles and organelles. The nerve terminal and distal Nodes of Ranvier take on a swollen appearence. Following this, demyelination or myelin retraction at Nodes of Ranvier occurs and the axon starts to breakdown with the loss of neurotubules and neurofilaments and finally the dissolution of the axolemma. As well as being pushed to the margins of the terminal as the terminals swell, synaptic vessicles may actually decrease in number and/or become trapped at the edge of the terminal. There have also been reports that acrylamide may interfere with the recycling of synaptic vesicles via inhibition of endocytosis (DeGrandchamp *et al.*, 1990). This may explain the increase in the size of the terminal as vesicular membrane is added into the axolemma aswell as some of the functional deficits described below.

1.5.2: FUNCTIONAL CHANGES IN NERVE FUNCTION FOLLOWING ACRYLAMIDE INTOXICATION:

Fullerton & Barnes conducted some of the earliest electrophysiological studies on the effects of acrylamide. They discovered that following acrylamide administration, the conduction velocities of action potentials along motor nerves innervating the hindpaw of rats was reduced. Rats showing severe behavioural abnormalities had reductions in conduction velocity of approximately 80%. However, this was not the case in less severely affected animals. These findings were confirmed by Leswing & Ribelin (1969) and they concluded that measurement of nerve conduction velocity was not a suitable diagnostic tool since it was not recorded until intoxication was evident clinically. Experiments conducted by Hopkins & Gilliat (1971) using baboons treated with relatively high doses of acrylamide showed that not only was nerve conduction velocity decreased but so to was the amplitude of the action potentials. These effects were greater in sensory nerves.

The studies published following on from the work described above focused more carefully on the nerve terminal as the location of the primary site of functional deficit following acrylamide intoxication. Schaumburg *et al.* (1974) demonstrated that the Pacinian corpuscles in the toe pads of cats showed a reduction in generator potential following administration of acrylamide which preceded any ultrastructural changes. Tsujihata *et al.* (1974) described a decrease in the releasable pool of acetylcholine at the neuromuscular junctions of rats and also claimed in unpublished data that acrylamide

reduced miniature endplate potential frequency and produced a greater suppression of endplate potential amplitude upon repetitive stimulation without an increase in quanta size of the miniature endplate potential. It has been demonstrated that the effects of acrylamide on nerve terminal function precede the observation of deficits in the conduction velocity of action potentials along nerves. Sumner & Asbury (1975) studied the initiation of impulses by muscle spindle and tendon organ afferent terminals following acrylamide treatment. They concluded that impulse initiation was affected by acrylamide prior to any effects being recorded on action potential conduction velocity. Lowndes & Baker (1976) studied stimulus bound repetition in soleus α-motoneurones of the cat following tetanic conditioning. The post-tetanic potentiation of the motoneurones was reduced following acrylamide administration which was attributed by the authors to a decline in stimulus bound repetition. They concluded that this was further evidence to support the idea that the primary functional alterations occurring following acrylamide were occurring at the nerve terminal. Subsequently, Lowndes *et al.* (1978) correlated the neurological effects of acrylamide in cats with the neurophysiological changes in the muscle spindles of the animals. They concluded that changes recorded in spindle function following the administration of acrylamide may cause the ataxia observed in the cats.

In conclusion it appears that there is considerable evidence to support the idea that the primary site of functional changes following acrylamide intoxication is the nerve terminal and that these changes may or may not be preceded by changes in axon ultrastructure. However, the precise mechanism by which acrylamide brings about these changes is still unclear although there several hypotheses which have been investigated. Some of these are presented below.

1.6: MECHANISMS OF ACRYLAMIDE NEUROTOXICITY:

The mechanisms underlying acrylamide induced neuropathy have been the subject of extensive reseach. This has resulted in three main areas of interest with regard to hypotheses aimed at explaining the morphological and functional abnormalities observed following acrylamide intoxication. These are firstly, the effect of acrylamide on the ability of the neuron to produce energy. Secondly, more generalised effects of acrylamide on protein formation and function and finally the function of the transport systems of the neuron.

1.6.1: THE 'ENERGY HYPOTHESIS' (INHIBITION OF GLYCOLYTIC ENZYMES):

This hypothesis was proposed by Spencer, Schaumburg and their collegues and is based around the observations that:

1) The distal regions of axons are more succeptable to the effects of acrylamide than more proximal regions.

2) Ten nM neurofilaments accumulate at distal Nodes of Ranvier.

3) There is a decrease in conduction velocity of action potentials associated more frequently with large myelinated fibres.

These observations are consistent with those seen with other compounds such as hexane, 2,5-hexanedione and carbon disulfide (Spencer & Schaumburg, 1978; Spencer *et al.*, 1979). This similarity suggested that there may be a common mechanism by which the compounds named above cause neuropathy. The energy hypothesis targets the glycolytic pathway as the common site of action of these compounds. It is hypothesised that impairment of this pathway would lead to an energy deficit within the axon which would be greatest in the distal regions since they rely on the neuronal cell body for their maintenance. The accumulation of neurofilaments may be the result of impairment of axonal transport following inhibition of glycolitic enzymes. In support of this, fast axonal transport has been demonstrated to be sensitive to the inhibition of glyceraldehyde-3-phosphate dehydrogenase (Ochs *et al.*, 1969; Ochs & Smith, 1971; Sabri & Ochs, 1971, 1972). Impairment of energy production would also impair nerve conduction since the Node of Ranvier has special energy requirements due to its role in saltatory conduction of action potentials. The apparent sensitivity of nervous tissue to the effects of acrylamide prompted Howland (1985) to study its effect *in vitro* on a nerve specific isoenzyme of enolase, a glycolytic enzyme. He demonstrated that acrylamide inhibits this enzyme

particularly in distal regions and concluded that this may explain some of the specific vulnerability of nervous tissue.

The energy hypothesis which is perhaps more accurately described as the 'glycolytic hypothesis', appears to offer a plausible mechanism underlying the neuropathy produced by acrylamide and related compounds with some good experimental evidence to back it up. However, there are aspects of the experimental evidence which may argue against the hypothesis. The most important of these is that the concentrations of acrylamide required to produce *in vitro* inhibition of glycolytic enzymes are extremely high when compared to the likely *in vivo* concentrations even when clinically observable neuropathy is severe. It must also be remembered that neither glyceraldehyde-3-phosphate dehydrogenase or nerve specific enolase would be rate limiting steps in glycolysis (Howland, 1985). This would explain reports that acrylamide does not cause a decrease in oxygen consumption or an increase in glycolytic intermediates (Hashimoto & Aldridge, 1970; Johnson & Murphy, 1977). It has also been demonstrated that rapid axoplasmic transport (discussed below) is affected following acrylamide administration even when energy reserves in the axon are still high (Sickles & Pearson, 1987).

Thus, it seems that the glycolytic hypothesis may not fully explain the effects of acrylamide on the axon but it may be possible that this represents part of the other possible biochemical lesions discussed below.

1.6.2: OTHER BIOCHEMICAL LESIONS:

As stated at the beginning of this chapter, acrylamide is able to bind covalently to biological sulphydryl and amine groups. Acrylamide would thus be expected to affect many enzymes and biological processes. Aswell as the 'energy hypothesis' described above, there have been many other attempts to explain the accumulation of neurofilaments in the distal regions of axons which is generally regarded as a primary indicator of acrylamide neurotoxicity.

It is known that phosphorylation and proteolytic cleavage are important in the normal function of neurofilaments. Tanii, Hayashi & Hashimoto (1988) have demonstrated that acrylamide is capable of inhibiting a thiol protease called calcium-activated neutral protease (CANP) which is responsible for the breakdown of neurofilaments in the terminal regions of the axon where it is activated by increased local levels of calcium. It has also been suggested that acrylamide may inhibit this enzyme by decreasing calcium influx into the presynaptic terminal (DeGrandchamp *et al.*, 1990). Inhibition of CANP by leupeptin, a known protein inhibitor, has been shown to result in similar neurofilament accumulations to those seen following the administartion of acrylamide (Roots, 1983). This reduction in neurofilament breakdown may be doubly important since it has been suggested that the proteolytic fragments produced by the action of CANP may act as a chemical signal between the distal axon and the perikaryon (Schlaepfer, 1987; Edwards *et al.*, 1984).

It has also been demonstrated by Lapadula *et al.* (1989) that acrylamide is able to bind directly to cytoskeletal proteins including those of neurofilaments and microtubules (specifically microtubule associated protein-1 and -2). Other biochemical effects such as the *in vitro* alteration of amino acid incorporation into proteins (Hashimoto & Ando, 1973) and the perturbation of protein phosphorylation and phospholipid metabolism (Berti-Mattera *et al.*, 1990) serve to further demonstrate the extremely widespread effects of acrylamide on the biochemical function of the neuron. It should also be clear from these observations that the precise biochemical mechanisms underlying acrylamide induced neuropathy are far from clear. It should also be emphasised that the dosing regimes employed to demonstrate biochemical lesions *in vivo* are sufficient to cause severe clinical signs of neurotoxicity and that the *in vitro* concentrations of acrylamide required to alter protein formation or function would be difficult to acheive in an animal model. This may be an artefact arising from the sensitivity of the detection techniques which may require relatively large scale changes when compared to the level of change sufficient to trigger the degeneration of a neuron. Whilst the primary lesion caused by acrylamide is biochemical in nature, perhaps it is best to establish primary functional changes in the systems which maintain the axon in order to narrow down the wide spectrum of acrylamides' biochemical effects to those which are the initiators of the neuropathy. As has already been mentioned above, the cytoskeleton appears to be one site at which acrylamide has biochemical potential for disruption. One of the funtions of this system is the transport of essential materials from the cell body to the most distal regions of the neuron. The study of neuronal transport systems following acrylamide administration has yielded some interesting hypotheses which may indicate which of its many potential biochemical insults are the most important in the disfunction and ultimately failure of the axon.

1.6.3: THE EFFECTS OF ACRYLAMIDE ON AXONAL TRANSPORT:

A considerable amount of investigation has been performed into the effects of acrylamide administration on the various categories of anterograde and retrograde axoplasmic transport. There have been a number of conflicting observations made during the course of these studies which may be a result of the fact that acrylamide causes progressive deficits which may be both causative or reactive in origin.

Cavanagh (1964) was one of the first to speculate that the dying back neuropathy which follows acrylamide exposure, was due to a block of the transport of essential macromolecules in the axon. Following this many studies considered the effect of acrylamide upon slow and fast anterograde transport systems in chronically intoxicated animals (Pleasure *et al.*, 1969; Bradley & Williams, 1973; Sumner *et al.*, 1976; Griffin & Price, 1976; Rasool & Bradley, 1978; Weir *et al.*, 1978; Sahenk & Mendell, 1981; Chreitien *et al.*, 1981; Souyri *et al.*, 1981; Couraud *et al.*, 1982; Sidenius & Jakobsen, 1983). These studies showed minor transport deficits which were not of sufficient magnitude to confirm the hypothesis proposed by Cavanagh. Studies conducted into the

effect of acrylamide on retrograde transport have been made using both chronically and acutely exposed animals (Sahenk & Mendell, 1981; Jakobsen & Sidenius, 1981; Miller *et al.* 1983; Miller & Spencer, 1984). The results of these studies showed that a single dose of acrylamide had a greater effect on retrograde transport than can be demonstrated after multiple (chronic) exposure. Studies conducted by Sickles (1989) using acute exposure showed that the same was true for fast anterograde transport. This was to be expected since Hollenbeck (1986) has proposed that the mechanisms of retrograde and anterograde transport are similar. This prompted the formation of the idea that acrylamide is capable of causing a significant but intermittent block of axoplasmic transport. It has been demonstrated that the transported quantity of proteins is reduced to approximately 50% of control levels within 3 hours of exposure followed by rapid recovery within 24 hours although only a 10-20% decrease in the rate of transport was recorded (Sickles, 1989,1991).

Since the effects of acrylamide on transport systems outlined above can be demonstrated to occur rapidly after exposure to relative low doses it is reasonable to propose that a direct action of acrylamide on the rapid transport systems of the axon may be a primary step in the initiation of axonal degeneration. However, several studies have shown accumulation of transported materials such as organelles (Cavanagh, 1982) and proteins (Souyri *et al.*, 1981) in nerve terminal regions prior to axonal degeneration. This contradiction has resulted in the hypothesis that it is the loading/unloading of the transport systems which the subject of disruption following acrylamide exposure (Edwards *et al.*, 1991). Materials newly synthesised in the perikaryon associate with the anterograde transport system. Complex exchanges of materials occur in the axon as anterograde transport occurs with stationary and mobile phases created. In the distal regions of the nerve, materials are removed by secretion and degradation or are loaded onto the retrograde transport system. An effect of acrylamide on the loading system throughout the neuron would result in both a decrease in the quantity of materials transported and also lead to accumulations of material in the terminal regions as loading onto the 'return' mechanism was impeded (Edwards *et al.*, 1991). It is of note that most rapidly transported proteins participate in the exchange between the stationary and mobile phases in the axon (Munoz-Martinez, 1982). This could explain the shift from the transported to the stationary phase reported for some forms of acetylcholinesterase (Couraud *et al.*, 1982).

The biochemical lesion which is responsible for these alterations to transport systems remains unclear due to the many interactions of acrylamide with molecules within the neuron.

1.7: A BRIEF INTRODUCTION TO ECOTHIOPATE:

Ecothiopate is a quaternary organophosphorous anticholinesterase having peripheral action due to its inability to cross the blood-brain barrier. Ecothiopate was first synthesised by Tammelin (1958). Compounds of this type were first identified in the middle of the

nineteenth century. Included in this category of anticholinesterases are insecticides such as parathion and paraxon aswell as warfare agents such as sarin and tabun. In addition to these potentially deadly uses, organophosphates have also found uses in medicine and have been utilised in the treatment of glaucoma and myasthenia gravis (Schaumann & Job, 1958).

Anticholinesterase drugs 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 and have a similar chemical structure to that of acetylcholine (e.g. edrophonium). Compounds with a more stable enzyme-inhibitor complex comprise the second group which are sub-divided into carbamates and organophosphates.

The harmful or therapeutic effects of these compounds results from their ability to slow the hydrolysis of the neurotransmitter acetylcholine via inhibition of acetylcholinesterase in the synaptic cleft of the neuromuscular junction. This results in the prolongation of the lifespan of acetylcholine molecules within the synaptic cleft leading to multiple interactions of acetylcholine molecules with receptors and/or diffusion of some acetylcholine out of the cleft where it can interact with pre-synaptic receptors (Boyd & Martin, 1956).

These abnormal receptor interactions result in the post anticholinesterase observations of repetitive firing of muscle cells following the stimulation of a single nerve action potential, spontaneous muscle fasciculation, muscle cell fibrillation and the generation of antidromic discharges in the motor nerve. The precise mechanisms behind these observations remain the subject of some debate and are discussed in more detail later in this thesis. Perhaps the best understood of these phenomenon is that of repetitive firing of the muscle cells following a single evoked nerve action potential which leads to the increase in amplitude of muscle twitches following exposure to anticholinesterases. This is thought to be the result of a prolonged and enhanced endplate potential which outlasts the refractory period of the endplate region of the muscle cell membrane responsible for the generation of the muscle action potential such that further action potential generation occurs from the same endplate potential yielding what amounts to an asynchronous tetanus of short duration. Figure 1.2 shows a hard copy of an oscilloscope recording made during early work on this project using an intracellular microelectrode positioned near the motor endplate region of a muscle cell in a diaphragm taken from a mouse, pinned out in a bath containing physiological saline and treated *in vitro* with 500 nm ecothiopate for 7 minutes and maintained at 37°C. A single 10 x threshold stimulus (indicated by the stimulus artefact S) was then applied to the phrenic nerve which innervates this muscle. This resulted in the generation of an action potential (1) followed by two repetitive action potentials (2 and 3). The repetitive action potentials arose from the prolonged endplate potential which can still be seen after them (EPP).

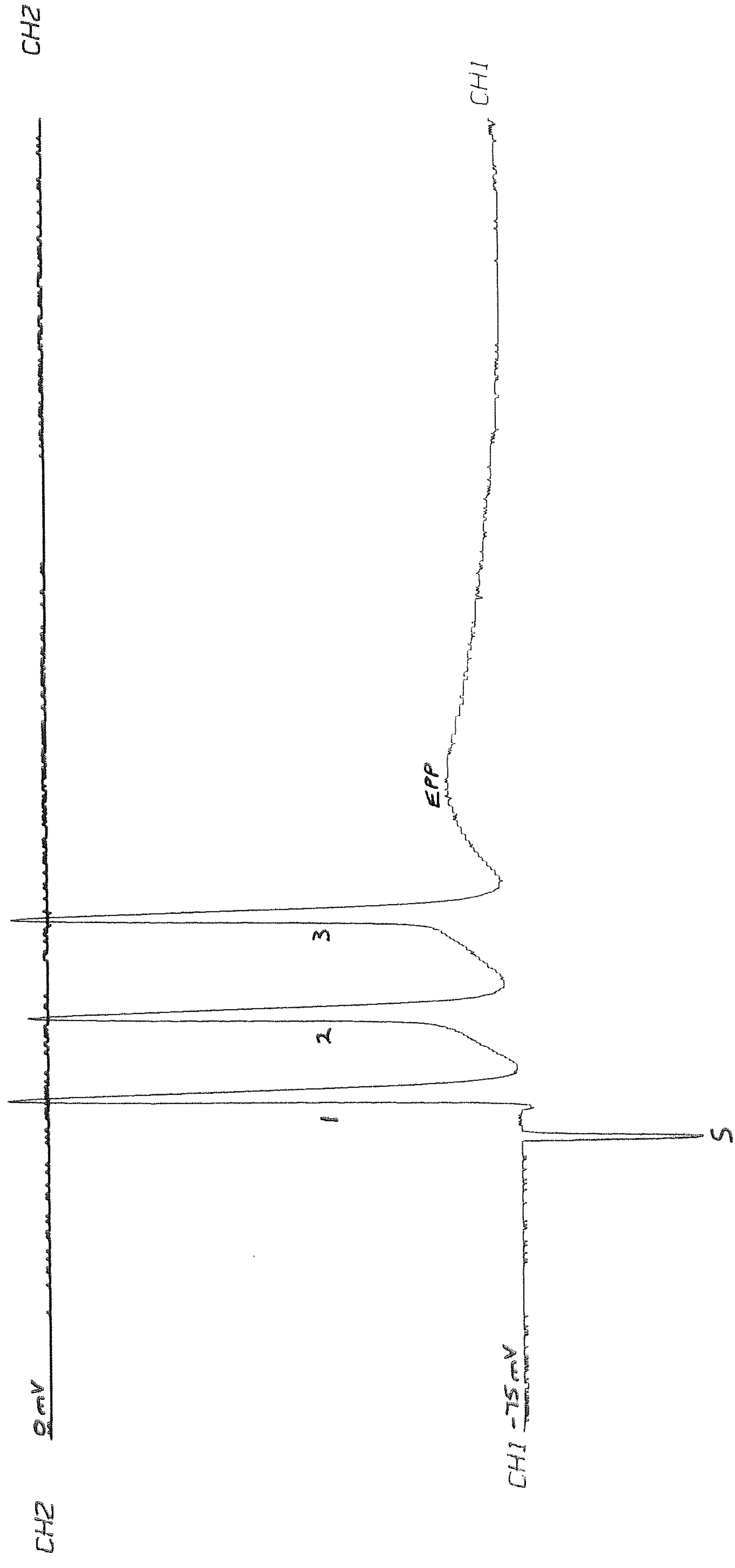
In the absence of an applied nerve stimulation anticholinesterase treatment results in the generation of spontaneous twitching of motor units within a muscle (Hobbiger 1976). The generation of a synchronus twitch within a motor unit is due to antidromic conduction of

action potentials in the nerve which innervates the cells of that unit. However the process or processes underlying the generation of this action potential remain uncertain although much evidence has been described to support a number of different possibilities.

Ecothiopate was used in the investigation presented in this thesis as a tool to assess the functional state of the neuromuscular junctions of mice exposed to acrylamide. The rationale for these experiments is explained in detail later in this thesis but centres around the lability of the effects of ecothiopate on stimulated and spontaneous muscle twitches which have been shown to be sensitive to small changes in temperature, ion concetrations in bathing solutions (e.g. calcium and magnesium) and the presence of other drugs such as (+)-tubocurarine (Ferry, 1988). It is therefore reasonable to hypothesise that any morphological or functional changes induced by acrylamide treament may affect this sensitive response.

Overleaf:

Fig. 1.2: Repetitive action potentials following a single evoked nerve action potential in the phrenic nerve recorded using an intracellular microelectrode placed near the endplate region of a muscle fibre within a mouse hemidiaphragm preparation bathed in physiological saline, gassed with carbogen and maintained at 37°C. The preparation was exposed to physiological saline containing 500 nM ecothiopate for 7 minutes prior to recording. S = stimulus artefact. 1 = evoked muscle twitch. 2 = first repetitive non evoked twitch. 3 = second repetitive non evoked twitch. EPP = prolonged endplate potential resulting from anticholinesterase action of ecothiopate.



200mV/DIV 5.00ms/DIV
50.0mV/DIV 5.00ms/DIV
CH2
CH1
0mV
CH1 -75mV
CH2
EPP
1
2
3
S

CHAPTER 2

MATERIALS AND METHODS:

2.1: ANIMALS:

Male albino mice, purchased from Bantin and Kingman, Hull and fed *ad libitum* on the companies breeding diet were used in all experiments. They were 6-7 months of age and 35-40 g in weight. Kelly (1978) and Banker *et al* (1983) have indicated that at this age CFHB rats and CBF-1 mice have stable neuromuscular characteristics (spontaneous and evoked transmitter release, receptor number, neuromuscular junction ultra structure and resting membrane potential) and it seemed reasonable to assume that the mice used in this study might exhibit similar stability.

2.2: DRUGS:

2.2.1: ECOTHIOPATE:

Ecothiopate iodide was made up from the dry powder portion of phospholine iodide BNF eye drops (Ayerst) which contained 12.5 mg ecothiopate iodide and 40 mg potassium acetate. 3.26 ml of distilled water was added to this to provide a 10^{-2} M solution of ecothiopate. A 100 fold dilution of this solution yielded a 10^{-4} M stock solution which was stored as 1 ml aliquots at -20°C in order to prevent freeze-thaw cycles. The stock solution contained 6 μM potassium acetate which was considered to have a negligible effect on the potassium concentration when added to the physiological saline in *in vitro* experiments.

Ecothiopate was administered *in vivo* by subcutaneous injection in the scruff of the neck using a 26 gauge needle at a concentration of 500 nmoles/kg *in vivo* with the injection solution being made up with 0.9% sodium chloride and 0.14 mM atropine in addition to the ecothiopate. The atropine counteracts the muscarinic effects of the ecothiopate. The injection volume was 0.1 ml per 20g of body weight.

2.2.2: ACRYLAMIDE:

Monomeric acrylamide (molecular weight 71.08) of 99.92 % minimum assay in crystalline form was obtained from BDH laboratory supplies (Poole, England). This was dissolved in distilled water to give acrylamide solutions of 0.14 M (20 mg/2.0 ml) and 0.28 M (40 mg/2.0 ml) which when administered at 0.1 ml per 20g body weight corresponded to doses of 50 and 100 mg/Kg respectively. All dosing solutions were made up fresh.

Acrylamide was administered orally using a 21 gauge x 38 mm curved gavage with a beaded tip to aid passage of the tube down the throat and reduce the risk of damage to the oesophagus. Control animals received oral doses of distilled water of equivalent volume.

2.3: BEHAVIOURAL TESTING:

2.3.1: OBSERVATIONAL BEHAVIOURAL TESTING:

Mice were observed prior to humane killing in order to assess whether they showed any deficit in their behavioural characteristics following administration of compounds.

This was carried out in accordance with the functional observational battery (FOB) currently in use at the Central Toxicology Laboratory of Zeneca (Macclesfield, Cheshire) whereby the treated animal is compared to a normal animal with regard to a list of possible abnormalities. This FOB is based upon that of Irwin (1978) but uses a three point scale of severity for assessing the level of deficit shown by an animal. Deficits may be termed slight, moderate or severe (see appendix 3).

This method has obvious limitations. It was not possible to compare, with any precision, the degree of disability of different groups of mice unless they were assessed at the same time, since the acuteness of observation and decisions on categorisation are likely to vary over a period of time. This observational testing was secondary to the main electrophysiological/pharmacological studies and only utilised the animals used in these experiments. The comparison of a large number of animals on the same day was not possible.

Thus the use of a more quantitative measure of behavioural deficit was desirable and particularly one which focused on the physical manifestations of the peripheral neuropathy associated with acrylamide. The test of climbing performance devised by Coughenour *et al* (1976) was chosen for this purpose.

2.3.2: CLIMBING PERFORMANCE TEST:

Following observation as described above, mice were subjected to a test of their limb strength and co-ordination using a method based on that of Coughenour *et al* (1976). They demonstrated that the ability of an animal to climb to the top surface of a wire mesh grid from its underside was directly related to its ability to perform more complex tests such as the rotarod test but with the benefit that no training of the animal was required before testing thus making it less time consuming. In addition to this there is also the benefit that the animal needs little encouragement to perform the task it has been set since climbing to the safer upper surface of the wire mesh is very instinctive. With tests such as the rotarod it may be necessary to use unpleasant stimuli in order to get the animals to stay on the treadmill. This is undoubtedly due to the repetitive nature of the training required for the rotarod test leading to other behaviours such as memory and

shock avoidance becoming involved. The climbing method also avoids complexities inherent in a situation where a learning curve is present.

The use of the landing foot splay test (Edwards and Parker, 1977) whereby animals are dropped onto a soft surface and the separation of their hind limbs on landing (measured by analysis of the imprints in the landing surface) is used to quantify the extent of neuropathy was considered. However, it was discovered that dropping mice from a consistent starting position was very difficult in comparison to rats upon which the original study was based. It was therefore concluded that the climbing test would be the most suitable test for detecting physical signs of motor neuropathy in this study. The procedure for the climbing test is described below.

The mouse under study was placed in the centre of a 30 x 30 cm woven 4 gauge (4 wires per inch) stainless steel wire mesh which was raised to a height of 30 cm above a cushioned potential landing area before being inverted. The time, in seconds, for the mouse to traverse to the edge of the mesh and climb over to the top side was recorded. If the mouse fell from the mesh during the course of this process then the time for this to occur was recorded. There was a default time of 120 seconds after which the mouse was removed from the mesh if it had failed to climb to the top but had managed to hang on and prevent a fall.

2.4: PREPARATION OF TISSUES FOR FURTHER EXPERIMENTATION:

2.4.1: DISSECTION OF PHRENIC NERVE-HEMI DIAPHRAGM:

The hemi diaphragm preparations from the mouse used in all but the patch clamp study were obtained as follows. The mouse was killed by cervical dislocation following a blow to the back of the head which was sufficient to render the animal unconscious. It was then held under a warm running tap for approximately 30 seconds in order to saturate the fur with water. This was found to aid later experimentation in that stray fur was not as likely to get stuck to the diaphragm upon its removal. The mouse was then laid out on its back and the skin removed from the ventral region of the thorax. The sternum and ribs were removed above the point where the fourth rib nearest the diaphragm joined the sternum leaving the diaphragm supported by the remaining ribs. The phrenic nerves were then identified where they run either side of the heart and ligatures tied leaving a thread of around 1 inch in length for later threading of the nerves through electrodes. The nerves were then sectioned above the ligatures and cut away from their connective tissue so that their only contact was at their insertion into the diaphragm. Any connective tissue on either side of the diaphragm was then removed and the remaining ribs and diaphragm were cut laterally as close to the dorsal wall of the body as possible with care being taken not to cut the phrenic nerves. The whole diaphragm preparation was then placed in approximately 200 mls of ice cold modified Lileys physiological saline which had been gassed with carbogen (95% O_2 / 5% CO_2)

for 20 minutes prior to use (see appendix). The diaphragm was divided into left and right hemi-diaphragms in a large glass petri dish which was filled with physiological saline. Any excess tissue associated with the ribs or connective tissue on the diaphragm was removed at this point. The diaphragm was divided down the centre of the central tendon and through the ribs either side of what remained of the sternum. This produced two hemi diaphragms each with innervation by a phrenic nerve.

2.4.2: DISSECTION OF *LUMBRICALES* MUSCLES:

The mouse was killed as above and the hind limbs removed. These were then pinned firmly to a cork dissection board such that the sole of the foot was uppermost. The skin of the lower leg and foot was removed revealing the musculature below. In order to reach the *lumbricales* it was necessary to remove the overlying tendons and muscles of the *flexor digitorum brevis* and the *flexor digiti quinti brevis*. Following this the *lumbricales* could be clearly seen and removed easily. They were then transferred to physiological saline as in the dissection of the diaphragm before further treatment.

2.5: MATERIALS AND METHODS USED IN PHARMACOLOGICAL EXPERIMENTATION:

2.5.1: EXPERIMENTS TO STUDY THE TWITCH RESPONSE IN THE MOUSE HEMI-DIAPHRAGM:

The experiments performed in this part of the study looked at the possible modification by dosing *in vivo* with acrylamide of the known response of a hemi-diaphragm preparation to an *in vitro* dose of the organophosphorous anticholinesterase compound ecothiopate.

2.5.2: DATA ACQUISITION:

Hemi-diaphragms (HD) were removed from mice given acylamide or distilled water only. The HD was then pinned out by the ribs and central tendon using entomological pins on Sylgard in a glass petri dish. A small spring clip was attached to the central tendon with care being taken not to impinge upon the muscle cells of the HD itself as this would result in damage to the cells and a subsequent loss of propagation of action potentials and contraction due leakage of intracellular potassium ions. A length of braided silk thread was then tied to the spring clip. The HD was transferred onto a specially made holder which held the HD by the ribs via 2 stainless steel spikes embedded in a perspex block supported by a metal arm which was also used to mount the stimulating electrodes. This was then placed in an organ bath containing modified Lileys physiological saline (see appendix) which was gassed continuously with carbogen. The

silk thread was then attached to a force transducer (Dynamometer UF-1) mounted above the organ bath on a rack and pinion adjuster so it could be moved in a vertical plane. This permitted the tension on the HD preparation to be adjusted. The phrenic nerve was threaded through a tubular stimulating electrode connected to a Digitimer DS2 isolated stimulator. Temperature within the organ bath was maintained at 37°C ±0.2 at all times via circulation of heated water through the water jacket of the organ bath. Figure 2.1 overleaf shows this arrangement:

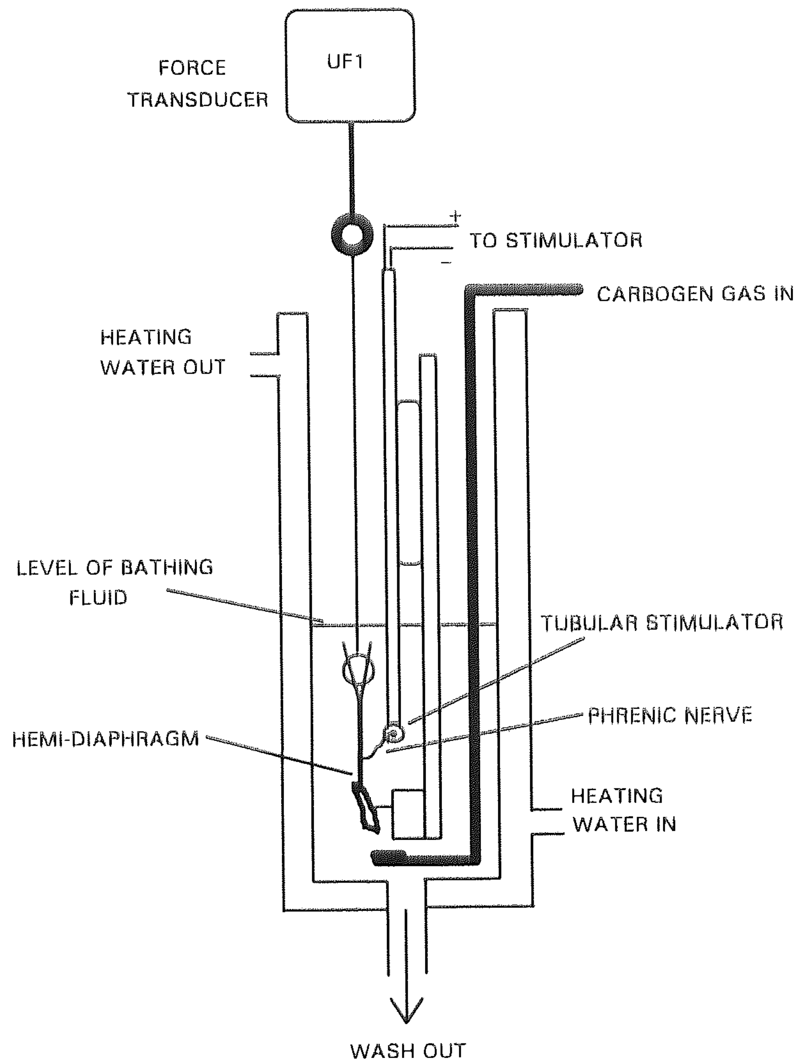


Figure 2.1: Diagram to show the organ bath and associated apparatus from which stimulated and spontaneous twitch activity in mouse hemi-diaphragm preparations was recorded following washout after the administration of 500 nM Ecothiopate for 7 minutes at 37°C.

The output from the force transducer was amplified and displayed on a pen recorder (Washington oscillograph 400 MD/2). This was calibrated for each experiment by hanging known weights (1, 2, 4 and 10g) from the force transducer with the tissue in place and at its experimental resting tension. Resting tension in the hemi-diaphragms was defined as the tension on the tissue at which the twitch response to a single supramaximal stimulus had the greatest amplitude. A supramaximal stimulus was defined as being 10x threshold. The recorder output was always found to be linear. Once the tension on the hemi-diaphragms had been set they were allowed to equilibrate whilst being stimulated supramaximally at a frequency of 0.01 Hz (0.02 mS duration) until the baseline viewed on the pen recorder had stabilised. This usually took approximately 20 minutes. Throughout these experiments the frequency of stimulation was controlled by a digital timing device (Devices Digitimer) which triggered the stimulator.

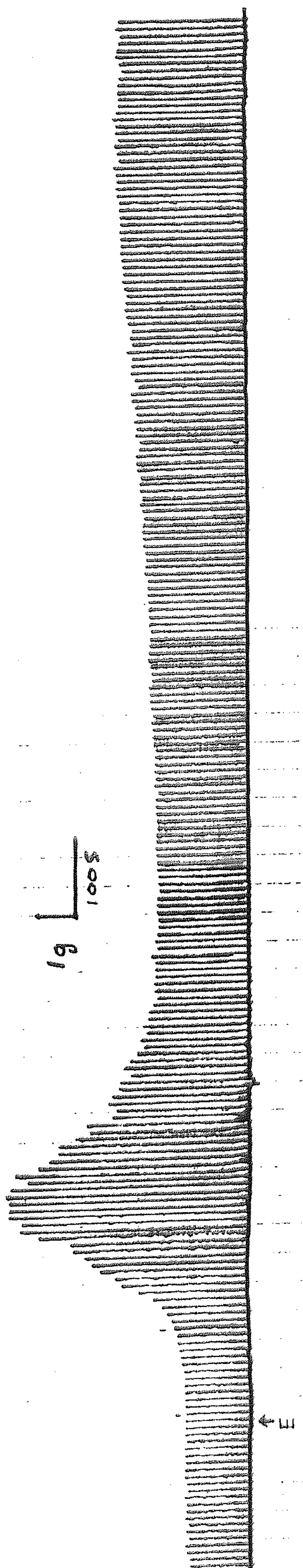
Two HDs from the same animal were experimented upon at once and this allowed one preparation to be used to record indirectly stimulated twitches (supramaximal/0.2 mS/0.1 Hz) and the other to record spontaneously occurring twitches induced by the addition of 500 nM ecothiopate to the organ bath for 7 minutes after which time the drug solution was rapidly replaced by fresh saline. The ecothiopate was not washed out in the experiments to record evoked twitches since the maximum amplitude of potentiated twitches was to be measured. Maximum potentiation did not always occur within 7 minutes.Washing the preparation in a fresh physiological saline after 7 minutes has been shown to produce and maintain the potentiation of evoked twitches. If this washing is not carried out when recording spontaneous twitches, the effect of the ecothiopate may wax and wane in a variable manner and it was decided that this would introduce an unnecessary source of variation into an already variable response. Spontaneous twitches were also recorded on tape by a Racal Store 4 tape recorder (0.5 V peak, $1^{7}/_{8}$ I.P.S.) for later computer analysis. Recording continued for 20 minutes following the washing of the preparation.

2.5.3: DATA ANALYSIS:

2.5.3.1: STIMULATED TWITCHES:

As described above, the twitches initiated by the stimulation of the phrenic nerve were recorded on paper via a chart recorder and an example of one of these records is shown overleaf:

Overleaf: Fig 2.2 Mouse dosed orally with distilled water only. Record of contractions of hemi-diaphragm preparation in response to supramaximal stimulation of the phrenic nerve at 0.1 Hz. Due to the slow time base each contraction is recorded as a vertical line. Calibration 1g, 100 S. At ↑ 500 nM ecothiopate was added . Note prolonged localised contractions indicated by thickening of line at base of the response.


1g
100s
E

From these paper records the following measurements were made manually:

1) The time from the addition of the ecothiopate to the first twitch showing an increase in amplitude relative to those preceding it. This was termed **time to first potentiation (TFP).**

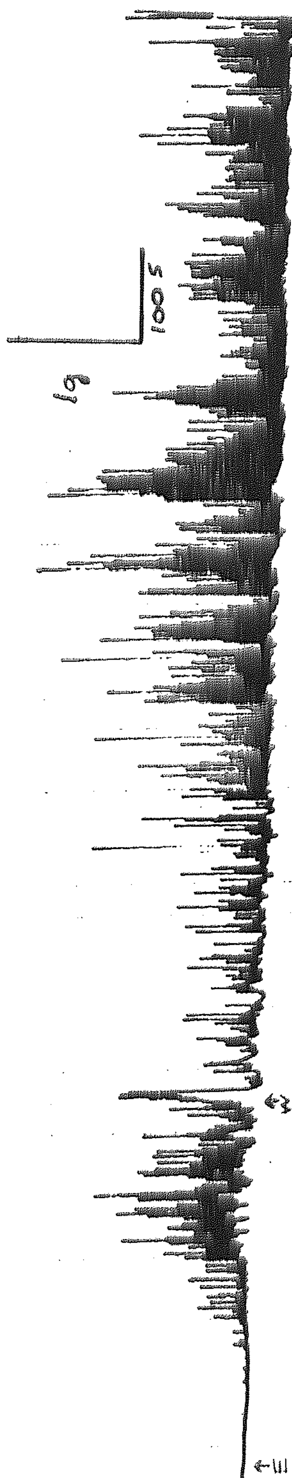
2) The time from the addition of the ecothiopate to the twitch showing the greatest increase in amplitude relative to all other twitches. This was termed **time to peak potentiation (TPP).**

3) The amplitude of the largest twitch following addition of ecothiopate and the amplitude of the twitch immediately preceding its addition. The former was expressed as a percentage of the latter and termed **maximum potentiation (MP).**

2.5.3.2: SPONTANEOUS TWITCHES:

Spontaneous twitches were recorded both on paper and on tape. Overleaf is a pen recorder trace showing typical spontaneous twitches from a control animal:

Overleaf: Fig 2.3 Mouse dosed orally with distilled water only. Record of contractions of hemi-diaphragm preparation. No stimulation of phrenic nerve. Due to the slow time base each contraction is recorded as a vertical line. Calibration 1g, 100 S. At ↑ 500 nM ecothiopate was added and the preparation was washed 7 mins later at W.



From this record it can be seen that spontaneous twitches exhibit highly variable amplitude and frequency. This makes manual measurement of these traces difficult. During the course of these experiments it became apparent that attempts should be made to quantify the total amount of spontaneous activity. An initial attempt was made to do this by trying to measure the block of light afforded by the trace on a transilluminated paper chart record using a photo-electric meter. However, it was not possible to find a sensitive enough meter to give a resolution greater than that possible by observing the records and subjectively scoring the activity as high, medium or low. These methods would also give little information on individual variations in twitch frequency or amplitude.

The recording of the spontaneous twitches on tape did give some other options. One was to simply replay the tape at a lower speed back to the pen recorder thus giving better separation of the twitches allowing manual measurement. This is the same as running the pen recorder at a higher speed. The problem with this idea is the number of twitches to be measured as can be seen later in the results section. It was obvious that some form of mechanised analysis was needed. The digitisation of the data and some form of computer based analysis was therefore decided upon.

2.5.3.3: COMPUTERISED ANALYSIS OF SPONTANEOUS TWITCHES:

Two computerised systems were utilised in the analysis of the twitching dependant upon the type of information required:

1:THE VIRTUAL OSCILLOSCOPE:

It was necessary during the study of spontaneous twitching to determine if the time course of the twitches showed any relationship with their size and if there was any effect of acrylamide. This data was gathered via the use of virtual oscilloscope software (Scope V 3.1 Analog Digital Instruments, Australia) run on a Macintosh classic following digitisation of the input signal by a MacLab/4 12 bit analogue to digital converter (Analog Digital Instruments). The software sampling frequency was set to run at 10 KHz and this gave 2560 data points per screen at a sweep speed of 200 mS per screen. Triggering was via a positive going threshold trigger which was set manually on screen just above the background noise.

Each captured twitch was stored as a single screen from which time and amplitude measurements could be made by use of a cursor which was calibrated for amplitude in g and time in mS. For each twitch the time to reach peak amplitude from rest (**TTP**), the time from peak amplitude to half decay (**T½D**) and the amplitude were recorded.

Due to the fact that once triggered the data acquisition process went off line whilst that screen of data was written to a hard disk, 20 minutes of taped responses only yielded

approximately 100 recorded twitches. This was sufficient to give a reasonable sampling of twitch sizes for the purpose of determining if there was any correlation between amplitude and **T½D** but as can be seen from figure 2.3 there were many more twitches than this occurring in the 20 minute period. Another consequence of the non-continuous sampling was that frequency data for the twitches was not available from this analysis. It was therefore necessary to analyse the data in such a way as to increase the number of twitches captured and derive amplitude and frequency data from them automatically. Continuous sampling was also desirable so that all the twitches occuring during the sampling period could be analysed.

2: AUTOMATED ANALYSIS OF SPONTANEOUS TWITCHES USING THE LABVIEW SOFTWARE SYSTEM:

In order to measure the amplitude and instantaneous frequency (the reciprocal of the interval between consecutive twitches) of all the twitches occurring in the 20 minute experimental run it was necessary to run the tape recorded data through a more complex software application on a machine capable of simultaneous analogue to digital conversion, measurement and data storage. These facilities were not available in the laboratory but through collaboration with Dr. David Davis of Birmingham University a suitable program written within the Labview software application (National Instruments) was adapted and this is described below.

Tape recorded data was first passed through the D.C. filter (unit model no. NL106) of a Neurolog amplifier (National Instruments) in order to maintain a constant baseline for voltage input into the next section of the system. This was a 12 bit analogue to a digital converter (National Instruments NB-MIO-169 data acquisition card) running at 10 KHz and installed in a Macintosh II ci computer. The application which actually performed the measurements was written within Labview and used a single channel software voltage comparator to discern the twitches from the baseline. The measurement process was triggered by a threshold trigger set just above the baseline noise and glitches were removed via the use of maximum and minimum duration triggers. This was possible since earlier analysis with the virtual oscilloscope had shown that the spontaneous twitches varied little with respect to rise or decay time, i.e. no repetitive firing of the muscle cells was occurring (Brown *et al.* 1936).

For each experiment this system produced a spreadsheet containing the amplitude and instantaneous frequency of each twitch captured and also gave the total number of twitches captured which were divided into 10 second time bins. This enabled the production of the following data:

1) Spontaneous twitch amplitude: This was expressed both as a mean value per experiment and also as a histogram to show the amplitude distribution of the twitches.

2) Instantaneous twitch frequency: For each experiment the mean and maximum frequency was obtained together with a distribution.

3) Number of twitches: The number of twitches occurring in 10 second time bins (90 bins = 15 min.) was manipulated to give 5 min. time bins to observe if there was any change in twitch output during the 15 min. sampling period. The total number of twitches occurring in 15 min. was also calculated.

2.6: METHODS AND MATERIALS FOR ELECTROPHYSIOLOGICAL EXPERIMENTATION:

2.6.1: THE RECORDING OF INTRACELLULAR ACTION POTENTIALS, ENDPLATE POTENTIALS AND MINIATURE END PLATE POTENTIALS:

Hemi-diaphragms were pinned out in a Sylgard based perspex bath attached to the stage of a microscope through which the preparation could be viewed via transillumination at a magnification of x40. The bath was constantly perfused with modified Lileys' physiological saline (see appendix) gassed with 5% CO_2 in 95% O_2 and maintained at 37°C ±0.2. Approximately 300-400 ml of saline was perfused through the bath in 1 hour. The phrenic nerve of the preparation was threaded through a perspex tube embedded in the wall of which was a pair of silver stimulating electrodes. These were connected to a stimulator (Devices model DS2) which provided supramaximal stimulation pulses of 0.02 mS duration (10x threshold). This was triggered when required, as in the case of recording jitter, by a digital timing device (Devices Digitimer type 3290) which was set to run at 30 Hz.

All intracellular records were made using glass capillary microelectrodes filled with 3M KCl. These had an initial input impedance 20-30 mΩ after pulling but this was reduced to 5-6 mΩ following bevelling of the tip at 45° on a settled slurry of 0.005 μM alumina powder in 3 M KCl (Lederer *et al.* 1979). This was done to make the tip of the electrode sharper and enable it to penetrate the cell membrane causing as little damage as possible and thus facilitate the holding of membrane potential over the recording period. The microelectrode was connected to a microprobe amplifier (Model M-707,W.P.I. Inc., USA) with an input impedance of 10^9 Ω. This was also connected to a silver/silver chloride pellet type electrode which earthed the saline in the bath. The x1 output from this was amplified by a Devices type 3290 a.c. amplifier with the adjustable low and high frequency response set at 0.16 Hz and 10 kHz respectively. These settings did not affect the rise times or general shape of the responses recorded but reduced some of the background noise. All responses were recorded on magnetic tape (Racal Store 4 DS

running at 32 inches per second) for later analysis. All recordings were made at the endplate region with the detection of miniature endplate potentials (MEPPs) with the fastest possible rise time being used as confirmation of the microelectrode position.

In all uncut cells, the recording of data only proceeded if resting membrane potential (RMP) was equal to or more negative than -65 mV. Cut fibre preparations, in which the muscle cells were cut either side of the end plate region to block propagation of action potentials along the cells (via disruption of the potassium ion gradient across the membrane), had RMPs in the region of -45 mV and were not subject to such strict criteria. Cells were cut in order to record endplate potentials (EPPs).Following cutting of the cells the action potential recorded at the endplate was seen to diminish in amplitude and eventually fail leaving only the EPP after approximately 30 minutes. During this time the rate of perfusion of physiological saline was increased in order to wash away any excess potassium ions liberated from the cells. If the RMP dropped by more than 5 mV during any recording period the cell was rejected. Action potentials (APs) were required to have a rise time in excess of 100 V/sec and to overshoot RMP.

MEPPs were recorded for 3 minutes when they were of suitable quality (rise time <1 ms and clearly discernible above baseline noise). After this time RMP sometimes became unstable. RMP was noted in all fibres used to record other data. Trains of indirectly evoked APs were recorded in order to analyse their jitter using the method of Kelly, Ferry & Bamforth (1990). These were stimulated at a frequency of 30 Hz via the digitimer and stimulator for a period of approximately 2 seconds. This was sufficient time to record the train of 30 APs required for analysis of jitter. The tetanic contraction of the HD induced by this stimulation tended to pull the microelectrode out of the cell under study or for the cell to be damaged by the electrode. This problem was counteracted by pinning the diaphragm out very tightly and using extra pins in close proximity to the recording area. This controlled excessive movement of the preparation. An identical protocol was used for the analysis of EPP jitter although the movement of the preparation was less of a problem since contraction in the recording area had ceased due to the cutting of the cells from which recording occurred. MEPPs, APs and EPPs were not recorded in the same cells.

2.6.2: ANALYSIS OF INTRACELLULARLY RECORDED POTENTIALS:

2.6.2.1:MEPP FREQUENCY:

MEPP frequency was obtained by simply replaying them from magnetic tape into a MacLab/Macintosh computer system of the same specification as previously described in the analysis of spontaneous twitches but this time running a virtual chart recorder program (Chart V3.2, AD Instruments). This software enabled the 3 minutes of MEPPs to be recorded and then compressed so that they fitted onto only 2 screens. From these screens the number of MEPPs clearly discernible from baseline was counted and

frequency calculated by dividing the number of twitches occurring by the time in seconds in which they occurred. Any glitches were detected by briefly examining the traces through a 'zoom window' in which they could be seen not to have the characteristic shape of a MEPP. Due to the time compression used it is important to undertake this procedure as a glitch on the compressed screen was indiscernible from the MEPPs which appeared as a vertical line.

2.6.2.2: JITTER OF APs AND EPPs:

If a single muscle cell is stimulated indirectly by a supramaximal stimulus to the nerve there is a delay between the initiation of an action potential in the nerve and the recording of the resultant action potential in the muscle cell. This delay or latency is due to conduction of the AP along the nerve, terminal depolarisation and transmitter release, postsynaptic depolarisation (the generation of an endplate current) and the excitation and propagation of the subsequent muscle AP to the recording electrode. Within a train of stimuli this latency is variable and is in the order of tens of μS. The variation in latency has been termed 'jitter'. In the normal nerve/muscle system, with suprathreshold stimuli, the variability in transmission time along the nerve and along the muscle fibre (measured using direct stimulation) is less than 3μS (Stalberg and Trontelj, 1979). Therefore the main source of jitter in the normal nerve/muscle system following indirect stimulation is assumed to be the endplate. The term jitter was coined from technical literature describing the instability of an oscilloscope display caused by a varying trigger or unstable time-base generator.

This variability can be expressed mathematically as the standard deviation around the mean interpotential interval. However, there are sometimes slow trends superimposed on the short-term random variation which tend to increase the standard deviation making it erroneous. An alternative to this is to express the jitter as the mean consecutive difference (MCD) over a train of action potentials (see section 2.6.2.3 for definition). This parameter measures short term variability and is relatively unaffected by slow trends making it a more suitable quantification of jitter (Ekstedt *et al.*, 1974; Stalberg and Trontelj, 1979).

2.6.2.3: THE COMPUTERISED ANALYSIS OF JITTER:

In this study trains of 30 APs or EPPs at 30 Hz were analysed using computer programs devised by Dr. S. S. Kelly which were run on a Digital Systems PDP 11/03 mini computer.

The first stage of the analysis process was to digitise the analogue data from the magnetic tape. This was achieved by running the program JITOT. The taped records were replayed into the 12 bit AD converter of the PDP (± 5 V input range) at a speed of $^{15}/_{16}$ of an inch per second. Original recording took place at 30 inches per second. This

gave a record replay ratio of 32:1. The JITOT AD conversion software was programmed to run at 20 KHz and this combined with the record:replay ratio gave an effective sampling frequency of 640 KHz. This method effectively speeded up the computer ensuring that enough data points were obtained during the analysis of each AP/EPP in the train of 30 to give sufficient resolution of their rise times in order that any changes in their latencies could be quantified. The software was configured such that for each AP/EPP there was a window for the sampling of data consisting of 650 data points each covering 50 μS. Allowing again for the record:replay ratio this represents approximately 1 mS of 'real time' and thus the positioning of the window over the AP/EPP is critical if an accurate representation of the AP/EPP is to be captured. As can be seen from fig. 2.4 below, the window needed to be positioned before the start of the AP/EPP but after the stimulus artefact. This was done via the use of a programmed delay which retarded the start of the window relative to an initiating trigger pulse which was recorded on the second channel of the tape recorder during the data acquisition. This pulse (detected by the PDP via a Schmitt trigger) was emitted at time zero on the digitimer with the pulse to the stimulator occurring exactly 1 mS later. The delay of the sampling window was input manually, in 0.1 mS units, into the software with the aid of the fact that the distance between the initiating trigger pulse and the beginning of the stimulus artefact represented 1 mS. By replaying a sample response to an oscilloscope and callibrating the divisions on the screen it was possible to calculate the correct delay of the window in order for it to cover the desired response after the stimulus artefact had passed. Once the programmed delay had been set, the tape was cued to the beginning of the train of responses and the first 30 consecutively digitised by the AD program.

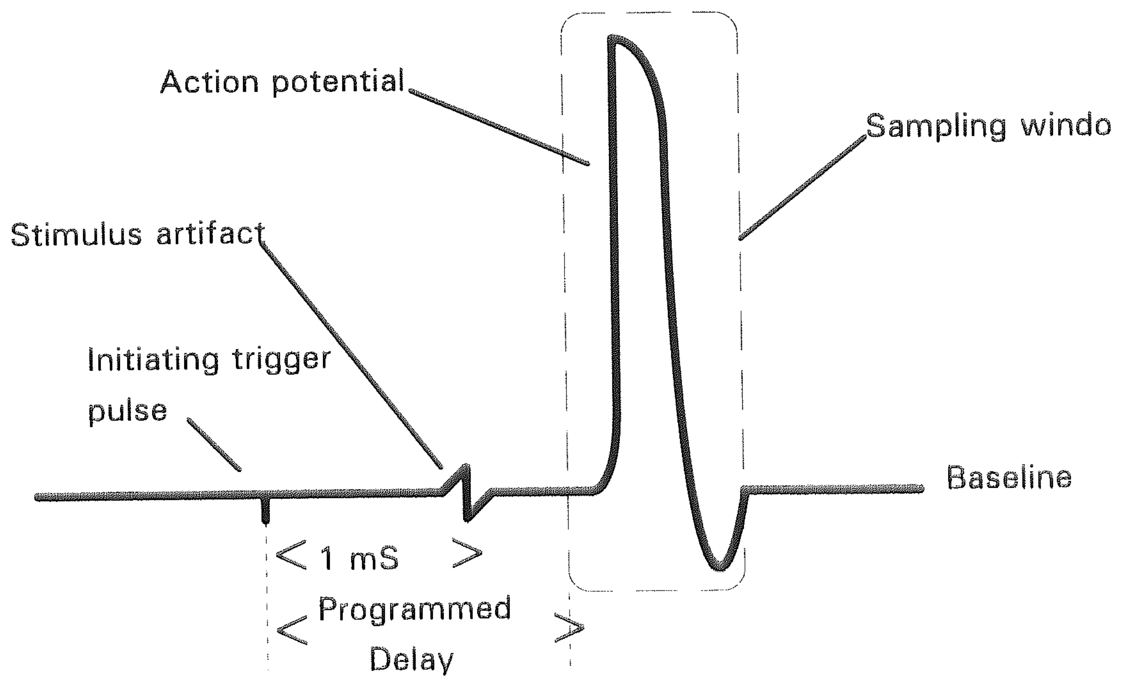


Fig 2.4: Representation of an action potential to show the position of the initiating trigger pulse, stimulus artefact and sampling window relative to it. The programmed delay was input manually in order to position the 1 mS sampling window over the action potential but after the stimulus artefact. The 1 mS separation of the initiating trigger pulse and the stimulus artefact was used to calculate the programmed delay via an oscilloscope.

The position of the baseline for each response was digitised as an average of 50 data points (80 μS real time) directly after the initiating trigger pulse from the digitimer. A sufficient delay between the initiating trigger pulse and the stimulus was necessary in order to allow enough time for baseline measurement. These data produced by JITOT were stored as raw data files on the PDP and analysed by further programs described below.

Raw data files were next analysed by a program called JITREB. This processed the raw files to yield simplified data files containing the values amplitude, rise time and latency of each AP/EPP within the train of 30 responses. Latency was defined as the time interval between the initiating trigger pulse and a point 10 % up the rising phase of the response, the peak of which having been calculated from the maximum value of a five data point rolling average taken throughout the sampling window.

Data files were then converted to summary data files by the program JITCAL. The data summaries could then be printed out to yield the following data:

RUN I.D. : A coded identification number for each cell recorded giving information on the treatment of the animal from which it came and the stimulation protocol.

RESTING MEMBRANE POTENTIAL: From manual input during the recording/analysis process in mV.

STIMULATION FREQUENCY: From manual input during the recording/analysis process in Hz.

THRESHOLD AND PEAK LATENCIES: Of each of the first 16 responses relative to the first with threshold being 10 % up the rising phase of the response (in μS).

PLATEAU MEAN PEAK: Mean amplitude in mV of APs 11 to 30 in the train.

MEAN TIME FROM THRESHOLD TO PEAK: in μS.

DELAY: The latency of AP/EPP 16 in the train minus that of the first.

MEAN CONSECUTIVE DIFFERENCE: This was calculated using the formula below:

$$MCD = ([L_{11}-L_{12}]+[L_{12}-L_{13}]+......[L_{29}-L_{30}]) \div 19$$

Where L_n = Latency AP or EPP n.

Of these data the values of MCD, delay and the threshold latency of the first 16 responses were recorded in a spreadsheet for further analysis. The partitioning of the data at AP/EPP 11 for the calculation of jitter is due to the nature of the of the change in the latency observed during the train of 30 responses at 30 Hz. Fig 2.5 below shows a representation of this:

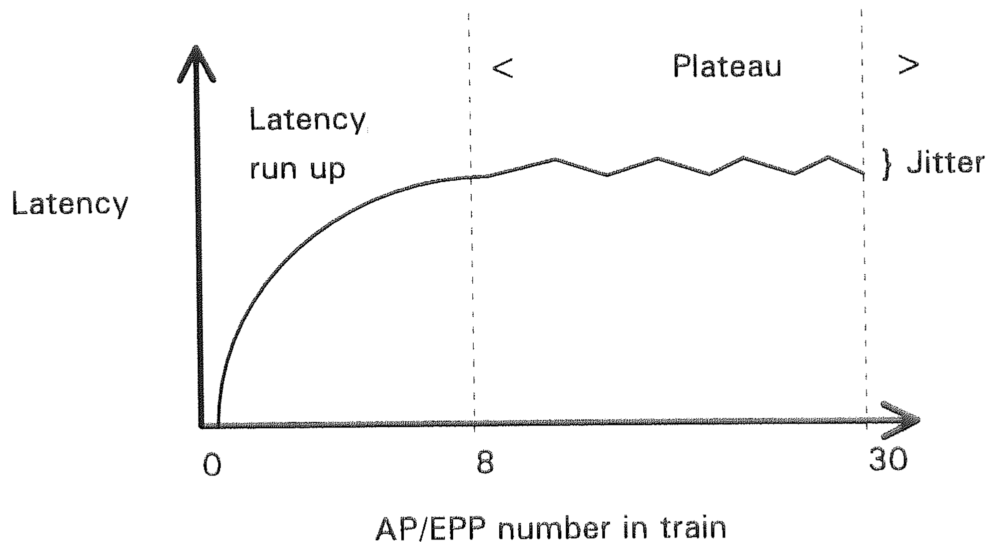


Fig 2.5: Diagram showing the typical variation of latency during a train of 30 indirectly evoked responses in mouse hemi-diaphragm preparation.. Note the rise and plateau in latency in a train of 30 responses (APs or EPPs) evoked at 30 Hz in the mouse hemi-diaphragm.

It can be seen from this that between responses 1 and 6 there is a 'run up' of latency after which a 'plateau' is reached. This plateau region contains small fluctuations in latency and it is this which constitutes jitter and is quantified by the calculation of MCD between responses 11 and 30. Delay is a measure of the systematic increase in latency between the first response and that achieved at the plateau and is measured by comparing the first and sixteenth response. This ensures that the latency of the first response is compared to the latency of a response which is within the plateau region. Analysis of the latencies of the first 16 responses in this study has shown that the plateau was always reached by response 11 and thus the jitter analysis undertaken by the computer programs is valid in this respect.

2.6.2.4: CALIBRATION OF THE JITTER ANALYSIS SYSTEM:

The calibration of the analogue to digital input was achieved via a separate program on the PDP11/03. Ten pulses of 20 mS duration and 100 mV amplitude were produced by a pulse generator and recorded to magnetic tape (Racal Store 4 DS) running at 30 inches per second. The duration of these pulses was relatively long in order to ensure a steady voltage during the calibration. These pulses were then replayed into the PDP at real time with the input threshold set just above the background noise of the system. The software yielded the number of analogue to digital units equivalent to 1 mV. This value was input into the analysis programs manually as requested.

An assessment of the systems inherent jitter was made by using a pulse generator to simulate APs by giving 100 mV/0.5 mS pulses at a frequency of 30 Hz. These were then

analysed as usual and a figure of 2.3 μS was obtained for the MCD. This represents the error in the record/replay/analysis process.

2.6.3: ACCEPTANCE CRITERIA FOR CELLS USED TO RECORD JITTER:

Cells were rejected from the recording/analysis process if they failed to satisfy the following criteria:

1) A resting membrane potential of at least -65 mV.(This criterion was waived when recording from cut fibre preparations e.g. EPP jitter.

2) A rise time of 100 V/S or greater.

3) Not more than a 5 mV drop in membrane potential during the recording of the train of evoked responses.(This was monitored visually via L.E.D. display during recording.)

4) A positive membrane potential at the peak of the AP, i.e. the amplitude of the AP must be greater than the resting membrane potential.

5) No failures (missing responses) during the train.*

* Failures were detected by watching for each response on an oscilloscope display during the playback of the recorded trains of APs/EPPs. Any failures were immediately obvious due to the slow replay speed of the recorded responses.

2.6.4: EXTRACELLULAR RECORDING OF MINIATURE ENDPLATE POTENTIALS:

Extracellular miniature endplate potentials ($MEPP_O$s) were recorded using the same hardware as for intracellular MEPPs with the exception that the microelectrode was filled with physiological saline and was of a patch type with an approximately 1 μm tip. The microelectrode had an input resistance of approximately 10 MΩ. The recording electrode was positioned by gently lowering it on to the surface of the HD in the endplate region so that it just brushed the surface of the cells. It was then moved across the surface of the HD until the negative going deflections characteristic of extracellularly recorded MEPPs were observed on the oscilloscope. The electrode was then finely manoeuvred to a position where the amplitude and rise time of the $MEPP_O$s was greatest. The $MEPP_O$s were then recorded onto magnetic tape (Racal Store 4 DS running at 7 inches per second) for 3 minutes for later analysis.

Focally placed microelectrodes when closely applied to the endplate region record negative-going deflexions whose time course is faster than internally recorded events

(Fatt and Katz, 1952; Liley, 1956; Head, 1983). This is due to membrane capacitance where a lag in the timing of the events is caused by the membrane charging and discharging across its lipid bilayer. The miniature endplate current flows through a small area of membrane and it should be noted that whilst external measurements of time course are more accurate than intracellular records, amplitude measurements need to measured intracellularly since only a small variance in the position of an external recording electrode can greatly reduce the size of the MEPP recorded (Del Castillo and Katz, 1956). It is for this reason that the frequency analysis described earlier used intracellularly recorded MEPPs so that if the recording electrode moved, small MEPPs would not be lost in the background noise and thus contribute to an error in frequency measurement. The externally recorded miniature endplate potential can be considered as a measure of the endplate current since the voltage measured is directly proportional to the current flowing through the resistance of the saline between the recording and earth electrode. The diagram below shows a representation of the miniature endplate current and the extracellular microelectrode used to record the potential it creates:

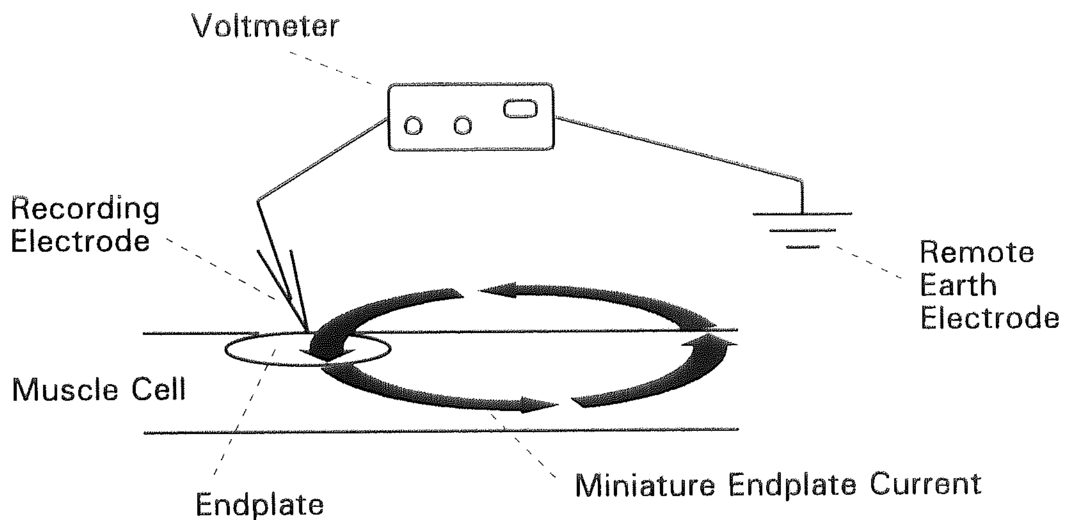


Fig 2.6: Diagram to show the arrangement of extracellular microelectrodes used for the recording of extracellular miniature endplate potentials in mouse diaphragm muscle cells.

The potentials measured in these experiments had amplitudes of approximately 0.5 mV and the resistance of the physiological saline between the electrodes was in the order of 100 KΩ and thus the size of the endplate current can be calculated as follows:

$$I = 0.5 \times 10^{-3} \div 10^{5} = 5 \text{ nA}.$$

This value for the amplitude of the endplate current is in agreement with other published values (Head, 1983).

2.6.5: ANALYSIS OF EXTRACELLULARLY RECORDED MEPPs:

The $MEPP_0$s recorded to magnetic tape were replayed into the virtual oscilloscope program on the Macintosh computer previously described in the analysis of spontaneous twitches (see section 2.5.3.3). The sweep time was set at 5 mS with 640 sampling points recorded during each sweep. This gave a sampling rate of 100 KHz. The trigger was set just above the baseline noise and the captured responses were averaged on line to produce one record representing the average $MEPP_0$ for each cell. From this the cursor was used to calculate the rise time (Trise) and the time from the peak of the $MEPP_0$ to its half decay ($T^{1/2}D$).

2.6.6: THE DETERMINATION OF STIMULUS STRENGTH/DURATION CURVES FOR EXCITATION OF NERVE TERMINALS AND MUSCLE CELLS:

Strength duration curves (SDCs) for the excitation of nerve cells were obtained as follows. Hemi-diaphragms were obtained as described previously and placed in the same experimental bath as for all other electrophysiological experiments and maintained at the same temperature and rate of saline flow through the bath. An extracellular microelectrode of 2-4 MΩ filled with 2 M NaCl was positioned close to a nerve terminal by observing negative going extracellularly recorded MEPPs on an oscilloscope screen as described earlier in section 2.6.4. The electrode was thus assumed to be at or near an endplate on the muscle cell surface. This recording electrode was then disconnected from the voltage recording apparatus and connected to a stimulator (Devices DS2) via soft connecting wires. These damped out any motion which might otherwise have been transmitted to the electrode causing it to move.. The switch from recording to stimulating configurations had an undetectable effect on the electrode position with regard to the amplitude of the MEPPs recorded upon a switch back to voltage recording. With the stimulating electrode now in position, voltage pulses of varying duration (0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 1.5 and 2.0 mS) were applied to drive current inwards at the electrode. At each duration of stimulus the voltage output of the stimulator required to elicit remote muscle cell contractions within the field of view was recorded. This indicated that the nerve had been stimulated since the stimulation of a muscle cell only yields contractions local to the electrode. This could be demonstrated by moving the stimulating electrode away from the nerve terminal region following observation of remote contraction. This resulted in only local contraction of muscle cells even if the stimulus was increased. It is of note that failure of nerve conduction of action potentials would result in the inability of this system to distinguish between muscle or nerve excitation since remote contractions would not be observed in either case.The threshold of the responses was always approached from a sub threshold value since experimentation with methodology

showed this to produce the most consistent value for the same nerve terminal. The determination of the threshold was carried out in ascending order of duration. Shorter durations required larger voltages and varying the duration in this manner meant that the threshold determinations made at longer duration pulses and thus of lower voltage, were made later in the run at each nerve terminal. This acted as an indicator that electrode position relative to the endplate was steady since high values of voltage output from the stimulator at the longer durations correlated with the loss or reduction in amplitude of MEPP amplitude. MEPP amplitude was monitored following each determination of a SDC and the data was rejected if this value appeared altered. The diaphragm was pinned out as tightly as possible in these experiments in order to reduce the likelihood of the site of stimulation moving relative to stationary stimulating electrode. The output of the stimulator was measured at the beginning and end of each experimental session but was always found to be consistent.

The determination of the SDC in muscle cells was somewhat simpler in that the position of the electrode was not as critical as in the protocol above. The stimulating electrode was lowered on to a muscle cell such that it just brushed the surface of it. The electrode was then raised to the height that it just cleared the cell surface. Threshold was judged to be the voltage required at a given stimulus duration to cause the cell directly below the electrode to twitch. There was no widespread twitching observed because there was no nervous tissue being excited. With these exceptions the protocol was the same as for the nerve terminals.

2.7: MATERIALS AND METHODS USED IN BIOCHEMICAL ANALYSIS:

2.7.1: THE DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY:

Acetylcholinesterase (AChE) activity is relatively easy to measure as shall be described later in this section but the more challenging part of this study is the extraction of the AChE to be analysed. It is desirable not only to extract this enzyme from tissue samples but also to learn something of its distribution within the sample. During the course of this study work carried out by Miss Anna Ancilewski on sequentially extracting AChE from the hemi diaphragm based on the method Younkin *et al* (1982), led to greater resolution of the distribution of AChE and in particular its different molecular forms. This method is referred to as **sequential extraction**. Prior to this work, the extraction of the AChE was more crude. This method is referred to as **conventional extraction**. Described below are both methods for extraction of AChE since both were undertaken during the course of this work as the more advanced method was not immediately available.

2.7.1.1: THE CONVENTIONAL METHOD FOR EXTRACTION OF AChE:

The aim of this method was to assay the activity of AChE in the diaphragm. To this end it would have been possible to homogenise the whole muscle and assay total AChE from the homogenate. However, this yields little data on the distribution of the AChE within the muscle. In this method the hemi diaphragms (HD) were subdivided in an attempt to obtain some idea as to the location of the AChE.

The hemi diaphragms used in this work were obtained as described in the dissection section. Each HD was then pinned out flat via the central tendon and ribs in a Sylgard based dish using small entomological pins. The dish contained a small quantity of the Liley's physiological saline used in the dissection. The HDs were then closely inspected and any remnants of connective tissue, adipose tissue or blood clots removed. The phrenic nerve was then removed by cutting it as close to the surface of the HD as possible. This 'cleaned' HD was examined under a dissection microscope and the region of it most likely to contain the motor endplates identified by looking for the near terminal branches of the phrenic nerve. This is a fairly subjective means of identifying the endplate region within a diaphragm but is aided by experience of observing their position in cholinesterase stained preparations. Once identified the region of the HD thought to contain the endplates was cut from the muscle with a scalpel and usually consisted of a strip of tissue approximately 3 mm wide which ran parallel to the ribs across the middle of the HD. This region of the muscle was designated the junctional region (J) and was assumed to contain the majority of the motor endplates in the HD. The remainder of the HD consisting of the two strips of muscle which were located on either side of the junctional region were separated from the ribs or the central tendon and together were designated the non junctional (NJ) region which was assumed to contain relatively few endplates. The diagram below shows this division of the HD:

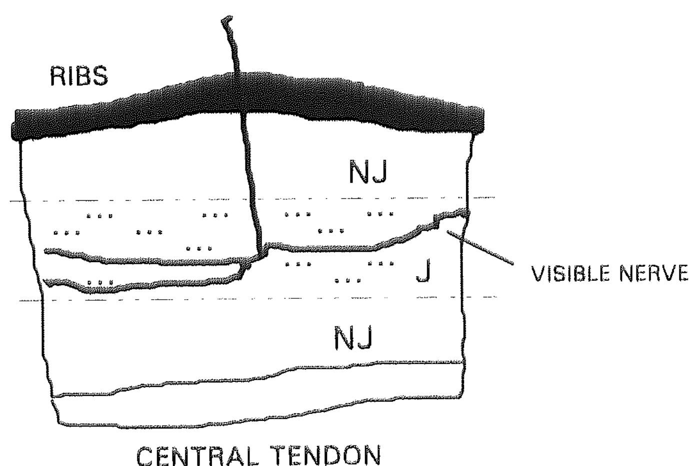


Fig 2.7: Diagram to show the division of a mouse hemi-diaphragm into Junctional (J) and non junctional (NJ) regions. This was in order to allow for the calculation of activity specific to the endplate during the determination of acetylcholinesterase activity within the diaphragm.

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The J and NJ regions were blotted dry and weighed on a five decimal place balance. The weights of the two regions varied with the extent of the branching of the phrenic nerve with less widespread branching leading to lower J tissue weights. J regions weighed between 50 and 80 mg with the NJ regions being approximately double this.

AChE was extracted from these samples as follows. The J or NJ region from each HD was placed in a centrifuge tube containing 2 ml of ice cold 0.1M pH 8 phosphate buffer and homogenised for 1 minute using an Ultra Turrax T25 homogeniser running at a speed of 24000 RPM. The homogeniser was rinsed off with 3 ml of ice cold buffer which was added to the homogenate to give a final sample volume of 5 ml. Due to the potential for a build up of heat in the tubes during this process which might damage the AChE, they were kept constantly embedded in ice. The 5 ml homogenate samples were then sonicated using a high intensity ultrasonic processor (Jencons 50W model). A total of 2 minutes sonication on a 30 seconds on / 30 seconds off regime was applied with samples still remaining on ice. This sonication programme has been shown within the laboratory to give the best balance between extraction and apparent degradation of the AChE.

Finally, the homogenate was centrifuged for 15 minutes at $4^{0}C$ at a speed of 2700 RPM in a Beckmann T.J. bench centrifuge. The resulting supernatant was carefully pipetted out of the tube and retained for assay whilst the pellet was discarded.

2.7.1.2: THE SEQUENTIAL METHOD FOR EXTRACTION OF AChE:

The conventional extraction method with division of the diaphragm into junctional and non junctional regions goes a little way to elucidating the location of the AChE in the diaphragm preparation but the AChE under investigation here has many different molecular forms and it would obviously be of great benefit to determine which molecular forms occur where in the diaphragm and whether this distribution is modified by acrylamide administration. To some extent this is made possible by the use of a sequential extraction process involving the differential solubility of certain AChE molecular forms in high or low ionic strength buffers. Globular forms of AChE are soluble in low ionic strength buffer (LIB) whereas asymmetric forms are soluble in high ionic strength buffer (HIB). This difference in solubility is conferred on the molecular forms by the absence or presence respectively of collagen tails in their structure. This method has been adapted from the method of Younkin *et al* (1982) who used rats as the study animal. Determination of the molecular forms in each sequential homogenate in their study was via the use of velocity sedimentation on sucrose gradients with subsequent assay for AChE activity of fractions removed from the gradient. Peaks in AChE activity of fractions corresponding to the appropriate molecular weight of the various forms of AChE are used to identify the molecular forms of AChE found in a particular sequential homogenate. This process has been repeated within the laboratory

for the use of the mouse as the study animal and the results have shown this method of extraction of AChE to be valid despite the species difference with corresponding molecular forms found in the same sequential homogenates. Once the sequential homogenates have been characterised for the molecular forms of AChE they contain and assuming the same protocol for extraction is adhered to then the use of the lengthy and complicated velocity sedimentation is no longer necessary. The extraction procedure and resulting molecular form content are described below.

PROCEDURE:

Junctional or non junctional strips of hemi diaphragm were prepared and weighed as before and placed in a centrifuge tube containing LIB (10mM phosphate buffer, pH 7.0 containing 1% Triton x100). The tissue was then homogenised in the tube using an Ultra Turrax T25 homogeniser running at a speed of 24000 RPM for 1 minute. During this and all subsequent processes the samples were maintained on ice or kept refrigerated in an attempt to prevent proteolytic breakdown of the asymmetric forms of the AChE. The homogenate was then transferred to a centrifuge and spun for 30 minutes at 39000g.

This process produced a supernatant fraction termed S1 which was carefully removed from the tube and kept on ice for assay. S1 is known to contain globular forms of AChE. The pellet remaining in the tube was re-suspended in LIB and re-homogenised prior to being centrifuged as before. The supernatant fraction from this process was termed S2 and is known to contain globular forms of AChE which are called residual globular forms due to the second homogenisation necessary to extract them. Together the S1 and S2 fractions are considered to contain the majority of the AChE activity attributable to globular forms of the enzyme.

The pellet was again re-suspended and homogenised but this time in HIB (LIB + 1.0 mM sodium chloride). The resulting supernatant fraction after centrifugation in this case was termed S3 and contained asymmetric molecular forms of AChE. The process was repeated again with the pellet to yield an S4 supernatant fraction containing residual asymmetric forms. The S3 and S4 fractions are considered to contain the majority of the AChE activity attributable the aymmetric forms of the enzyme.

The final stage of the process was to re-suspend and homogenise the pellet from the penultimate stage to give what was termed the H5 sample which contains non extractable forms of AChE. These are thought to represent that AChE which is most tightly associated with the synaptic cleft (integral membrane protein) and thus probably the most functional component of the total AChE present in the tissue sample as far as hydrolysis of acetylcholine transmitter is concerned. Fig. 2.8 is a summary of the sequential extraction procedure.

DIAPHRAGM MUSCLE
(J OR NJ)

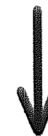
Homogenise in LIB + centrifuge (39000g /30 mins)

PELLET + **S1** (*Globular molecular forms*)
Homogenise pellet in LIB + centrifuge

PELLET + **S2** (*Residual globular forms*)
Homogenise pellet in HIB + centrifuge

PELLET + **S3** (*Asymmetric molecular forms*)
Homogenise pellet in HIB + centrifuge

PELLET + **S4** (*Residual molecular forms*)
Homogenise pellet in HIB



H5 (*Non extractable forms*)

Fig. 2.8: Flow diagram to show the stages in the sequential extraction of the different molecular forms of acetylcholinesterase from the diaphragm of the mouse. LIB and HIB are low and high isotonic strength buffers respectively.

2.7.2: THE SPECTROPHOTOMETRIC ASSAY FOR THE ESTIMATION OF ACETYLCHOLINESTERASE ACTIVITY:

A spectrophotometric assay technique based on that of Ellman *et al* (1961) was used to determine the activity of AChE in samples prepared as described already in this section. The method is simple, sensitive and reproducible and has been widely used for routine determination of AChE activity.

The principal behind the technique is to substitute acetylthiocholine for acetylcholine as the substrate for the extracted AChE so that the breakdown products resulting from the hydrolysis catalysed by the AChE are acetate and thiocholine. Also present in the reaction vessel is the reagent DTNB which contains 5,5,-dithiobis-2-nitrobenzoate ions. These ions react with the thiocholine to produce a yellow anionic product, 5-thiol-2-nitro benzoic acid. This product absorbs ultra-violet light of the wavelength 412 nm and thus the change of absorbance at this wavelength is proportional to the AChE activity producing the thiocholine.

In this study the activity of the extracted AChE was assayed using the principal above and the change in absorbance was measured using a Philips PU8700 series UV/VIS fixed bandwidth spectrophotometer in conjunction with a PU8700 cell programmer with a multicell holder.The cell programmer enabled the absorbance of a sample to be tracked continuously for periods of five seconds with an interval between readings of 1 to 10 minutes. This allowed 4 replicates to be run for each sample. These replicates were then averaged. Temperature was closely controlled and monitored during the assay process with a heated water bath being used to warm samples and reagents before the addition of acetylthiocholine and DTNB to the extracted samples and also to circulate water to the cell holder which was equiped with a water jacket. The temperature was set at 30^{0}C at all times.

Prior to assay the spectrophotometer was blanked by running a cuvette containing phosphate buffer through the instrument for reference. This established the zero for the subsequent run. 1 ml each of sample, 0.5mM acetylthiocholine and DTNB colour reagent were mixed in a test tube in the water bath and then a 1 ml sample of the mixture was rapidly transferred to a disposable cuvette which was placed in the spectrophotometer for assay. This was repeated for each of the four replicates and since J and NJ samples were run at the same time this meant that 8 cuvettes in all were loaded in the machine at any time. The change in absorbance at 412 nm was then recorded every 1.5 minutes for 9 minutes. These data were then plotted to check that there was a straight line relationship between time and increase in absorbance over the 9 minute period. This relationship shows that temperature was constant throughout the run and also that other limiting factors such as substrate exhaustion were not occurring. Absorbance change per minute was then calculated from the gradient of these graphs.

2.7.3: PURIFIED ENZYME EXPERIMENTS:

In some experiments purified type VI-S acetylcholinesterase from the electric eel (Sigma) with a unit hydrolysis rate of 1 μmole of acetylcholine per minute at pH 8 and 37°C was dissolved in pH 8 phosphate buffer at concentration such that there was 1 enzyme unit per 10 ml. The solid form of the enzyme contains 225 units per mg. This solution replaced the sample solution described in the assay procedure following the addition of 10^{-6} M acrylamide and an incubation of 45 minutes at 37°C in a water bath. This was a very high concentration of acrylamide relative to that which one would expect following the *in vivo* dosing and was used to ensure that if acrylamide was having a direct effect upon AChE activity then it would be detected by the assay.

2.7.4: BIOCHEMICAL CALCULATIONS:

The Beer-Lambert law states that the absorbance (A), or the extent of radiation absorbed by a medium, is proportional to the concentration of that medium and the thickness of the layer. Since absorption is equal also to the reciprocal of the transmittance, the law can be expressed in terms of absorption as follows:

$$A = \text{Log} \frac{Io}{It} = ECL$$

Where: A = Absorbance.

Io = Intensity of incident radiation.

It = Intensity of transmitted radiation.

E = Molecular extinction coefficient for the absorbing medium ($dm^3\ mol^{-1}\ cm^{1}$).

C = Concentration of the absorbing solution (molar).

L = Light path in the absorbing material (cm).

The molar extinction coefficient for DTNB is $1.36 \times 10^4\ dm^3\ mol^{-1}\ cm^{-1}$. For experimental purposes E is required in millilitres and hence:

$$E = 1.36 \times 10^4 \times 10^3 \times 10^{-9} = 1.36 \times 10^{-2}\ ml\ nmole^{-1}\ cm^{-1}$$

The rate of hydrolysis (R) of acetylthiocholine substrate can be calculated from the following equation:

$$R = \frac{\text{Change in absorbance (A) per min}}{1.36 \times 10^{-2}}\ nmol\ min^{-1}\ ml^{-1}$$

The units are calculated as follows:

$$R = \frac{\text{Change in absorbance x nmol x cm}}{\text{min x cm x ml}}$$

$$= \text{Change in absorbance nmol min-1 ml-1}$$

If the concentration of tissue in a given sample is X mg ml^{-1}, the cuvette volume V ml and the volume of tissue sample in the cuvette is Y ml then R can be expressed as follows:

$$R = \frac{\text{Change in absorbance x V ml}}{1.36 \times 10^{-2}\text{min x cm x } \frac{\text{X mg}}{\text{ml}} \text{ x Y ml}} \quad \text{nmol min}^{-1}\text{ mg}^{-1}$$

2.7.5: CALCULATION OF ENDPLATE SPECIFIC ACTIVITY (EPSA):

The division of the HD into junctional and non junctional regions enables an estimate to be made of the activity which is specific to the endplate region. If it is asumed that the AChE activity associated with muscle cells in the junctional region is the same as that in the non junctional region then subtraction of the non junctional quantities of AChE from the junctional quantities should allow an estimate to be made of AChE associtiated with the synapse. This might include the functional AChE associated with the clefts of the synapse and any other local accumulations. This fraction of the total AChE activity has been termed the endplate specific activity and is calculated as shown below:

EPSA = Ja-NJa

Where: Ja = Junctional activity per mg of tissue.
NJa = Non juntional activity per mg of tissue.

2.8: MATERIALS AND METHODS USED IN HISTOLOGY:

2.8.1: HISTOCHEMICAL LOCALISATION OF ACETYLCHOLINESTERASE:

The method of Karnovsky and Roots (1964) was used for the histochemical localisation of AChE. This method has the advantages that it is visually controlled (approx. 1-2 hours), allowing remarkable subtlety in the degree of staining obtained and that colour is produced directly at the site of enzymatic activity.

The basis of the method is that thiocholine liberated by the hydrolysis of acetylthiocholine by the AChE reduces ferricyanide to ferrocyanide which combines with Cu^{2+} ions to form the insoluble product of copper ferrocyanide known as Hatchett's

Brown. The Cu^{2+} ions in the medium are complexed with citrate to prevent the spontaneous formation of copper ferricyanide. The formula for this stain is included in the appendix and the precise procedure is described below.

2.8.2: PROCEDURE FOR DETERMINATION OF ENDPLATE SHAPE:

Hemi-diaphragms (HD) were removed as described previously and pinned out flat by the ribs and central tendon on strips of dental wax using entomological pins. These preparations were then placed in glass vials and covered with approximately 40 ml of 0.1 M pH 8.0 phosphate buffer containing 5% glutaraldehyde to act as a fixer. The vials were then placed in a refrigerator for 4 hours. This treatment was sufficient to lightly fix the tissue. The tissues were then rinsed rapidly in approximately 100 ml of distilled water before being covered with 40 ml of the phosphate buffer without the gluteraldehyde. The preparations were then stored overnight in the refrigerator. This prolonged washing of the tissues was found to yield improved staining. The fixed and washed HDs were then cut from the ribs and any excess connective tissue or other debris was carefully removed along with the phrenic nerve. The HDs were then incubated at room temperature in freshly prepared staining medium made up as described in the appendix.

The staining medium was apple green in colour and remained so for several hours after being made. However, if it developed a brown tinge this was indicative of it having deteriorated and fresh medium was made. This was rare and was usually attributable to the deterioration of the potassium ferricyanide solution used as one of its constituents. For this reason stock solutions were stored at $4^{0}C$ for only a few weeks.

Staining with this relatively light fixation took between 1 and 2 hours after which time the HDs were washed in distilled water and mounted in an aqueous mounting medium (Immu-Mount, Shandon Scientific Ltd.) on a glass microscope slide covered by a cover slip.

2.8.3: MEASUREMENT OF END PLATE DIMENSIONS:

Slides prepared as described above were observed under a binocular microscope with x10 eyepieces (fitted with a calibrated graticule) and a x100 oil immersion objective. The band of the preparation containing the majority of the endplates was located and its left most limit was identified. From this point the slide was scanned unidirectionally to the right with the intention that no endplate should be measured twice. Measurement of the endplates consisted of measuring their length along the axis of their associated muscle fibre and their maximum width perpendicular to that axis. However, before an endplate could be measured it had to satisfy the following criteria:

1) The endplate must be located on a clearly discernable muscle fibre with borders which are in the same plane of focus as the endplate.
2) The outline of the endplate must be distinct, i.e. not understained and not so heavily stained that the borders have become fuzzy in appearance.
3) The endplate must be parallel to the plane of focus to allow accurate measurement of its dimensions.

Although each HD contains many endplates it was found that the number of endplates fitting the above criteria was small since many wrap around the muscle fibres. For this reason several animals were used at each dose in order to get sufficient data. This also helped to take account of any variation between animals.

2.8.4: PREPARATION OF MATERIAL FOR DETERMINING SARCOMERE LENGTH:

HDs were fixed as above with the exception that they were left in the fixing phase of the process for 24 hours in order to make the muscle fibres considerably stronger to allow for them to be separated without extensive damage by the processing described below.

The HDs were transferred to the AChE staining medium and staining took 3 to 4 hours following the extra fixing. Once the endplate region could be clearly identified by the appearance of brown speckles on the surface of the HD, small squares of tissue approximately 4 mm on each side were cut from the HD in the areas appearing richest in endplates. Each of these squares was transferred to a glass vial containing 30 ml of the AChE staining medium. An Ultra Turrax T25 homogeniser running at a speed of 10500 RPM was then used to break up the small square of tissue until it appeared that only single cells remained. Approximately 5 minutes of homogenisation was necessary to disassociate the fibres. This process was performed in AChE staining medium so that end plates not previously stained due to their position away from the surface of the HD could be stained and thus increase the potential number of cells with stained endplates for later extraction. It is of note that since the stain was only being used to show the location of the endplates over staining of endplates was not a problem. Following 30 minutes of incubation of the cells in the AChE stain 5 ml samples of the cell suspension were filtered using a 25 mm diameter round polycarbonate filter with a pore size of 0.8 μm (Nuclepore Corp. Pleasanton, California). 5 ml of distilled water was then flushed through the filter to wash the staining medium from the trapped cells and thus halt any further staining.The filter was then observed under a dissection microscope and single cell fragments with brown stained endplates could be seen. With the use of fine nosed watch makers forceps these cell fragments (approx. 2 mm in length) were transferred to

a drop of aqueous mounting medium on a glass microscope slide. Once 10 to 15 cell fragments were transferred a coverslip was placed over the mount and it was allowed to set.

2.8.5: MEASUREMENT OF SARCOMERE LENGTH:

The length of the sarcomeres in the muscle fibre fragments was measured using a microscope fitted with Nomarski differential interference optics which gave clearer definition of the muscle striations. These appeared as alternating light and dark bands perpendicular to the long axis of the muscle fibres. 10x eyepieces were used in conjunction with an 100x objective for the measurements once appropriate fragments had been located at low power. The right hand eyepiece was fitted with a graticule which was calibrated using a slide micrometer etched with 100 divisions at a separation of 0.01 mm. It was calculated that one eyepiece graticule division was equivalent to 0.001 mm.

For each fragment with clearly visible striations the separation of adjacent dark bands was measured by identifying 3 to 5 adjacent repeats of the banding sequence and measuring their total width and then dividing by the number in the group as this reduces errors associated with determining the centre of the bands. This was done in two locations for each fibre. Firstly, directly beneath the endplate and then as far away from this point as possible without impinging upon any cut ends or damaged parts of the fibre where distortion may have ocurred. In some cases the fragment was lying in such a way that the darkly stained and therefore opaque endplate was parallel to the plane of focus and thus blocking the view of the striations below. These fragments could not be measured but were found to be rare. Endplates more often appeared to be on one side of the fibre or the other with the striations visible across approximately half the fibre.

The measurement made away from the endplate acted as a control against which the endplate measurement was compared in each fibre. Thus each endplate spacing had its own control from within the same fibre. Results are expressed as endplate striation separation in μm and as a percentage of that of the control value.

2.8.6: THE COMBINED STAINING OF ACETYLCHOLINESTERASE AND MOTOR NERVE TERMINALS:

The staining undertaken in this section is based on a modification of the method Pestronk and Drachman (1978) made by Pollard (1984).

The use of a combined silver/cholinesterase stain was of interest in this study because it was desirable to observe the extent of visible nerve damage occurring in the terminal regions of the motor nerves and at the nerve terminals themselves. The clarity and reliability of the stain permit quantitative measurements of neuromuscular junctions in order to determine pathological changes.

PROCEDURE:

Hemi-diaphragms (HD) were pinned out flat on to strips of dental wax with entomological pins via the ribs and central tendon and then placed in glass vials. The vials were then filled with 0.1 M pH 7.4 phosphate buffer/ 5% formaldehyde. The tissues were fixed for 3 hours and then unpinned, the ribs removed and any excess connective or adipose tissue carefully cut away. They were then washed in phosphate buffer for at least 1 hour followed by 2 washes in distilled water of 10 minutes each. The HDs were then incubated in acetylcholinesterase staining medium for approximately 10 minutes at room temperature in order to lightly stain the neuromuscular junction for later identification of the terminal arborisation of the motor nerves. The staining medium was then washed off with 2 quick changes of distilled water. The HDs were then immersed in 0.5 mM potassium ferricyanide solution for 10 minutes. This was removed by 3 x 5 minute washes with distilled water before the tissues were covered with absolute ethanol for 2 x 30 minute washes in order to extract most of the lipids from the tissues. The tissues were washed twice for 15 minutes in distilled water before being transferred to silver staining medium to stain nervous tissue for 1 hour with agitation every 20 minutes for 1 minute. This was followed by a 20 minute wash in distilled water. The silver stain requires the addition of a reducing solution in order for it to develop and turn nervous tissue black. This was done in small plastic petri dish which could be placed under a suitably powerful microscope so that the development of the stain on the axons of the HDs could be controlled visually. The development process was halted by the addition of a few drops of concentrated ammonium thiosulphate solution to a distilled water wash into which the tissues were placed when they had developed sufficiently. The tissues were then given a final wash in distilled water and stored overnight before being dehydrated and cleared using toluene. Slides of these preparations were then made using a toluene based mount (Shandon Scientific Ltd.). All glassware was washed in chromic acid and rinsed with distilled water before use. Tissues were only handled with plastic forceps and plastic spatulas were used throughout the preparation of reagents.

2.9: THE HAEMATOXYLIN AND EOSIN STAINING OF SCIATIC NERVES:

Segments of sciatic nerve approximately 2 cm in length were removed proximally to the 'sciatic notch' from mice following control or acrylamide treatment. The tissue was then fixed in 10% neutral buffered formal saline prior to being embedded in parafin wax. 5μm thick sections were then cut from the paraffin block to produce transverse sections of the sciatic nerve. These sections were then stained with Mayer's alum haematoxylin and eosin/phloxine by following the immersion steps listed below:

	Time:
1. Xylene I	1 minute
2. Xylene II	1 minute
3. Absolute alcohol I	1 minute
4. Absolute alcohol II	1 minute
5. 90% alcohol	1 minute
6. 70% alcohol	1 minute
7. Rinse in distilled water	
8. Stain with Mayer's haematoxylin	5 minutes
9. Wash in running distilled water	5 minutes
10. Stain with 0.25% eosin / 0.25% phloxine	5 minutes
11. Rinse in distilled water	
12. 70% alcohol	30 seconds
13. 90% alcohol	30 seconds
14. Absolute alcohol I	1 minute
15. Absolute alcohol II	1 minute
16. Xylene I	1 minute
17. Xylene II	1 minute
18. Mount in DPX	

2.10: STATISTICAL ANALYSIS:

Before statistical tests can be used to make inferences about the differences or similarities of populations of data it is important to understand their general characteristics and frequency distributions so that the correct test can be applied which is not compromised by those data it has been applied to.

All data in this study underwent primary analysis before statistical tests were applied. This consisted of production of general descriptive statistics and frequency distributions (Microsoft Exel analysis tools) in conjunction with simply looking at the data. Below is sample of the descriptive statistics output from the Microsoft Excel analysis tools pack (data set = 1,2,3,4,5,6,7,8,9,10):

Data X	
Mean	5.5
Standard Error	0.957427108
Median	5.5
Mode	#N/A
Standard Deviation	3.027650354
Variance	9.166666667
Kurtosis	-1.2
Skewness	0
Range	9
Minimum	1
Maximum	10
Sum	55
Count	10

This output gives a variety of information about the data including kurtosis (the degree of peak) and skewness. Used in conjunction with the frequency distributions it became clear that, with the exception of one set of data, the data was not normally distributed and could not be normalised. This was due to skewness of the data and/or the presence of outliers. Thus it was necessary to apply non parametric statistical tests to the data. In the one exception to the general non normality a standard t-test assuming unequal variance was applied from the Microsoft Excel analysis tools pack.

2.10.1: THE WILCOXON RANK SUM TEST:

This test has been reported to be equivalent to the Mann-Whitney test (Conover, 1980). It makes the assumptions that independent random samples are taken from 2 populations and provides a procedure for testing that 2 populations are identical but not necessarily normal. The test assumes a null hypothesis were the 2 populations are identical.

The test is performed by jointly ranking (from lowest to highest) the values of the 2 samples and then examining the sum of the ranks for the sample with the number of observations. If the sample size (n) of one of the data sets is less than 10 this value (T) is then compared to tabulated values of T_u and T_L (upper and lower limit values for T at chosen value of α). The null hypothesis is rejected if $T > T_u$ or $T < T_L$.

If the sample size of both data sets is greater than 10 the sampling distribution of T is approximately normal and this allows the use of a z statistic in the Wilcoxon rank sum test.

z is calculated as below:

$$z = (T - \mu_T) / \sigma_T \quad \text{where } \mu_T = \text{mean of } T = n_1(n_1+n_2+1) / 2$$

$$\sigma_T = \text{variance of } T = \text{ square root of } (n_1 n_2 / 12).(n_1+n_2+1)$$

The critical value of z for α =0.05 (z_α) was read from a table of upper tail areas for the normal curve and is 1.645. The null hypothesis was rejected if $z > z_\alpha$ or if $z < z_\alpha$ with the inference being that population 1 was shifted to the right or left population 2 respectively.

The theory behind the Wilcoxon rank sum test assumes that the population distributions are continuous so that there is zero probability that any two observations are identical. In practice there will often be ties in the ranking procedure with two or more observations with same value. For these situations, each observation in a set of tied values receives a rank score equal to the average of the ranks for the set. For example, if two observations are tied for the ranks of 3 and 4, each is given a rank of 3.5 and the next higher value receives a rank of 5. When there are ties, there is a correction for the variance formula shown below:

$$\sigma^2_T = (n_1 n_2 / 12)[(n_1+n_2+1)-(\Sigma_j t_j(t^2_j-1) / (n_1+n_2)(n_1+n_2 -1))]$$

where t_j denotes the number of tied ranks in the jth group.

Practically, unless there are many ties, the correction will have very little effect on the value of σ^2_T.

The Wilcoxon rank sum test is an alternative to the two sample t-test that requires fewer assumptions. In particular, Wilcoxon's test does not require normality for the two

populations, only that they be identical under the null hypothesis. When assumptions underlying a t-test hold it will be more likely to declare an existing difference. This is logical since the t-test uses the magnitudes of observations rather than just their relative magnitudes (ranks). When assumptions for a t-test are violated the Wilcoxon rank sum test is the more informative test and is more likely to declare a difference when it exists. This is particularly true when non normality of the populations is present in the form of severe skewness or extreme outliers.

CHAPTER 3

THE BEHAVIOURAL EFFECTS OF ACRYLAMIDE:

3: OBJECTIVES:

The objective of the experiments described in this chapter was to determine a dose of acrylamide that is below the threshold of detection of some existing behavioural techniques but may still cause deficits in the function of peripheral nerves. This provides a benchmark against which new potential screening techniques can be compared. Due to the varying nature of dosing protocols and species variation throughout the scientific literature it was necessary to conduct a small behavioural study in parallel with other work in order to determine the ability of the behavioural techniques used to detect deficits when applied to the specific animals and dosing protocols used in this project. The intention of this behavioural study was to find a dose of acrylamide which was sub-threshold for detection using simple, rapid behavioural techniques when applied to mice prior to their killing for the purpose of further experimentation *in vitro*.

The two techniques used in this study were those of functional observation and a climbing test of limb strength and co-ordination (see methods for details). These two techniques were both easy to conduct and required no training of the mice prior to behavioural assesment.

3.1.1: RESULTS OF FUNCTIONAL OBSERVATION TESTING:

Analysis of the records kept during the observation of animals shows that only mice receiving a cumulative acrylamide dose of 200 mg/kg (administered as 2 x 100mg/kg on consecutive days) showed any clinical signs of neuropathy when compared to untreated control animals. Mice receiving either 50 or 100 mg/kg of acrylamide showed no observable deficit when compared to untreated controls. Mice receiving 200 mg/kg of acrylamide displayed abnormalities of gait, with the hind feet being dragged slightly during the recovery phase of a stride, giving the animal a slightly unsteady appearance. This was especially evident when the mice walked on a smooth table top. This was observed only between days 8 and 16 following administration of the first dose of acrylamide. Overall activity and responsiveness of the animals appeared normal at all doses although no quantitative tests were carried out. No mice showed clinical signs with any of the doses of acrylamide 32 days after its administration. However, 10 animals (approximately 1% of mice receiving >100 mg/kg) either died or were killed 24 hours after acrylamide treatment. These animals developed severe tremors of the whole body within hours of dosing which became progressively worse up to 24 hours. At this time any surviving mice with severe symptoms were humanely killed.

This was highly atypical and no explanation has been possible for the cause of this abnormality. However, Fullerton & Barnes (1966) reported that rats receiving a single oral dose of 166-249 mg/kg acrylamide (the LD_{50} dose range) developed a fine tremor lasting 48 hours after which time they either recovered completely or died within 2-3 days. This may indicate a lower LD_{50} for the mice in this study with those dying representing particularly sensitive animals. This hypothesis is not supported by the study of Teal & Evans (1982) who report a LD_{50} value for CD-1 mice of approximately 600-700 mg/kg when injected i.p. at 100mg/kg/day. Mice which survived did not show tremors at any stage.

In conclusion it appears that the effects of a dose of 50 or 100mg/kg of acrylamide on the peripheral nervous system of the mice used in this study was undetectable using a functional observational battery. Detection was only possible 8 days after a cumulative dose of 200 mg/kg with recovery occurring by day 32.

3.2.1: RESULTS OF CLIMBING PERFORMANCE TEST:

Measurement of the ability of mice to balance on a rotating rod or cone is often used as a measure of impaired motor function. In most of these tests animals must be trained prior to the performance of the test. This is a very time consuming procedure and has the drawback that some animals, even with training, never pass the test and thus have to be excluded from further study. The test of the ability of mice to climb from the underside of an inverted wire screen described in this work is based on that of Coughenour *et al.* (1976) and has the advantages that it requires no training of the animals which also exhibit a lower control failure rate allowing fewer animals to be used in total. The apparatus used in the test is also much simpler and removes variables from the comparison of data such as rod diameter and rotation speed in the rotarod test.

The mice subjected to this test in this study could do one of three things. They could either climb to the upper side of the wire screen, fall from it whilst trying or remain on its underside for 120 seconds after which time they were removed. Only 5 out of 155 mice failed to either climb or fall during the course of this study. Two of these were animals not treated with acrylamide. They were excluded from the results since classifying them clearly as either climbers or fallers was impossible. Any arbitrary classification of this data was unacceptable since the large time values of 120 seconds could have easily skewed any other genuine trends in the data sets. These animals appeared to make little attempt to move towards the edge of the mesh and often just hung motionless from it after a limited attempt at moving across it. The use of this method with compounds which cause immobilization was contra-indicated for this reason by Coughenour *et al.* (1976). It is of interest that so few mice failed to climb or fall and this may indicate that the test was successful in motivating the mice to make an attempt to get to the perceived safety of the upper side of the screen and that immobilization for whatever reason was not a major problem. Coughenour *et al.* (1976) used the number of mice either failing to reach the top

of the wire screen or the number falling from it as their index of impaired motor function. The technique was modified for the experiments presented in this study such that the percentage of mice climbing to the top of the screen was recorded which is essentially the same as measuring the number that failed to climb but also recorded the times the mice took to either climb or fall. This was an attempt at increasing the the sensitivity of the test with a hypothesis that mice insufficiently intoxicated to fall from the screen might never the less climb to the top of it more slowly.

If mice subjected to this test were suffering from acrylamide induced neuropathy of sufficient severity one would expect to observe fewer animals to climb successfully. Those that did might be expected to do so more so slowly whilst those that fell should do so faster.

Table 3.1 below shows a summary of the climbing performance of mice following acrylamide administration compared to control mice.

Treatment	Day of study	Mean climb time /S	Mean fall time /S	% of mice climbing	N:
Control	1-32 Days	30.5 ±18.2	33.1±19.2	64	66
100 mg/kg ACR	1 Day	32.2 ±7.6	19.5±10.9*	60	15
100 mg/kg ACR	4 Days	38.1 ±16.97	31.0±11.6	69	13
100 mg/kg ACR	8 Days	29.8 ±17.6	21.8±14.2	40	10
100 mg/kg ACR	16 Days	41.2 ±34.0	31.5±5.0	75	8
100 mg/kg ACR	32 Days	36.8 ±8.2	36.7±15.28	63	8
200 mg/kg ACR	3 Days	40.0 ±31.2	21.3±10.03*	25#	4
200 mg/kg ACR	8 Days	37.0 ±9.89	22.6±13.6*	11#	19
200 mg/kg ACR	32 Days	41.9 ±27.8	35.3±32.6	14#	7

Table 3.1: Table showing mean times (± s.d.) for mice to either climb to the top side of an inverted wire mesh screen or fall from it. Mice were given oral acrylamide (ACR). 200 mg/kg ACR administered as 2 x 100 mg/kg doses on consecutive days. Control animals received distilled water only. * = values significantly different from control (Wilcoxon rank sum test, p=0.05).# = significantly different from control (χ^2 test, α =0.05). No statistically significant differences were observed between control animals studied at varying times after sham-dosing.

From these data it can be seen that the percentage of mice climbing to the top of the wire screen was significantly reduced in mice receiving 200 mg/kg acrylamide and studied 3 or 8 days later when compared to the control group (χ^2 test α=0.05). This effect was first recorded 3 days after treatment began. It was greatest 8 days after treatment and was still present in mice studied at 32 days. However, mice receiving 100 mg/kg were not significantly affected at any time. Mean climb time was not significantly increased although there is a trend towards it. Mean fall time appears to fit the expected outcome

with mice studied 3 and 8 days after 200 mg/kg acrylamide treatment falling from the screen significantly faster than control mice (Wilcoxon rank sum test, $p=0.05$). Again the lower dose of 100 mg/kg failed to produce evidence of any deficit with the exception of those mice studied 1 day after treatment. However, the figure for fall time at 8 days following this dose was very close to being significant. This may indicate that the level of deficit in the climbing ability of the mice receiving 100 mg/kg acrylamide is near but still below the threshold of detection of this test. The significant difference observed at the one day time point appears to be anomalous especially when there is no such difference observed 3 days later. This trend is not concurrent with the data for the 200 mg/kg dose group where the effect appears to be persistent. However, an early effect and recovery at this lower dose cannot be ruled out. Caution must be applied when looking at the data for climb/fall times since although the total number of observations made may be acceptable at first glance, one must remember that the mean times are based on the times for mice which actually climb or fall. For example, the mean climb time for mice treated with 200 mg/kg acrylamide and studied on day 8 is derived from only 2 mice (11% of 19) although the total number of mice tested at this dose was 19. Fig 3.1 overleaf shows histograms of the climb/fall times of mice in selected dose groups which illustrate this point.

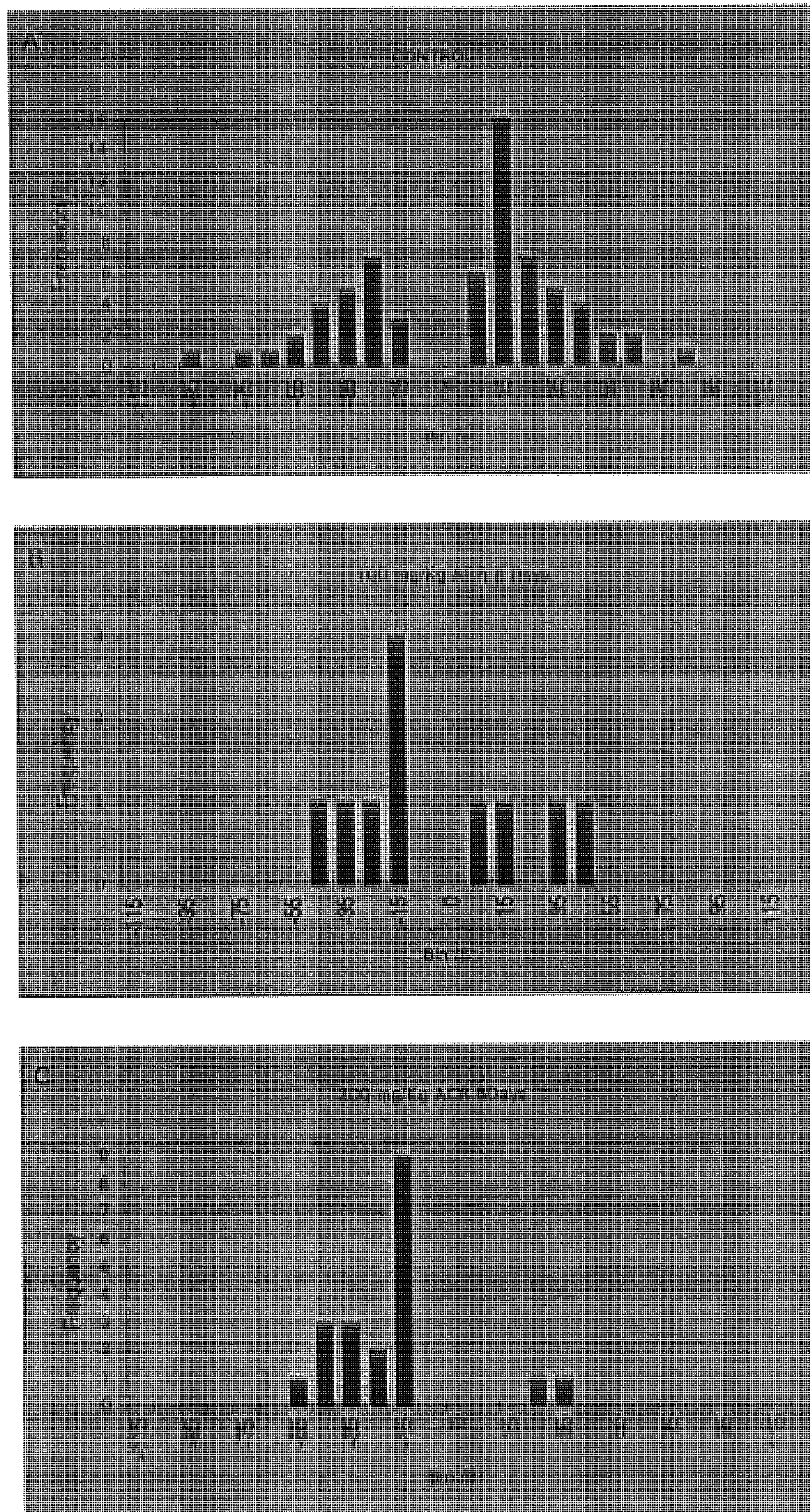


Fig 3.1: Histograms to show distributions of times (bins in seconds) for mice placed on an inverted wire mesh screen to either climb to the top side of it (+ve values) or fall from it (-ve values). A: Control. Distilled water only. B: 100 mg/kg acrylamide studied 8 days after treatment. C: 200 mg/kg acrylamide studied 8 days after treatment. (200 mg/kg administered as 2 x 100 mg/kg doses on consecutive days). Oral administration.

3.3: DOES A SINGLE ORAL DOSE OF 100 mg/kg ACRYLAMIDE PRODUCE NEUROPATHY WHICH IS BELOW THE THRESHOLD OF DETECTION OF FUNCTIONAL BEHAVIOURAL TECHNIQUES ?

The observational data and climbing performance data obtained from the experiments described above, indicate that the threshold dose for detection of signs of acrylamide induced neuropathy with both techniques lies between 100 and 200 mg/kg. With the exception of the anomalous data for mean fall time discussed above, a dose of 100 mg/kg appeared to have no observable effect in either of the tests performed. It is proposed that this dose is sub-threshold for detection by these methods. It is of interest at this point to compare this finding to those of other studies in order to see if this proposed threshold is in broad agreement with doses of acrylamide which have been shown to ellicit behavioural deficits.

3.3.1: A COMPARISON TO OTHER OBSERVATIONAL STUDIES OF THE BEHAVIOURAL EFFECTS OF ACRYLAMIDE:

Comparing the observational data obtained in the present experiments to other studies is complicated by the great variation in species and dosing protocols used. The total administered dose of acrylamide provides a convenient means of comparing various dosing schedules (Lowndes & Baker, 1977). However, it is unlikely that the total doses equate with total accumulated doses which remain in the animal. Edwards (1975) reported that the plasma half life of intravenously administered acrylamide is approximately 2 hours. Approximately 40% of a dose appears in the urine within 24 hours (Hashimoto & Aldridge, 1970). Thus acrylamide rapidly leaves the blood and is probably taken up by most tissues, but nervous tissue (brain, spinal cord and sciatic nerve) has been shown to contain considerable radioactivity 14 days after administration of ^{14}C labelled acrylamide (Hashimoto & Aldridge, 1970). Non-nervous tissues did not contain significant radioactivity at this time. Whether this radioactivity is attributable to [^{14}C] acrylamide or to a metabolite is not clear, nor whether it is acrylamide or a metabolite which is responsible for the neuropathic signs observed in animals. In either case the dose of acrylamide required to elicit neuropathic signs has been shown to be cumulative, for studies on cats show similar degrees of abnormality at a mean dose of 102 mg/kg acrylamide whether it be administered as daily doses of 1, 2, 5, 10, 15, 25, 40 or 50 mg/kg (Kuperman, 1958). He also found that the route of administration was not significant in the onset of observable behavioural deficits. Thus it appears that with regard to behavioural observations, comparisons between studies can be made on the basis of cumulative acrylamide dose. Table 3.2 shows data from a variety of studies using different species and dosing protocols.

Source and species:	Cumulative dose of ACR at which observations first indicated possible neuropathy:	Observations:
Kuperman (1958) / Cat	100-132 mg/kg (2-125 days)	Ataxia.
Schaumburg, Wisniewski & Spencer (1974) / Cat	130-150 mg/kg (13-15 days)	Weaving gate and hind limb unsteadiness.
Sumner & Asbury (1975) / Cat	200 mg/kg (20 days)	slight hindlimb ataxia.
Lowndes & Baker (1975) / Cat	105-135 mg/kg (7-9 days)	Ataxia, truncal sway when walking and lack of co-ordination.
Lowndes *et al.* (1978) / Cat	105 mg/kg (5-7 days)	Unsteady gate, wide legged stance, high stepping gait and truncal sway.
Goldstein (1985) / Cat	150 mg/kg (5 days)	Head tremor and hindlimb sway.
Spencer & Schaumburg (1977) / Rat	480 mg/kg (8-12 days)	Unsteady shaky gait.
Jennekens *et al.* (1979) / Rat	400 mg/kg (19 days)	slightly unsteady gait.
Jakobsen & Sidenius (1983) / Rat	200 mg/kg (14 days)	Feet diverged or converged with overlapping toes. Gait was steady
DeGrandchamp & Lowndes (1990) / Rat	140-175 mg/kg (10 days)	Exaggerated limb splay when quickly picked up the tail.
Moser *et al.* (1992) / Rat	360 mg/kg (30 days)	Altered gait.
Gold *et al.* (1992) / Rat	375 mg/kg (10 days)	Splaying of the toes and mild weakness of the hindlimbs.
LoPachin *et al.* (1993) / Rat	351 mg/kg (15 days)	Mild foot splay and ataxia.
Souyri *et al.* (1981) / Chicken	200 mg/kg (7-9 days)	slight ataxia.

Table 3.2: Table to show some of the the variation in the cumulative dose of acrylamide (ACR) required for the first observation of neuropathic signs in a variety of species. Times in parenthesese indicate the time from the start of dosing for observations of behavioural deficit to be made.

From this table it can be seen that the cat appears to develop clinical signs of neuropathy at a cumulative dose of 100-200 mg/kg whereas rats require 140-480 mg/kg. It is generally accepted that the cat is the species most sensitive to experimental intoxication with acrylamide. The large variation in the dose required to produce the first observable signs of neuropathy might be explained by the subjective nature of observational battery testing as well as any difference between different species or strains of the same species. The figure of 200 mg/kg obtained in this study appears to place the mice used in the overlap between cats and rats with regard to their sensitivity to acrylamide. Alterations of the gait of intoxicated animals is the most common early sign of neuropathy and there have been many attempts to explain this phenomenon. Kuperman (1958), who provided the first observations of acrylamide neuropathy in cats, found no signs of alteration in peripheral motor or sensory nerves, neuromuscular junctions, voluntary muscle or spinal reflex pathways. He proposed that the observations he made were caused by alterations in brain stem function but other structural studies soon implicated peripheral nerves as a site of acrylamide-induced neuropathy (Prineas, 1969; Schaumburg *et al.*, 1974). Of interest was the fact that sensory nerve terminals showed pathological change before motor nerve terminals. This suggests that early observable neuropathic signs might be the result of sensory dysfunction prior to any motor abnormality. Sumner & Asbury (1975) appeared to confirm this when they discovered that acrylamide reduces the number of muscle spindle endings and tendon organs responding to muscle stretch in cats. Lowndes *et al.* (1978) demonstrated that the onset of this sensory deficit correlated well with the observation of early clinical signs of neuropathy in cats and suggested that altered spindle function might underlie these observations. This hypothesis is made all the more appealing by the previous observations of Lowndes & Baker (1976) in which they reported that at the same time as clinical signs became evident there were no changes in the neuromuscular function of motor neurones measured by generation of stimulus-bound repetition and post-tetanic potentiation. This remained true even if the dose of acrylamide was doubled for a given time point of study. At later time points these measures did show that motor nerves were altered and one must question whether the lack of correlation between motor nerve terminal deficit and the observation of neuropathic signs is merely a result of lack of sensitivity of the detection technique compared with that used in the analysis of sensory nerve function. However, the hypothesis that proprioceptor dysfunction induced by acrylamide is the cause of the alterations in gait observed in intoxicated animals remains appealing. The study of Lowndes *et al.* (1978) only indicated the effects of acrylamide on the state of muscle spindles with respect to their function as sensors of static muscle position. These structures are also known to provide information about muscle length changes thus indicating the velocity of muscle movements not just their position. This plays a vital role in the generation of co-ordinated movement. Other muscle receptors are also affected such as tendon organs (Sumner & Asbury, 1975) and Pacinian corpuscles (Schaumburg *et al.*, 1974). Thus it would appear that a peripheral

sensory deficit accounts for the early observational signs of acrylamide neuropathy and other studies by Fullerton and Barnes (1966) and Hopkins (1970) are consistent with this view. However, there are some authors who propose some central nervous system involvement in observational abnormalities observed following acrylamide administration.

Schaumburg *et al.* (1974) observed truncal ataxia in cats as an early observation of acrylamide neuropathy which might indicate a cerebellar disturbance. In support of this are the degenerative changes in the granular layer of the cerebellar vermis and extensive destruction in the dorsal spinocerebellar tracts observed in some animals (Spencer & Schaumburg, 1974; Prineas, 1969). Hopkins (1970) has also reported a baboon which despite being tetraplegic after acrylamide administration, had little peripheral histological change and had normal motor conduction. He concluded that this was indicative of additional central nervous system damage.

In conclusion it seems that the early neuropathic signs of acrylamide induced neuropathy should be mainly attributed to disturbances in terminal regions of sensory neurones although one cannot rule out the possibility of an effect on the central nervous system.

3.3.2: A COMPARISON OF CLIMBING PERFORMANCE TEST DATA TO OTHER STUDIES:

It appears from the data that a single oral dose of 100 mg/kg of acrylamide causes insufficient peripheral neuropathy to be clearly detected by a test of climbing performance. The inability of a mouse to climb to the top of the wire screen was attributed by Coughenour *et al.* (1976) to attenuation of the grasping reflex and of the more complex motor co-ordination required. They implied that climbing to the top of the screen was a more complex behaviour than simply hanging from it and that a lower dose of a compound producing impaired motor function was required to alter the number of mice climbing compared to that required to increase the number of mice falling from the screen. The recording of the climb or fall times during this study did not support this idea. One can hypothesise that climb times should be more sensitive to acrylamide than fall times. The data shows the opposite to be true with fall times decreasing whilst climb time was not significantly altered. The data concerning recovery of the mice is also interesting when considering this argument, for 32 days after dosing the mean fall time of the mice had recovered whilst the percentage of mice climbing to the top of the screen had not. This observation supports the idea that the ability to climb in a co-ordinated way may be more sensitive to acrylamide in that recovery is slower whereas the grasp reflex responsible for the ability of the mice to hang from the screen for longer before eventually falling recovers faster.

In the results described above, the figure of 200 mg/kg for the cumulative dose of acrylamide required to elicit a significant drop in climbing performance compares well with the findings of other studies. Coughenour *et al.* (1976) concluded that their wire

screen test was directly comparable to the rotarod test and thus it is appropriate to consider data from rotarod experiments as comparable with the wire screen test. Work carried out by Gilbert & Maurissen (1982) showed that BALB/c mice failed the rotarod test at a cumulative dose of acrylamide of 205 mg/kg on the eighth day of the study. Increased hindlimb splay on landing following a drop from a height of 15 cm was observed in this study at a cumulative dose of 155 mg/kg on day 6. This result indicates that the landing foot splay test proposed by Edwards and Parker (1977) is more sensitive in this case than the rotarod test. Attempts were made to use this method in this project but consistent results were not obtained with control animals due to problems with releasing them cleanly and it was abandoned (see methods). Kaplan & Murphy (1972) reported that rats failed the rotarod test 6.4 days after 320 mg/kg or 4.6 days after 345 mg/kg of acrylamide. Mice appear to be more sensitive to acrylamide than rats. Since there are no data available for cats subjected to a rotarod test it is not possible to say whether, as with the observational study, mice fall somewhere between cats and rats with regard to acrylamide sensitivity.

3.3.3: IS THERE NEUROPATHY BELOW THE DETECTION THRESHOLD OF BEHAVIOURAL TECHNIQUES ?:

Since one of the aims of the work presented in this thesis was to attempt to apply sophisticated electrophysiological techniques to the early detection of peripheral neuropathy, the question arises as to whether a dose of acrylamide which does not cause any deficits in behavioural tests actually causes any neuropathy ?

DeGrandchamp & Lowndes (1990) demonstrated that despite the absence of any clinical signs, rats receiving 105 mg/kg showed widespread pathological changes at the neuromuscular junction (NMJ) of the motor nerves innervating the soleus muscles. Mean endplate length was increased as were the number of terminal branch points per neuromuscular junction. The number of NMJs with at least one degenerating terminal branch showed a marked increase (from 9 to 63%) whilst the number of degenerating terminals per NMJ doubled. There was also an increase in the number of NMJs with terminal sprouts. These observations appear to suggest that distal neuropathy can be assumed to be occurring prior to the observation of clinical signs. However, in the same study, lumbrical muscles showed much less evidence of the effects of acrylamide. Whether the diaphragm muscle and its associated phrenic nerve showed any pathology at the 100 mg/kg level of acrylamide was unknown until later in the studies contained in this thesis when similar staining techniques to those employed by DeGrandchamp & Lowndes were applied to hemi-diaphragm preparations. Work by Lowndes & Baker (1976) with cats showed that the ability of motor nerve terminals to generate and maintain stimulus bound repetition was compromised after a cumulative dose of 150 mg/kg acrylamide. The first clinical signs of neuropathy were observed 1 day earlier at 135 mg/kg. On balance it appears reasonable that motor nerve terminals of the mouse diaphragm should show some

deficit in function at a dose below that required to elicit clinical signs of neuropathy. This deficit would then progress with an increase in cumulative dose to the point where clinical abnormality would become apparent.

The experiments described in the remainder of this thesis attempted to show (and subsequently explain) that there were detectable functional differences at the neuromuscular junction prior to the observation of clinical signs of neuropathy using a variety of techniques of which most were novel to this area of investigation. It appears from the experiments and literature described so far that a dose of 100 mg/kg of acrylamide was clinically sub-threshold yet may cause subtle changes in nerve morphology and hence function. The presence of these changes at higher doses where clinical signs of neuropathy have been demonstrated would help to validate the subthreshold effects.

CHAPTER 4

DOES ACRYLAMIDE AFFECT THE JITTER OF INDIRECTLY EVOKED ACTION POTENTIALS AND ENDPLATE POTENTIALS IN THE MOUSE HEMIDIAPHRAGM?

4: OBJECTIVES:

The behavioural experiments performed in the early stages of this study demonstrated that a single oral dose of 100 mg/kg acrylamide was insufficient to cause mice to exhibit the characteristic early clinical signs of central /peripheral distal axonopathy (see introduction and behavioural data). However, the behavioural deficits induced by acrylamide suggest changes in peripheral nerve function which might be detected using electrophysiological techniques (Anderson, 1981). Assuming that some sort of deficit in the function of peripheral nerves is the precursor or cause of the appearance of clinical signs of neuropathy, sensitive electrophysiological measures should be able to detect any abnormality prior to clinical signs becoming apparent both temporally and with regard to cumulative dose. The nerve terminal and the immediate pre-terminal axon have been shown to be the regions in which structural and functional axonopathy first occur following the administration of acrylamide. (Cavanagh, 1982; Chretien *et al.*,1981; DeGrandchamp & Lowndes 1990; Prineas, 1969; Schaumburg *et al.*, 1974; Spencer & Schaumburg, 1974 & 1977; Sumner & Asbury, 1974,1975). Therefore, any early detection of neuropathy using electrophysiological techniques must concentrate on aspects of nerve terminal and pre-terminal axon function. It was hypothesised that electrophysiological detection of neuropathy should be possible prior to the observation of clinical signs which probably represent relatively large deficits in function of the peripheral nerves. The terminal regions of sensory neurons have been shown to be more sensitive to the effects of acrylamide than equivalent regions in motor neurons with regard to the rapidity of onset of functional deficits. However, this distinction may be the result of the techniques used to assess the integrity of the motor nerve terminals. DeGrandchamp & Lowndes (1990) reported pathological changes in motor nerve terminals at the neuromuscular junctions of soleus muscles in the rat prior to any clinical signs of acrylamide intoxication thus implying that the earliest effects of acrylamide may not be limited to sensory nerve endings only.

The objective of the experiments presented here was to test the hypothesis that it is possible to detect functional deficits in the terminal regions of peripheral motor nerves by applying the electro-physiological technique of 'jitter analysis' to the neuromuscular junction of mouse diaphragm muscle. Furthermore, it was hypothesised that these deficits can be demonstrated to occur following an acrylamide dosing protocol which is insufficient to cause observable clinical signs of neuropathy.

4.1: THE EFFECT OF ACRYLAMIDE ON RESTING MEMBRANE POTENTIAL:

The resting membrane potential (RMP) of muscle cells was recorded during the course of jitter experiments requiring the use of intracellular microelectrodes since it is a sensitive indicator of any damage caused by their insertion. A fall in RMP during the course of an experiment is a criterion for excluding a muscle fibre from subsequent analysis. This is an obvious parameter to analyse first since gross deviations in the resting potential of an electrically excitible cell will effect its function in more subtle evalutions of its state. As can be seen from table 4.1, RMP appeared to be unaffected by administration of acrylamide or by ecothiopate (the positive control compound used in the 'jitter' experiments described later). No significant statistical differences were found between mice dosed with distilled water only (control) and those receiving acrylamide treatment.

Treatment	Day of study	Mean RMP ± S.D. /mV
Control	1-16 Days	70.7 ±4.7
50 mg/kg ACR	4 Days	71.9 ±3.9
50 mg/kg ACR	8 Days	69.1 ±3.2
100 mg/kg ACR	1 Day	70.1 ±3.3
100 mg/kg ACR	4 Days	72.0 ±5.3
100 mg/kg ACR	8 Days	71.9 ±4.6
200 mg/kg ACR	8 Days	72.5 ±4.5
200 mg/kg ACR	16 Days	72.1 ±3.3
500 nmoles/kg ECO	2 Days	68.6 ±3.5
500 nmoles/kg ECO	3 Days	69.8 ±3.9

Table 4.1: Table to show resting membrane potentials in mouse hemi-diaphragm muscle cells at the endplate region following oral administration of acrylamide (ACR) or ecothiopate (ECO) administered as a subcutaneous injection. Measurements were made at the various times shown following treatment. 200 mg/kg dose of ACR administered as 2 x 100mg/kg doses on consecutive days. Control animals received distilled water only. In all cases, n>30.

These data confirm previous accounts of the lack of any effect of acrylamide or ecothiopate in causing alterations in RMP of muscle cells. Ecothiopate has been shown to have no effect on RMP on several previous occasions (Kelly & Ferry, 1994; Tattersall, 1990.). This indicates that treatment with acrylamide or ecothiopate does not compromise the ability of the muscle cells of the diaphragm to maintain the potassium ion gradients across their membranes. It is of note that data was only recorded from cells with a RMP of greater than -65 mV with the exception of cut fibre preparations used in the analysis of

EPPs (see methods). Cells exhibiting gross alterations in RMP would thus have been excluded from analysis and the data presented here represents a sub population of fibres excluding cells either damaged by the insertion of the micro-electrode or showing gross abnormality of RMP. Cells with low initial RMP were rare. The majority of the cells into which electrodes were inserted had RMPs which initially satisfied the rejection criteria but then became unstable and declined rapidly. This could be interpreted as damage to the muscle cell membrane caused by the insertion of the electrode. Treatment with acrylamide did not seem to have any obvious effect on the number of cells showing this characteristic, i.e. acrylamide pretreatment did not seem to cause an increase in the succeptability of cells to microelectrode damage.

4.2: THE EFFECT OF ACRYLAMIDE ON THE LATENCIES OF INDIRECTLY EVOKED ACTION POTENTIALS AND ENDPLATE POTENTIALS:

4.2.1: OBJECTIVE:

Treatment with acrylamide has been shown to cause distinct pathology at the neuromuscular junctions of rats at lower dose and/or earlier time points than with the observation of clinical signs of neuropathy (DeGrandchamp & Lowndes, 1990). Four days after the start of dosing at 35 mg/kg/day they observed degenerating terminal branches, diminished synaptic vesicle content, terminal neurofilament accumulations and tubulo-vesicular profiles at the neuromuscular junctions in soleus muscles. These observations were repeated in lumbrical muscles although the effect did not appear to be as great at this early time point indicating possible differential sensitivity of motor nerves.

Jitter, the variability in the latency of a series of action potentials or endplate potentials, has been demonstrated to be a sensitive indicator of neuromuscular disease (Stalberg & Trontelj, 1979). Previous work in the laboratory with regard to this technique has concentrated on the effects of anti-cholinesterase drugs using the method for jitter analysis devised by Kelly *et al* (1990). It was decided to apply the measurement of jitter to the detection of acrylamide-induced neuropathy since it appeared reasonable that the alterations to nerve terminal structure reported elsewhere might adversely effect some aspect of action potential conduction in the non myelinated pre-terminal axon or the release of the neuromuscular transmitter.

4.2.2: RESULTS OF EXPERIMENTS ON THE JITTER OF ACTION POTENTIALS:

As described earlier in this thesis (Section 2.6.2.3), the computerised analysis of the latencies of indirectly evoked action potentials (APs) yields data on MCD of APs 11-30 and on delay, i.e. the increase in latency of the 16th AP in a train of 30 relative to that of the first AP . Table 4.2 below shows a summary of the delay and MCD data:

Treatment	Day of Study	Mean Delay /µS	Mean MCD /µS	No. of cells
Control	1-16	36.8 ±14.7	10.4 ±9.6	67
50 mg/kg ACR	4	28.8 ±10.7	6.7 ±2.3	15
50 mg/kg ACR	8	40.1 ±23.8	11.9 ±10.5	31
100 mg/kg ACR	4	42.3 ±17.9	11.3 ±7.3	28
100 mg/kg ACR	8	57.4 ±23.2 *	11.8 ±6.6	28
200 mg/kg ACR	3	39.9 ±11.4	8.6 ±3.6	28
200 mg/kg ACR	8	41.7 ±12.4 *	8.6 ±3.3	50
200 mg/kg ACR	16	44.6 ±10.2 *	9.0 ±3.5	34
500 µM/kg ECO	2	55.3 ±21.1 *	13.5 ±9.2 *	29
500 µM/kg ECO	3	58.2 ±23.9 *	11.7±4.4 *	27

Table 4.2: Summary data for the delay and MCD of trains of 30 indirectly evoked action potentials at 30 Hz recorded at the endplate region of muscle fibres of mouse diaphragm following acrylamide (ACR) or ecothiopate (ECO) treatment (oral / subcutaneous injection administration respectively). 200 mg/kg ACR treatment consisted of 2 doses of 100 mg/kg on consecutive days. Control animals received distilled water only. Values ± S.D.* = significant difference from control (Wilcoxon rank sum test, $p<0.05$).

From this table it can be seen that acrylamide has no significant effect on the MCD of APs 11-30 in a train of 30 responses at 30 Hz. This is in contrast to ecothiopate which at 2 and 3 days after its administration caused a significant increase in MCD. However, delay was affected by the administration of acrylamide with significant increases occuring 8 days after administration of 100 and 200 mg/kg of acrylamide. It is interesting that a dose of 200 mg/kg did not produce an increase in delay on day 3. This may indicate that the effect of acrylamide upon delay is time rather than dose dependant. A dose of 50 mg/kg of acrylamide was insufficient to produce an increase in delay. Figure 4.1 below shows the progressive increase in latency with respect to the first AP (AP1) of the first 16 APs of a train of 30 stimulated at 30 Hz.

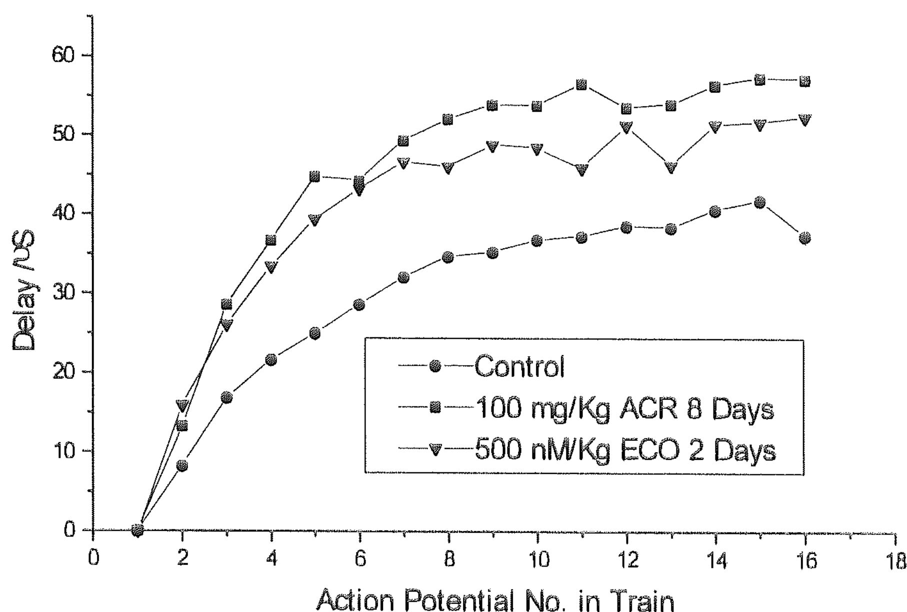


Fig 4.1: Graph to show the progressive increase in mean latency with respect to AP1 of the first 16 indirectly evoked action potentials of a train of 30 recorded at the endplate and stimulated at a frequency of 30 Hz. The data is the average from more than 28 cells in total sampled from the diaphragms of at least 5 animals for each treatment. Note the increase in latency 8 days after oral administration of acrylamide (ACR) and 2 days after subcutaneous injection of ecothiopate (ECO). The ecothiopate data also exhibits greater jitter (MCD) in the plateau region beginning at action potential 10. Control animals received distilled water only.

This graph clearly shows the increased delay of AP16 relative to that of AP1 following the administration of acrylamide or ecothiopate as described above. It also shows the increase in 'jitter' as measured by MCD following administration of ecothiopate. This manifests itself in the graph as variations in the delay of APs 10-16. It can be seen that following ecothiopate there is considerably more variation in the values of delay for APs 10-16 than after the control (distilled water only) and acrylamide treatments. However, this variation in latency can be problematic. From figure 4.1 above it can be seen that the mean latency of AP15 is higher than that of AP16. Thus, the selection of AP16 as the comparison point with AP1 might cause a significant difference in delay to be recorded as a consequence of a low value (even though it may be the mean of several experiments) which does not truly represent the 'plateau' of the latency increase curve. This may also be thought of as a phase difference between the oscillations of the latency within the train of APs. In the case of figure 4.1 an analysis of delay using AP15 as the comparison point to AP1 (i.e. latency = latency of AP15 - latency of AP1) yielded the following results:

Treatment	Day of Study	Mean delay /μs	No. of cells
Control	1-16	41.9±17.4	67
100 mg/kg ACR	8	57.5±27.8*	28
500 nM/kg ECO	2	51.9±14.0*	29

Table 4.3: Summary data for the delay of trains of 30 indirectly evoked action potentials (APs) at 30 Hz using the difference in latency between the first and fifteenth AP to calculate delay. APs were recorded at the endplate region of muscle fibres of mouse diaphragm following acrylamide (ACR) or ecothiopate (ECO) treatment (oral / subcutaneous injection administration respectively). Control animals received distilled water only. Values ± s.d.* = significant difference from control (Wilcoxon rank sum test, $p<0.05$).

From this table it can be seen that in this case, changing the number of the AP in the train to be compared to the first from 16 to 15 does not affect the result of the analysis despite the apparently large change in the value depicted in figure 4.1. However, the observation of this potential artefact does have consequences with respect to the resolution of the analysis system, i.e. its ability to distinguish small increases in delay values. To this end it is reasonable to suggest that future investigations should be carried out using modified computer software which is capable of averaging several 'plateau' latencies before comparison to that of the first AP.

Figures 4.2 to 4.6 below show histograms to show the frequency distributions of the values of delay of AP16 relative to AP1 recorded from a train of 30 APs at 30 Hz following various treatments:

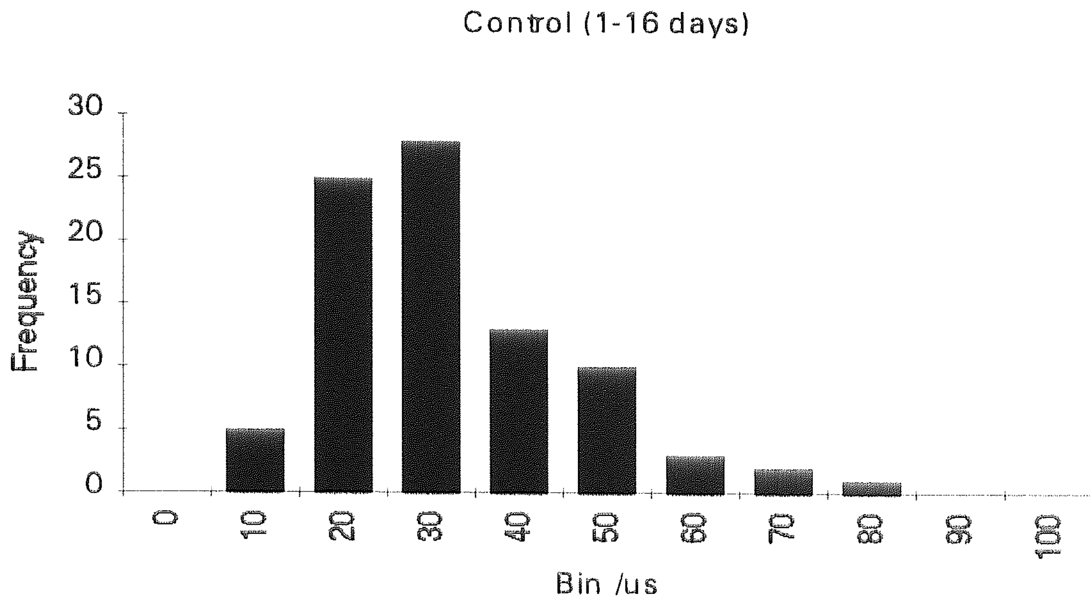


Figure 4.2: Histogram to show the frequency distribution of values of the delay of action potential (AP) 16 relative to AP1 calculated from data recorded in the endplate region during trains of 30 APs indirectly evoked at a frequency of 30 Hz in the phrenic nerve/diaphragm preparation of mice. Data from control animals (treated with distilled water only).

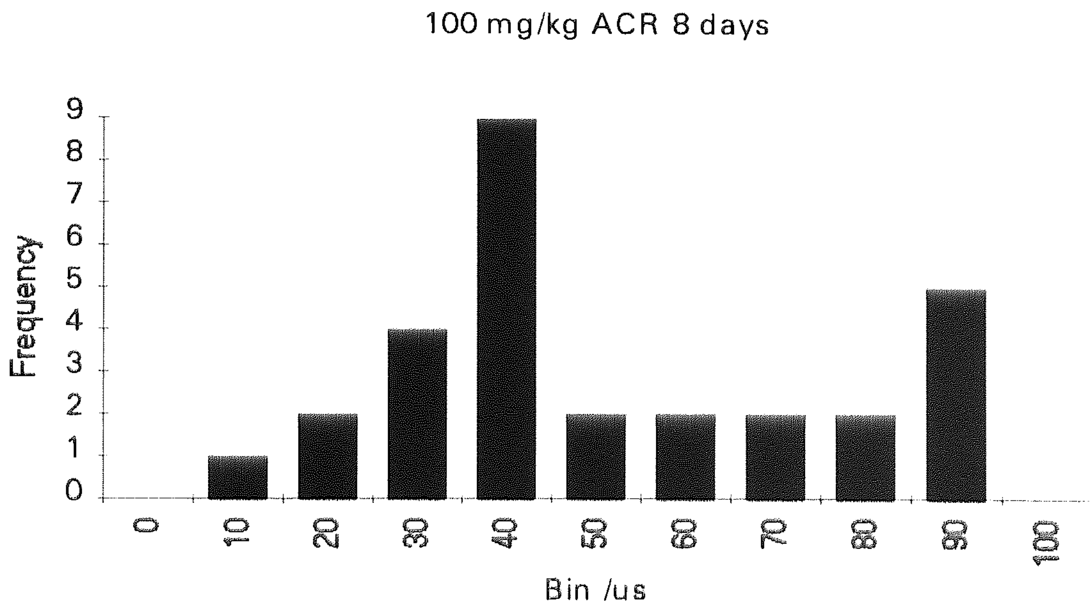


Figure 4.3: Histogram to show the frequency distribution of values of the delay of action potential (AP) 16 relative to AP1 calculated from data recorded in the endplate region during trains of 30 APs indirectly evoked at a frequency of 30 Hz in the phrenic nerve/diaphragm preparation of mice. Data recorded from animals 8 days after treatment with 100 mg/kg acrylamide administered orally.

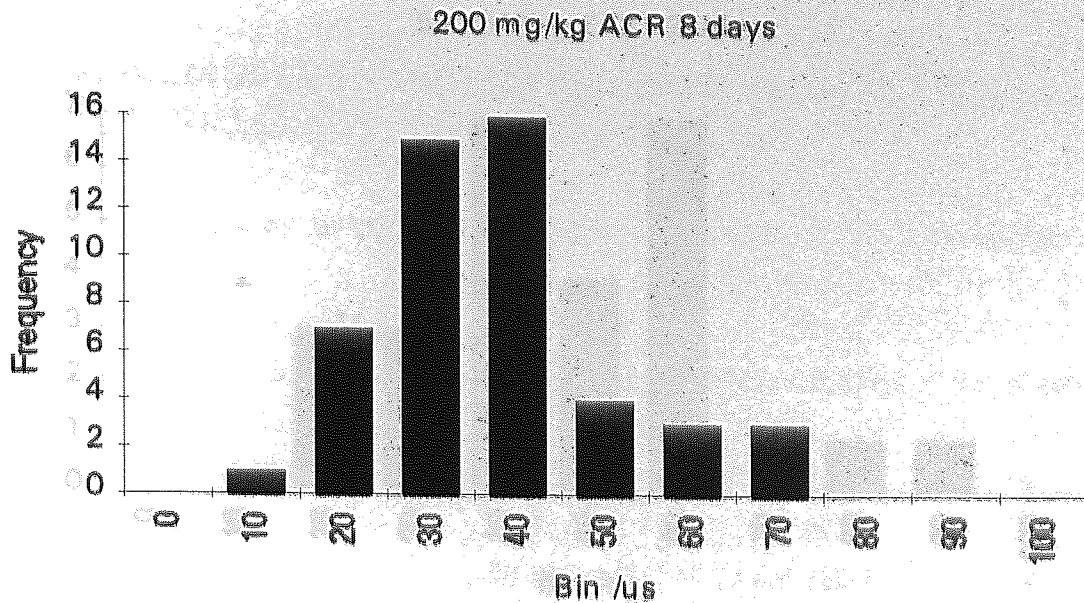


Figure 4.4: Histogram to show the frequency distribution of values of the delay of action potential (AP) 16 relative to AP1 calculated from data recorded in the endplate region during trains of 30 APs indirectly evoked at a frequency of 30 Hz in the phrenic nerve/diaphragm preparation of mice. Data recorded from animals 8 days after treatment with 200mg/kg acrylamide administered orally.

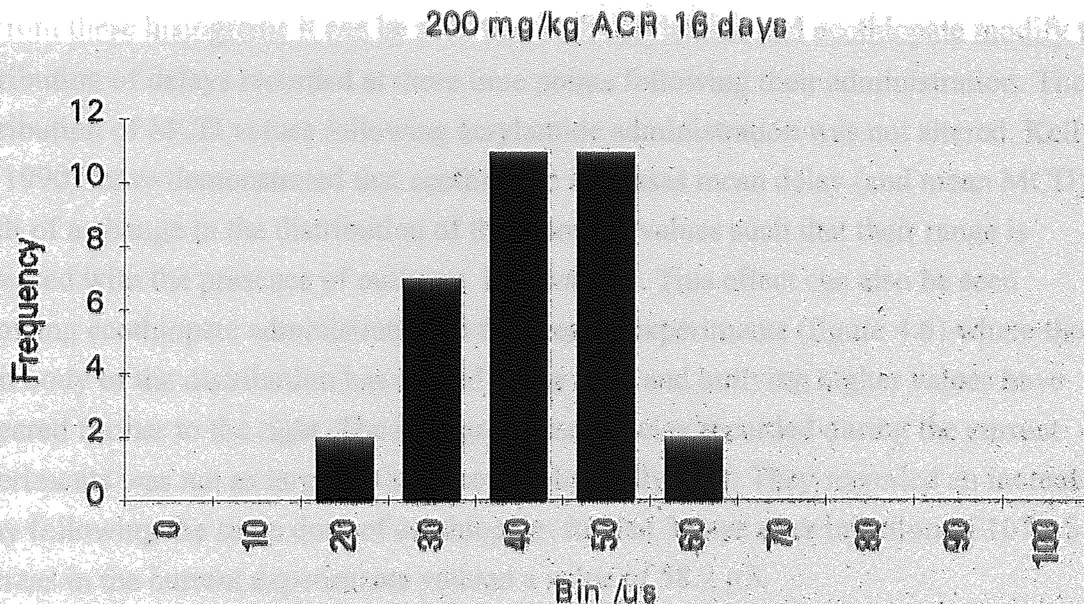


Figure 4.5: Histogram to show the frequency distribution of values of the delay of action potential (AP) 16 relative to AP1 calculated from data recorded in the endplate region during trains of 30 APs indirectly evoked at a frequency of 30 Hz in the phrenic nerve/diaphragm preparation of mice. Data recorded from animals 16 days after treatment with 200 mg/kg acrylamide administered orally.

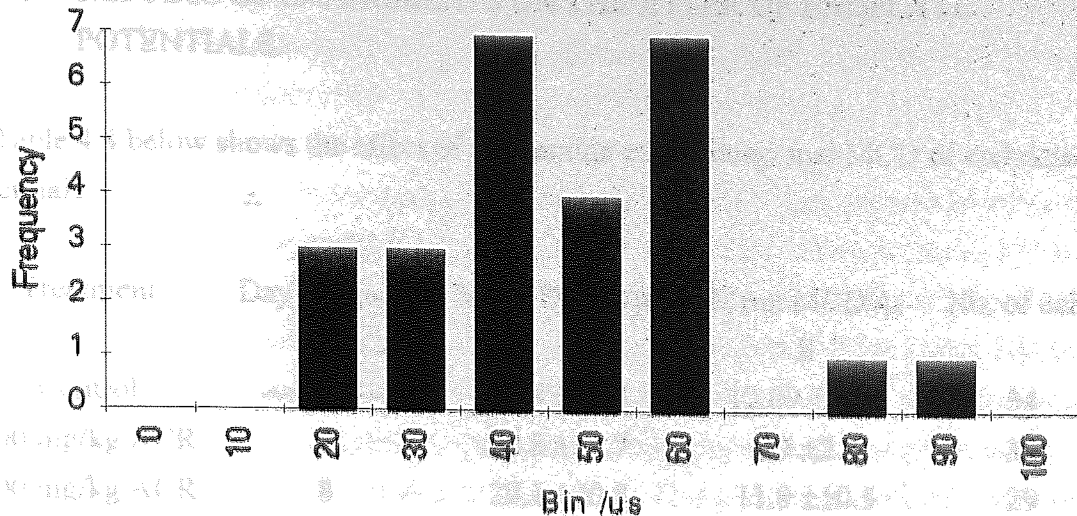


Figure 4.6: Histogram to show the frequency distribution of values of the delay of action potential (AP) 16 relative to AP1 calculated from data recorded in the endplate region during trains of 30 APs indirectly evoked at a frequency of 30 Hz in the phrenic nerve/diaphragm preparation of mice. Data recorded from animals studied 2 days after treatment with 500 nM/kg ecothiopate administered via subcutaneous injection.

From these histograms it can be seen that both acrylamide and ecothiopate modify the distribution of delays recorded at these time points following their administration. The distribution of MCD values following acrylamide administration was not altered. Kelly *et al.* (1990) have demonstrated that ecothiopate increases mean delay (and mean MCD) as a result of a change in the distribution of the recorded values such that their range is increased with the presence of outlying high values . This effect can also be seen following ecothiopate administration in the present experiments (figure 4.6) where the main body of the distribution has moved to the right and outlying higher values have appeared further to the right. The increase in mean delay recorded during the current experiments was not as large as that recorded by Kelly *et al.* They recorded an increase in delay following the same dose of ecothiopate studied 3 days after injection of 107 μS whereas in the current experiments yielded a value of 58.2 μS.

Animals receiving a dose of 100 mg/kg of acrylamide and studied 8 days later yielded a frequency distribution of delay values which was comparable to that seen following ecothiopate administration (figures 4.3 and 4.6). Animals receiving 200 mg/kg of acrylamide and studied 8 days later yielded similar data although there were not distinct outlyers in the population (figure 4.4). Sixteen days after 200 mg/kg of acrylamide the change in mean latency appears to be due to a more generalised shift in the recorded values to the right (figure 4.5). It may be possible that the lower dose of acrylamide affects only a sub-population of succeptable cells resulting in a more bimodal distribution whereas higher doses affect a larger population in a more graded manner.

4.2.3: RESULTS OF EXPERIMENTS ON THE JITTER OF ENDPLATE POTENTIALS:

Table 4.4 below shows the effect of acrylamide on the delay and MCD of endplate potentials:

Treatment	Day of Study	Mean Delay /μS	Mean MCD /μS	No. of cells
Control	-----------	31.4 ±14.4	12.09 ±3.05	54
100 mg/kg ACR	8	30.8 ±12.7	6.7 ±2.3	30
200 mg/kg ACR	8	29.1 ±20.8	11.9 ±10.5	29

Table 4.4: Summary data of delay and MCD of trains of 30 indirectly evoked endplate potentials at 30 Hz recorded in the endplate region of muscle fibres of mouse diaphragm following acrylamide (ACR) administered orally. 200 mg/kg ACR treatment consisted of 2 doses of 100 mg/kg on consecutive days. Control animals received distilled water only. Values ± S.D. No significant differences (Wilcoxon rank sum test, $p<0.05$).

This table shows that acrylamide had no significant effect on the delay or MCD of endplate potentials at a time when the delay of action potentials was significantly increased.

4.2.4: DISCUSSION:

In section 4.2.1 it was suggested that from previous reports of early signs of nerve terminal degeneration following administration of relatively low doses of acrylamide it was reasonable to assume that the characteristics of pre-terminal nerve action potential conduction, release of acetylcholine and postsynaptic generation of muscle action potentials may be affected.

The finding that MCD of action potentials was not increased by acrylamide administration implies that it was unlikely that there were deficits in post-synaptic function at the neuromuscular junction. Ecothiopate was included in these experiments as a positive control for MCD. The effect of ecothiopate has been proposed to be post-synaptic in origin possibly caused by alterations in the process of generation of the APs at the end plate via increased variability of time to threshold and/or changes in the locus of generation of the action potential. The threshold for excitation may also vary (Kelly & Ferry, 1994). These authors also concluded that increased jitter was not caused by a general effect on the plasma membrane of the muscle fibres and that since jitter of endplate potentials was not

increased by ecothiopate administration, altered latency of transmitter release could be excluded. Thus it appears that acrylamide causes no functional post-synaptic deficits at this early stage of neuropathy and that since jitter of endplate potentials is not affected (table 4.4), it has no observable presynaptic effect on the latency of transmitter release.

Acrylamide did have an effect on the delay of action potentials. In the case of ecothiopate administration this has been attributed to the increased run down of endplate potential amplitude early in the course of a train of responses. The increase in delay would be caused by the endplate potentials reaching the threshold for action potential initiation nearer their peak and this would introduce a systematic increase in delay (Kelly & Ferry 1994). It is interesting to note that the delay of endplate potentials 8 days after 100 or 200 mg/kg ACR was not increased relative to that of control values. Action potential delay was measured at 10 % of peak amplitude which at the endplate, represents a point on the rising phase of the endplate potential which gave rise to it. This point is about half way between the resting membrane potential and the threshold for depolarization. Endplate potential delay was measured at 10% of peak amplitude but this represents a point within 2 mV of the resting potential. These data might imply that the rise time of the endplate potential varies and if endplate potential amplitude runs down early in the train so that the point of measurement 10% up the action potential is nearer the peak of the endplate potential, this would be the case. However, the increased run down in endplate potential amplitude in ecothiopate treated preparations can be attributed to an increase in the amplitude of the first endplate potential in the train due to the anticholinesterase action of ecothiopate. The possibilty that acrylamide has some effect on cholinesterase levels cannot be ruled out (see: Effects of acrylamide on miniature endplate potentials). This effect may be a secondary effect arising from some other toxic insult since the increase in delay is not apparent until 8 days after dosing even at the higher 200 mg/kg dose. Delay may also be attributed to the slowing of action potential propagation within a train of responses in the non-myelinated terminal axon branches where it can be hypothesised that the lack of myelin sheathing permits large numbers of potassium ions to leave the neuron. The high frequency of stimulation within the train may lead to an intracellular depletion of potassium ions with a resultant drop in membrane potential. This situation may be further enhanced by the reciprocal increase in extracellular potassium concentration which may impede activation of sodium channels. It is possible that acrylamide may exacerbate this situation by affecting the neuronal membrane or causing demyelination in the region of distal nodes of Ranvier exposing paranodal fast potassium channels (Black *et al.* 1990). However, this idea is inconsistent with the data since no increase in delay of endplate potentials was observed.

A classical electrophysiological observation in acrylamide induced neuropathy is the reduction in conduction velocity along nerves attributed to the loss of larger diameter and hence faster conducting fibres (Fullerton & Barnes, 1966). The possibility that the increase in delay observed in the current experiments could in some way be related to this phenomenon seems very unlikely since the reduction in conduction velocity previously

measured has been shown to be due to the complete loss of AP conduction in larger diameter fibres. Delay measures the systematic increase in conduction time regardless of what the initial value for conduction velocity might be. The measurement of conduction velocity over the same path as that used for the determination of delay and MCD was not possible. This is because it is impossible to accurately measure the conduction distance involved since the path of the phrenic nerve fibres in the diaphragm is highly convoluted. Finally, effects on conduction velocity have been reported to occur very late after the observation of clinical signs of neuropathy (Lowndes *et al.* 1978) while the effect observed here on delay occurs at a 100 mg/kg dose which is not sufficient to cause clinical signs in the mice under study.

In conclusion it appears that at this early stage of neuropathy, acrylamide has no significant effect on the post synaptic function of neuromuscular junctions in the diaphragm of the mouse as measured by MCD. Any early effects on transmitter release caused by changes in terminal ultrastructure such as the reported decrease in the number of synaptic vessicles (DeGrandchamp & Lowndes, 1990) may be masked by the high safety factor for neuromuscular transmission. The effect of acrylamide on delay could not have been due to changes in conduction velocity of action potentials in the non-myelinated pre-terminal axon because of the abscence of an increase in endplate potential delay, but could be due to an increased run down in endplate potential amplitude possibly due to an indirect effect on the metabolism and/or efficiency of functional acetylcholinesterase. The abscence of an effect of acrylamide on MCD in association with changes in delay makes it difficult to draw firm conclusions from these experiments. Delay has been demonstrated to be a variable parameter with respect to factors such as the tension placed on the hemidiaphragm preparations and as such is of limited use as an indicator of pre and post synaptic events.

The experiments presented in chapter 6 initially focussed on the duration of miniature endplate potentials since this is a sensitive index of any reduction in cholinesterase function which may be implied by the increase in delay following acrylamide administration.

CHAPTER 5

DOES ACRYLAMIDE HAVE AN EFFECT ON THE TWITCH RESPONSES OF THE MOUSE HEMI-DIAPHRAGM FOLLOWING ANTICHOLINESTERASE EXPOSURE ?

5: OBJECTIVES:

The objective of the experiments presented in this section was to ascertain whether or not acrylamide pre-treatment, at a level insufficient to cause clinical signs of neuropathy, was able to modify the known response of the hemidiaphragm / phrenic nerve preparation to an *in vitro* exposure to the organophosphorous anticholinesterase compound ecothiopate. That is, to use ecothiopate as a 'probe' for the condition of the nerve terminal regions of the phrenic nerve. Ecothiopate, like other anticholinesterases, has been demonstrated to cause the potentiation of indirectly evoked muscle twitches and also to cause the generation of spontaneous twitches or fasciculation (Ferry, 1988; Morrison, 1977; Burd & Ferry, 1987). These responses are sensitive to temperature and the magnesium ion concentration in the bathing physiological saline and are thought to be events specifically related to the neuromuscular junction and the immediate pre-terminal axon (Randic & Straughan, 1964). There are thought to be both pre and post-synaptic components to the generation of twitch potentiation and fasciculation. For these reasons it was hypothesised that any acrylamide induced deficits in the function of the nerve terminals may reflect themselves in a modification of the responses of hemidiaphragm preparations to ecothiopate exposure. A similar rationale was employed by Lowndes *et al.* (1974) who studied the affects of di-isopropyl flourophosphate (DFP) pre-treatment on the evoked twitch responses of soleus nerve-muscle preparations following the administration of edrophonium. In the experiments reported in this thesis both evoked and spontaneous twitches were studied separately in a mixture of left and right hand side hemidiaphragm preparations. No differences were observed in the response of left or right hand side hemidiaphragms to ecothiopate.

5.1: THE EFFECT OF ACRYLAMIDE ON INDIRECTLY EVOKED TWITCHES IN THE MOUSE HEMIDIAPHRAGM FOLLOWING ECOTHIOPATE EXPOSURE:

If acrylamide is capable of reducing the efficiency of acetylcholinesterase at the neuromuscular junction (see chapter 6) then it may be possible to detect a reduction in the affect of ecothiopate upon the potentiation of indirectly evoked twitches of the mouse hemidiaphragm. Another possible explanation for such a result might be that acrylamide administration results in partial functional denervation. The possibilty that acrylamide in

some way interferes with the generation of repetitive firing in the nerve and muscle cells of the non denervated hemidiaphragm could also explain such observations.

In the experiments described below, phrenic nerve/hemidiaphragm preparations were dissected from mice which had been pre-treated *in vivo* with acrylamide or distilled water. These preparations were placed in organ baths containing physiological saline which was maintained at 37°C and gassed with carbogen. Tension on the preparations was adjusted such that a single supramaximal stimulus to the phrenic nerve yielded a twitch of the greatest magnitude. The preparations were allowed to equillibrate at a stimulus frequency of 0.01 Hz until a baseline resting tension was firmly established. Ecothiopate (500 nM) was then added to the saline in the organ bath.

Two parameters of the indirectly evoked muscle twitches (0.1Hz) were studied in the experiments. The first was the timing of the onset of the first potentiated twitch (TFP) and the second that of the maximally potentiated twitch (TPP) following the addition of ecothiopate (500 nM) to the organ bath. Figure 2.2 in chapter 2 (Materials and Methods) shows an example of the data obtained in these experiments. Table 5.1 below shows the data obtained from these experiments.

Treatment	Time of Study	Mean TFP /S	Mean TPP /S	Mean TPP-TFP /S	N:
Control		233 ±71	601 ±187	376 ±129	8
100 mg/kg ACR	1 Hour	205 ±125	540 ±205	335 ±107	5
100 mg/kg ACR	4 Hours	275 ±86	612 ±162	338 ±80	5
100 mg/kg ACR	8 Hours	200 ±73	410 ±161	210 ±88*	5
100 mg/kg ACR	12 Hours	316 ±57	680 ±76	363 ±71	4
100 mg/kg ACR	1 Day	287 ±33	640 ±200	352 ±173	7
100 mg/kg ACR	4 Days	186 ±61	511 ±166	325 ±113	6
100 mg/kg ACR	8 Days	228 ±75	576 ±164	348 ±90	7
100 mg/kg ACR	16 Days	228 ±78	517 ±158	288 ±83	7
100 mg/kg ACR	32 Days	232 ±80	523 ±171	301 ±85	4
200 mg/kg ACR	8 Days	205 ±80	725 ±198*	520 ±187*	5

Table 5.1: Summary data table to show the effect of orally administered acrylamide (ACR) pre-treatment on supra maximally stimulated twitches (0.1 Hz) of the mouse hemi-diaphragm preparation following *in vitro* exposure to 500 nM ecothiopate (ECO) with regard to the time course of twitch amplitude potentiation. TFP = the time from addition of ECO to the first muscle twitch showing potentiation of amplitude. TPP = the time from addition of ECO to the muscle twitch showing the maximum potentiation of amplitude. 200 mg/kg ACR administered as 2 x100 mg/kg on consecutive days. Control animals received pre treatment with distilled water only. Values ± S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

From these data one can see that a 100mg/kg dose of acrylamide was insufficient to cause any significant alteration in the timing of the first potentiation (TFP) of the evoked twitch response of the hemidiaphragm following the addition of 500nM ecothiopate to the organ bath. The time taken to reach the maximum or peak twitch potentiation (TPP) was also not significantly altered at this dose of acrylamide.

However, TPP was significantly increased 8 days after an acrylamide pre-treatment of 200 mg/kg. This is a dose of acrylamide which is sufficient to cause clinical signs of neuropathy in the mice studied at this time point (see chapter 2).

It is of note when examining these data that TFP could be affected by variations in the position of addition of the ecothiopate as well as variations between preparations in the amount of turbulence in the physiological saline in the organ bath caused by the bubbling of carbogen gas through it from the base of the organ bath. There could also be inter-preparation variation in the ability of the ecothiopate to diffuse to the location of the anticholinesterase at the neuromuscular junction. Care was taken to add the ecothiopate to the organ bath at the same position and at the same rate in each experiment but the other factors mentioned above were beyond close control. Thus, the TFP data must be viewed with regard to these variables.

Subtraction of TFP from TPP yields the time taken from the first potentiated twitch to that of the maximally potentiated twitch. This may be a better indicator of the rate of onset of the action of ecothiopate. These data show that an 8 hour *in vivo* pre-treatment of 100 mg/kg acrylamide significantly reduces this value. The 200 mg/kg / 8 day dose of acrylamide significantly extends this time. These results indicate that at a low dose and/or an early time point, acrylamide may actually facilitate the onset of the effects of ecothiopate in some way whereas higher doses of acrylamide retard these effects.

The second parameter of the of the indirectly evoked twitches to be measured was their amplitude. For each hemidiaphragm the baseline (pre-ecothiopate) twitch amplitude was recorded following the adjustment of the tension on the muscle such that the maximum twitch amplitude was attained. Following the addition of ecothiopate the amplitude of the maximally potentiated twitch was recorded and from these two values the percentage increase in the twitch amplitude was calculated. Table 5.2 below shows the data derived from these experiments:

Treatment	Time of study	Mean twitch amplitude pre ECO. /g	Mean max. twitch amplitude post ECO. /g	Mean % MP	N:
Control		3.3 ±01.1	10.2 ±3.5	313 ±45	8
100 mg/kg ACR	1 Hour	3.1 ±01.3	9.6 ±4.1	322 ±131	5
100 mg/kg ACR	4 Hours	3.7 ±2.0	9.5 ±3.9	310 ±167	5
100 mg/kg ACR	8 Hours	3.0 ±1.9	14.9 ±3.4	601 ±247*	5
100 mg/kg ACR	12 Hours	4.7 ±1.2	14.4 ±5.3	303 ±38	4
100 mg/kg ACR	1 Day	3.6 ±1.0	8.0 ±1.4	229 ±54*	7
100 mg/kg ACR	4 Days	2.8 ±1.8	10.8 ±2.4	493 ±224	6
100 mg/kg ACR	8 Days	4.1 ±2.0	10.7 ±4.9	266 ±45*	7
100 mg/kg ACR	16 Days	4.1 ±0.6	11.3 ±2.0	279 ±49	7
100 mg/kg ACR	32 Days	4.0 ±1.0	9.6 ±2.4	239 ±40*	4
200 mg/kg ACR	8 Days	3.2 ±0.9	8.1 ±3.5	245 ±42*	5

Table 5.2: Summary data table to show the effect of orally administrated acrylamide (ACR) pre-treatment on supra maximally stimulated twitches (0.1 Hz) of the mouse hemi-diaphragm preparation following *in vitro* exposure to 500 nM ecothiopate (ECO) with regard to twitch amplitude. MP = maximum potentiation and is expressed as a percentage of the pre ECO twitch amplitude. 200 mg/kg ACR administered as 2 x100 mg/kg on consecutive days. Control animals received distilled water only. Values ± S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$)

From table 5.2 it can be seen that baseline twitch amplitude varied greatly even when taking the mean value of several experiments. This is due to variation in the size of the hemidiaphragms obtained from the mice. The calculation of the percentage increase in twitch amplitude following ecothiopate exposure for each experiment makes some allowance for this variation and allows the comparison of results from different sized hemidiaphragms.

The data show that 1, 8 and 32 days after pre-treatment with 100 mg/kg acrylamide, there is a significant decrease in the ability of ecothiopate to potentiate evoked muscle twitches. The higher acrylamide pre-treatment of 200 mg/kg with an 8 day incubation also significantly reduced the potentiating effect of ecothiopate.

5.1.1: CONCLUSIONS AND DISCUSSION:

These experiments show that acrylamide pre-treatment, at a level sufficient to cause clinical signs of neuropathy, slows the onset of the maximum twitch potentiation following ecothiopate exposure. There is also evidence to suggest that a lower dose of acrylamide may actually facilitate the onset of the maximum twitch potentiation.

The data also show that a dose of acrylamide insufficient to cause clinical signs of neuropathy can reduce the maximum amplitude of post-ecothiopate potentiated twitches. This effect is also repeated at the higher dose of acrylamide.

The potentiation of indirectly evoked muscle twitches by anticholinesterases has been well documented. This 'enhancement' of the twitch is the result of repetitive firing of muscle cells which has been attributed to the persistent presence of acetylcholine at the endplate resulting in the prolongation of the endplate potential from which repetitive muscle action potentials arise (Brown *et al*, 1936; Eccles *et al*, 1942; Ferry, 1988). The large and synchronized compound action potential of the muscle produces a current flow which is capable of re-exciting some of the fine motor nerve endings, giving rise to what has been termed the ephaptic back-response (Lloyd, 1942; Brown & Matthews, 1960). This back-response then propagates in both ortho and antidromic directions resulting in the re-excitation of the muscle cells. Thus, generation of repetitive firing would appear to be a post-synaptic event. However, in the presence of low (0.1mM) magnesium ion concentration in the bathing physiological saline it has been demonstrated that there is an additional pre-synaptic mechanism for the generation of repetitive firing involving synchronised release of acetylcholine perhaps due to re-excitation of the terminal region of the nerve which in turn leads to the antidromic propagation of nerve action potentials to other nerve terminals in the same motor unit (Masland & Wigton, 1940; Clark *et al*, 1980 & 1984; Ferry, 1988). Masland and Wigton (1940) demonstrated that when acetylcholine was injected intra-arterially into cat hind limbs, a burst of antidromic nerve activity could be recorded. This lead to their hypothesis that it was the action of acetylcholine pre-synaptically which triggered antidromic nerve activity and hence twitch potentiation. On the other hand, Riker and his co-workers questioned the role of acetylcholine in twitch potentiation entirely and claimed that the anticholinesterase itself acted pre-synaptically to yield twitch enhancement via antidromic nerve activity (Werner & Kuperman, 1963; Riker & Okamoto, 1969). One further mechanism has been proposed. The 'potassium hypothesis' was first proposed by Katz (1962) and has been championed by Hohlfeld *et al.* (1981). This theory proposes that the prolonged and increased transmitter action occurring after the administration of an anticholinesterase increases local efflux of potassium from the depolarised area of the muscle fibre and that these potassium ions then depolarise nerve endings resulting in the generation of action potentials. The experiments described here were performed with 1mM magnesium in the bathing fluid and it was demonstrated by Ferry (1988) that under these conditions the majority of the generation of repetitive firing is post-synaptic in origin due to large and prolonged endplate potentials re-exciting the

muscle membrane. Therefore, these experiments could reasonably be expected to detect post-synaptic functional deficit following acrylamide administration although the role of pre-synaptic generative events cannot be excluded.

There are a number of possible explanations for the post acrylamide changes in the parameters measured during the course of the experiments carried out on the modification of stimulated twitch responses by *in vitro* exposure to ecothiopate. The increase in the onset time of the maximally potentiated twitch may be explained by a decrease in the release of acetylcholine. A similar effect has been noted by Clark *et al.* (1983) following modification of bathing saline around hemidiaphragm preparations such that calcium:magnesium ratios were reduced. Decreased twitch enhancement following acrylamide administration (i.e. decreased repetitive firing) could be due to decreased excitability of muscle membrane or decreased release of acetylcholine which would limit the effect of or the size/duration of the endplate potential respectively. Another possibility is that acrylamide is in some way depleting a proportion of the functional cholinesterase in the synaptic cleft. If this was the case then the effect of ecothiopate on twitch potentiation would be reduced because there would not be as much active cholinesterase for it to inhibit and thus the change in the cleft concentration of acetylcholine would not be as great resulting in reduced size and duration of the endplate potentials which give rise to the repetitive firing which is responsible for the increase in the magnitude of the twitch. If acrylamide was capable of affecting cholinesterase then it should be possible to observe that twitches recorded prior to the addition of ecothiopate were larger in diaphragms taken from acrylamide treated animals than from the untreated controls. However, this hypothesis assumes that all the hemidiphragms are the same size. This is clearly not the case since animals vary considerably and thus no such effect of acrylamide could seen in the data. This problem was solved by examining the time course of miniature endplate potentials (see chapter 6).

5.2: THE EFFECT OF ACRYLAMIDE ON SPONTANEOUS TWITCHES OF MOUSE HEMIDIAPHRAGM FOLLOWIG ECOTHIOPATE EXPOSURE:

As described in chapter 2 (materials and methods), spontaneous muscle twitches occuring in the hemidiaphragm preparations following their exposure to ecothiopate were analysed using two computer systems. The first of these, the virtual oscilloscope, yielded data on the amplitude, rise time and half decay time of a sample of twitches. The second 'Labview' based system produced data on the amplitude and frequency characteristics of all the spontaneous twitches occurring in a 15 minute period beginning 7 minutes after the addition of ecothiopate to the organ bath.

5.2.1: VIRTUAL OSCILLOSCOPE ANALYSIS OF ECOTHIOPATE INDUCED SPONTANEOUS TWITCHES OF THE MOUSE HEMIDIAPHRAGM PREPARATION FOLLOWING ACRYLAMIDE PRE-TREATMENT:

These data were used to show if acrylamide was able to modify the time profile of the contraction of ecothiopate induced spontaneous twitches of the mouse hemidiaphragm preparation. Figure 5.1 below shows an example of the output from the virtual oscilloscope following the capture of a spontaneous twitch.

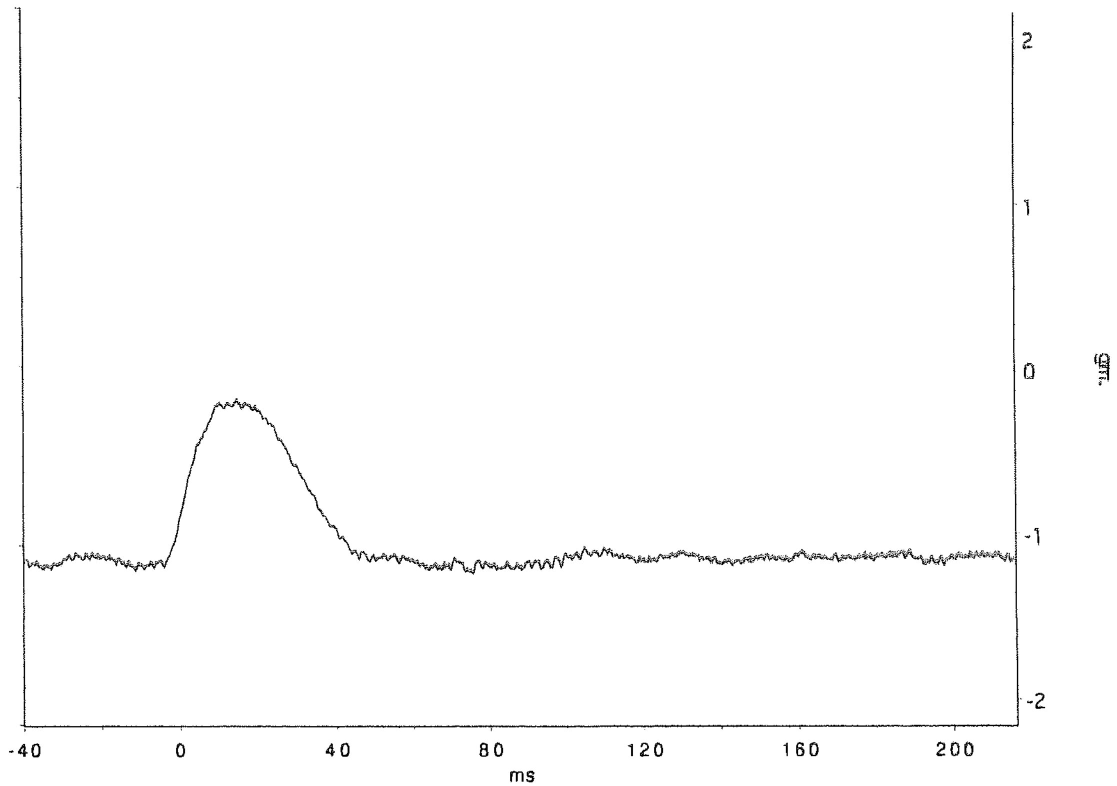


Figure 5.1: An example of an ecothiopate induced spontaneous twitch from a control mouse previously recorded to magnetic tape (Racal Store 4) and captured on replay by Scope V virtual oscilloscope software running on a Macintosh Classic computer. Analogue to digital conversion was via a Maclab/4 12 bit AD converter. (Scope V and Maclab/4 supplied by Analog Digital Instruments, Australia). Measurements of time and amplitude characteristics were made using the callibrated cursor of the software. The abscissa was callibrated using known weights attached to the force transducer usually connected to the muscle preparation.

Figures 5.2 and 5.3 below show scattergrams of the data gathered for investigating the effect of *in vivo* acrylamide pre-treatment on the time from the base to the peak of the rising phase of the spontaneous twitches occurring following *in vitro* ecothiopate exposure.

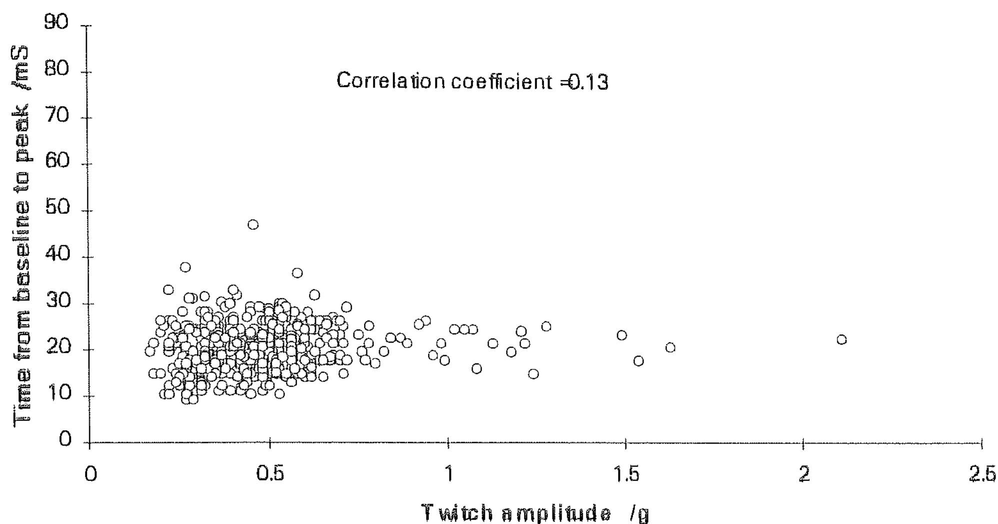


Fig 5.2: Scattergram to show the relationship between amplitude and time from baseline to peak of spontaneous twitches in the mouse hemi-diaphragm muscle following pre treatment with orally administered distilled water (control) and *in vitro* exposure to 500 nM ecothiopate (recording started 7 minutes after the addition of ecothiopate).

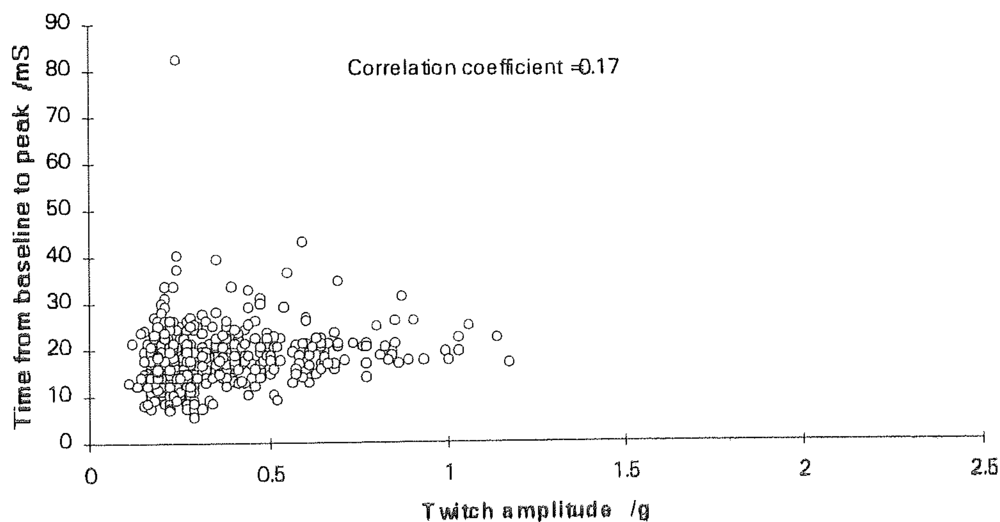


Fig 5.3: Scattergram to show the relationship between amplitude and time from baseline to peak of spontaneous twitches in the mouse hemi-diaphragm muscle following pre treatment with orally administered acrylamide (100 mg/Kg studied 8 days after dosing) and *in vitro* exposure to 500 nM ecothiopate. Recording started 7 minutes after the addition of ecothiopate.

From these data one can see that there is no general correlation between the amplitude of a spontaneous twitch and its rise time. This indicates that repetitive firing is not the cause of the variation in the amplitudes observed since this would cause there to be a positive correlation between rise time and amplitude of the twitches. The larger twitches would have arisen from the summation of the mechanical responses to two or more closely spaced (3-5 μs; Ferry, 1988) repetitive firings of the nerve. The rise time of the summated twitch would thus be longer than that of a twitch resulting from a single nerve action potential. The variation in the spontaneous twitch amplitude is most likely to be the result of variation in the size of motor units in the hemidiaphragm. Eight days after a dose of 100 mg/Kg of acrylamide no effect on the rise times of the twitches can be observed. This finding was repeated even when the dose of acrylamide was doubled. However, one can see that the small number of larger amplitude twitches present in the control data are absent in the data from acrylamide treated hemidiaphragms. The low number of twitches captured per experiment by this data-capture system made the clarification of this observation very difficult.

A similar picture is seen with regard to the time from peak to half decay of these twitches. The correlation coefficients for control and a 100 mg/Kg / 8 day dose of acrylamide of -0.04 and -0.01 respectively indicate that there is no relationship between the size of a twitch and its half decay time and that acrylamide does not effect this parameter.

5.2.2: CONCLUSIONS:

It can be concluded from the data presented above that acrylamide pre-treatment does not effect the rise or fall time of the spontaneous twitches of the mouse hemidiaphragm following exposure to ecothiopate. However, it appears that acrylamide does affect the amplitude of the twitches in some way. Further analysis of this data with the virtual oscilloscope would be difficult since its capture rate of the twitches was insufficient to give a large enough sample of them to facillitate meaningful analysis. Therefore, a more powerful data capture system was employed which was able to acquire data on the frequency (which was as yet unknown) and amplitude of all the spontaneous twitches occurring in the 15 minutes following the 7 minute ecothiopate pre-exposure.

5.2.3: LABVIEW ANALYSIS OF SPONTANEOUS TWITCHES:

This method of data capture yielded data on the frequency and amplitude of the spontaneous twitches of the hemidiaphragms occurring in a 15 minute period following the first 7 minutes of exposure to ecothiopate .

The frequency data took the form of the number of twitches occuring in ten second time bins from which frequency distributions were plotted. These could be compared to the original paper chart records of the experiments in order to provide some validation that the computerised analysis system was yielding frequency data which was not distorted by some unforseen quirk of the sampling process as was the case with virtual oscilloscope described above. The instantaneous frequency of each twitch relative to that which proceeded it was also measured by the software.

5.2.3.1: THE EFFECT OF ACRYLAMIDE ON SPONTANEOUS TWITCH FREQUENCY:

Figures 5.4 to 5.13 below show the frequency distributions (10 second time bins) of spontaneous twitches occurring in hemidiaphragm preparations during a 15 minute period following 7 minutes exposure to 500 nm ecothiopate. The mice from which these diaphragms were taken were pre-treated with either distilled water only (control) or 100 mg/Kg acrylamide. Acrylamide treated mice were studied at various times following dosing as indicated. These figures are included to represent the variability seen during the course of these experiments. For comparison, Figures 5.14 to 5.17 following these distributions show copies of chart recorder paper output produced at the time of the experiments as an inidicator of the activity occurring during the course of the experiment are included where the condition of the these records permits since the ink used in the recorders tended to fade or smudge very easily. Frequency distributions for which a paper record is shown are marked *.

Controls:

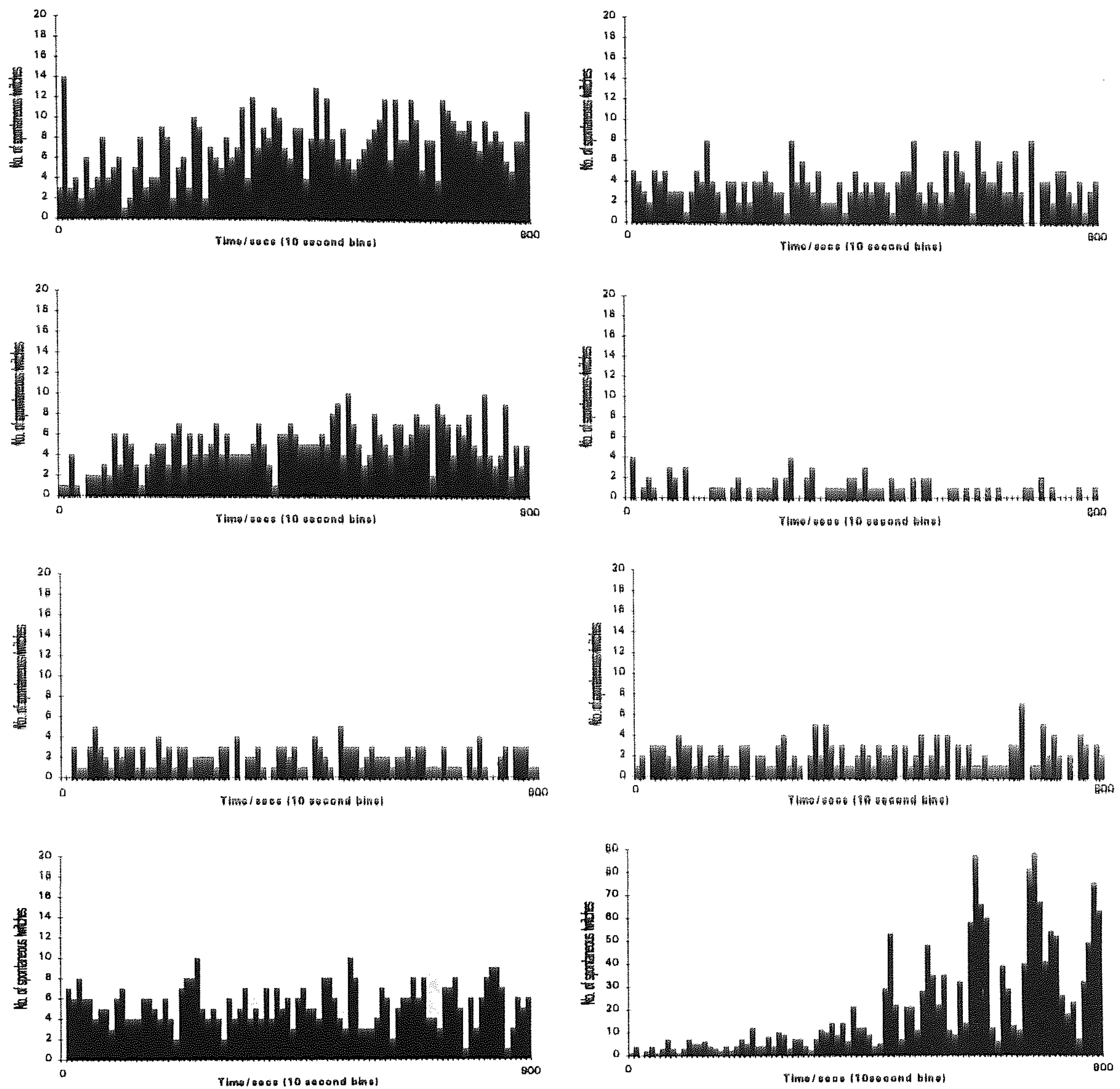


Figure 5.4: Frequency distributions (10 second time bins) to show the effect of distilled water (control) *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

100 mg/Kg Acrylamide/1 hour:

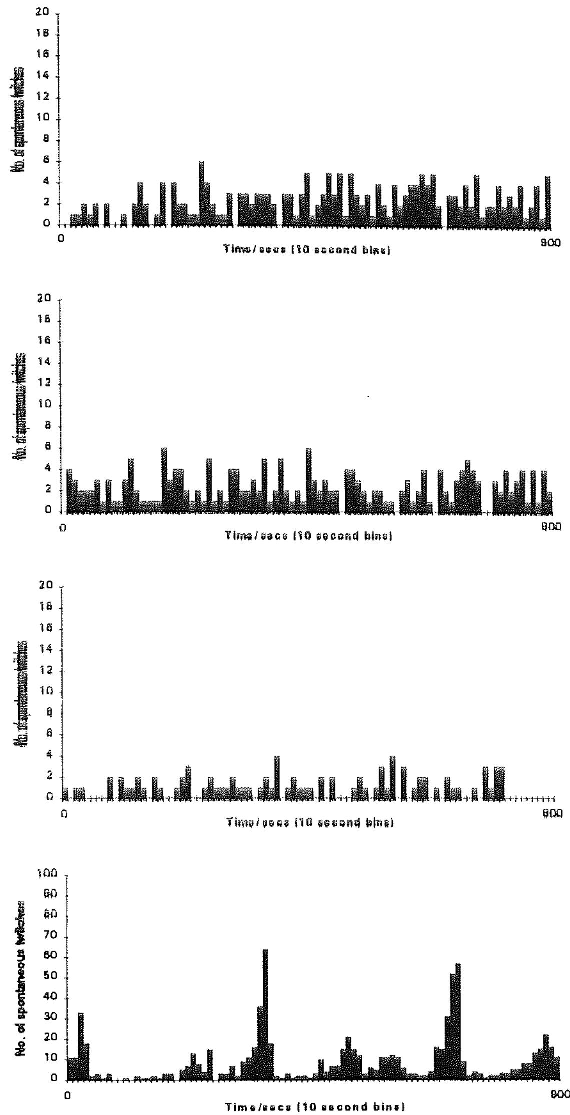


Figure 5.5: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 1 hour after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

100 mg/Kg Acrylamide/4 hours:

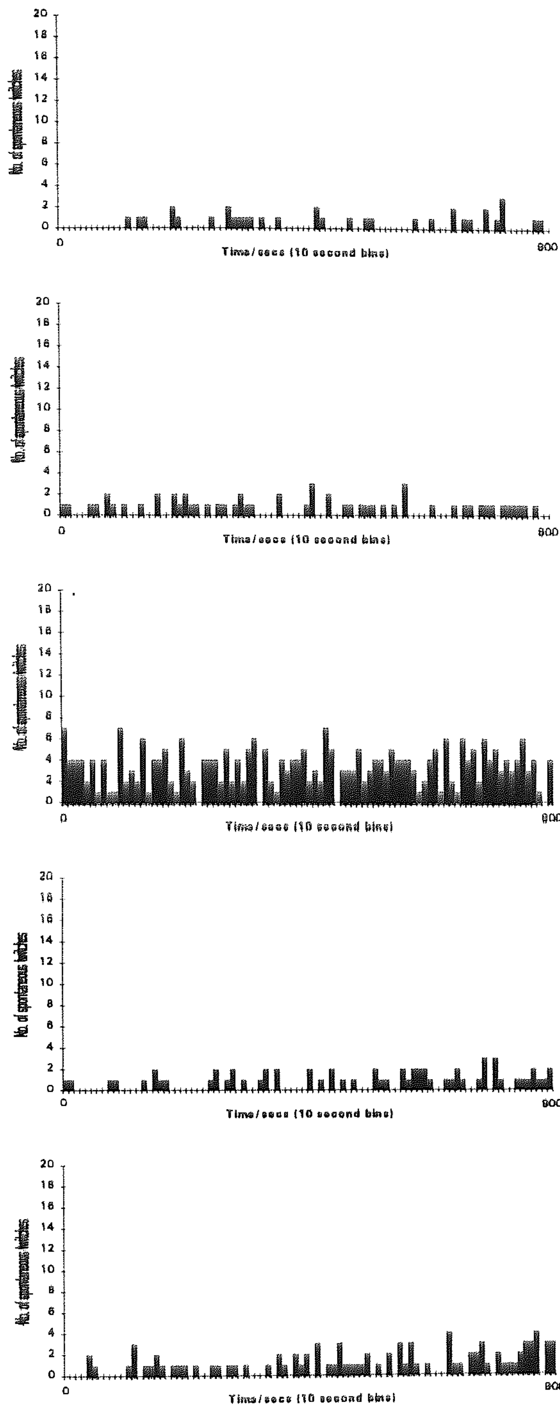


Figure 5.6: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 4 hours after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

100 mg/Kg Acrylamide/8 hours:

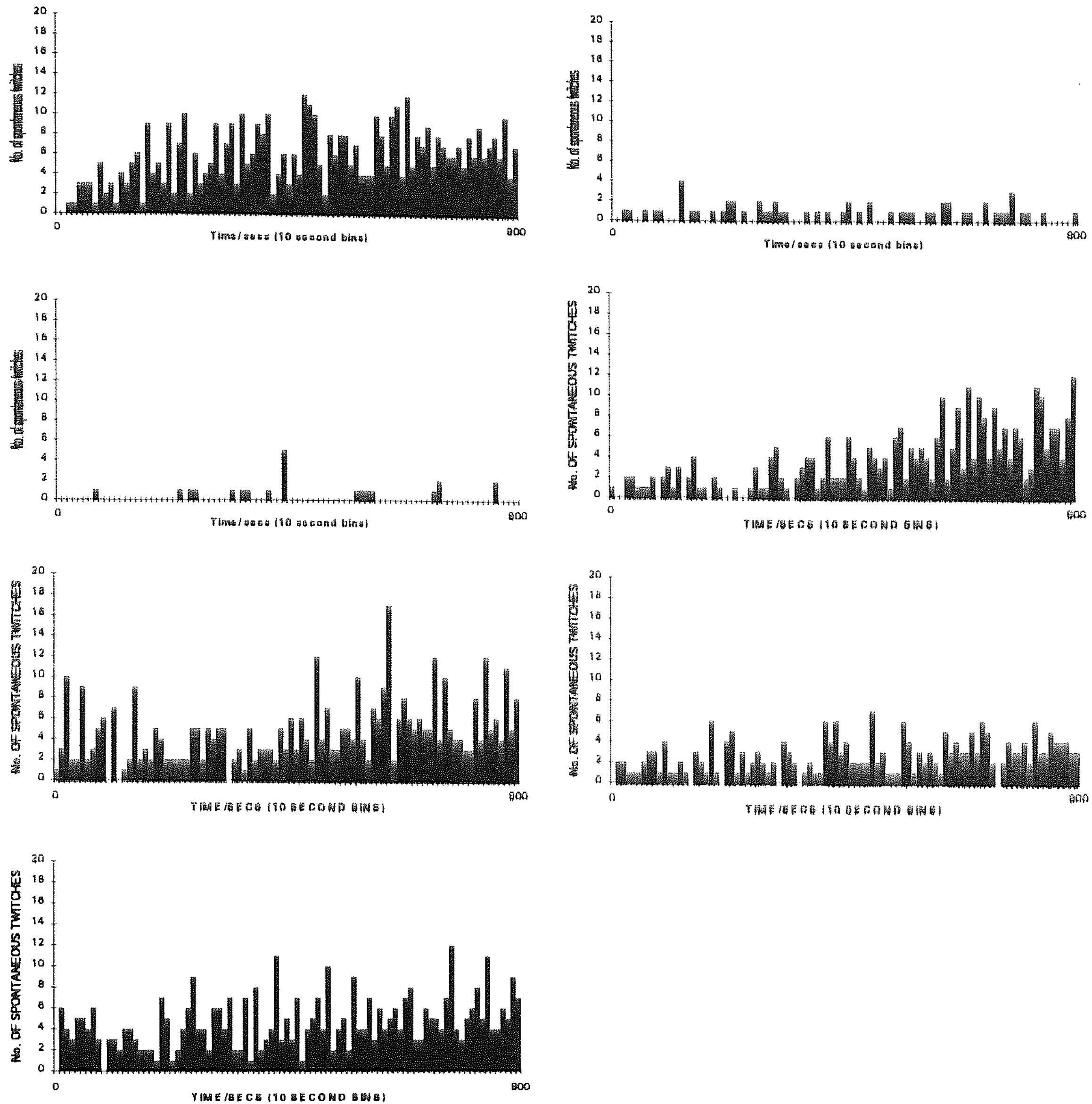
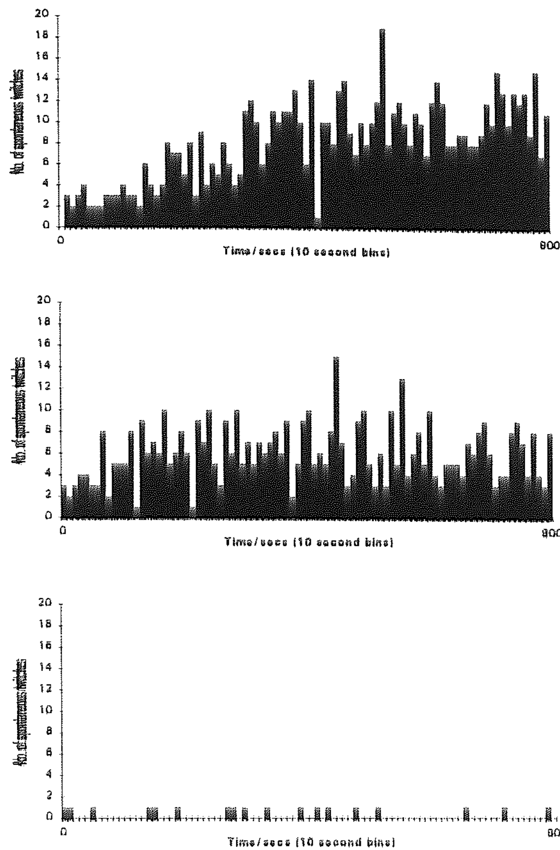


Figure 5.7: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 8 hours after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

100 mg/Kg Acrylamide/12 hours:



*

Figure 5.8: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 12 hours after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa. * = copy of chart recorder paper output made at time of experiment can be seen in figure 5.14.

100 mg/Kg Acrylamide/1day:

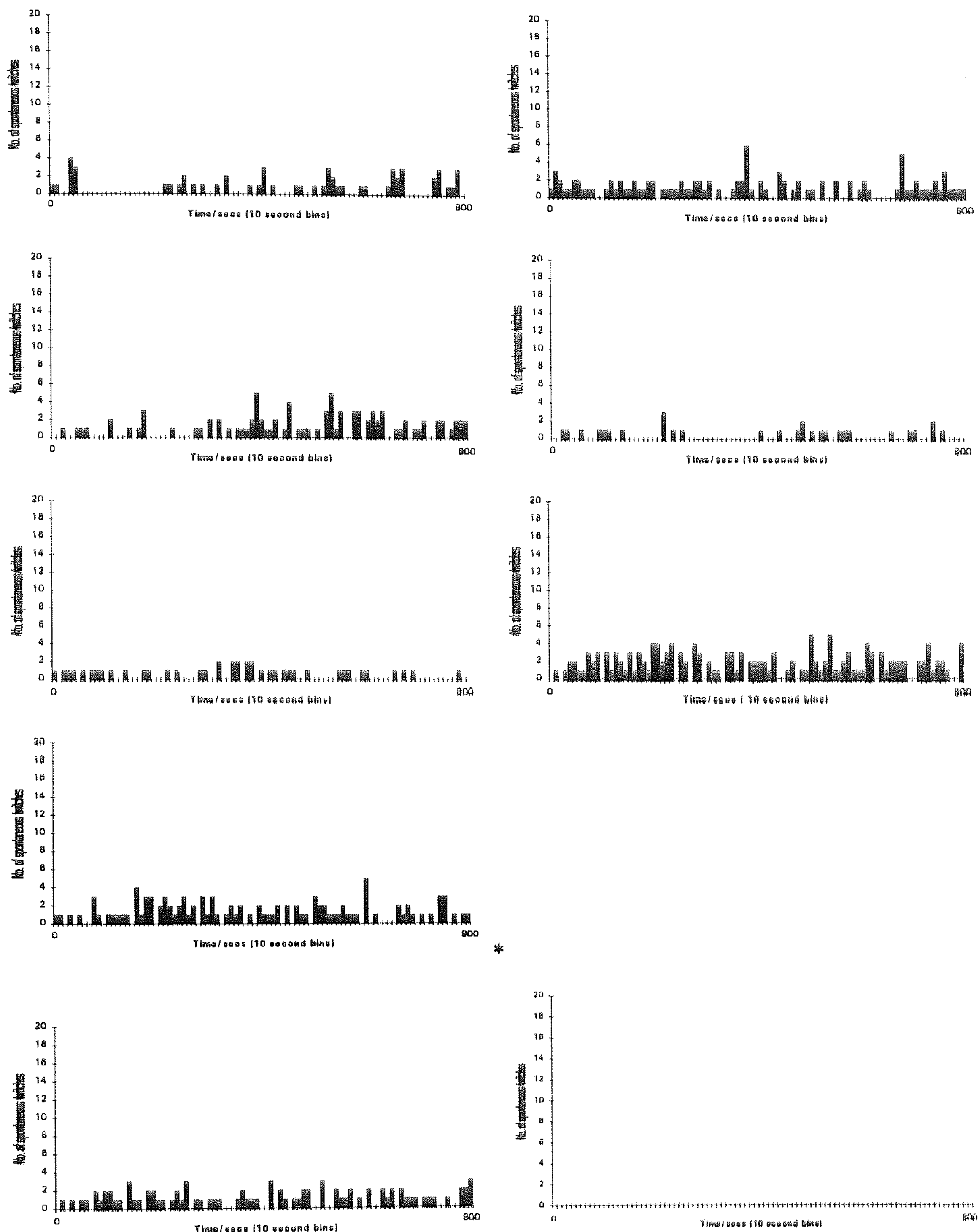


Figure 5.9: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 1 day after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. * = copy of chart recorder paper output made at time of experiment can be seen in figure 5.15.

100 mg/Kg Acrylamide/4 days:

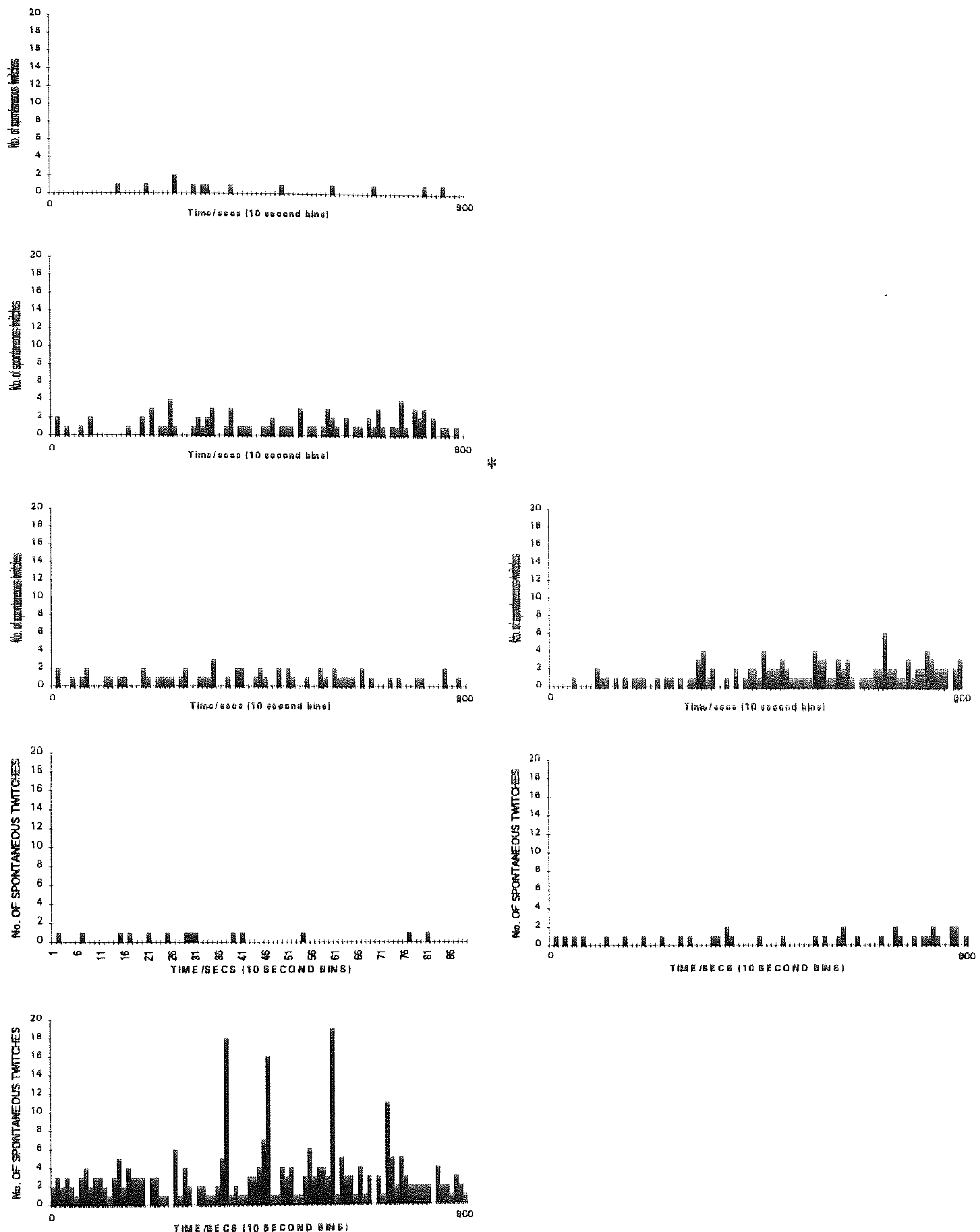


Figure 5.10: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 4 days after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa. * = copy of chart recorder paper output made at time of experiment can be seen in figure 5.16.

100 mg/Kg Acrylamide/8 days:

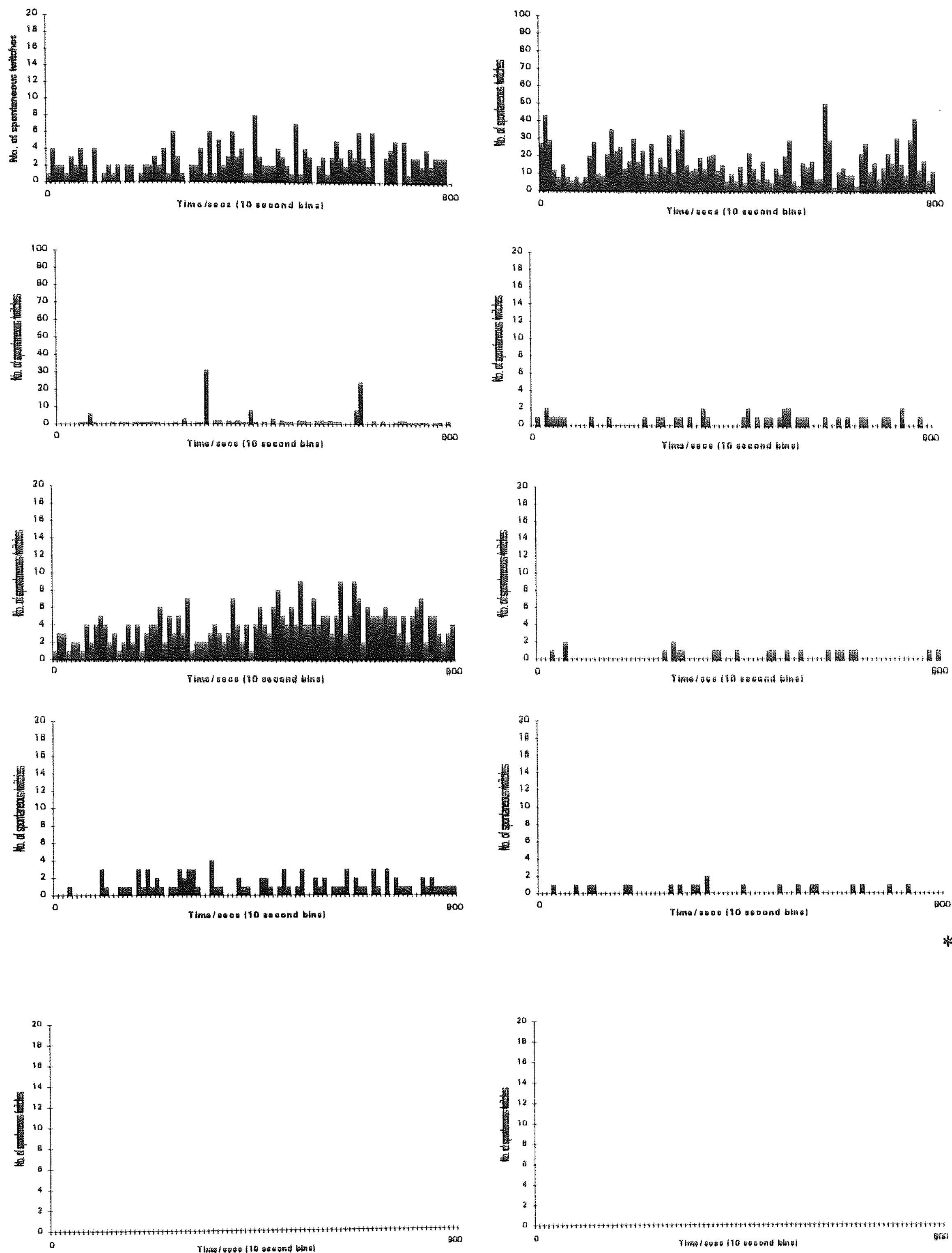


Figure 5.11: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 8 days after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa. * = copy of chart recorder paper output made at time of experiment can be seen in figure 5.17.

100 mg/Kg Acrylamide/16 days:

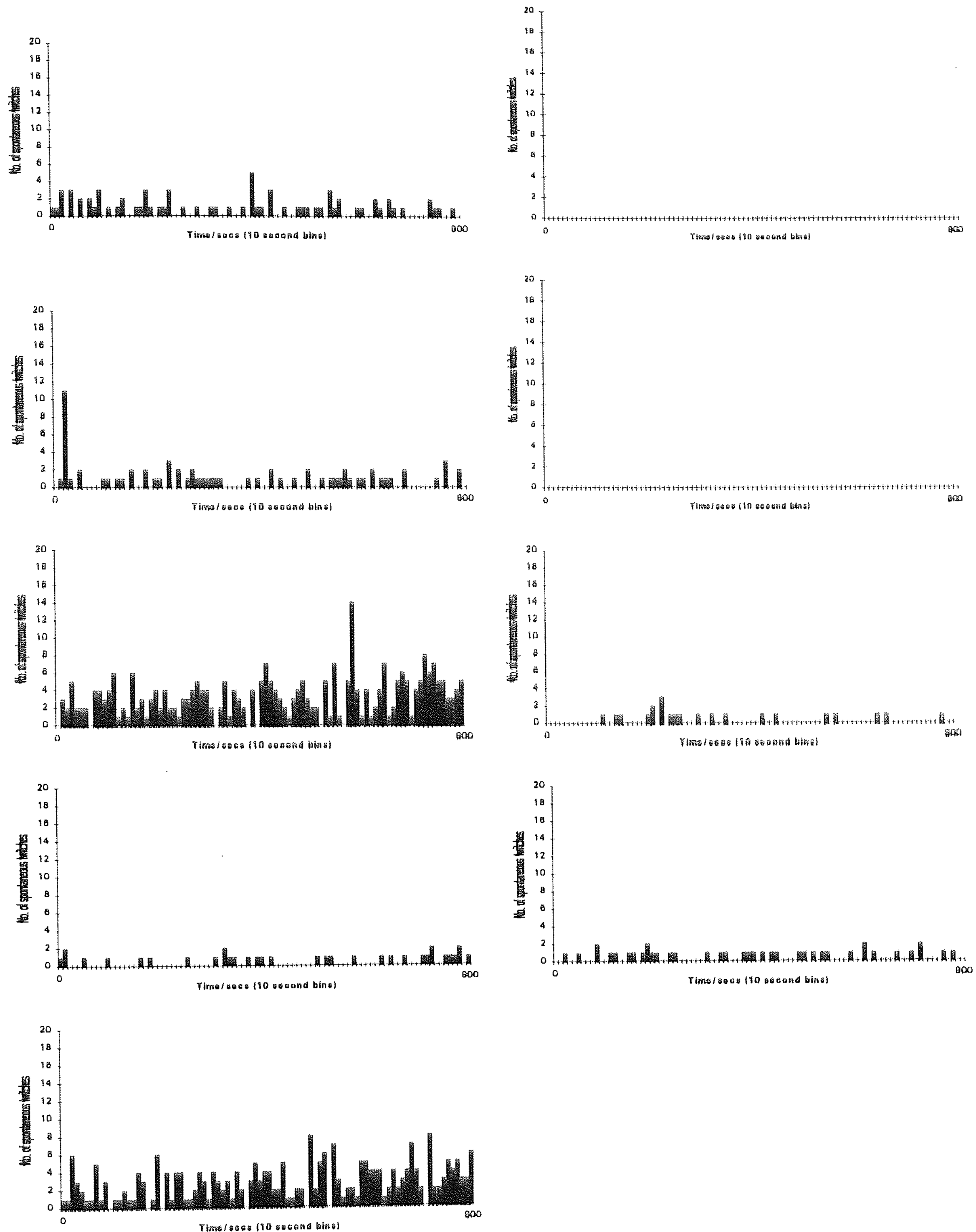


Figure 5.12: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 16 days after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

100 mg/Kg Acrylamide/32 days:

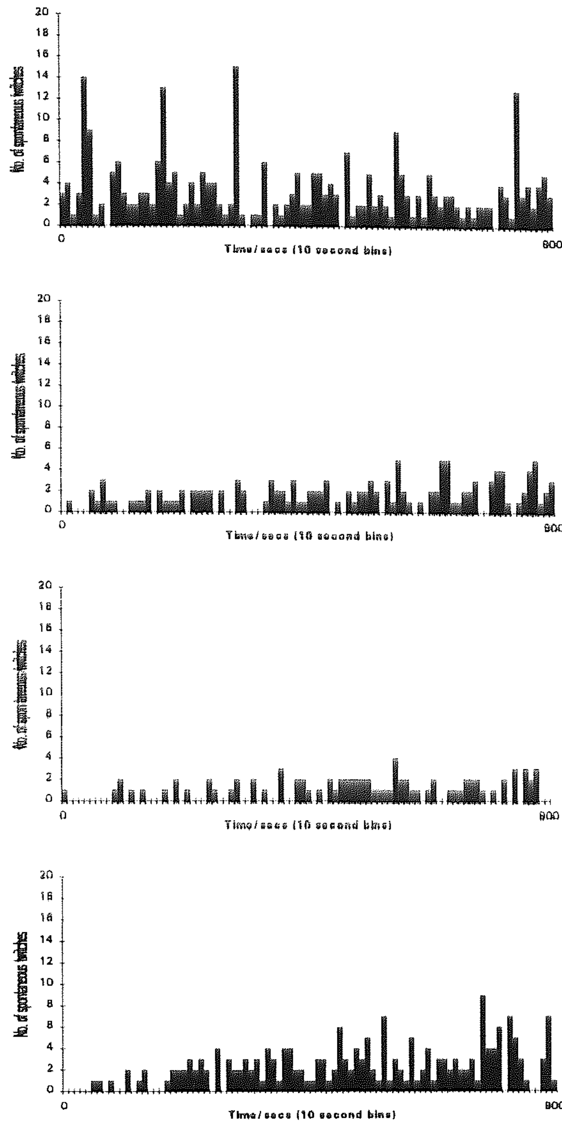


Figure 5.13: Frequency distributions (10 second time bins) to show the effect of 100 mg/Kg acrylamide *in vivo* pre-treatment on the frequency of spontaneously occurring twitches in the hemidiaphragms of mice following exposure to ecothiopate. Hemidiaphragm preparations were dissected 32 days after acrylamide dosing. Spontaneous twitches were sampled for 15 minutes beginning 7 minutes after the addition of ecothiopate to the physiological saline of the organ bath. Time 0 on abscissa.

From these figures it can be seen that there is a large amount of variability in the number of twitches occuring in the 15 minute data capture period. The control preparations show more consistent levels of activity although some preparations show less activity than others. There is noticably less activity in the hemidiaphragms of mice pre-treated with 100 mg/Kg acrylamide and studied 1, 4, 8 and 16 days after dosing. By 32 days after the 100 mg/kg dose there is the possibilty of some recovery. Figures 5.14 to 5.17 below show examples of chart recorder output for the frequency distributions above marked *.

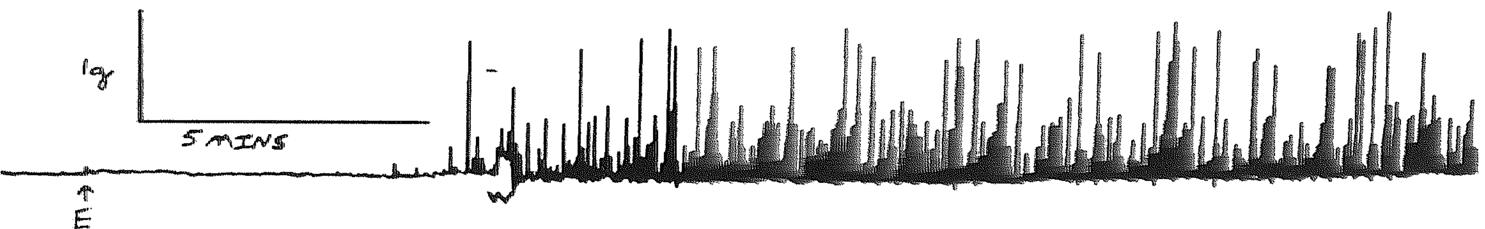


Figure 5.14: Chart recorder paper output made during the course of an experiment to record the spontaneous muscle twitches of a mouse hemidiaphragm preparation following 7 minutes exposure to 500 nM ecothiopate *in vitro*. The mouse from which these data were obtained was pre-treated *in vivo* by oral administration of 100 mg/kg of acrylamide and the diaphragm removed for study 12 hours after dosing. E = addition of ecothiopate. W = washout of ecothiopate.

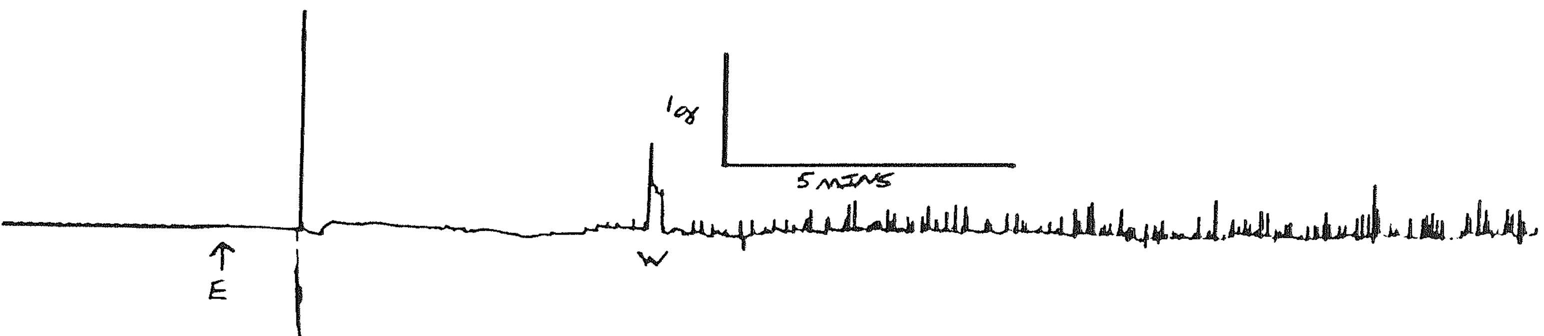


Figure 5.15: Chart recorder paper output made during the course of an experiment to record the spontaneous muscle twitches of a mouse hemidiaphragm preparation following 7 minutes exposure to 500 nM ecothiopate *in vitro*. The mouse from which these data were obtained was pre-treated *in vivo* by oral administration of 100 mg/kg of acrylamide and the diaphragm removed for study 1 day after dosing. E = addition of ecothiopate. W = washout of ecothiopate.

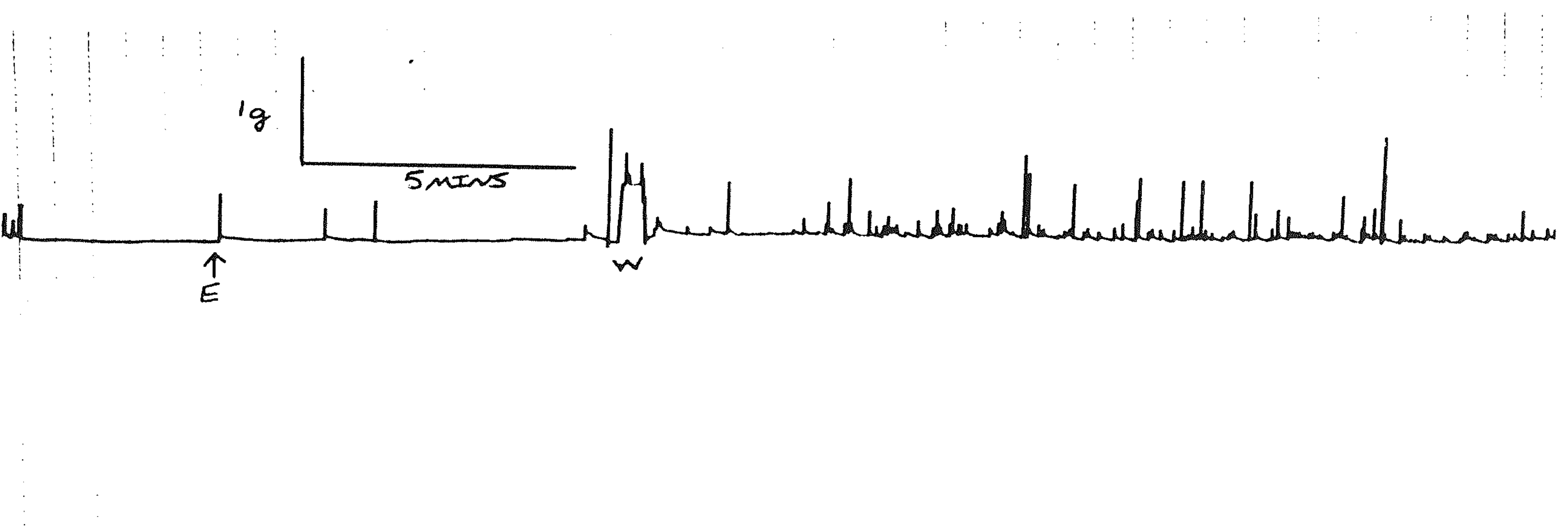


Figure 5.16: Chart recorder paper output made during the course of an experiment to record the spontaneous muscle twitches of a mouse hemidiaphragm preparation following 7 minutes exposure to 500 nM ecothiopate *in vitro.* The mouse from which these data were obtained was pre-treated *in vivo* by oral administration of 100 mg/kg of acrylamide and the diaphragm removed for study 4 days after dosing. E = addition of ecothiopate. W = washout of ecothiopate.

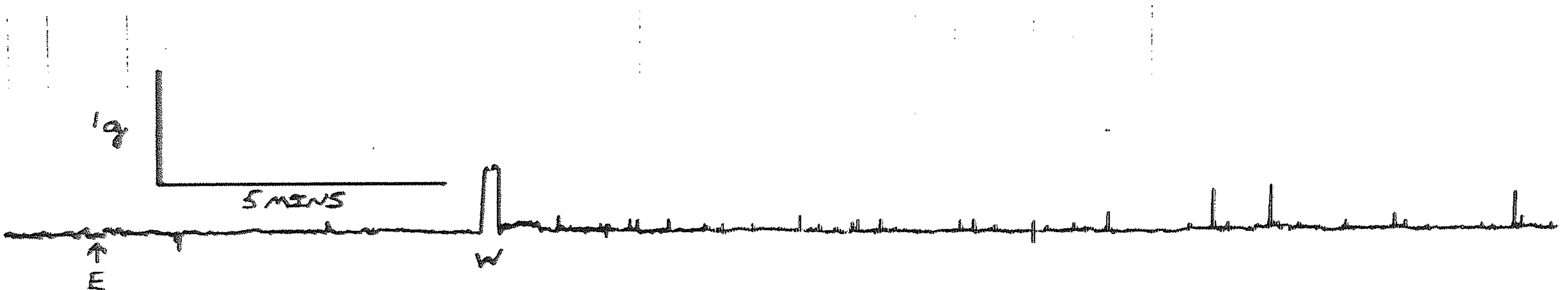


Figure 5.17: Chart recorder paper output made during the course of an experiment to record the spontaneous muscle twitches of a mouse hemidiaphragm preparation following 7 minutes exposure to 500 nM ecothiopate *in vitro*. The mouse from which these data were obtained was pre-treated *in vivo* by oral administration of 100 mg/kg of acrylamide and the diaphragm removed for study 8 days after dosing. E = addition of ecothiopate. W = washout of ecothiopate.

Figure 5.18 below shows the mean number of twitches captured during the 15 minute sampling period of these experiments.

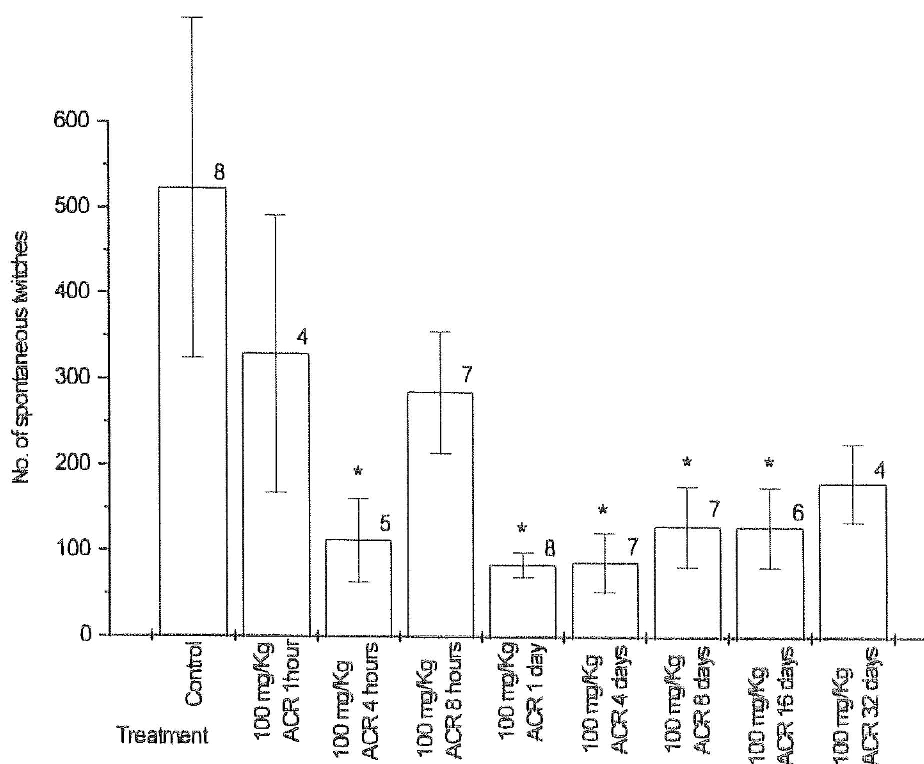


Fig 5.18: Graph to show the effect of acrylamide (ACR) pre treatment on the mean total number of spontaneous twitches of mouse hemi-diaphragm prepararions recorded in 15 minutes following the washout of an *in vitro* administration of 500 nM ecothiopate for 7 minutes. Control animals received distilled water only. *In vitro* temperature was maintained at $37^{o}C \pm 0.5$. Figures = number of observations. * = significant difference from control (Wilcoxon rank sum test, $p<0.05$).

From this figure it can be seen that there is a consistent significant reduction in the number of spontaneous twitches occurring 1 day after the administration of acrylamide. There is a significant reduction 4 hours after the administration of acrylamide but this is not repeated at the 8 hour time point.

Another feature of the data presented in figures 5.4 to 5.13 is the build up of spontaneous activity over the 15 minute data capture period in some preparations. Figure 5.19 below shows the mean spontaneous activity occurring in 5 minute time segments from 0-5, 5-10 and 10-15 minutes.

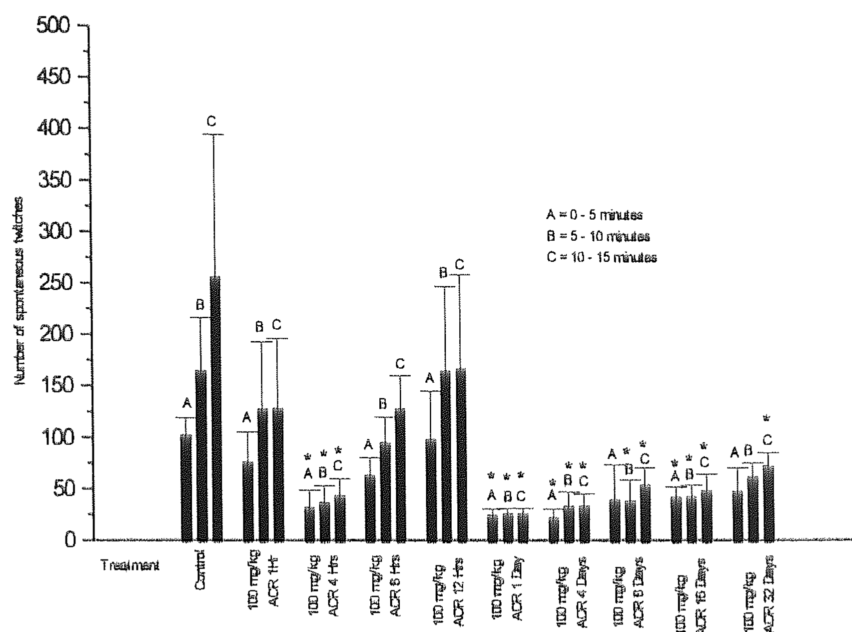


Fig 5.19: Graph to show the number of spontaneous twitches of mouse hemi-diaphragm preparation recorded during 15 minutes (separated into 5 minute time bins; A, B and C) following the washout of 500 nM / 7 min. *in vitro* ecothiopate. Animals were pre treated *in vivo* with orally administered distilled water (control) or acrylamide (ACR) and studied at the time point indicated after dosing. *In vitro* temperature was maintained at $37^{o}C \pm 0.5$ throughout all experiments.

These data show that at time points following the administration of acrylamide at which the total number of spontaneous twitches is significantly reduced, there is also a supression of the build up of the spontaneous activity throughout the 15 minute recording period. Thirty two days after the administration of acrylamide it can be seen that there are signs of a recovery of this build up.

The Labview analysis system also permitted the measurement of the instantaneous frequency of the spontaneous twitches. Instantaneous frequency is the reciprocal of the interval between consecutive twitches and this parameter provides another way of analysing the frequency of the spontaneous twitches. Figure 5.20 below shows the effect of acrylamide pre-treatment on the maximum instantaneous frequencies recorded for a given dose group.

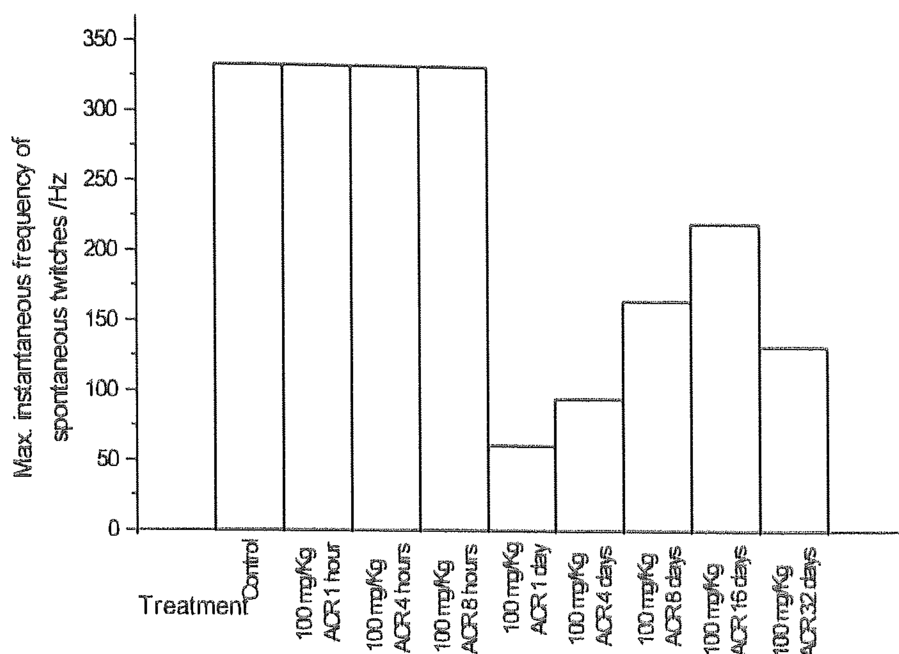


Fig 5.20: Graph to show the effect of orally administered acrylamide (ACR) pre treatment on the maximum instantaneous frequency of spontaneous twitches, recorded from all mouse hemidiaphragm preparations within a dose group, occuring within 15 minutes following *in vitro* exposure to 500 nM ecothiopate for 7 minutes at $37^{0}C \pm 0.5$.

From this figure it can be seen that maximum instantaneous twitch frequency recorded from all the experiments conducted at a particular dose is reduced 1 to 32 days after the *in vivo* pre-treatment with acrylamide. This correlates well with the reduction seen in the data for the total number of twitches occurring in the 15 minute data capture period (figure 5.18). It must be remembered that this data refers to twitches occuring in the many motor units throughout the hemidiaphragm and does not represent the ability of one motor unit to fire at a high frequency. Thus this measure is simply a function of the number of twitches occurring within the 15 minute data capture period. However it does indicate that when the total number of twitches occurring in the data capture period is reduced, the twitches are separated by a greater interval and thus not occurring in the bursts which can be seen in some of frequency distributions of figures 5.4 to 5.13.

The mean instantaneous twitch frequency following the various acrylamide pre-treatments is shown in figure 5.21 below:

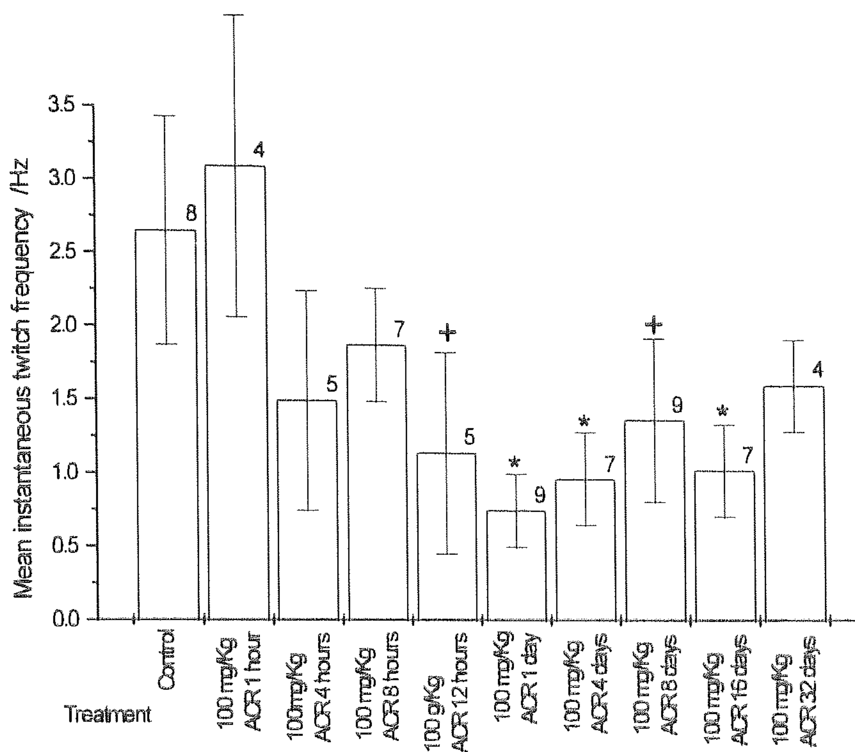


Fig 5.21: Graph to show the effect of orally administered acrylamide (ACR) pre treatment on the mean instantaneous frequency of spontaneous twitches in mouse hemi-diaphragm preparations following washout of *in vitro* ecothiopate (500nM / 7 min.). Control animals received distilled water only. Figures = number of observations. * & + = significant difference from control values (Wilcoxon rank sum test, $p<0.05$ & $p<0.1$ respectively).

Once again there is a correlation to the data of figure 5.18 in that acrylamide appears to have a significant effect on the frequency of twitches 1 day after its administration. This reduction in frequency is repeated at later time points. This data indicates again that the spontaneous twitches occurring within the whole hemidiaphragm are separated by greater time intervals when their frequency is reduced. This is important since it demonstrates that the bursts of spontaneous activity occurring in control hemidiaphragms are less likely to be present following acrylamide pre-treatment.

5.2.3.2: THE EFFECT OF ACRYLAMIDE ON THE AMPLITUDE OF SPONTANEOUS TWITCHES FOLLOWING ECOTHIOPATE EXPOSURE:

The Lab View data analysis system has already been shown in the previous section to greatly enhance the sampling of data from the magnetic tape recordings of the spontaneous twitch experiments. This lead to the confirmation of the subjective observations about the effect of acrylamide on spontaneous twitch frequency made from the paper chart records produced at the time of the experiment. It also appeared from these records that twitch amplitude was also being reduced by acrylamide pre-treatment and it was hoped that the greater sampling capacity of the Lab View system would yield data to confirm this observation.

Figure 5.22 below shows the data acquired by this analysis:

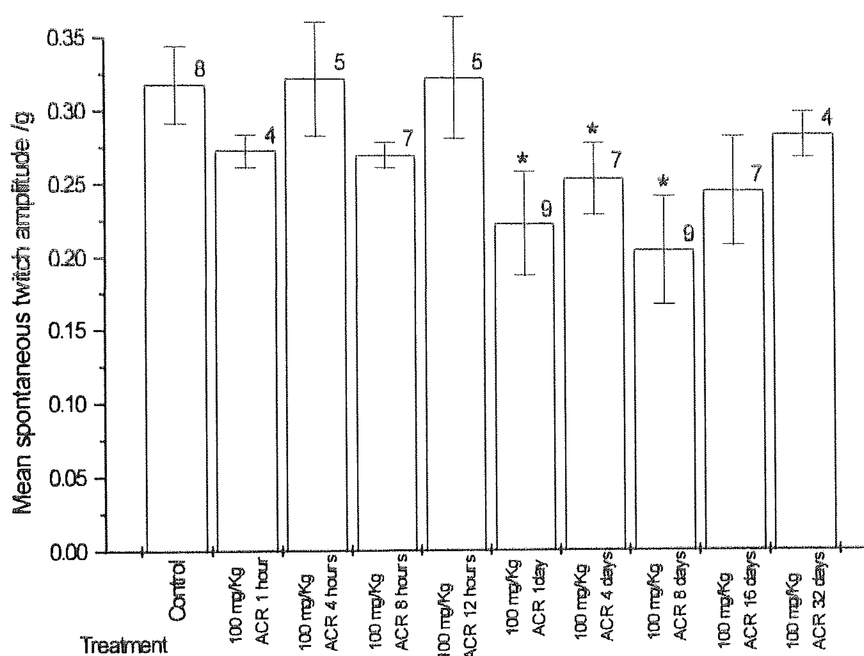
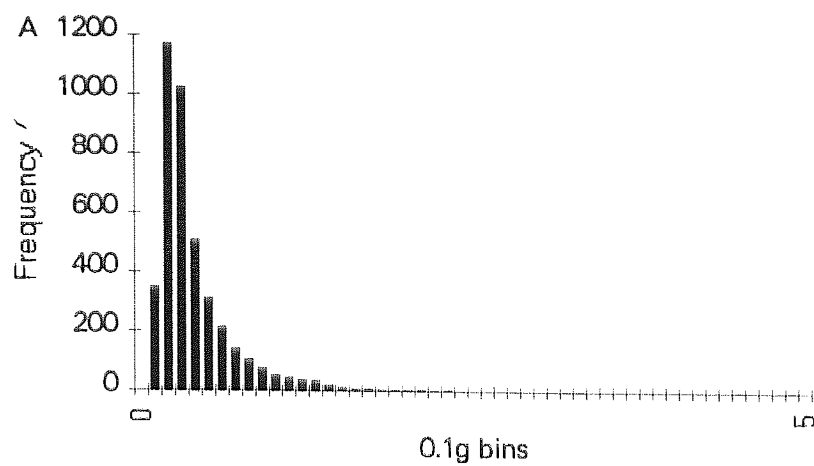


Fig. 5.22: Graph to show the effect of orally administered acrylamide (ACR) pre treatment on the amplitude of spontaneous twitches in mouse hemi-diaphragm preparations recorded for 15 minutes following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Control animals received distilled water only. Figures = number of observations. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

From these data it can be seen that there are slight but significant reductions in the mean amplitudes of ecothiopate induced spontaneous twitches 1, 4 and 8 days after the administration of acrylamide. However, the data appears to be very variable with the data

obtained 1 and 8 hours after acrylamide administration appearing only marginally different from that which is significant statistically. The non-parametric Wilcoxon rank sum test used to test this data relies on ranking the data and this may explain the statistical inferences made from the tests. It is possible that the reduction in the mean amplitude is due to the loss of a sub-population of larger twitches which thus alters the ranking of the smaller but more abundant twitches. Figures 5.23 to 5.28 below show frequency distributions for some of the time points shown in figure 5.22 above in an attempt to investigate this possibility.



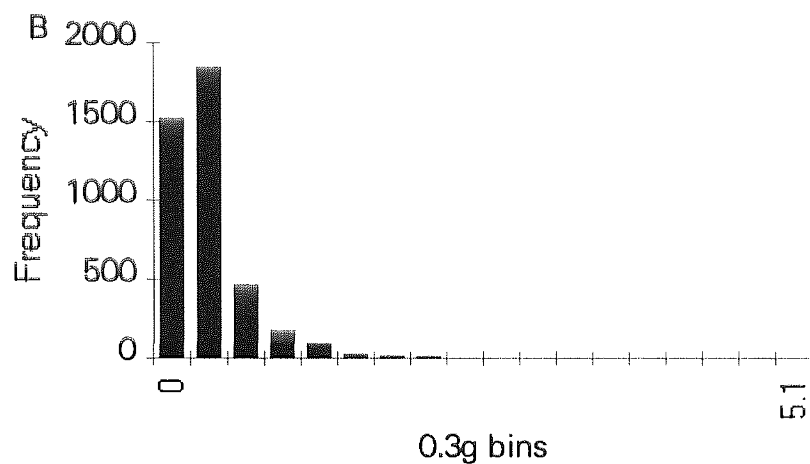
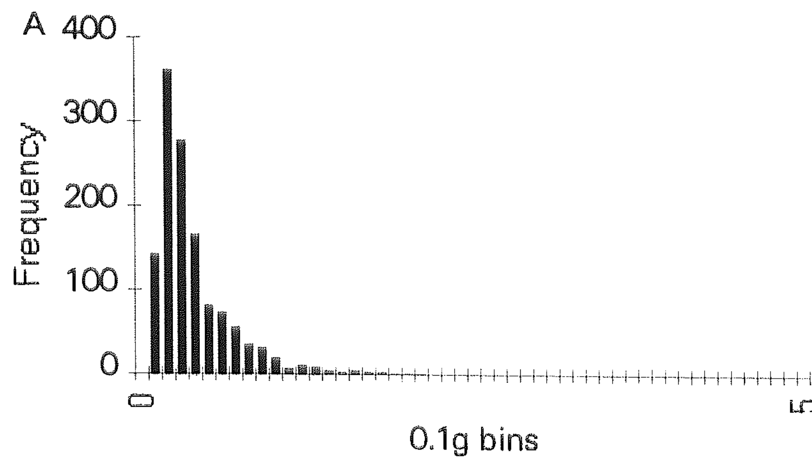


Fig. 5.23: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with distilled water only. A: 0.1g bins. B: 0.3g bins.



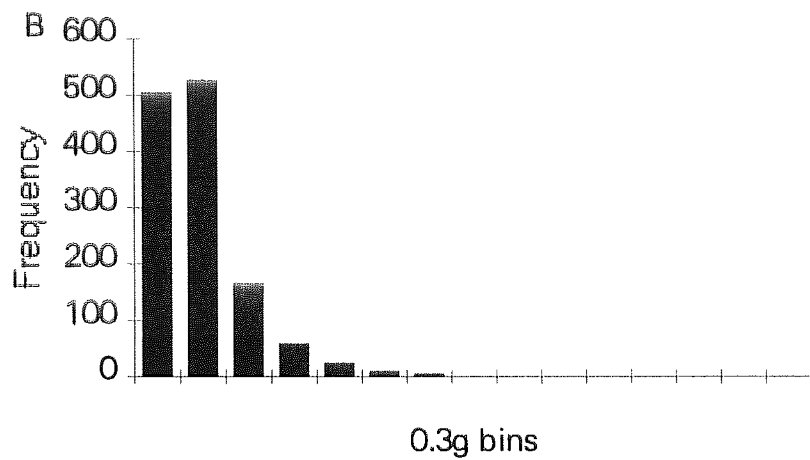


Fig. 5.24: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with 100 mg/Kg acrylamide and studied 12 hours later. A: 0.1g bins. B: 0.3g bins.

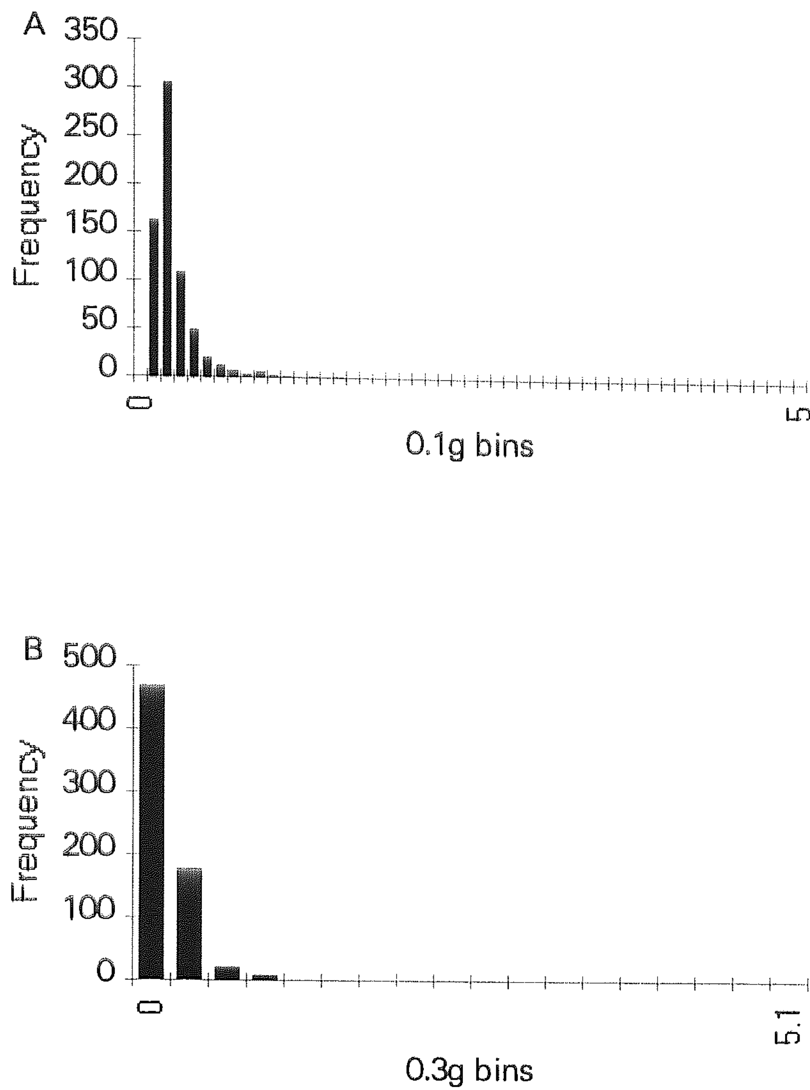
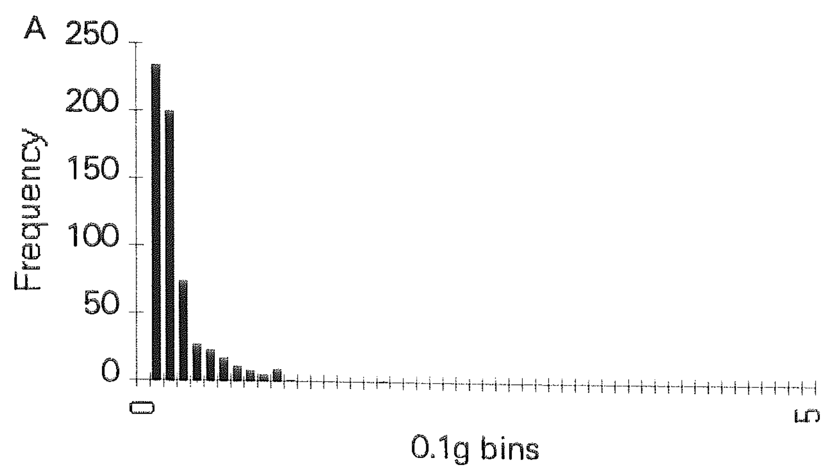


Fig. 5.25: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with 100 mg/Kg acrylamide and studied 1 day later. A: 0.1g bins. B: 0.3g bins.



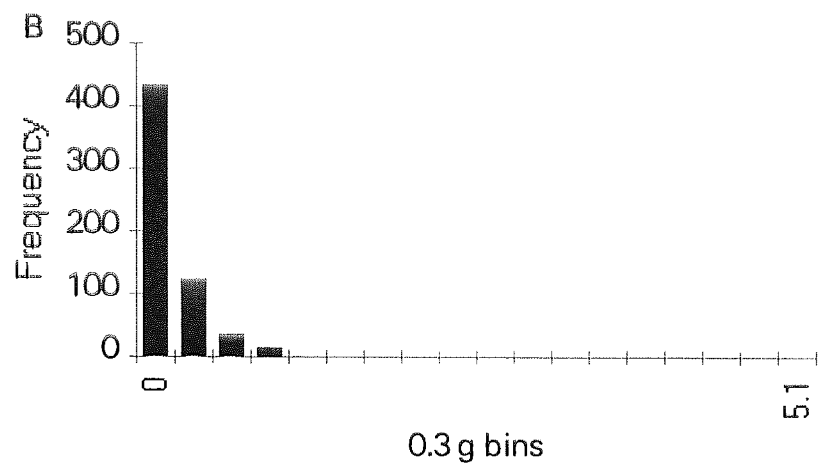
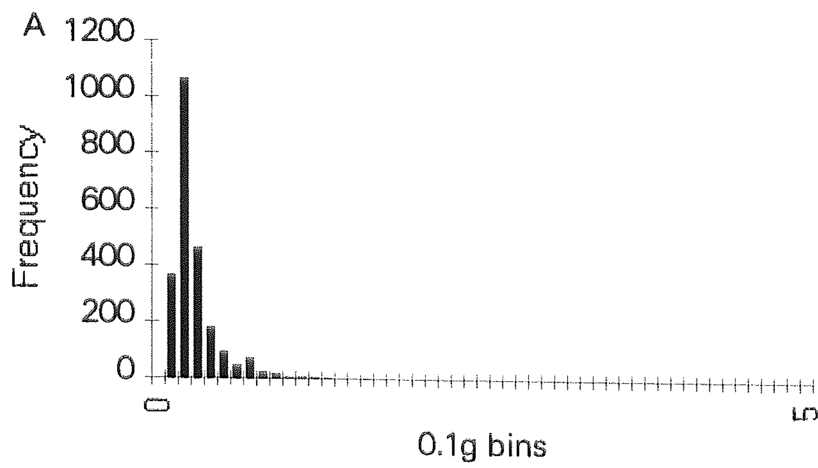


Fig. 5.26: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with 100 mg/Kg acrylamide and studied 4 days later. A: 0.1g bins. B: 0.3g bins.



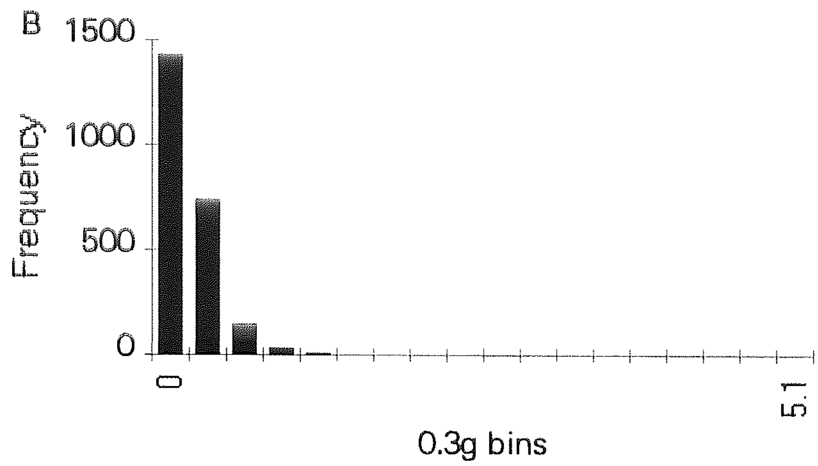
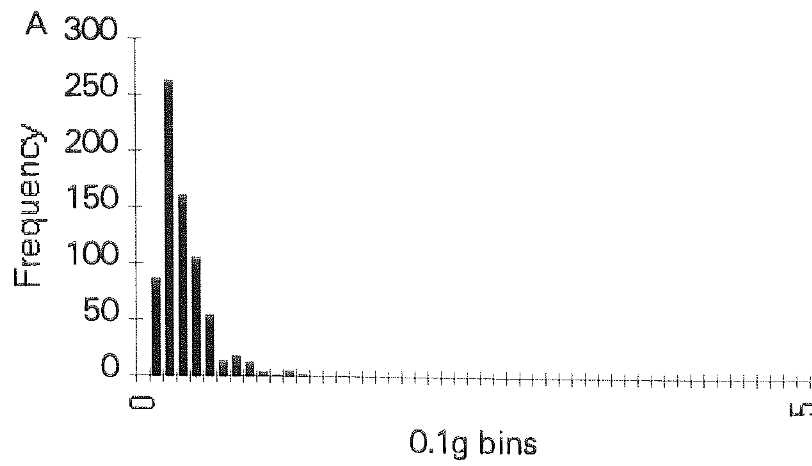


Fig. 5.27: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with 100 mg/Kg acrylamide and studied 8 days later. A: 0.1g bins. B: 0.3g bins.



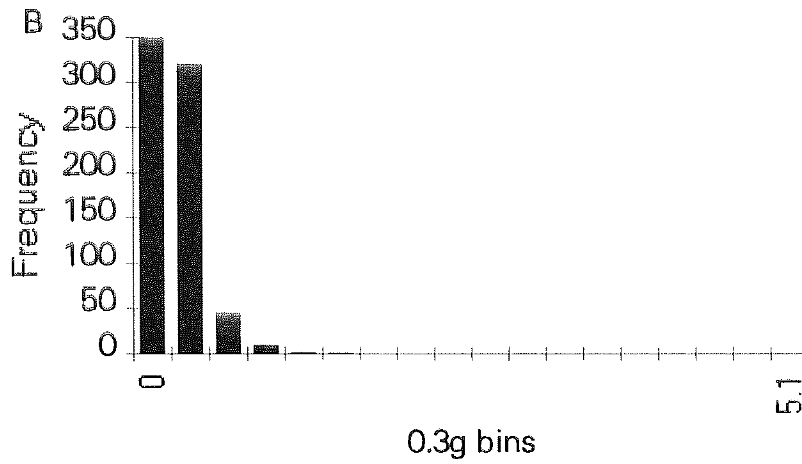


Fig. 5.28: Frequency distributions of the amplitudes of spontaneous muscle twitches recorded in the hemi-diaphragm preparation of mice for 15 mins. following *in vitro* exposure to 500 nM ecothiopate for 7 mins. Mice were pre treated orally with 100 mg/Kg acrylamide and studied 32 days later. A: 0.1g bins. B: 0.3g bins.

From these frequency distributions one can see that there is a reduction in the frequency of the larger twitches at the time points at which the statistical analysis showed significant differences between data from acrylamide treated and control animals for mean spontaneous twitch amplitude. The data shown for control and 12 hours post-acrylamide are typical for the no-effect time points. One can see that there are a small number of larger twitches which skew the distribution to the right. In the frequency distributions for time points where the mean twitch amplitude is significantly different from control (1, 4 and 8 days after 100mg/kg acrylamide) it can be seen that the frequency of these larger twitches is reduced. However, it can also be seen from these data that there is also a shift to the left in the distributions indicating that there is a greater number of smaller twitches. This might indicate that the large twitches observed prior to acrylamide pre-treatment are being attenuated following acrylamide administration by some unknown mechanism. It is possible to speculate that the spontaneous nerve action potential which is generated at or near the nerve terminal is unable to propagate antidromically and/or orthodromically within the motor unit(s) and thus is unable to recruit as many muscle cells for contraction. This may affect the large twitches only or may affect the majority of the spontaneous twitches occurring following ecothiopate administration. The data shown in figure 5.28 for the frequency distribution of twitches 32 days after acrylamide administration show that there may be a reversal of this trend with part B of the figure showing that there is an increase in frequency in the second (0.3) bin. However, this 'recovery' seems marginal but may be just sufficient to alter the ranking of the twitch amplitudes within the sample relative to those in the control group, to yield a non-significant difference between the groups.

5.2.3.3: CONCLUSIONS AND DISCUSSION:

From these data it can be concluded that 1 to 8 days after an *in vivo* acrylamide pre-treatment of 100 mg/Kg which is insufficient to cause behavioural signs of neuropathy in the mice under study, it is possible to detect a reduction in the amplitude of ecothiopate induced spontaneous twitches of mouse diaphragm muscle relative to those recorded from control animals.

The frequency of the spontaneous twitches is also reduced following acrylamide administration both grossly over the whole data sampling period and also with regard to the apparent 'bursting' behaviour observed in the control preparations. The build up of spontaneous twitch activity may also have been attenuated by the administration of acrylamide although the smaller number of twitches recorded post acrylamide may mask this affect. The affects on twitch frequency and amplitude appear to coincide temporally with the consistent reductions in activity occurring 24 hours after the administration of acrylamide.

Many questions arise from this data relating to the mechanism(s) underlying the effects reported above. The reduction in twitch amplitude observed following acrylamide administration may be due to a breakdown in antidromic action potential conduction within the neuronal aborisation of a motor unit or units resulting in a loss of the larger twitches. This idea is supported both by the reduction in the size of spontaneous twitches recorded and by the observations made during the course of the experiments that preparations which did not produce fasciculations of recordable magnitude could be seen to be fibrillating under a low power microscope. The antidromic conduction path around the axon reflex arc between motor nerve terminals of the same motor unit is such that an action potential must propagate along fine axons initially but must excite continually larger ones, possibly at branch points, in order to reach the point at which orthodromic conduction down to another nerve terminal can occur. It can be speculated that if the excitability of axons was compromised by acrylamide to a limited degree, this may be enough to block some antidromic propagation of action potentials at branching points since an action potential in a small nerve fibre may not be able to cause sufficient depolarisation of the relatively large branch point membrane area to excite it due its capacitance. This situation is reversed in orthodromic conduction and thus a large acrylamide induced deficit could be tolerated without any breakdown in propagation (this may also explain the abscence of any effects of acrylamide on the delay of orthodromically conducted evoked action potentials recorded in the muscle cells of the diaphragm - chapter 4). It proved very difficult to test the hypothesis that acrylamide was reducing the ability of spontaneous nerve action potentials generated at or near the nerve terminal to propagate around the 'axon reflex arc' to other nerve terminals and hence to other muscle cells. An attempt was made to repeat the jitter analysis described previously in chapter 4 but in a reverse direction with the point of stimulation being at or near a nerve terminal and the recording site being a remote muscle cell from the same motor unit, i.e. recordings were attempted around the axon reflex arc between two muscle cells of the same motor unit. The hypothesis underlying this experiment was that acrylamide is able to modify the probability of an action potential initiated at a given nerve terminal, reaching the other muscle cell(s) of the motor unit via antidromic/orthodromic conduction along the interconnecting nerve. This effect might manifest itself as an increased number of failures in transmission within the train of action potentials initiated at the terminal at 30 Hz. However, this experiment proved very difficult to conduct due to the large tetanic contraction produced by the frequency of stimulation which made the maintainence of stimulating electrode position impossible. It was not appropriate to use either TTX or DTC to stabilise the preparation with respect to this problem. An alternative method to examine this possibility would be to load the synaptic vesicles of the motor nerve terminals throughout a hemidiaphragm with a fluorescent dye which is incorporated into the vesicles during their recycling at the presynaptic membrane. This would create fluorescent nerve terminals upon the removal of the dye loading medium. Stimulation of such terminals would result in a loss of fluorescence as the contents of the vesicles was expelled

and diluted in the extracellular fluid. A single nerve terminal could then be stimulated and the resultant decrease in fluorescence resulting from the stimulation of motor nerve terminals within the same motor unit by antidromic propagation of the action potential could be quantified. If acrylamide was able to limit antidromic propagation of the action potentials it would be expected that on average there would be more fluorescence observed in preparations taken from treated animals compared to those from untreated animals. Despite the existence of suitable fluorescent dyes, this analysis would require extensive method development and quantification studies on the distribution and size of motor units within the hemidiaphragms together with development of appropriate statistical analysis. Development of such analysis would have required more time than was available for this project but remains an exciting prospect. However, the position of a stimulating electrode could be accurately maintained and monitored for single stimuli and it was therefore decided to examine the excitability of the terminal region of the motor nerves as described in chapter 2 (section 2.6.6) with regard to the hypothesis that acrylamide reduces the excitability of nerve terminals and it is this which accounts for the reduced size of twitches recorded following acrylamide administration via a reduction in the number of antidromic impulses generated by the nerve terminals and the subsequent reduction in recruitment of other muscle cells within the motor unit(s). The results of this investigation are presented in chapter 7.

Another possible effect of acrylamide which may explain some of the observations described above has been put forward earlier in this chapter in section 5.1.1. It was suggested that acrylamide may be able to reduce the effect of ecothiopate on the potentiation of stimulated twitches by inhibiting the action of a portion of the cholinesterase activity at the neuromuscular junction. Thus, the effects of ecothiopate would be reduced since it would be inhibiting a sub population of the cholinesterase that is available for inhibition at the neuromuscular junctions of control animals. Such an effect of acrylamide could also effect spontaneous twitches whereby repetitive firing of muscle fibres following the conventional stimulation of a nerve terminal by a nerve action potential originally generated spontaneously at another nerve terminal of the same motor unit, was reduced. This explanation seems unlikely since it was demonstrated in section 5.2.1 that repetitive firing was not responsible for the variation in amplitude of spontaneous twitches.

The processes leading to the generation of spontaneous twitches following anticholinesterase treatment are still the subject of some debate. It has been suggested that the process outlined in section 5.1.1 for the generation of repetitive firing whereby acetylcholine impinges upon pre-synaptic nicotinic acetylcholine receptors and thus brings about terminal depolarisation and the generation of nerve action potentials (which then propagate bi-directionally throughout a motor unit), is responsible (Webb & Bowman, 1974; Bowman, 1990; Masland & Wigton). An alternative to this is suggested by Ferry (personal communication) and by observations made during the course of experiments to record miniature endplate potentials using extracellular microelectrodes. It has been noted

whilst positioning electrodes to make these recordings that if the tip of electrode comes into contact with the nerve terminal and thus mechanically deforms it to some extent, that a large increase in the number of miniature endplate potentials is observed. One interpretation of this observation is that the nerve terminals can be mechanically depolarised yielding the release of acetylcholine at the synapse. This depolarisation may also initiate an action potential in the neuron which then propagates to other nerve terminals of the same motor unit. In the absence of an electrode it has been suggested that the sub synaptic hypercontraction described by Ferry & Cullen (1991) following anticholinesterase exposure may supply the mechanical stimulus required for the initiation of spontaneous twitching. However, the majority of opinion is currently in favour of the former explanation. The observation that acrylamide pre-treatment reduces the frequency of spontaneous twitches could thus have a number of different explanations. One of these might be that acrylamide is capable of reducing the excitability of the nerve terminals and/or the pre-terminal axon so that generation of action potentials by whatever method may be reduced. This possibility was investigated by the generation of strength duration curves (see chapter 7).

In conclusion, the experiments described in this chapter demonstrate that acrylamide is able to modify the response of the diaphragm muscle of the mouse such that ecothiopate induced spontaneous twitches are reduced in both amplitude and frequency. The possibilities that this may be the result of an anticholinesterase action of acrylamide and/or an effect on distal nerve excitability are investigated further in the following chapters.

CHAPTER 6

IS THERE ANY DIRECT EVIDENCE THAT ACRYLAMIDE HAS ANTICHOLINESTERASE PROPERTIES ?

6: OBJECTIVES:

The overall objective of the experiments presented in this chapter was to determine if acrylamide, at the doses and timepoints used in the experiments already reported, is able to impair the function of acetylcholinesterase at the neuromuscular junction of the mouse since this was suggested as a possible mechanism underlying the observations made on the 'delay' of indirectly evoked action potentials (chapter 4) and on the post-ecothiopate stimulated and spontaneous twitch responses of the mouse hemi-diaphragm preparation (chapter 5).

Two methods of investigation were employed in this section of work. The first was an analysis of the time course and frequency of miniature endplate potentials. The second took the form of biochemical assay of the acetylcholinesterase activity of mouse diaphragm muscles following various extraction techniques.

6.1: DOES ACRYLAMIDE AFFECT THE RISE AND HALF DECAY TIMES OR FREQUENCY OF MINIATURE ENDPLATE POTENTIALS ?

6.1.1: OBJECTIVE:

The affect of acrylamide administration on the delay of indirectly evoked action potentials and also on spontaneous and stimulated twitches following exposure to ecothiopate described in chapters 4 & 5 prompted the formation of the idea that acrylamide may reduce the effectiveness of functional acetylcholinesterase (AChE) at the neuromuscular junction.

This hypothesis can be tested by studying the duration of extra-cellularly recorded miniature endplate potentials ($MEPP_o$s). If acrylamide causes a reduction in the anticholinesterase capability at the neuromuscular junction, one would expect to see a prolongation of the decay of $MEPP_o$s as a result of the increased life span (and hence increased number of bindings with receptors) of the quantally released acetylcholine prior to its breakdown. The frequency of intracellular miniature endplate potentials ($MEPP_i$s) was also recorded in attempt to confirm unpublished accounts of a decrease in MEPP frequency following acrylamide administration.

6.1.2: RESULTS FROM $MEPP_O$ DURATION EXPERIMENTS:

Figure 6.1 and table 6.1 below show that *in vivo* acrylamide pre-treatment has no significant effect on the rise time of $MEPP_O$s. These data also show that the position of the extracellular microelectrode at or very near the motor endplate was consistent during the experiments performed since Fatt & Katz (1951) demonstrated that small variations in electrode position yielded large increases in the rise time of $MEPP_O$s.

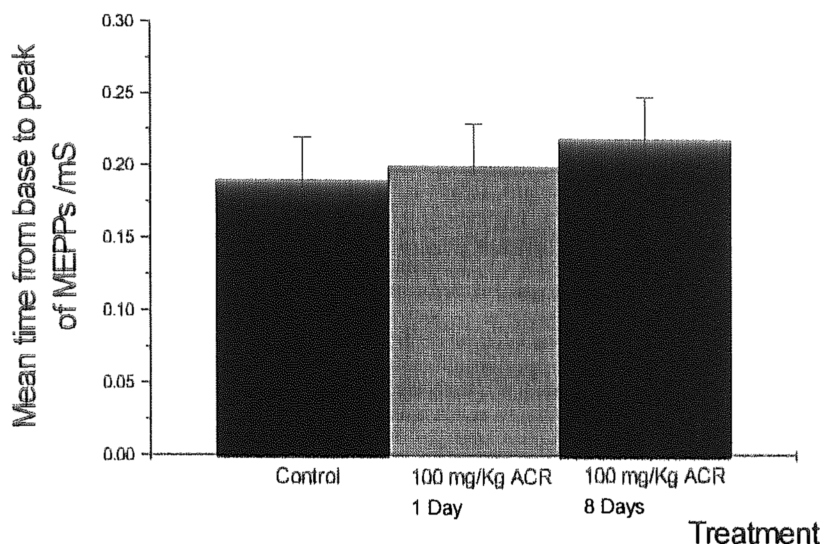


Fig 6.1: Graph to show the effect of orally administered acrylamide (ACR) on the mean time from base to peak of extracellularly recorded miniature endplate potentials in the mouse hemi-diaphragm preparation. Control animals received distilled water only. Error bars = S.D. No significant differences from controls (Wilcoxon rank sum test, $p<0.05$). Results are means from 3 mice per treatment (4-8 cells sample per mouse).

Treatment	Mean time from base to peak of MEPPs /mS (± s.d.)
Control (distilled water only)	0.19 ± 0.03
100 mg/kg ACR 1 Day	0.20 ± 0.03
100 mg/kg ACR 8 Days	0.22 ± 0.03

Table 6.1: The effect of orally administered acrylamide (ACR) on the mean time from base to peak of extracellularly recorded miniature endplate potentials in the mouse hemi-diaphragm preparation. Control animals received distilled water only. No significant differences from controls (Wilcoxon rank sum test, $p<0.05$). Results are means from 3 mice per treatment (4-8 cells sample per mouse).

However, figure 6.2 and table 6.2 below show that the time from peak amplitude to half decay of $MEPP_O$s is significantly increased 1 day after the administration of 100 mg/kg of acrylamide (0.29 ±0.04 increased to 0.35 ±0.08 mS) and that this effect is still present 8 days after this treatment (0.38 ±0.06 mS).

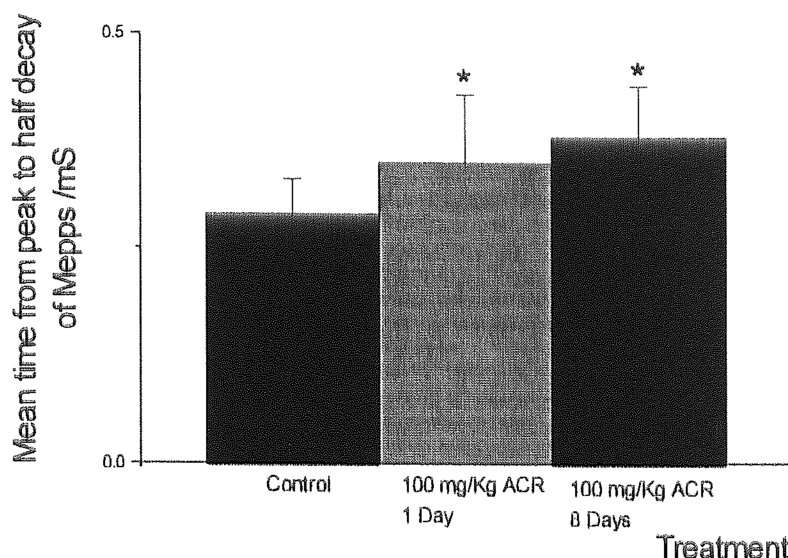


Fig 6.2: Graph to show the effect of orally administered acrylamide (ACR) on the mean time from peak to half decay of extracellularly recorded miniature endplate potentials in the mouse hemi-diaphragm preparation. Control animals received distilled water only. Error bars = S.D. * =significantly different from control (Wilcoxon rank sum test, $p<0.05$).Results are means from 3 mice per treatment (4-8 cells sample per mouse).

Treatment	Mean time from peak to half decay of MEPPs /mS (± s.d.)
Control (distilled water only)	0.29 ± 0.04
100 mg/kg ACR 1 Day	0.35 ± 0.08*
100 mg/kg ACR 8 Days	0.38 ± 0.06*

Table 6.2: The effect of orally administered acrylamide (ACR) on the mean time from peak to half decay of extracellularly recorded miniature endplate potentials in the mouse hemi-diaphragm preparation. Control animals received distilled water only. * = significantly different from control (Wilcoxon rank sum test, $p<0.05$). Results are means from 3 mice per treatment (4-8 cells sample per mouse).

6.1.3: CONCLUSIONS:

These results show that 24 hours after the administration of 100 mg/kg of acrylamide, the rise time of Mepps is unaffected whereas the half decay time is significantly longer.

These observations are repeated 8 days after the same dose with there being no significant difference between the two timepoints.

These data imply that acetylcholine has an increased life span in the synaptic cleft. Inhibition of AChE would yield data of this kind and a biochemical assay of AChE activity would be expected to corroborate this view. Such an assay was being developed for use in the laboratory during the course of these studies and was applied to this question (see below).

6.2: EFFECT OF ACRYLAMIDE ON MINIATURE ENDPLATE POTENTIAL FREQUENCY:

6.2.1: OBJECTIVE:

Acrylamide has been shown to decrease the frequency of miniature endplate potentials (Swift & Lambert, unpublished observations in Tsujihata *et al.*, 1974). Ultrastructural studies have shown that nerve terminals become swollen with 10 nM neurofilaments early in the course of neuropathy and that this can lead to the paucity and marginalisation of synaptic vesicles within affected terminals. DeGrandchamp, Reuhl & Lowndes (1990) described an increase in axolemmal surface area following acrylamide treatment, caused by a more ruffled surface with the length of these irregularities being equivalent to the circumference of synaptic vesicles. They suggested that these profiles represented synaptic vesicles arrested whilst fusing with the axolemma. The report of decreased frequency of miniature endplate potentials does not give a dose at which these observations were made although it was implied that this was an early effect.

Since intracellular miniature endplate potentials ($MEPP_i$s) were routinely observed during the positioning of electrodes for other work, some were recorded to magnetic tape and analysed for frequency as described in methods (section 2.6.1).

6.2.2: RESULTS FROM MINIATURE ENDPLATE POTENTIAL FREQUENCY EXPERIMENTS:

Acrylamide had no significant effect on the frequency of $MEPP_i$s as can be seen from figure 6.3 below:

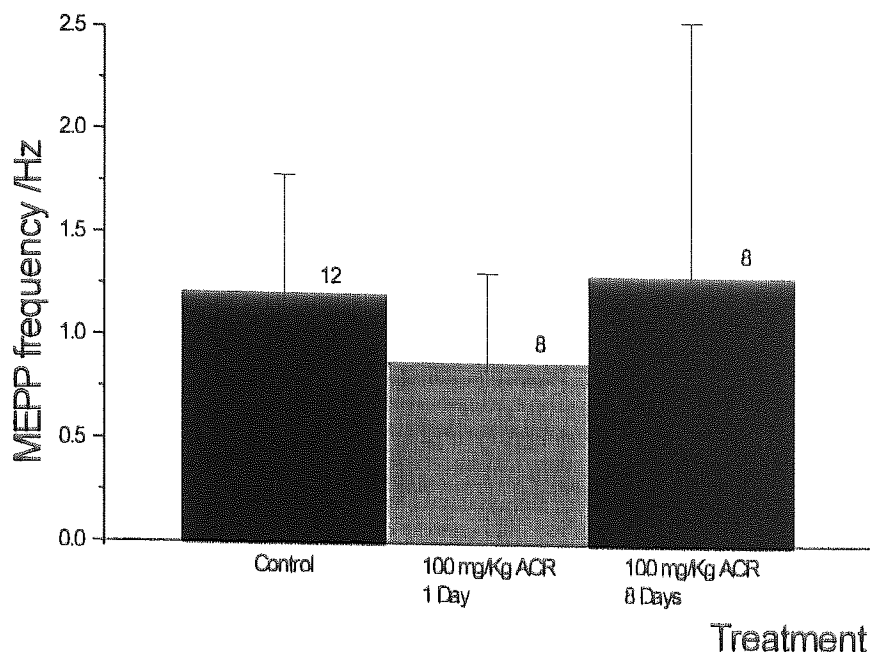


Fig. 6.3: Graph to show the effect of orally administered acrylamide (ACR) on the mean frequency of intracellularly recorded miniature endplate potentials (MEPPs) in the mouse hemi-diaphragm preparation. Control animals received distilled water only. Error bars = S.D. No significant differences (Wilcoxon rank sum test, $p<0.05$). Figures = number of cells from which data was recorded (3 animals per dosing group).

6.2.3: CONCLUSIONS:

No effects on this characteristic of nerve terminal function were observed at any of the other doses of acrylamide employed in this study, even those which caused the mice to exhibit clinical signs of neuropathy. This result contradicts the previous report of the effect of acrylamide on the frequency of MEPPs. However, it is possible that species differences coupled with the analysis of a different nerve and muscle combination may explain the lack of an effect in the present study. Another possibility related to this may be that the degree of neuropathy required before this observation can be made is greater than that acheived during the course of these studies.

6.3: IS IT POSSIBLE TO CONFIRM AN ANTICHOLINESTERASE ACTION OF ACRYLAMIDE USING A BIOCHEMICAL ASSAY ?

6.3.1: OBJECTIVES:

The objective of this section of work was to identify whether the effect of acrylamide in increasing the decay of MEPPs described above could be attributed to a direct inhibitory effect on AChE or whether its effects could be the result of a secondary effect following a primary toxic action which then affected the quantity of AChE available for the breakdown of acetylcholine. An effect of acrylamide detectable by biochemical assay might also corroborate the findings of the electrophysiological measurements and confirm that the effect of acrylamide on MEPPs is related to abnormalities in AChE levels or function and not some other physical deficit.

Both *in vivo* and *in vitro* administration of acrylamide was required in order to determine if acrylamide had a direct inhibitory effect on AChE. This is because a reduction in AChE activity following an *in vivo* dose could be explained by both a direct inhibition or a simple reduction in the quantity of enzyme in the synaptic cleft. If AChE activity was reduced following a brief *in vitro* exposure to acrylamide then it would be reasonable to assume that it was having a direct effect upon the function of the enzyme.

Before the activity of AChE can be assayed it is necessary to extract it from the study tissue. Two methods of extraction were undertaken in these experiments. The first method, termed conventional extraction (see methods), is one which has been common throughout scientific research for many years. This method yields a test sample which contains the gross quantity of AChE contained within the muscle regardless of its location within that muscle or its molecular form. The division of the study muscle into junctional (J) and non-juctional (NJ) regions gives some spatial resolution (allowing the calculation of endplate specific activity) but this is limited and subjective in nature since the J / NJ division is not a precise process. However, as a method of obtaining samples for the detection of gross changes in cholinesterase levels it has the advantages of being simple and thus rapid. The second extraction technique employed is termed 'sequential extraction' and through differential solubility of the molecular forms of AChE in low or high ionic strength salt solutions, it is possible to extract the different molecular forms into separate samples (see methods). Assay of these samples yields much more detailed information on the characteristics of the inhibition or reduction in quantity of AChE per unit weight of tissue. The conventional and sequential techniques were employed during the course of these studies since the sequential extraction procedure was under development within the laboratory and hence not immediately available.

6.3.2: THE EFFECT OF *IN VIVO* ADMINISTRATION OF ACRYLAMIDE ON ACETYLCHOLINESTERASE ACTIVITY FOLLOWING ITS CONVENTIONAL EXTRACTION:

The effect of various doses of acrylamide on the activity of AChE in J and NJ regions following conventional extraction are shown in table 6.3 and figure 6.4 below:

		Mean AChE activity /nmol min-1 mg-1				
Treatment	Time of study	Junctional	Non Junctional	EPSA	Total	No. of mice.
Control	---------	1.91 ±0.24	1.34 ±0.23	0.57 ±0.45	3.25 ±0.11	3
100 mg/kg ACR	1 Hour	1.95 ±0.20	1.71 ±0.19	0.24 ±0.31	3.67 ±0.22*	4
100 mg/kg ACR	1 Day	2.00 ±0.15	1.67 ±0.18	0.33 ±0.15	3.67 ±0.29*	4
100 mg/kg ACR	4 Days	2.26 ±0.20	1.65 ±0.12	0.61 ±0.35	3.31 ±0.28	4
100 mg/kg ACR	8 Days	1.67 ±0.23	1.45 ±0.31	0.22 ±0.08	3.12 ±0.54	3
10^{-5}M ACR *In Vitro*	20 mins.	1.81 ±0.12	1.18 ±0.23	0.62 ±0.21	2.99 ±0.30	3

Table 6.3: Summary data table of the effects of various orally administered acrylamide (ACR) treatments on the acetylcholinesterase (AChE) activity of mouse diaphragms following conventional extraction from junctional (J) and non junctional (NJ) regions. Endplate specific activity (EPSA) was calculated by subtraction of NJ values from J values. *In vitro* treatment consisted of 20 min. incubation of the diaphragm in acrylamide solution at 37°C. Control animals received distilled water only. Values ± S.D.* = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

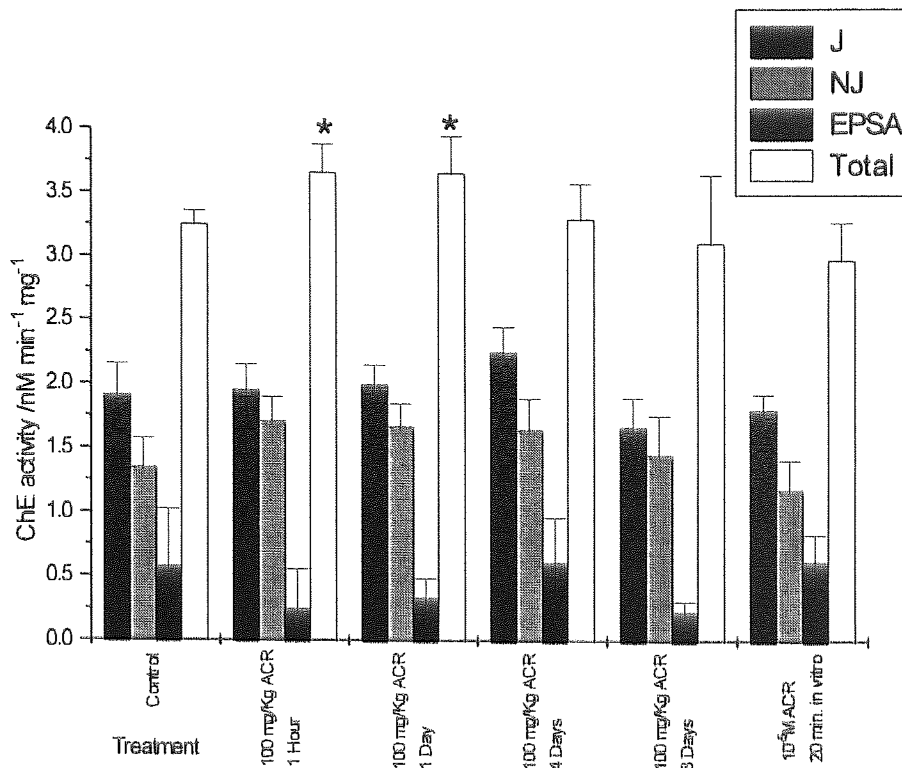


Fig 6.4: Graph to show the effects of various orally administered acrylamide (ACR) treatments on the acetylcholinesterase (AChE) activity of mouse diaphragms following conventional extraction from junctional (J) and non junctional (NJ) regions. Endplate specific activity (EPSA) was calculated by subtraction of NJ values from J values. *In vitro* treatment consisted of 20 min. incubation of the diaphragm in acrylamide solution at 37°C. Control animals received distilled water only. Error bars = ±S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

Total activity was calculated by simple addition of J and NJ values. The endplate specific activity (EPSA) which represents the fraction of the activity within the J region of the diaphragm which is located at or near the neuromuscular junction, is calculated by subtracting the NJ activity from the J activity.

It was hypothesised in the previous section that the decay times of MEPPs were increased following acrylamide administration due to a reduction in the activity of AChE. Thus one would expect to see decreased activity levels of this enzyme at the dose/time points at which MEPPs were prolonged, i.e. at 1 and 8 days following 100 mg/kg of acrylamide. If one looks at the total activity data presented above it appears that AChE activity is actually significantly higher 1 day after the acrylamide is administrated and that there is no difference at 8 days. The question thus arises as to whether the total activity derived from all areas of the diaphragm is the critical factor in determining the decay time of MEPPs which are a phenomenon associated very closely with the endplate region. The calculation of EPSA allows an estimate to be made of the activity which is associated specifically with the endplate region. The EPSA data shows no significant differences from control values but figure 6.4 does indicate that EPSA is lowered following

acrylamide administration. However, care must be taken when evaluating this apparently promising trend. From table 6.3 and figure 6.4 it can be seen that J activity remains relatively stable whilst NJ activity is consistently increased following acrylamide administration. Due to the nature of the calculation of EPSA, this observation accounts for the apparent decrease in EPSA. That is, the decrease in EPSA post acrylamide is due to an increase in NJ activity and not a fall in J activity. Therefore, the level of AChE activity in the synaptic cleft is probably no lower and thus should be able to bring about the usual rapid breakdown of acetylcholine and hence the normal decay of MEPPs.

6.3.3: CONCLUSIONS:

In conclusion it appears that a method of studying the activity of AChE which is closely associated with the synaptic cleft of the neuromuscular junction is desirable in order to determine if the hypothesis proposed in this section is true. The finding that there is an increase (although not statistically significant) in NJ activity of AChE is of interest. This might represent a response of the tissues to an effect of the acrylamide or may be a primary effect itself. It is of note that the *in vitro* incubation with 10^{-5}M acrylamide for 20 minutes did not produce results of a similar pattern to those of the *in vivo* experiments. This may support the idea that any effect of acrylamide on AChE is a secondary one arising from some other deficit.

6.3.4: THE EFFECT OF *IN VIVO* ADMINISTRATION OF ACRYLAMIDE ON ACETYLCHOLINESTERASE ACTIVITY FOLLOWING ITS SEQUENTIAL EXTRACTION:

As mentioned above, this method of extraction of AChE permits the separation of the molecular forms of the enzyme into globular, asymmetric and so called 'non extractable' forms. This has the advantage over the conventional extraction procedure that one is able to attribute AChE activity to specific forms of the enzyme which are known to be the ones responsible for the breakdown of acetylcholine at the neuromuscular junction. Of particular interest in this respect are the so called non extractable forms of the enzyme. These have been shown to be asymmetric in structure and concentrated at the neuromuscular junction as well as being almost exclusively external resulting in the conclusion that they represent the enzyme associated with basal lamina and thus are most closely associated with hydrolysis of acetylcholine (Younkin *et al.*, 1982, Busker *et al.*, 1994). Therefore, it can be hypothesised that following doses of acrylamide sufficient to prolong the half decay times of MEPPs, the activity of non extractable forms of AChE should be reduced, if an action on AChE is responsible.

Table 6.4 and figure 6.5 below show the data obtained from the assay of sequentially extracted samples:

		Mean acetylcholinesterase activity /nmol min-1 mg-1		
Region of diaphragm	Form of cholinesterase	Control	100 mg/kg ACR 1 Day	100 mg/kg ACR 8 Days
Junctional	Globular	3.46 ±0.51	1.40 ±0.21*	3.31 ±0.56
	Asymmetric	1.39 ±0.15	0.61 ±0.18*	1.12 ±0.33
	Non extractable	0.72 ±0.11	0.17 ±0.06*	0.85 ±0.08
	Total	5.59 ±0.62	2.18 ±0.20*	5.28 ±0.97
Non Junctional	Globular	2.11 ±0.22	0.99 ±0.31*	2.13 ±0.57
	Asymmetric	0.89 ±0.16	0.29 ±0.15*	0.49 ±0.25
	Non extractable	0.31 ±0.09	0.07 ±0.05*	0.44 ±0.13
	Total	3.32 ±0.37	1.34 ±0.47*	3.06 ±0.93
EPSA	Globular	1.37 ±0.51	0.41 ±0.15*	1.18 ±0.02
	Asymmetric	0.49 ±0.24	0.32 ±0.14*	0.63 ±0.10
	Non extractable	0.41 ±0.13	0.11 ±0.08*	0.40 ±0.05
	Total	2.27 ±0.72	0.84 ±0.32*	2.21 ±0.08

Table 6.4: Summary data table to show the effects of orally administered acrylamide (ACR) on the acetylcholinesterase (AChE) activity of mouse diaphragm in junctional (J) and non junctional (NJ) regions extracted by the sequential extraction method giving a division of the total activity into that attributable to globular, asymmetric and non extractable forms. Endplate specific activity (EPSA) was calculated by subtraction of NJ values from J values. Control animals received distilled water only. Values ± S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$). Results are the means from 5 mice per treatment.

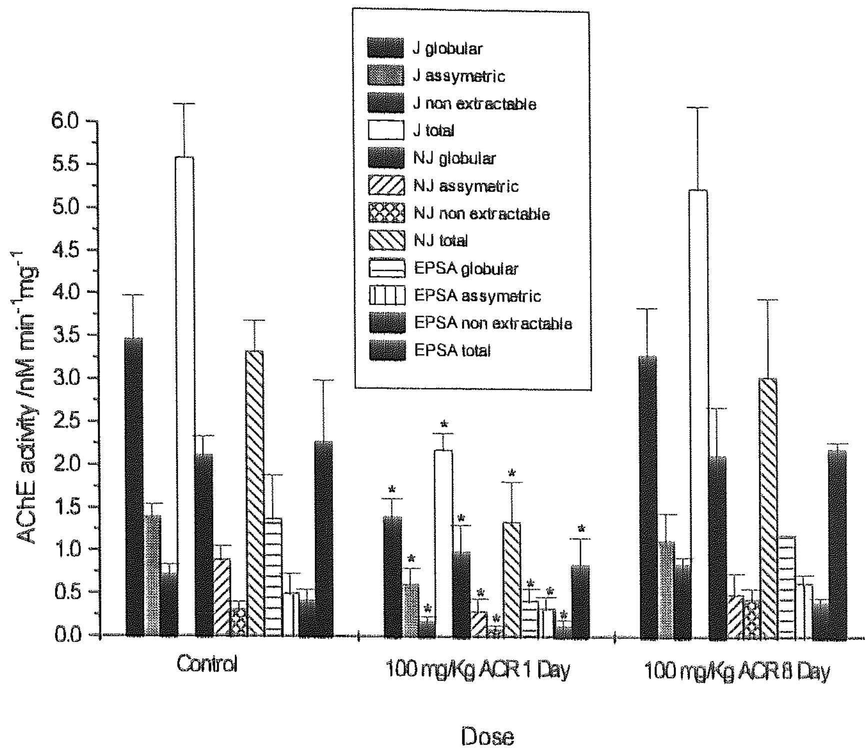


Fig. 6.5: Graph to show the effects of orally administered acrylamide (ACR) on the acetylcholinesterase (AChE) activity of mouse diaphragm in junctional (J) and non junctional (NJ) regions extracted by the sequential extraction method giving a division of the total activity into that attributable to globular (G), asymmetric (A) and non extractable (NE) forms. Endplate specific activity (EPSA) was calculated by subtraction of NJ values from J values. Control animals received distilled water only. Error bars = S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

These data show that 1 day after *in vivo* pretreatment with 100 mg/kg acrylamide there is a significant reduction in AChE activity. This reduction appears to be independent of the molecular form of the enzyme with all 3 of the extraction groups (globular, asymmetric and non-extractable) showing a significant decrease in activity. The data of most interest in table 6.4 is that which relates to EPSA since this relates most closely to the activity of AChE in the endplate region. Total activity is reduced to 37% of the non treated value. Further calculations show that the activity of globular, asymmetric and non-extractable forms of the enzyme are reduced to 30%, 65% and 26% of the control (distilled water only) values respectively. This can be more clearly seen from figure 6.6 below:

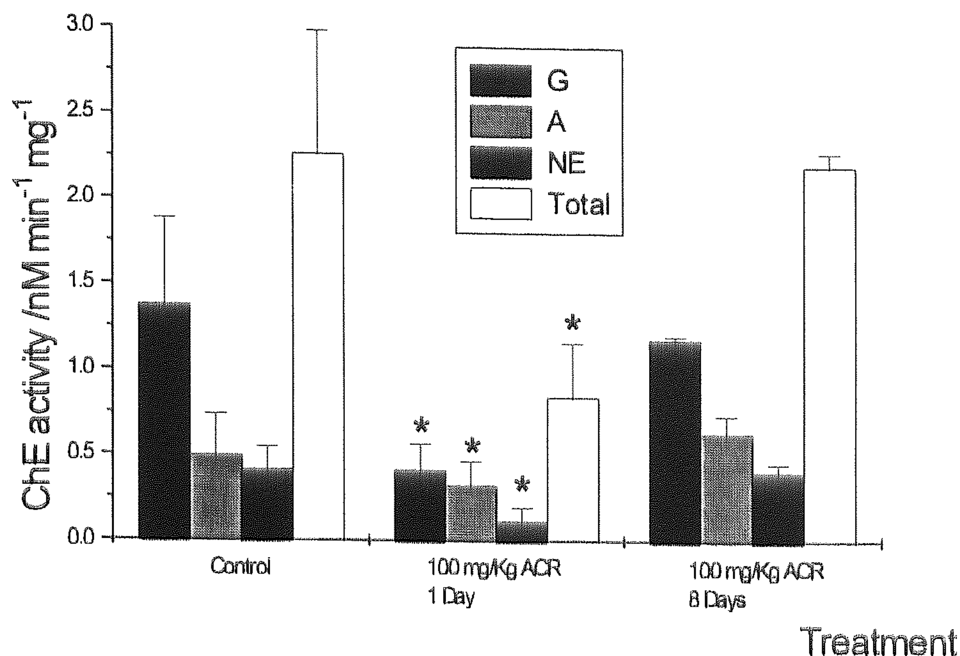


Fig. 6.6: Graph to show the effects of orally administered acrylamide (ACR) on the endplate specific acetylcholinesterase (AChE) activity of mouse diaphragm separated into that attributable to globular (G), asymmetric (A) and non extractable (NE) forms. Endplate specific activity (EPSA) was calculated by subtraction of NJ values from J values. Control animals received distilled water only. Error bars = S.D. * = significant difference from control values (Wilcoxon rank sum test, $p<0.05$).

No significant reduction in AChE activity was observed 8 days after the *in vivo* administration of 100 mg/kg acrylamide.

6.3.5: CONCLUSIONS:

The sequential extraction data is in marked contrast to that obtained with the conventional extraction technique where acrylamide treatment caused a significant increase in total AChE activity at the one day time point. With regard to the hypothesis that the increase in the half decay time of MEPPs following acrylamide treatment is caused by a decrease in the function of AChE, one must ask the question as to which extraction method (conventional or sequential) ultimately yields the most valid data with regard to accepting or rejecting it. The significant increase in total activity assayed following conventional extraction was the result of a non significant increases in the activity of J and NJ activities. This method of extraction is also non-specific with regard to the molecular forms of the enzyme being assayed. The idea has already been introduced above that the critical enzyme activity concerned with the length of MEPPs is that most closely associated with the neuromuscular junction. Conventional extraction of AChE is

unable to provide this level of resolution. It is therefore proposed that the data which best represents the functional integrity of AChE at the neuromuscular junction with regard to breakdown of the spontaneously released quanta of acetylcholine is the data from the sequential extraction experiments yielding activity of the endplate specific non-extractable form of the enzyme. The sequential extraction procedure also yielded results which were more consistent with regard to statistical significance.

Therefore, if the hypothesis was true, one would expect that the dose and time points at which the half decay time of MEPPs were extended would correspond with the dose and time points showing a decrease in the endplate specific activity of non-extractable forms of AChE. One day after the administration of 100 mg/kg of acrylamide the data correlates in favour of concluding that the hypothesis is true. However this is not the case at the 8 day time point following the same dose. Thus the hypothesis is only partially supported by the data but there is some evidence to suggest that acrylamide administration may be having an effect on the performance of the functional AChE at the neuromuscular junction of the mouse diaphragm.

6.3.6: THE EFFECT OF *IN VITRO* EXPOSURE TO ACRYLAMIDE ON THE ACTIVITY OF PURIFIED ACETYLCHOLINESTERASE:

It was stated in the objectives of this section that a decrease in the activity of AChE could be caused either by inhibition of the enzyme or by a decrease in the actual quantity available at the neuromuscular junction for reaction. In order to elliminate inhibition as a possible cause, the activity of type VI-S purified AChE from the electric eel was assayed following incubation at $37^{o}C$ for 45 minutes with either normal physiological saline (control) or physiological saline containing $10^{-5}M$ acrylamide. If acrylamide was capable of causing inhibition or direct, rapid breakdown of AChE then the activity of the sample incubated with acrylamide would be lower than that of the control sample. Figure 6.7 below shows the effect of acrylamide on AChE activity post incubation compared to control. Since a known quantity of AChE activity was added to both the control and the acrylamide treated samples prior to incubation and assay, the activity of the AChE can be expressed simply as the change in absorbance of light of 412 nm wavelength by 5-thiol-2-nitro benzoic acid against time.

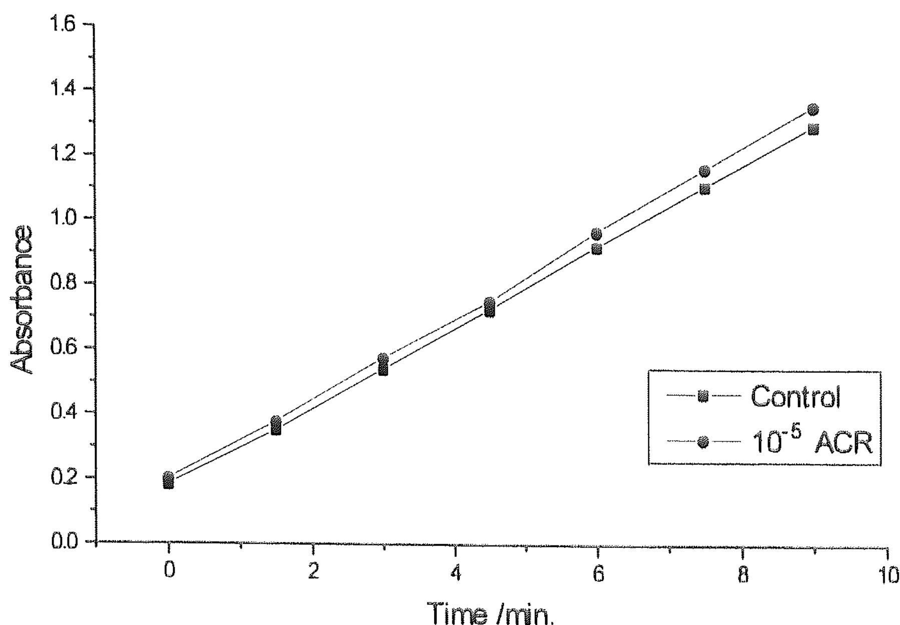


Fig. 6.7: Graph to show the effect of acrylamide (ACR) (45 min. *in vitro* incubation at $37^{0}C$ in $10^{-5}M$ ACR / physiological saline solution) on the activity of type VI-S purified acetylcholinesterase from the electric eel, as indicated by absorbance at 412 nm by 5-thiol-2-nitro benzoic acid, the product resulting from the breakdown of acetylthiocholine by acetylcholinesterase in the presence of DTNB. Controls were incubated in physiological saline only.

From figure 6.7 it can be seen that the rate of hydrolization of acetylthiocholine, represented by the slope of the lines in the graph, is not affected by the incubation of the test AChE with $10^{-5}M$ acrylamide. This result indicates that it is unlikely that acrylamide is able to directly inhibit AChE.

6.4 DISCUSSION:

The absence of any effect of acrylamide on the frequency or rise time of MEPPs implies that the pre and post synaptic events involved in the generation of these potentials is unaffected. Thus, it can be concluded that the doses of acrylamide employed in these experiments were insufficient to cause a paucity of synaptic vesicles or affect there release (DeGrandchamp & Lowndes, 1990; DeGrandchamp *et al.*, 1990; Tsujihata *et al.*, 1974).

Once the acetylcholine contained within these vesicles reaches the post synaptic membrane it interacts with the acetylcholine receptors as normal to yield MEPPs of normal rise time. The absence of any effect of acrylamide on the acetylcholine receptor mediated ion channels of the post synaptic muscle membrane was confirmed by some brief experiments performed by Martin Gosling of the University of Aston using the technique

of cell attached patch clamping. Patch electrodes containing 10^{-6}M acetylcholine were applied to the endplate region of separated mouse *lumbricales* muscles and the mean open duration times and amplitudes were measured. It was found that the *in vivo* and *in vitro* dosing regimes applied to the investigation of half decay time of MEPPs had no effect on these parameters. The *lumbricales* muscles were used instead of those of the diaphragm due to the restrictions made by the patch clamp technique on cell size and the absence of a technique for the dissociation of intact muscle fibres from the diaphragm.

It seems reasonable to conclude from the data on the half decay times of MEPPs that acrylamide is marginally prolonging the life span of acetylcholine in the synaptic cleft. An increase in the half decay time of the magnitude seen in these experiments has been demonstrated within the laboratory to be equivalent to an inhibition of 10% or less of the functional AChE (Bamforth,1989; Crofts, 1996; Ancilewski, 1996). If acetylcholine life span is being increased then the question arises as to how this might be occurring.

One possible explanation that was proposed earlier in this chapter was a direct inhibition of AChE by acrylamide. However, the results of the experiments described in section 6.3.6 following the *in vitro* exposure of purified AChE to acrylamide suggest that this is unlikely.

An alternative explanation may be that *in vivo* acrylamide exposure results in a decrease in the amount of functioning AChE available within the synaptic cleft. The precise mode of such an action by acrylamide is not clear but may involve the defects in transport systems or direct/indirect metabolic disruption which have been suggested as mechanisms underlying the neurotoxicity of acrylamide (Spencer & Schaumburg, 1978; Spencer *et al.*, 1979, Bisby & Redshaw, 1987; Gold *et al.*, 1985; Jakobsen & Sidenius, 1983; Souyri *et al.*, 1981; Moretto & Sabri, 1988) since the synthesis and location of AChE has been demonstrated to be at least partially under the control of the motor nerve (Guth, 1968; Drachman, 1972, 1976) despite continuing debate over the site(s) of AChE synthesis. This may occur in the nerve cell followed by secretion from the nerve terminals or it may occur in the basement membrane and sarcolemma following induction by the nerve cell (Bowman, 1990). The axoplasmic transport rate of AChE was studied by Rasool & Bradley (1978). They concluded that acrylamide significantly reduced the rate of transport of AChE (by approximately half to 287 mm/h). The prolongation of the half decay time of MEPPs and the decrease in endplate specific AChE activity following its sequential extraction 1 day after *in vivo* exposure to 100 mg/kg acrylamide are consistent with an indirect reduction in the quantity of functional AChE available for the breakdown of acetylcholine in the synaptic cleft. However, the suppression of AChE activity was not present at the 8 day time point following *in vivo* exposure to acrylamide whilst the half decay time of the MEPPs was still significantly extended. In addition to this, the conventional technique for the extraction of AChE yielded data which was the opposite of that attained using the more sophisticated sequential extraction technique. These anomalies may be the result of the inability of the extraction techniques employed in these experiments to resolve the AChE specific to the curtailment of MEPPs, i.e. the calculation

of endplate specific activity does not differentiate between extracellular and intracellular compartments of AChE. With these limitations in mind it is not surprising that the analysis of MEPP duration and assay of extracted AChE activity do not give parallel results. Interpretation of this data is further complicated by the fact that acrylamide treatment may lead to subtle changes in the morphology of the neuromuscular junction (DeGrandchamp & Lowndes, 1990; DeGrandchamp *et al.*, 1990; Tsujihata *et al.*, 1974) which may effect the diffusion characteristics of acetylcholine released into the synaptic cleft thus possibly creating pools of differing acetylcholinesterase concentration. Areas of high acetylcholine concentration in addition to a possible reduction in the cleft concentration of AChE may lead to its repetitive binding to its receptors thus prolonging the MEPPs recorded. The results of experiments to investigate the possibility that a single dose of 100 mg/kg of acrylamide may alter the morpholgy of the neuromuscular junction are presented in chapter 9.

In conclusion, the results presented in this section do not enable one to confirm that the increase in the half decay time of MEPPs is caused by a decrease in the capacity of AChE at the neuromuscular junction to breakdown acetylcholine. However, some of the results indicate that there may be a possibilty that this is the case. The results of the *in vitro* experiments suggest that acrylamide does not directly inhibit AChE and that therefore, any effect of acrylamide on the ability of AChE to breakdown acetylcholine is probably of a secondary nature following a primary toxic effect of acrylamide in the peripheral nerve..

CHAPTER 7

DOES ACRYLAMIDE HAVE AN EFFECT ON THE EXCITABILITY OF THE TERMINAL REGIONS OF THE MOTOR NERVES IN THE MOUSE HEMIDIAPHRAGM PREPARATION.

7: OBJECTIVES:

The objectives of the work presented in this chapter were to investigate the possibility that acrylamide is able to reduce the excitability of mouse motor nerve terminals by the production and comparison of strength/duration curves for the threshold of excitability. Curves were derived for both nerve and muscle in order to demonstrate that any change in the threshold characteristics following acrylamide administration were specific to nervous tissue. In addition to this, a histopathological examination of the motor nerve terminals was carried out in an attempt to correlate any functional abnormality with structural changes.

7.1: THE EFFECT OF ACRYLAMIDE ON THE STRENGTH/ DURATION RELATIONSHIP OF THRESHOLD EXCITATION AT MUSCLE CELLS AND THE TERMINAL REGIONS OF THE MOTOR NERVES OF THE MOUSE HEMIDIAPHRAGM.

Figure 7.1 below shows the mean strength/duration curves obtained from the direct electrical stimulation of the terminal region of the motor nerve of the mouse hemidiaphragm and the muscle cells. The extracellular stimulating electrode was positioned close to the terminal region of a nerve by first recording extracellular miniature endplate potentials using the protocol described in section 2.6.6 in chapter 2.

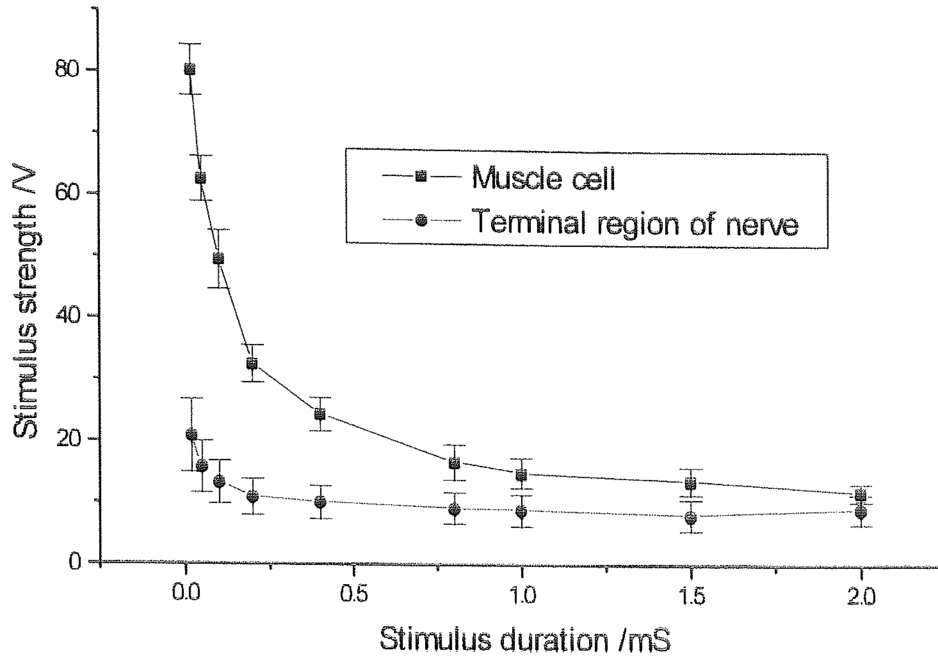


Fig. 7.1: Graph to show mean strength/duration curves for the direct electrical excitation of the nerve terminal region of neurones and of muscle cells of the hemi-diaphragm preparation from mice 8 days after the oral administration of distilled water only. Error bars = S.E. Data from 15 cells from 5 mice.

From this graph it can be seen that the two different cells have very different profiles of excitability with the motor nerve being much more sensitive than the muscle cell to stimuli of shorter duration.

Figures 7.2 and 7.3 below show the effect of *in vivo* acrylamide pre-treatment 8 days before experimentation on the mean strength/duration curves of muscle cells and the terminal region of the motor nerve.

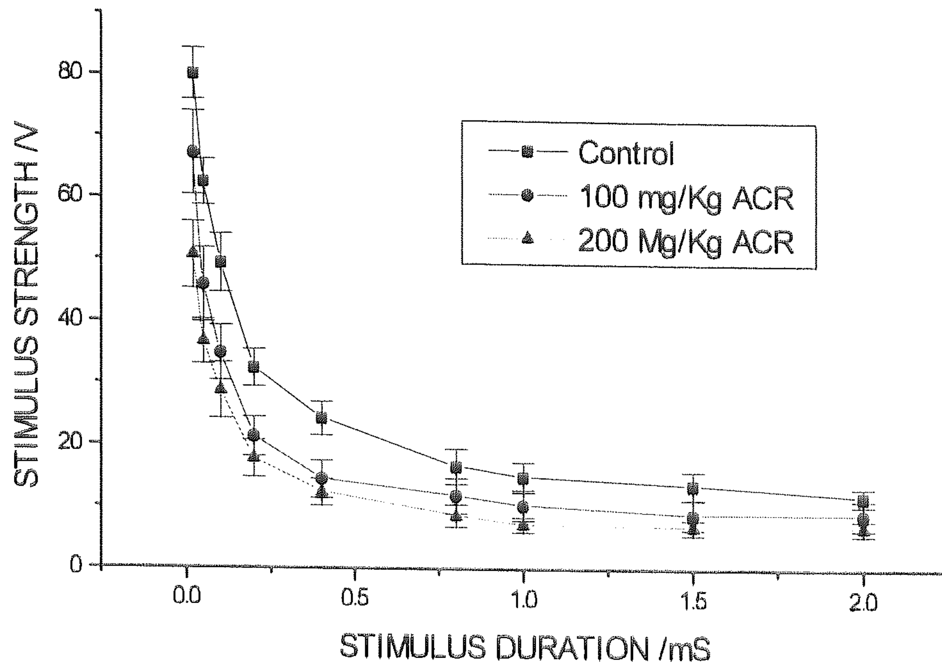


Fig 7.2: Graph to show mean strength/duration curves for the direct electrical excitation of muscle cells of the mouse hemi-diaphragm preparation 8 days after the oral administration of distilled water (control), 100 mg/Kg acrylamide (ACR) or 200 mg/Kg (100 mg/Kg x 2 on consecutive days) ACR. Error bars = S.E. Data from 15 cells from 5 mice.

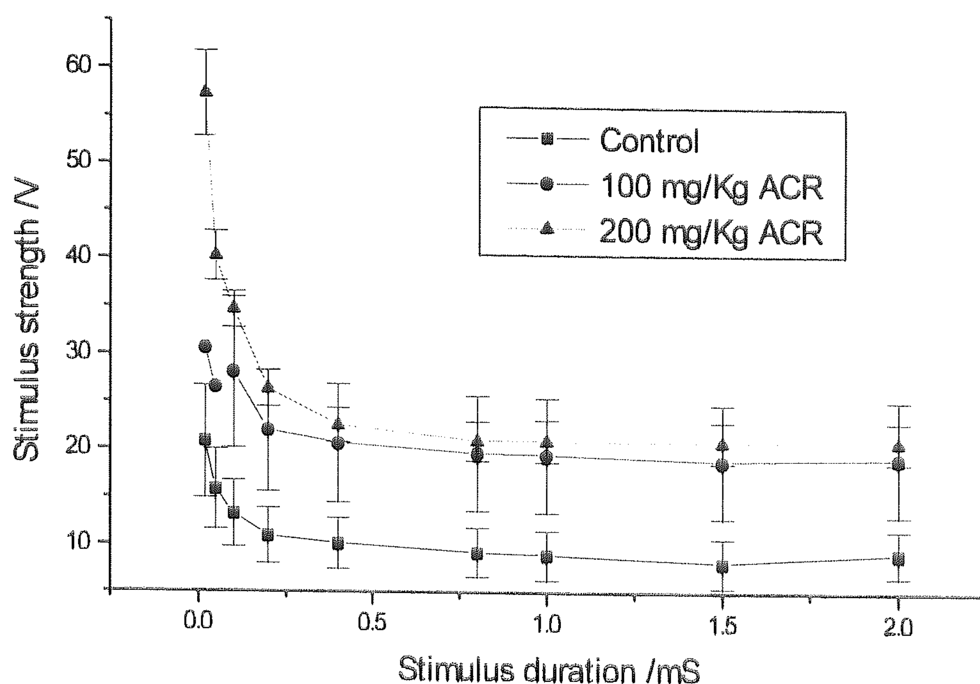


Fig 7.3: Graph to show mean strength/duration curves for the direct electrical excitation of the nerve terminal region of neurones of the mouse hemi-diaphragm preparation 8 days after the oral administration of distilled water (control), 100 mg/Kg acrylamide (ACR) or 200 mg/Kg (100 mg/Kg x 2 on consecutive days) ACR. Error bars = S.E. Data from 15 cells from 5 mice.

From these figures it can be seen that acrylamide has no marked effect on the position of the strength/duration curves derived from the muscle cells of the mouse hemidiaphragm. Note that the error bars in these and the subsequent curves represent standard error and not standard deviation as has been the case in preceding figures. This change was made in order to give improved clarity to the data presented. No significant differences were found between the data from control and treated groups following analysis using the t-test. However, figure 7.3 shows that acrylamide did have an effect on the strength/duration curves derived from the terminal regions of the motor nerves. One can see that following acrylamide treatment the curves are shifted upwards. The data recorded following the 100mg/Kg dose was not found to be significantly different from that of the controls. Following the 200 mg/Kg dose, all the data points were significantly different from the controls ($p<0.05$, t-test). The lack of statistical significance for the data recorded following the 100 mg/Kg dose is probably due to the greater variation in the values contributing to the mean value and this may be indicative of differential susceptibility of the nerve terminal regions to acrylamide induced neuropathy at this dose level. The upward shift in the curves indicates that acrylamide is able to modify the threshold of excitation of the nerves.

One question to arise from this data was whether the upward shift in the strength/duration curves recorded for the nerves was uniform or whether only certain durations were being affected; i.e. was the total charge required to cause excitation constant. A change in the shape of the curves would be just as interesting as the shift in the curves position. This would also be true in the case of the muscle cells. To this end the data was normalised to the rheobasic value and replotted. The rheobasic value for a strength/duration curve is defined as the minimum value of stimulus strength capable of excitation of the nerve or muscle cell. Figures 7.4 and 7.5 below show the replotted strength/duration curves following normalisation to the rheobasic value of each curve.

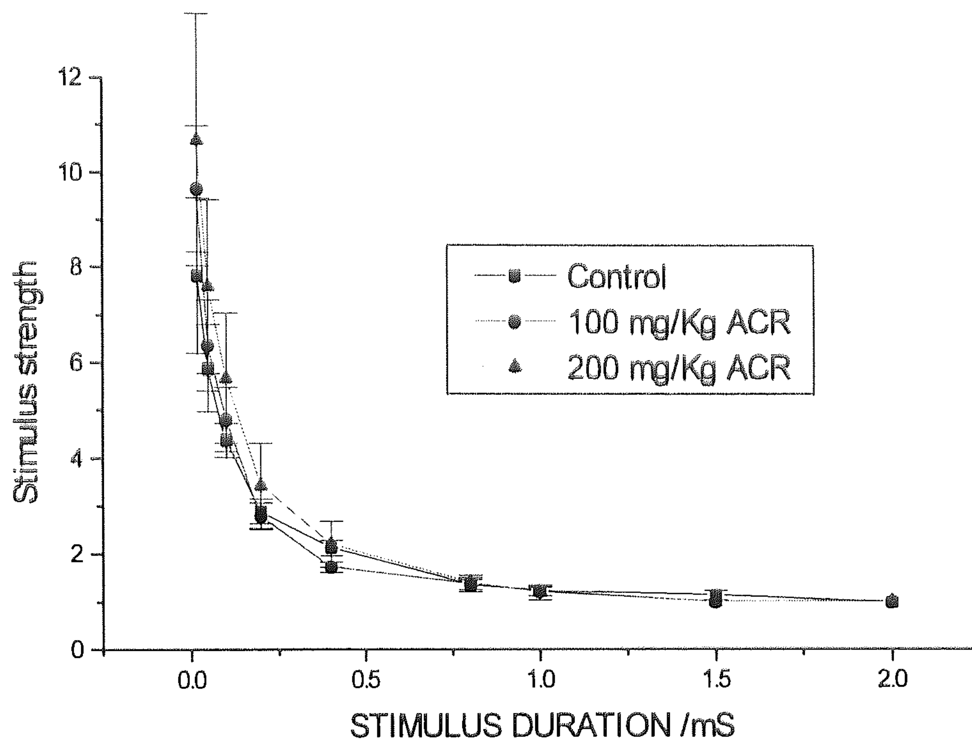


Fig 7.4: Graph to show mean strength/duration curves following normalisation to the rheobasic value for the direct electrical excitation of muscle cells of the mouse hemi-diaphragm preparation 8 days after the oral administration of distilled water (control), 100 mg/Kg acrylamide (ACR) or 200 mg/Kg (100 mg/Kg x 2 on consecutive days) ACR. Error bars = S.E. Data from 15 cells from 5 mice.

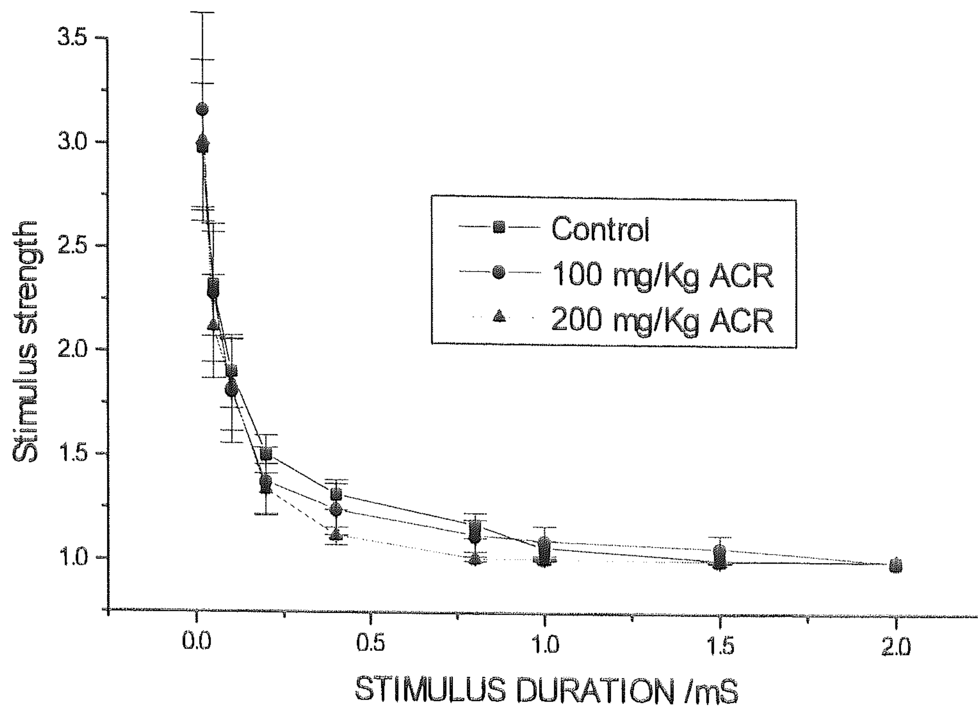


Fig 7.5: Graph to show mean strength/duration curves following normalisation to the rheobasic value for the direct electrical excitation of the nerve terminal region of neurones of the mouse hemi-diaphragm preparation 8 days after the oral administration of distilled water (control), 100 mg/Kg acrylamide (ACR) or 200 mg/Kg (100 mg/Kg x 2 on consecutive days) ACR. Error bars = S.E. Data from 15 cells from 5 mice.

From these graphs one can see that the shape of the strength/duration curves is not altered by the administration of acrylamide indicating that there are no fundamental differences in the stimulus strength/duration relationship and that in the case of the nerve terminals, the only difference post-acrylamide is that threshold for electrical excitation is uniformly increased across the range of stimulus durations.

An explanation of this phenomenon is difficult to speculate upon but one clue might be given by the work on mathematical modelling of the effects of membrane parameters on the strength/duration curve by Bostock (1983). In this work the thresholds to applied current pulses were determined for the myelinated nerve model of Goldman & Albus (1968) from which strength duration curves were plotted and compared to three strength/duration equations which have been proposed in the past (Weiss, 1901; Lapicque, 1907; Hill, 1936). The main questions to be answered by the model were how was the shape of the strength/duration curve related to the active and passive properties of the fibre and could the shape of the curve and its changes with membrane parameters, be described usefully in terms of a simple strength/duration model. The latter question yielded the answer that the model which best fitted the computed data was that of Weiss who interestingly obtained accurately timed, short duration stimuli by cutting two wires in quick succession with a bullet fired from a liquid carbon-dioxide carbine. Bostock varied

12 membrane parameters sequentially in order to determine the effects of active and passive properties on the characteristics of the strength/duration curve which was plotted as charge (stimulus strength x duration) versus duration. Figure 7.6 below shows such a graph for the data obtained from the stimulation of the nerve terminal region in the experiments described above.

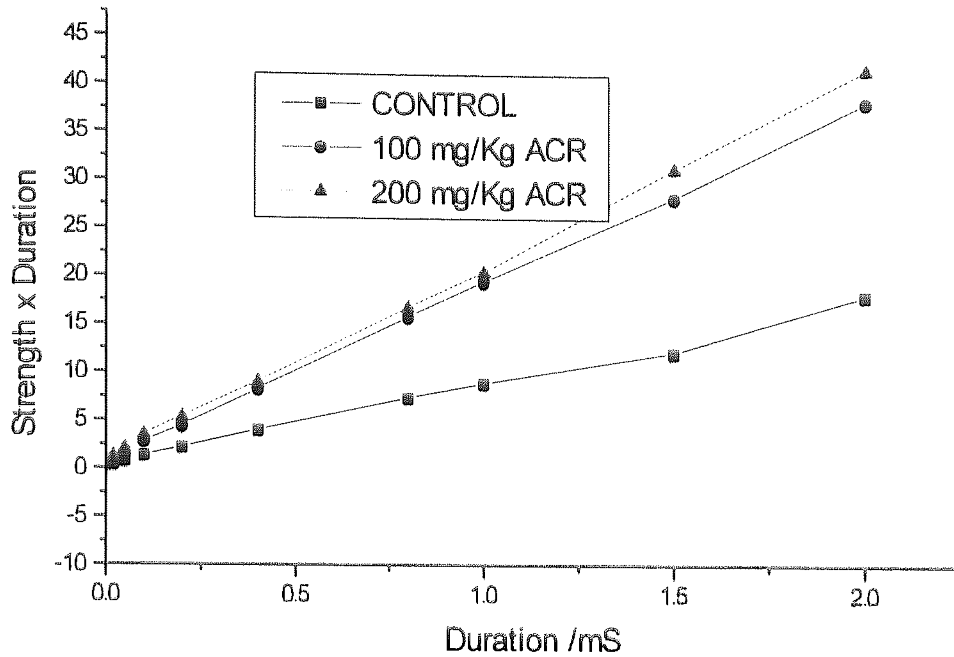


Fig 7.6: Graph to show strength/duration data plotted as charge versus duration as suggested by Bostock (1983) for the direct electrical excitation of the nerve terminal region of neurones of the mouse hemi-diaphragm preparation 8 days after the oral administration of distilled water (control), 100 mg/Kg acrylamide (ACR) or 200 mg/Kg (100 mg/Kg x 2 on consecutive days) ACR.

The only difference clearly visible on this graph between the data from control and acrylamide treated experiments is the increase in the gradient of the lines. Bostocks model attributes this effect to an increase in the rheobasic value which is consistent with figure 7.3. An analysis of the results obtained by Bostock following changes in membrane parameters indicates that the most likely cause of the gradient change proposed above, considering the lack of any other changes such as y-axis intercept value (representing the strength/duration time constant defined by Noble & Stein (1966) as the ratio between the charge threshold for very short stimuli and the rheobase), is a decrease in axoplasmic resistivity.

7.2: CONCLUSIONS AND DISCUSSION:

It can be concluded from these experiments that acrylamide is capable of increasing the rheobasic value of strength duration curves derived from the stimulation of the terminal regions of the motor nerves in the mouse diaphragm and that this increase in threshold of excitability is consistent throughout the strength duration curve. No such effects are seen in the muscle cells innervated by these nerves indicating that acrylamide is neuropathic and not myopathic. Drawing firm meaning from this result is, however, very difficult since the characteristics of a strength/duration curve can be influenced by many factors including: nodal capacitance, leak conductance, myelin capacitance, myelin conductance, axoplasmic resistivity, maximum sodium and/or potassium conductance, sodium activation/inactivation, nodal width and temperature. The mathematical modelling of Bostock indicates that the effect of acrylamide on the threshold of excitation of the terminal regions of motor nerves may be comparable to changes in axoplasmic resistivity in computer simulations. However, the value of this finding is questionable since the model is only an approximation to the myelinated nerve. Whilst it is possible that the actual point of stimulation in the real life experiments described previously is in the region of the distal Nodes of Ranvier, it cannot be assumed that this is the case and recordings may well have been made from the pre-terminal non-myelinated regions of the nerves or the terminals themselves. Thus the model may not be valid. There has also been some debate as to whether mammalian nerve terminals are electrically excitable (see Konishi, 1985).

Changes in the excitability of nerve terminals or pre-terminal axons following acrylamide administration may be the result of disruption of subcellular elemental regulation which has been demonstrated in experiments performed by LoPachin *et al.* (1992), although at doses sufficient to cause the presence of clinical signs of neuropathy. Changes in axoplasmic concentrations of ions and water may result in the changes in resistivity predicted by the Bostock model.

One method of gaining further information as to the cause of the effects described above might be to perform some histopathology on the terminal regions of the motor nerves in an attempt to correlate morphological changes with the functional changes already observed. For example, swollen nerve terminals may have an increased capacitance compared to normal terminals which would correlate with the increase in threshold of stimulation observed. The results of histological experiments concerned with this possibility are presented in chapter 8.

CHAPTER 8

DOES ACRYLAMIDE CAUSE MORPHOLOGICAL CHANGES IN THE TERMINAL REGIONS OF MOTOR NERVES INNERVATING THE MOUSE DIAPHRAGM ?

8: OBJECTIVES:

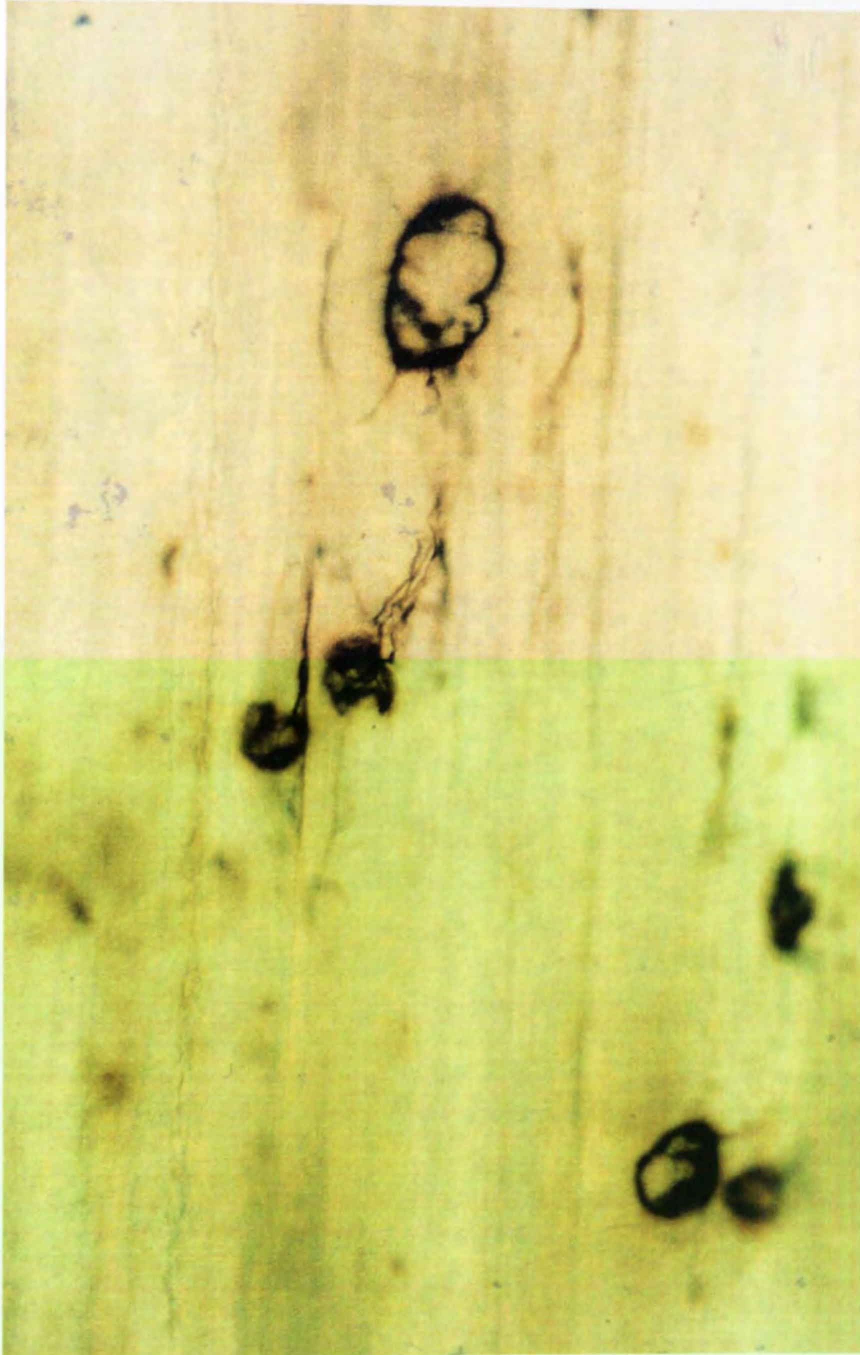
Several novel effects of acrylamide have been demonstrated in the experiments presented in the preceding chapters. These effects have been demonstrated to occur following doses of acrylamide which were insufficient to cause clinical signs of neuropathy in the mice used. Clinical signs have classically been linked to the presence of histopathological abnormalities of the peripheral nervous system. The objective of the histological experiments described in this chapter was to investigate whether there were any pathological abnormalities of the terminal regions of the phrenic nerve which could indicate a possible explanation for the novel effects recorded using other techniques following the administration of acrylamide. Three staining techniques were used in these experiments. The first was that of cholinesterase staining of the motor endplate region. Measurements made form these stained endplates and the sarcomeres within the striated muscle below could indicate any distortion of this region arising from terminal swelling due to accumulation of neurofilaments (see introduction - chapter 1) or an anticholinesterase effect of acrylamide. Silver staining of the pre-terminal aborisations of the phrenic nerve was performed in order to confirm or deny the presence of terminal swellings of the phrenic nerve motor terminals. Swollen distal Nodes of Ranvier could also be detected using this technique. Finally, more proximal sections of the sciatic nerve were stained with haematoxylin and eosin (HE) to confirm that any pathological defects observed at the terminals were not the result of a more generalised neuropathy within the animals under study. The HE staining of sciatic nerves is a technique commonly used in screening for peripheral neuropathy.

8.1: THE EFFECT OF ACRYLAMIDE ON THE MORPHOLOGY OF THE TERMINAL ABORISATION OF THE PHRENIC NERVE IN THE MOUSE DIAPHRAGM:

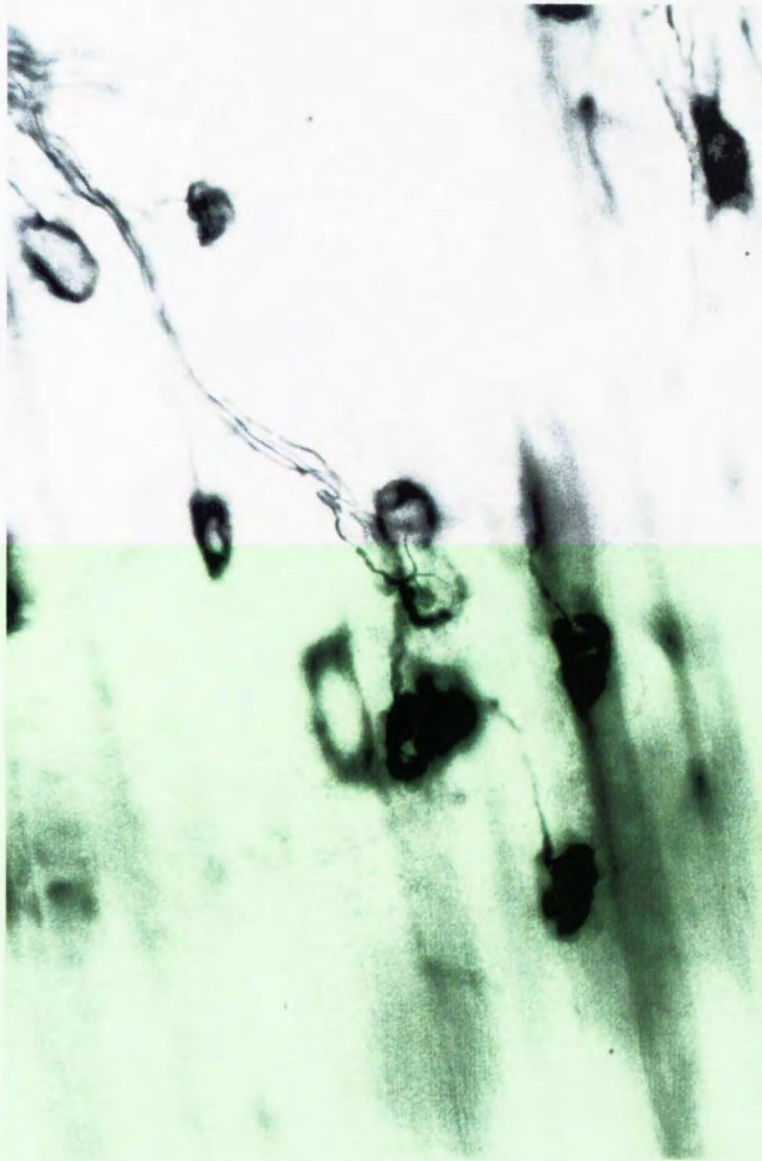
Plates A, B and C below are photomicrographs of silver/cholinesterase stained preparations of the terminal regions of the phrenic nerve in diaphragms taken either from mice receiving an oral administration of 100 mg/Kg of acrylamide or from those recieving distilled water only (control). The preparations were made 8 days after dosing. In the colour plates the brown areas of staining represent the motor endplates at which there is a high concentration of cholinesterase compared to the background. Within these areas it is possible to see the darkly stained terminal aborisations of the phrenic nerve. It is of note that only one endplate region is in focus in each photomicrograph. This is because the endplates on the surface of the diaphragm muscle cells are not all in the same plane of focus. Indeed, it was apparent from the study of the microscope slides that a single endplate was rarely found to be in the same focal plane across its entire length or width. This made the production of photomicrographs difficult. Slides were routinely scanned through many focul planes at each endplate and in this way it was possible to build up a 3 dimensional picture of the terminal aborisation of the nerve within ones mind. Close examination of these and other preparations has shown that a dose of 100 mg/Kg of acrylamide was insufficient to cause there to be any abnormalities in the morphology of the terminal regions of the phrenic nerve at any time up to 32 days after dosing which was the upper limit of this investigation. Positive observations for neuropathy would take the form of swellings on the pre-terminal axons and swelling of the nerve terminals themselves. Furthermore, a dose of 200 mg/Kg was also insufficient to cause there to be any alteration of morphology. This dose was sufficient to cause clinical signs of neuropathy and a deficit in performance of a climbing test in these mice.

Overleaf:

Plates A, B and C: Photomicrographs to show silver and cholinesterase stained preparations of the terminal region and neuromuscular junction of the phrenic nerve in the mouse diaphragm following oral administration of distilled water (A & B) or 100 mg/Kg acrylamide solution (C). Preparations were made following killing of the mice 8 days after dosing. Magnification = x40. The brown areas in the colour plates indicate the presence of cholinesterase in high concentration in the motor endplate region. Within these stained areas it is possible to see the terminal aborisations of the phrenic nerve.



A

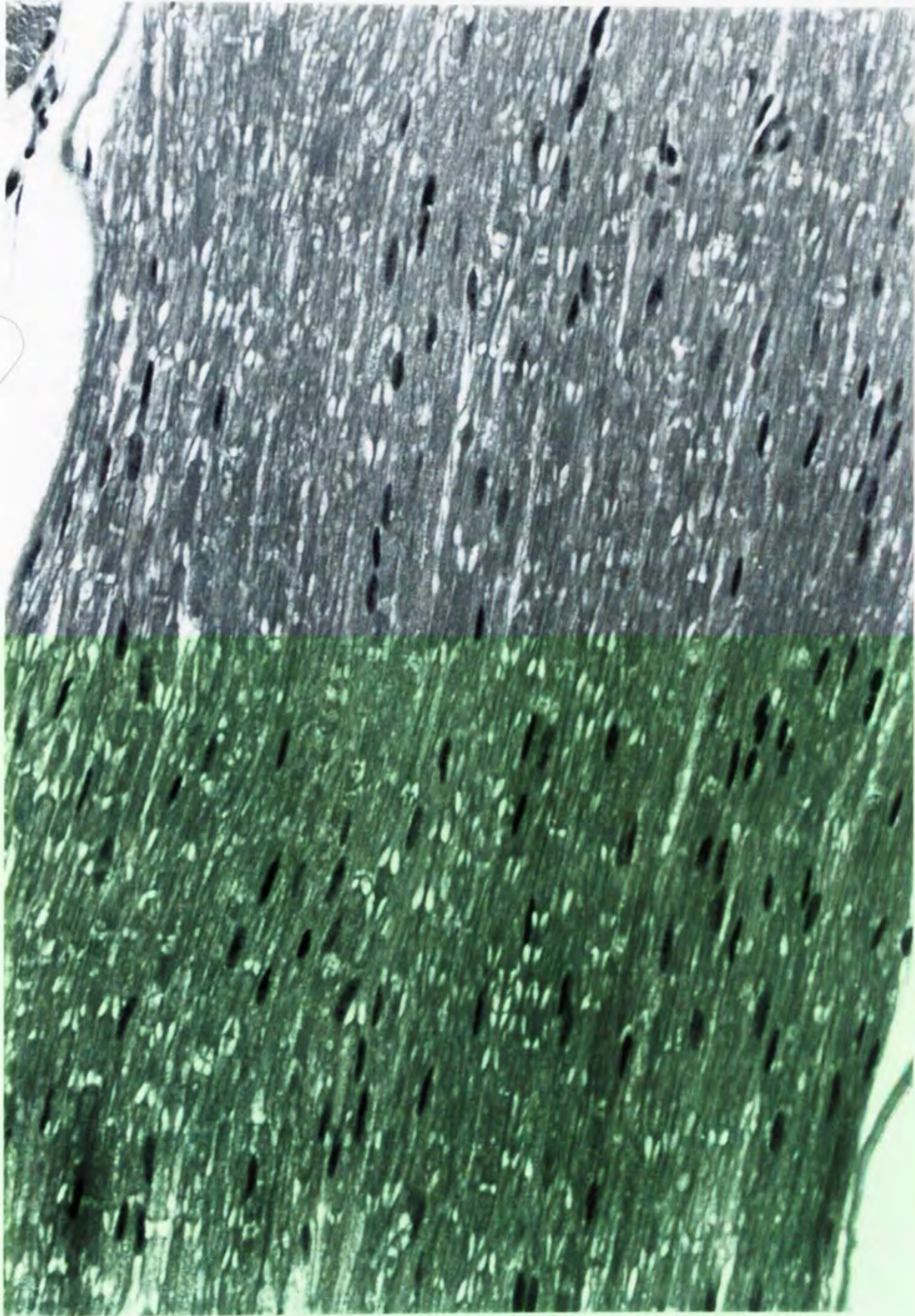


B

Plates D and E below show photomicrographs of HE stained longitudinal sections of the sciatic nerve taken from just below the sciatic notch.

Overleaf:

Plates D and E: Photomicrographs of haematoxalin and eosin stained longitudinal sections of the sciatic nerve of the mouse. Sections were prepared 8 days after the mouse was treated with either distilled water only (D) or 100 mg/Kg of acrylamide (E).



D

E

These photomicrographs and the other sections obtained during these experiments also fail to show any evidence of neuropathy following the administration of acrylamide. Positive observations for neuropathy in these sections would have taken the form of myelin breakdown possibly with the presence of digestion chambers, oedema and inflamation associated with tissue disruption.

This data would appear to support the hypothesis that the effects of acrylamide reported elsewhere in this thesis occur at a dose level which is insufficient to cause any visible alteration to the morphology of the perihperal nerves. It is of note that the methods used in this study would only detect gross alterations of the nerves and that an ultrastructural study of the same region may have yielded evidence of an effect on the neurons. Unfortunately this level of analysis was not available during the course of this investigation. Interestingly, DeGrandchamp & Lowndes (1990) applied the same silver staining method to neuromuscular junctions of the rat following acrylamide treatment and observed degeneration of terminal nerve branches in soleus and lumbrical muscles following a cummulative dose of 115 mg/Kg. Gross morphological changes were accompanied by ultrastructural changes. They also pointed out that the lumbrical muscles were less sensitive to acrylamide than the soleus. This suggests that the mouse and/or the diaphragm may be less sensitive to acrylamide with regard to its effects on the morphology of terminal nerve regions.

8.2: THE EFFECT OF ACRYLAMIDE ON ENDPLATE SHAPE AND SUB-ENDPLATE SARCOMERE SPACING:

8.2.1: OBJECTIVES:

The objective of the experiments described here was to apply the technique of cholinesterase staining to the endplate region following acrylamide administration in an attempt to observe any myopathic changes associated with any early morphological changes of the motor nerve terminals. The possible anticholinesterase action of acrylamide which has been hypothesised earlier in this thesis may also manifest itself as a deformation of the endplate region. Ferry & Cullen (1991) have shown that following treatment with the anticholinesterase ecothiopate, it is possible to observe a hypercontraction of sarcomeres in the endplate region of dissociated muscle fibres of the diaphragm. This localised contraction is also detectable as a deformation of the shape of the motor endplate whereby the width to length ratio of the cholinesterase stained endplate is reduced. This gives the stained endplates a more rounded shape compared to the oval profile of the untreated control, where the long axis of the endplate is aligned with the long axis of the muscle fibre upon which it is located. It was hypothesised that if acrylamide was somehow reducing the effectivness of acetylcholinesterase at the neuromuscular junction then

endplate deformation and local hypercontration of sarcomeres should be observed following its administration.

8.2.2: THE EFFECT OF ACRYLAMIDE ON ENDPLATE SHAPE:

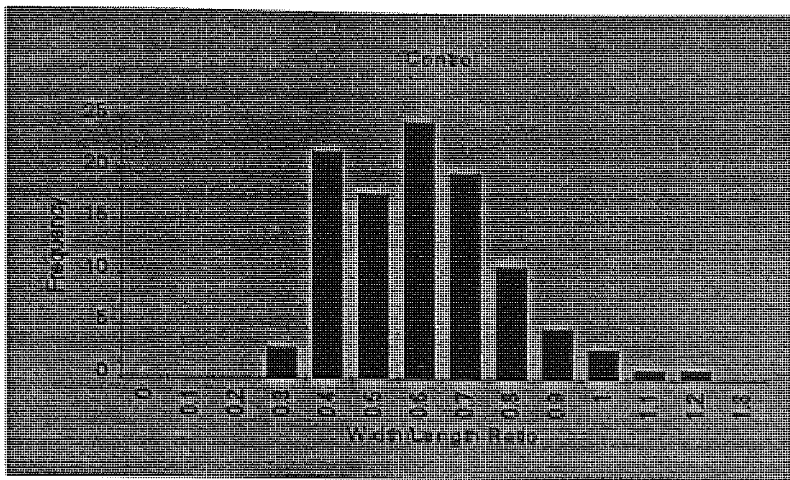
As mentioned above, the aim of these experiments was to assertain if there was any histological evidence of acrylamide causing endplate deformation due to changes in nerve terminal morphology or anticholinesterase activity. The width and length of cholinesterase stained endplates were measured and from this the width to length ratio was calculated. It has been demonstrated in the laboratory that following the administration of an anticholinesterase this ratio is reduced. This indicates that the endplate is becoming compressed along its long axis due to a localised hypercontraction beneath it in the muscle cell.

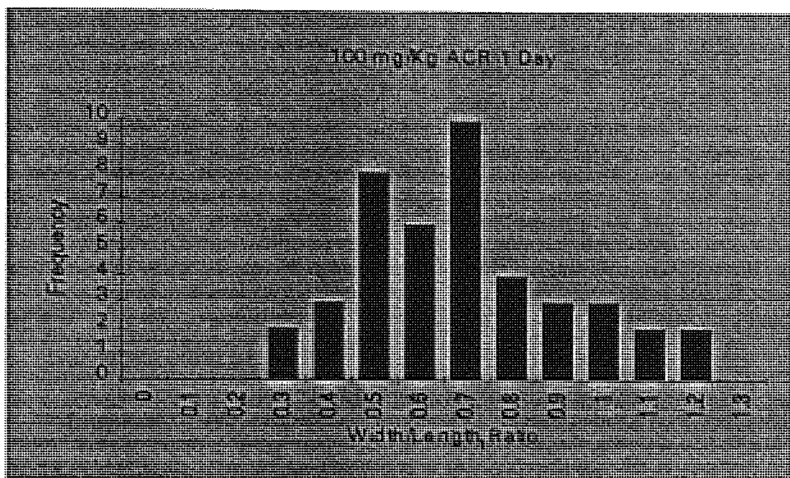
Table 8.1 below shows the results obtained from this analysis when applied to endplates from diaphragms of acrylamide treated or control (distilled water only) mice.

Treatment	Endplate characteristic: Mean Width /mm	Mean Length /mm	Mean Width/length Ratio	No. of mice
Control	0.054±0.015	0.083±0.016	0.66±0.18	5
100 mg/kg ACR 1 Day	0.061±0.014	0.085±0.013	0.73±0.23	5
100 mg/kg ACR 8 Days	0.055±0.010	0.091±0.024*	0.64±0.19	5

Table 8.1: Summary data table to show the effect of orally administered acrylamide (ACR) on the width, length and calculated width/length ratio of endplates of the mouse diaphragm visualised via staining of their acetylcholinesterase. Control animals received distilled water only. Values ± S.D. * = significant difference from control (Wilcoxon rank sum test, $p<0.05$).

Figure 8.1 below shows the frequency distributions for the width to length ratio data:





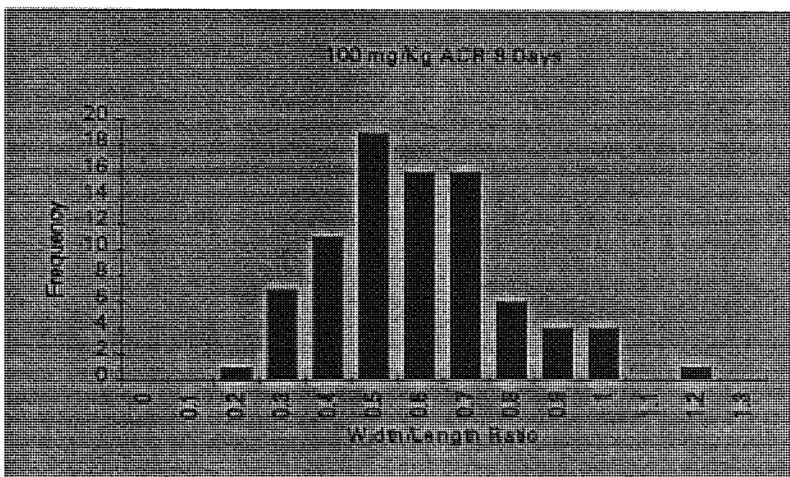
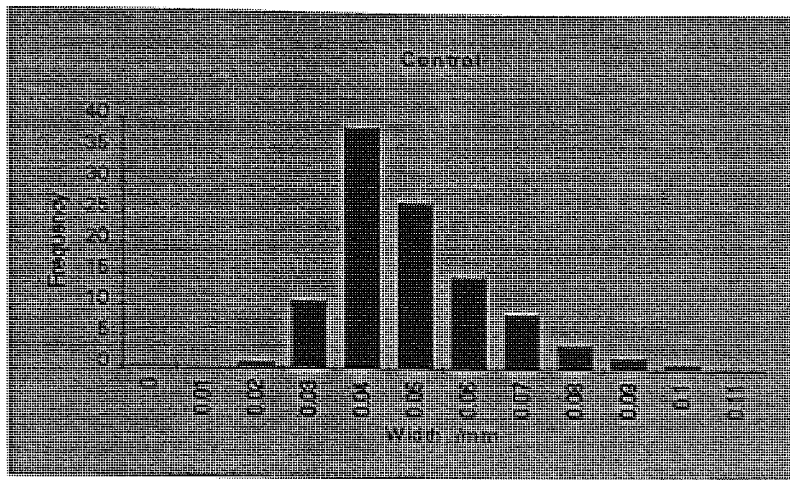
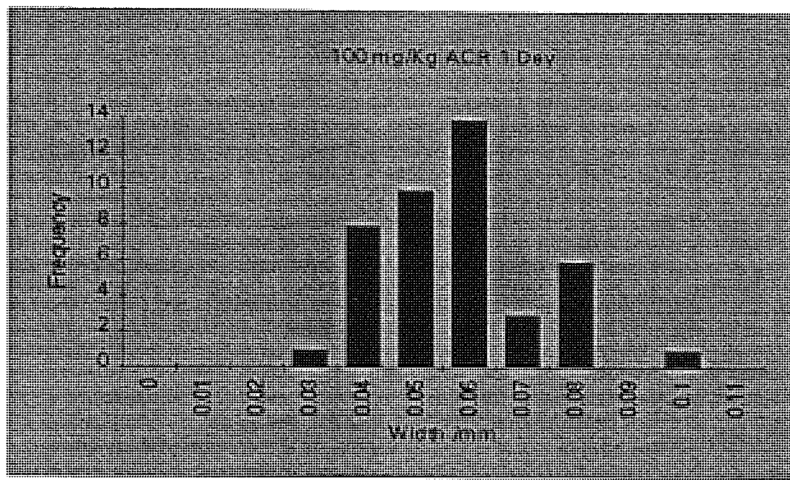


Fig.8.1: Histograms to show the effect of orally administered acrylamide (ACR) on the distribution of width/length ratios of acetylcholinesterase stained mouse diaphragm motor endplates. Control animals received distilled water only.

From these data it can be seen that the width to length ratio of the endplates is not significantly modified following acrylamide administration. It was proposed at the beginning of this section stated that if acrylamide was able to inhibit the activity of acetylcholinesterase then one would expect to see the width to length ratio of the endplates to decrease. Since there is no significant change in the ratio it cannot be concluded that

acrylamide has an anticholinesterase action. However, the frequency distributions shown in figure 8.1 do indicate that a small number of endplates demonstrate a reduced width/length ratio following acrylamide treatment. The endplate width data in table 8.1 and figure 8.2 below is also interesting in this respect since 1 day after acrylamide treatment the width of endplates appears to be slightly increased. This observation is not repeated at the 8 day time point and may indicate a mild transient effect of acrylamide on acetylcholinesterase which is not extreme enough to cause a significant difference in the mean width to length ratio data.





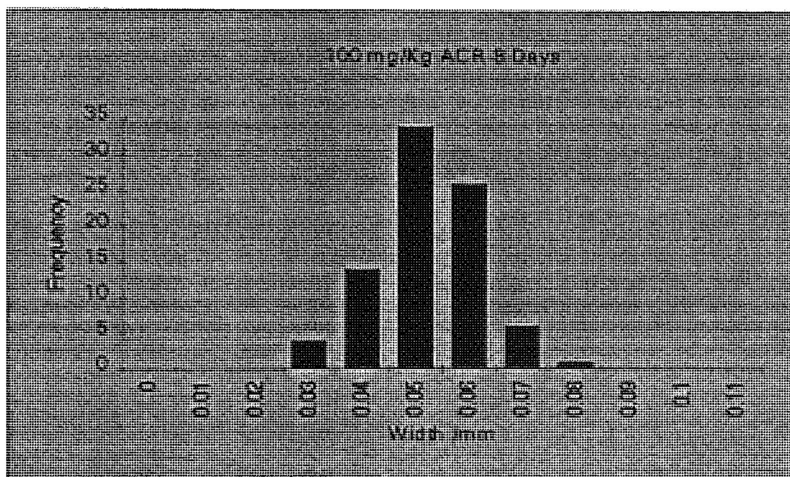


Fig.8.2: Histograms to show the effect of orally administered acrylamide (ACR) on the distribution of widths of acetylcholinesterase stained mouse diaphragm endplates. Control animals received distilled water only.

Table 8.1 shows that 8 days after acrylamide treatment there is a significant increase in the length of endplates. This is also apparent from the frequency distributions in figure 8.3 below:

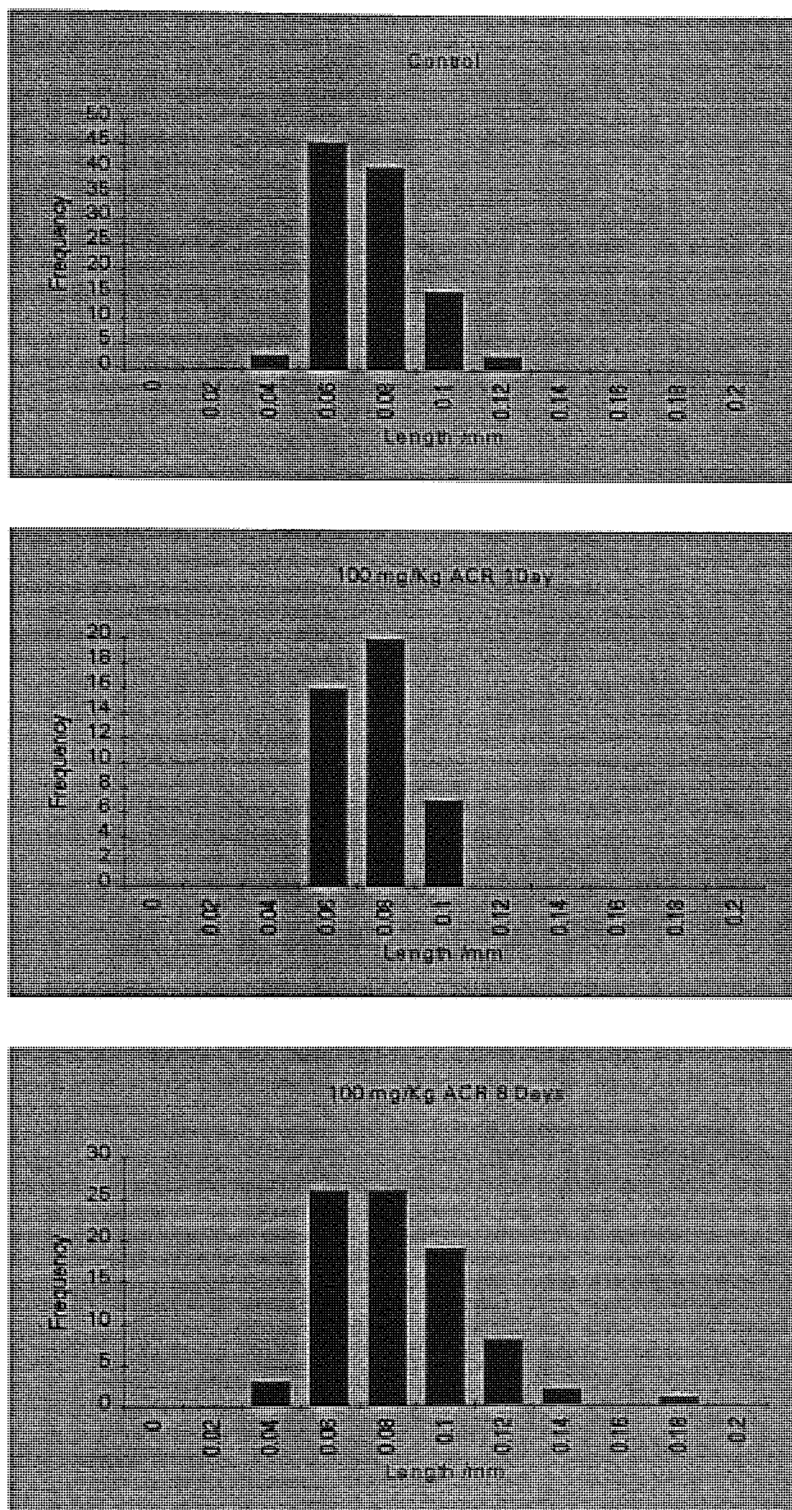


Fig.8.3: Histograms to show the effect of orally administered acrylamide (ACR) on the distribution of lengths of acetylcholinesterase stained mouse diaphragm endplates. Control animals received distilled water only.

8.2.3: THE EFFECT OF ACRYLAMIDE ON SARCOMERE SPACING:

It is possible to conceive that if acrylamide causes denervation supersensitivity (as indicated above) and hence an increase in endplate length, that any change in endplate width to length ratio caused by an anticholinesterase may be masked by this phenomenon, i.e. the compression of the endplate by the anticholinesterase induced local hypercontraction may be 'corrected' by the increase in length of the endplate caused by the appearance of new extrajunctional acetylcholinesterase which will stain positively.

One solution to this problem is to try to observe the underlying hypercontraction via measuring the length of the sarcomeres in the endplate region and comparing them to the sarcomeres in a region of the same muscle fibre away from the endplate. Table 8.2 below shows the data obtained using this method:

	Mean sarcomere length /µm			
Treatment	Endplate (E)	Non endplate (NE)	E/NE Ratio	No. of Mice
Control	2.2 ±0.46	2.0 ±0.40	1.10	3
100 mg/kg ACR 1 Day	2.3 ±0.55	2.4 ±0.36*	0.96	3
100 mg/kg ACR 4 Days	2.5 ±0.26*	2.3 ±0.25	1.09	3
100 mg/kg ACR 8 Days	2.7 ±0.31*	2.6 ±0.35*	1.04	3

Table 8.2: Summary data table to show the effect of orally administered acrylamide (ACR) on the mean sarcomere separation in the endplate and non endplate regions of muscle cells of the mouse diaphragm.Control animals received distilled water only. Values ± S.D. * = Significant difference from control values (t-test, $p<0.05$). No significant differences were observed between endplate and non-endplate regions.

These data show that there is no significant change in the length of sarcomeres immediately beneath the endplate compared to those in non-endplate regions within the same cell. This is evident from the ratio of endplate to non-endplate sarcomere lengths (E/NE ratio). It is of note that 1 day after the administration of 100 mg/kg acrylamide there was a slight but non- significant decrease in this ratio indicating that acrylamide may have some very limited effect on the length of sarcomeres in the endplate region. From these results it can only be concluded that acrylamide does not have a significant effect on the length of sarcomeres in the endplate region of muscle fibres of the diaphragm and that therefore it is unlikely that acrylamide is significantly affecting the acetylcholinesterase located at the endplates.

In addition to the findings above, statistical analysis of these data has also shown there to a significant increase in sarcomere length following acrylamide administration both at and away from the endplate region (see table 8.2). The fact that the sarcomeres are longer both at and away from the endplate does not interfere with the detection of a localised contraction at the endplate since calculation of the E/NE ratio for each fibre would take this into account. The reason for this observation is unclear but it is possible that this effect is artefactual arising from differences in the tension of the pinned out muscles during the fixing of the tissues prior to dissociation of the cells.

8.3: CONCLUSIONS AND DISCUSSION:

The endplate shape data reported above shows that the only effect of acrylamide is to cause an increase in the length of endplates 8 days after the oral administration of 100 mg/kg. This observation is in agreement with previous work by DeGrandchamp & Lowndes (1990) who also found a post acrylamide increase in endplate length. They concluded that this was attributable to muscle fibres becoming, to some extent, functionally denervated following neurofilament accumulation in the nerve terminals which resulted in a paucity of synaptic vesicles and a decrease in the readily releasable pool of acetylcholine. These events were concluded to have caused denervation supersensitivity and thus an increase in the length of the endplate as new acetylcholine receptors formed outside the existing endplate region. Supersensitivity is historically defined as the appearance of new extrajunctional acetylcholine receptors but histochemical studies have demonstrated that both acetylcholine receptors and esterase share a common morphological distribution even in pathological states (Pestronk & Drachman, 1978a, 1978b; Bjornskov, Norris & Mower-Kuby, 1982). This observation could also be explained by terminal sprouting which is common following the administration of acrylamide (DeGrandchamp & Lowndes, 1990). However, no evidence of sprouting was observed at this time point in silver stained diaphragms. Another possibility is that the increase in endplate length is artefactural in origin since at the 8 day time point following acrylamide administration mean sarcomere length both beneath the endplate and in the non

endplate region was significantly increased. This may indicate variations in the tension applied to the muscle during fixation. This view is corroborated by the abscence of an effect of acrylamide on the resting membrane potential (chapter 4).

There is no evidence in the data presented above that acrylamide is able to cause a local hypercontraction at the endplate and thus it must be concluded from these experiments that acrylamide does not have a strong anticholinesterase action if indeed it has any at all.

CHAPTER 9

CONCLUDING DISCUSSION

The major aim of the work presented in this thesis was stated at the beginning of chapter one to be to compare existing behavioural techniques for the detection of peripheral neurotoxicity with some novelly applied electrophysiological methods. In addition to this, a pharmacological method was also applied to this end. It was hoped that these techniques may produce more sensitive indicators of peripheral neuropathy than those currently observed by using functional observational batteries or other behavioural tests. However, any demonstrable deficit in peripheral nerve and/or muscle electrical function would serve to further validate the existing behavioural techniques especially if a mechanism behind any novel effect could be demonstrated.

9.1: CONVENTIONAL METHODS OF DETECTION OF PERIPHERAL NEUROPATHY:

In order to search for more sensitive detection of peripheral neuropathy it was first necessary to establish the sensitivity of existing behavioural techniques to a known neurotoxic agent. As has been described in chapter one, acrylamide has long been known to cause a progressive peripheral 'dying back' neuropathy and was selected as a suitable tool for modelling neuropathic changes of the kind which were of interest in the experiments described in this thesis. The first experiments performed during the course of this investigation were to establish a simple acrylamide dosing regime which was thought to be able to cause peripheral neuropathy but was below the detection threshold of existing behavioural techniques. The results of the functional observation and climbing performance tests presented in chapter 3 showed that a single orally administered dose of acrylamide of 100 mg/kg was not detectable by these methods but that a dose of 200 mg/kg (administered as 2 x 100 mg/kg on consecutive days) was. Thus, the threshold dose of acrylamide for detection by these techniques lies between 100 and 200 mg/kg for the mice used in these experiments. As shown in chapter 3 (table 3.2 and section 3.3.2), this result appears to fit in well with other studies although the range of different dosing regimes and species used complicates the placing of the data obtained during these studies within this historical framework. However, the figure obtained for the threshold dose of conventional detection of >100 but <200 mg/kg is in the correct range. The functional observational battery used in these experiments was very closely based on that used at the Central Toxicology Laboratories of Zeneca plc. The climbing performance test has been demonstrated to be equivalent to the rotarod test. These tests represent examples of commonly used tests (or tests analogous to them) for detecting neuropathy in animals during the screening of new compounds for neurotoxicity. Tests of this kind are simple and relatively inexpensive to perform since they require little in the way of expensive

equipment and are not particularly labour intensive but they do rely on the generation of relatively severe neuropathy based on histopathological examination of tissue taken from animals classed as intoxicated by them. There is also a large degree of subjectivity involved in observational methods.

Histological studies have shown that peripheral neuropathy is present in animals which show no clinical signs (e.g. Degrandchamp & Lowndes, 1990) and this is the observation which makes it reasonable to consider that there may be possibilities for improving the sensitivity of detection techniques for this form of distal neuropathy using techniques which focus on the functional status of the peripheral nerve and neuromuscular junction. The first technique to be newly applied in the search for improved sensitivity was that of the analysis of the 'jitter' of latencies recorded in trains of evoked action potentials.

9.2: IS JITTER ANALYSIS A SUITABLE BASIS FOR A NEW SCREENING TECHNIQUE FOR THE DETECTION OF PERIPHERAL NEUROPATHY ?

It was reasonable to assume at the outset of this investigation that administration of acrylamide would result in deficits in the normal function of the neuromuscular junction and that these deficits may be pre- or post-junctional (see chapter 4). The analysis of the 'jitter' of trains of action potentials evoked in peripheral nerve and recorded in the muscle cell at the endplate would appear be an attractive method for detecting any such deficits since it potentially measures effects on preterminal action potential conduction in the axon, neuromuscular transmission and postsynaptic generation of muscle action potentials. The technique also has the potential for identifying more closely which of these sites is the major contributor to any abnormality (e.g. via analysis of endplate potential jitter following the cutting of muscle cells). It is important to remember that jitter analysis would not detect a simple decrease in conduction velocity of action potentials along the axon since changes in the latency of action potentials is calculated relative to that of the first. Only changes in the latency during a train of responses is measured. This precludes the measurement of classic decreases in action potential velocity recorded following acrylamide administration such as those of Fullerton & Barnes (1966). However, it is unlikely that such changes in this parameter would occur following the relatively low doses of acrylamide used in the experiments reported here. Such effects are usually observed after the onset of clinical signs of acrylamide neurotoxicity.

The use of ecothiopate in this work as a positive control was essential in order to confirm that the experimental systems involved in obtaining the data were functioning to the levels previously reported within the laboratory and also to confirm the competence of the operator of the equipment. The presence of increased delay and MCD following *in vivo* exposure of mice to ecothiopate confirms that the technique was functioning satisfactorily prior to the investigation of the effects of acrylamide.

The results obtained following *in vivo* administration of acrylamide were disappointing since even at doses sufficient to cause clinical signs of neuropathy in the animals, no effect

was seen on the mean values of MCD. Delay showed some increases both at 100 mg/kg and 200 mg/kg but this was not observed until 8 days after the administration of acrylamide which may indicate that this was a secondary effect to some other undetectable primary action. It was also interesting to note that 4 days after 50 mg/kg of acrylamide the delay may possibly be reduced. However, a change in delay on its own is generally not considered to be strong evidence for any effects of a compound on the processes of neuromuscular transmission as is demonstrated by variations in delay which can be recorded if the tension the hemidiaphragm is pinned out to is varied. However this increase could be the result of the effects described in section 4.2.4 of chapter 4. These are increased run down of endplate potential amplitude within a train of responses or the systematic slowing of nerve conduction velocity in the preterminal nerve branches. The former of these two explanations has been proposed to explain the increase in delay observed following exposure of animals to ecothiopate which is a known organophosphorous anticholinesterase. This has raised the question as to whether acrylamide is capable of exerting a similar effect either by direct inhibition of acetylcholinesterase or by indirect effects on its production via metabolic or transportational mechanisms. The anticholinesterase hypothesis is discussed at more length later in this discussion following the observations made using pharmacological techniques.

Thus, it appears that the analysis of the jitter of evoked action potentials and/or endplate potentials are of little use as a detection technique for the early pre-clinical or even for later stages of acrylamide induced neuropathy. This may be because this technique examines variances in normal orthodromic action potential conduction and neuromuscular transmission. These events are known to have large inbuilt safety factors to ensure the function of the system even when relatively stressed. As a result, it is perhaps not surprising that even a dose of acrylamide sufficient to cause clinical signs of neuropathy (perhaps due to modification of function of sensory nerve endings - chapter 1) does not have a significant effect on this function. The abscence of an effect of acrylamide treatment on the resting membrane potential of muscle fibres (table 4.1) also points towards the lack of any denervation and in turn any effects on transmitter release. These conclusions resulted in the need to investigate other facets of the function of the neuromuscular junction which are more labile and perhaps more sensitive to minor disruptions of its state.

9.3: CAN ECOTHIOPATE BE USED AS A TOOL TO PROBE THE FUNCTIONAL STATE OF THE PERIPHERAL NERVE AND NEUROMUSCULAR JUNCTION ?

As stated above, the failure of the analysis of jitter to show any strong evidence for a pre-clinical effect on the function of the mouse neuromuscular junction necessitated the use of more labile and hence potentially more sensitive responses associated with the

function of the neuromuscular junction. Using the hemidiaphragm / phrenic nerve preparation it was hoped that the known potentiation of stimulated muscle twitches and characteristics of the associated spontaneous twitching (fasciculation and fibrillation) observed following exposure to ecothiopate might be altered by low level acrylamide pre treatment.

The data obtained from the experiments performed on the stimulated twitches showed that acrylamide treatment sufficient to cause clinical signs in the animals (200 mg/kg / 8 days) prolonged the time taken for the generation of peak (maximum) potentiation of the twitches from the point at which potentiation first occurred following exposure to ecothiopate. Data captured 8 hours after a dose of 100 mg/kg showed a facilitatory effect with a decrease in the time from first to peak potentiation. It is interesting to note this effect with reference to the facilitation (non significant) of delay recorded during the analysis of jitter of action potentials described above. These data also show that the sub-clinical dose of 100 mg/kg of acrylamide resulted in an increase (8 hours after dosing) followed by a decrease (1, 8 and 32 days) in the amplitude of the maximally potentiated twitch.

Spontaneous twitches recorded following sub-clinical acrylamide pre-treatment and *in vitro* ecothiopate exposure showed that acrylamide caused a decrease in frequency and to a lesser extent, amplitude. In some cases measurable twitches were abolished altogether although microscopic examination of the muscles used in these experiments showed that fibrillation was still occurring. These effects were observed 1 to 16 days after acrylamide administration which is in the same time range as the effects seen on the stimulated twitches.

In sections 5.1.1 and 5.2.3.3, a number of possible explanations were proposed for the post-acrylamide effects described above. These centred around three main possibilities. Firstly, that acrylamide was in some way reducing the acetylcholinesterase capacity in the synaptic cleft. Secondly, that acrylamide was interfering with the processes leading to the generation of spontaneous nerve activity and hence spontaneous twitching. Thirdly, that acrylamide may be able to disrupt antidromic action potential conduction within the 'nervous reflex arc' of motor units.

The potential for an anticholinesterase action of acrylamide was investigated in the experiments described in chapter 6. A prolonged miniature endplate potential half decay time was demonstrated following sub-clinical acrylamide treatment (100 mg/kg / 1 day, 8 day) and this represents functional evidence for a decrease in the anticholinesterase capacity in the synaptic cleft. However, as discussed in section 6.4, the prolongation of the half decay time recorded equates to an inhibition of acetylcholinesterase of no more than 10% and this probably accounts for the inability of biochemical techniques, employed later in the chapter, to corroborate this observation conclusively. In spite of this, the experiments performed in section 6.3.6 showed that there was no direct inhibitory effect of acrylamide on acetylcholinesterase. Therefore it seems likely that acrylamide has an indirect effect on the acetylcholinesterase available in the synaptic cleft. This may be the

result of impaired synthesis, assembly or transport of the enzyme as a result of a metabolic disturbance caused by acrylamide. Another possible explanation is that the normal diffusion of acetylcholine in the synaptic cleft is altered by changes in cleft morpholgy resulting from highly localised changes in nerve terminal morphology. This would mean that molecules of acetylcholine might undergo multiple interactions with post synaptic receptors and produce extended miniature endplate potentials. Such an effect may be highly localised with the majority of potentials being normal. This could result in only a minor affect on the length of the average potential produced by the method of analysis used in this experiment. However, with no evidence of any abnormal terminal morphology and the very early time point at which this effect would have to occur this possibility is highly unlikely.

The possibility that acrylamide was interfering with the generation of spontaneous activity in the nerve terminals and hence reducing the number of spontaneous twitches recorded following ecothiopate exposure was difficult to investigate since there are a number of potential mechanisms for the generation of such activity (see sections 5.1.1 and 5.2.3.3). However, all these mechanisms ultimately excite the nerve/nerve terminal to the point where an action potential is produced. It was therefore decided to investigate the electrical excitability of the terminal regions of the nerves following acrylamide administration. These experiments demonstrated a reduced excitability of these regions following acrylamide administration although the precise reason for this change remains unknown but may be the result of modification of the elemental composition of the axoplasm of the nerve cells or perhaps due to some modification of the axolemma relating to the flow of ions or simply its extent. The latter point is probably precluded by the lack of swelling of the nerve terminals or pre-terminal axons described in chapter 8.

The possibility that acrylamide is able to disrupt the antidromic conduction of nerve action potentials following their generation and thereby potentially reduce the size of the resulting spontaneous twitches and also the size of potentiated post ecothiopate stimulated twitches, proved impossible to investigate during the course of this thesis for the reasons outlined in section 5.2.3.3 although attempts were made using a 'reverse jitter technique'. However, the presence of fibrillation in preparations which appeared not to be fasciculating makes this an attractive possibility and one which deserves further investigation using the technique proposed in section 5.2.3.3 involving loading synaptic vesicles with a fluorescent dye in order to ascertain if transmission of action potentials around the nervous arc of motor units in the muscle is occurring normally following acrylamide administration.

The results obtained investigating the modification of the response of the phrenic nerve/hemidiaphragm to *in vitro* ecothiopate exposure following *in vivo* pre treatment with acrylamide suggest that ecothiopate can be used as a probe to test for deficits in the function of the neuromuscular junction although the mechanisms behind such changes remain unclear and should be the subject of further investigation.

9.4: CONCLUDING COMMENTS:

The aim of this project at the outset was to investigate the possibilities for the development of new techniques which could be applied to the screening of potentially neurotoxic compounds with actions focusing on the distal regions of peripheral nerves. The first attempt using 'jitter analysis' proved to be unsuccessful but a second attempt using ecothiopate as a probe for the functional condition of the neuromuscular region was more successful. However, existing commercial screening techniques such as those used to establish the sub clinical dose of acrylamide used in this study, have a number of features which differ from the more esoteric attempts made in this thesis. These include speed, simplicity and cost. The technique described in this thesis using ecothiopate requires a great deal of complicated and expensive equipment which is demanding and time consuming to use. The data it produces shows a great deal of variability requiring large samples of data to perform complex analysis on. These factors would make such a technique commercially unattractive bearing in mind the current alternatives. However, the ecothiopate approach does yield evidence of a compromise of peripheral nerve function at a very early time point and lower dose than the existing techniques such that the animals under study do not need to show any clinical abnormality. This helps to validate the existing commercial techniques. It must be remembered though, that only acrylamide has been shown to cause the effects described in this thesis and thus the technique is far from established as a possible screening test.

Further work on this project would be very interesting in order to investigate the effects of substituting acrylamide for other similar neurotoxins (e.g. 2,5 hexanedione). It may also prove interesting to substitute other known anticholinesterases for ecothiopate such as edrophonium. The variation in the responses of different nerves and muscles to acrylamide has been reported in the scientific literature and thus any firm understanding of the mechanism behind the observations reported in this thesis would also require detailed investigation of this variation with particular relation to differential metabolism (e.g. analysis of variation in the isozymes of cytochrome P-450) together with a more accurate study of the toxicokinetics of acrylamide in the specific test animals used.

This further investigation might yield clues which could help to answer many of the questions which this work has raised but only begun to study.

APPENDIX 1

REFERENCES

Ancilewski, A. (1996). PhD thesis, Aston University (in press).

Ando, K. & Hashimoto, K. (1972). Accumulation of (14-C) acrylamide in mouse nerve tissue. *Proc. Osaka Prefec. Inst. Public Health,* **10,** 7-12.

Auld, R. B., & Bedwell, S. F. (1967). Peripheral neuropathy with sympathetic overactivity from industrial contact with acrylamide. *Can. Med. Assoc. J.,* **96,** 652-654.

Bamforth, J. (1989). The effects of persistant anticholinesterase action at the neuromuscular junction. PhD thesis, Aston University.

Banker, B. Q., Kelly, S. S. & Robbins, N. (1983). Neuromuscular transmission and correlative morphology in young and old mice. *J. Physiol.* **339,** 355-375.

Berti-Mattera, L., Eichberg, J., Schrama, L. and LoPachin, R. (1990). Acrylamide administration alters protein phosphorylation and phospholipid metabolism in rat sciatic nerve. *Toxicol. App. Pharmacol.* **103**. 502-511.

Bikales, N., M. (1970). Acrylamide and related amides. In: High polymers, 24, 1, Vinyl and Diene Monomers (ed. E. C. Leonard) 81-0104, John Wiley and Sons Inc.

Bikales, N., M. & Kolodny., E. R. (1963). Acrylamide. In: Encyclopaedia of Chemical Technology, 2nd ed. A. Standen, 274-284, Interscience, New York.

Bisby, M. and Redshaw, J. (1987). Acrylamide neuropathy: Changes in the composition of proteins of fast axonal transport resemble those observed in regenerating axons. *J. Neurochem.* **48**. 924-928.

Bjornskov, E. K., Norris, F. H. & Mower-Kuby, J. (1982). Histochemical staining of the acetylcholine receptor, acetylcholinesterase and the axon terminal. *Muscle & Nerve,* **5**, 140-142.

Black, J. A., Kocsis, J. D. & Waxman, S. G. (1990). Ion channel organization of the myelinated fibre. *TINS,* **13,** 48-54.

Bostock, H. (1983). The strength-duration relationship for excitation of myelinated nerve: computed dependence on membrane parameters. *J. Physiol.* **341,** 59-74.

Bowman, W. C. (1990). Pharmacology of neuromuscular function. 2nd edn, Wright & Sons, London.

Boyd, I. A. & Martin, A. R. (1956). The endplate potential in mammalian muscle. *J. Physiol.,* **134,** 74-91.

Bradley, W. G. & Asbury, A. K. (1970). Radioautographic studies of schwann cell behaviour. I. Acrylamide neuropathy in the mouse. *J. Neuropath. Exp. Neurol.* **29,** 500-506.

Bradley, W. G. & Williams, M. H. (1973). Axoplasmic flow in axonal neuropathies. *Brain,* **96,** 235.

Brown, M. C. & Matthews, P. B. C. (1960). The effect on a muscle twitch of the back response of its motor nerve fibres. *J. Physiol.,* **150,** 332-346.

Brown, G., Dale, H. and Feldberg, W. (1936). Reactions of normal mammalian muscle to acetylcholine and to eserine. *J. Physiol.* **87**. 394-424.

Burd, P.and Ferry, C. (1987). A prolonged contraction at the endplate region of the diaphragm of rats and mice after anticholinesterases in vitro. *J. Physiol.* **391**, 429-440.

Busker,R. W., Zijlstra, J. J., van der Wiel, H. J. & van Helden, H. P. M. (1994). The functional role of molecular forms of acetylcholinesterase in neuromuscular transmission. *Neurochem. Res.,* **19,** 713-719.

Cabe, P. A., & Colwell, P. B. (1981). Toxic effect of acrylamide in Japanese Quail (*Coturnix coturnix japonica). J. Toxic. Envir. Hlth.,* **8,** 935-940.

Cavanagh, J. B. (1964). The significance of the 'dying back' process in experimental and human neurological disease. *Int. Rev. Exp. Pathol.* **3,** 219-264.

Cavanagh, J. B. (1982). The pathokinetics of acrylamide intoxication: a reassessment of the problem. *Neuropath. Appl. Neurobiol.,* **8,** 315-336.

Cavins, J.F., and Friedman, M. (1967a). Specific modification of protein sulfhydryl groups with α, β-unsaturated compounds. *Fed. Proc.,* **26,** 822.

Cavins, J.F., and Friedman, M. (1967b). New amino acids derived from reactions of E-amino groups in proteins with α, β-unsaturated compounds. *Biochem,* **6,** 3766-3770.

Chretien, M., Patey, G., Souyri, F. and Droz, B. (1981). Acrylamide induced neuropathy and impairment of axonal transport of proteins. II. Abnormal accumulations of smooth endoplasmic reticulum as sites of focal retention of fast transported proteins. Electron microscope radioautographic study. *Brain Res.* **205**, 15-28.

Clark, A. L., Hobbiger, F. & Terrar, D. A. (1980). Intracellular recording of the anticholinesterase-induced repetitive responses of mammalian muscles to single indirect stimuli. *J. Physiol.,* **302,** 26-27P.

Clark, A. L., Hobbiger, F. & Terrar, D. A. (1983). The relationship between stimulus-induced antidromic firing and twitch potentiation produced by paraoxon in rat phrenic nerve-diaphragm preparations. *Br. J. Pharmacol.,* **80,** 17-25.

Clark, A. L., Hobbiger, F. & Terrar, D. A. (1984). Nature of the anticholinesterase-induced repetitive response of rat and mouse striated muscle to single nerve stimuli. *J. Physiol.,* **349,** 157-166.

Coughenour, L., McClean, J. and Parker, R. (1976). A new device for the rapid measurement of impaired motor funtion in mice. *Pharmac. Biochem. Behav.* **6**, 351-353.

Couraud, J. Y., Di Giamberardino, J., Chretien, M., Souyri, F. & Fardeau, M. (1982). Acrylamide neuropathy and changes in the axonal transport and muscular content of the molecular forms of acetylcholinesterase. *Muscle and Nerve.* **5**, 302-312.

Crofts A. (1996). PhD thesis, Aston University (in press).

DeGrandchamp, R. and Lowndes, H. (1990). Early degeneration and sprouting at the rat neuromuscular junction following acrylamide administration. *Neuropathology and applied neurobiology.* **16**, 239-254.

DeGrandchamp, R., Reuhl, K., & Lowndes, H. (1990). Synaptic terminal degeneration and remodelling at the rat neuromuscular junction resulting from a single exposure to acrylamide. *Toxicol. Appl. Pharmacol.,* **105,** 422-433.

Del Castillo, J. and Katz, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *J. Physiol.* **132**, 630-649.

Dixit, R. Hasain, R. Seth, P. K., & Mukhtar, H. (1980). Effects of diethyl maleate on acrylamide-induced neuropathy in rats. *Toxicol. Lett.,* **6,** 417.

Drachman, D. B. (1972). Neurotrophic regulation of muscle cholinesterase: effects of botulinum toxin and cholinesterase. *J. Physiol.,* **226,** 619-627.

Drachman, D. B. (1976). Trophic interations between nerves and muscles: the role of cholinergic transmission (including usage) and other factors. In: Biology of cholinergic function (eds Goldgerg, A. & Hanin, I.), Ravens Press, New York, pp 162-201.

Eccles, J. C., Katz, B. & Kuffler, S. W. (1942). Effect of eserine on neuromuscular transmission. *J. Neurophysiol,* **5,** 211-230.

Druckrey, H., Consbruch, U., and Schmähl, D. (1953). Effects of monomeric acrylamide on proteins. *Zeit. für Natur.,* **86,** 145-150.

Edwards, P., Sporel-Ozakat, R. and Gispen, W. (1991). Neurotoxic acrylamide and neurotrophic melanocortin peptides- can contrasting actions provide clues about modes of action?*Neuropathology and applied neurobiology.* **17**, 91-104.

Edwards, P. (1975). Neurotoxicity of acrylamide and its analogues and effects of these analogues and other agents on acrylamide neuropathy. *Brit. J. Ind. Med.* **32**. 31-38.

Edwards, P. and Parker, V. (1977). A simple, sensitive and objective method for early assessment of acylamide neuropathy in rats. *Toxicol. App. Pharmacol.* **40**. 589-591.

Edwards, P., Sporel-Ozakat, R. and Gispen, W. (1984). Peripheral pain fiber function is relatively insensitive to the neurotoxic actions of acrylamide in the rat. *Toxicol. Appl. Pharmacol.* **111**, 43-48.

Edwards, P., Sporel-Ozakat, R. and Gispen, W. (1991). Neurotoxic acrylamide and neurotrophic melanocortin peptides - can contrasting actions provide clues about modes of action? *Neuropath. Appl. Neurobiol.,* **17,** 91-104.

Ellman, G., Courtney, K. and Andres, V. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7. 88-95.

Ekstedt, J., Nilsson, G. & Stalberg, E. (1974). Calculation of the electromyographic jitter. *J. Neurol. Neurosurg. Psychiatry.* **37,** 526-539.

Evans, H. & Teal, J. J. (1981). Appetitive behaviours as models of the neurotoxicity of acrylamide. *Fedn. Proc.*, **40**, 677.

Fatt, P. & Katz, B. (1951). An analysis of the endplate potential recorded with an intra-cellular electrode. *J. Physiol.* **115**, 320-370.

Fatt, P. & Katz, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109-128.

Ferry, C. B. (1988). The origin of the anticholinesterase induced repetitive activity of the phrenic nerve-diaphragm preparation of the rat in vitro. *Br. J. Pharmacol.* **94**, 169-179.

Ferry, C. B. and Cullen, M. (1991). Myopathic changes in indirectly stimulated mouse diaphragm after ecothiopate in vitro. *Int. J. Exp. Path.* **72**, 329-343.

Flock, H. G. & Rausch, E. G. (1973). Application of polyelectrolytes in municipal waste treatment. In: Water-soluble polymers, ed. N. M. Bikales, Polymer Science and Technology, 2, 21-73, Plenum Press, New York.

Fullerton, P. M., & Barnes, J. M. (1966). Peripheral neuropathy in rats produced by acrylamide. *Brit. J. Ind. Med.*, **23**, 210-221.

Garland, T. O., & Patterson, M. (1967). Six cases of acrylamide poisoning. *BMJ.*, **4**, 134-138.

Gilbert, S. G., & Maurissen, J. P. J. (1980). Assessment of the effects of three neurotoxic compounds on motor functions in mice. *Soc. Neurosci.*, 807A.

Gold, B., Griffin, J. W. and Price, D. L. (1985). Slow axonal transport in acrylamide neuropathy: different abnormalities produced by single-dose and continuous administration. *J. Neuroscience,* **5**, 1755-1768.

Gold, B., Griffin, J. W. and Price, D. L. (1992). Somatofugal axonal atrophy preceeds development of axonal degeneration in acrylamide neuropathy. *Arch. Toxicol.* **66**. 57-66.

Goldman, L., & Albus, J. S. (1968). Computation of impulse conduction in myelinated fibres: theoretical basis of the velocity-diameter relation. *Biophys J.* **8**, 596-607.

Goldstein, B. D. (1985). Acrylamide preferentially affects slowiy adapting cutaneous mechanoreceptors. *Toxicol. Appld. Pharmacol.* **80**, 527-533.

Griffin J. W. & Price, D. L. (1976). Axonal transport in motor neuron pathology. In : Recent research trends. Eds: Johnson & Brazier. New York, Academic Press. pp 33-54.

Guth, L. (1968). 'Trophic' influences of nerve on muscle. *Physiol. Rev.,* **48,** 645-687.

Hamblin, D. O. (1956). The toxicity of acrylamide-a preliminary report. In: Hommage au doyen rene fabre, Membre de l'institute, Professeur de toxicologie a la faculte de pharmacie de paris, 159-199, SEDES., Paris.

Hashimoto, K. and Aldridge,W. (1970). Biochemical studies on acrylamide, a neurotoxic agent. *Biochem. Pharmacol.* **19**. 2591-2604.

Hashimoto, K., Sakamoto, J. & Tannii, H. (1981). Neurotoxicity of acrylamide and related compounds and their effects on male gonads in mice. *Arch. Toxicol.* **47,** 179-189.

Head, S. D. (1983). Temperature and end-plate currents in rat diaphragm. *J. Physiol.* **334**, 441-459.

Hill, A. V. (1936). Excitation and accommodation in nerve. *Proc. R. Soc. B.,* **119,** 305-355.

Hobbiger, F. (1976). Pharmacology of anticholinesterase drugs. In: *Neuromuscular junction. Handbook of experimental pharmacology*, **42,** (ed. E. Zaimis), Springer-Verlag, Berlin, pp 487-581.

Hohlfeld, R., Sterz, R. and Peper, K. (1981). Presynaptic effects of anticholinesterase drugs at the end plate. *Pflugers Arch.* **391**, 213-218.

Hollenbeck, P. (1986). Moving in different directions: organelle transport. *Nature.* **319,** 724-725.

Hoover, M. F. (1970). Bactericidal quaternary diallyl ammonium polymers. *US Patent 3,539,684 (Calgon Corporation).*

Hopkins, A. (1970). The effect of acrylamide on the peripheral nervous system of the baboon. *J. Neurol. Neurosurg. Psychiat.,* **33,** 805-816.

Hopkins, A. & Gilliat, R. (1971). Motor and sensory nerve conduction velocity in the baboon: Normal values and change during acrylamide neuropathy. *J. Neurol. Neurosurg. Psychiatry,* **34,** 415-426.

Howland, R. D. (1985). Biochemical studies of acrylamide neuropathy. *Neurotoxicol.*, **6,** 7-16.

Igusu, H., Goto, I., Kawamura, Y., Kato, M., Izumi, K., & Kuroiwa, Y. (1975). Acrylamide encephaloneuropathy due to well water pollution. *J. Neurol. Neurosurg. Psychiatry.,* **38,** 581-584.

Jakobsen, J. & Sidenius P. (1983). Early and dose dependent decrease of retrograde axonal transport in acrylamide intoxicated rats. *J. Neurochem.* **40,** 447-454.

Jennekens, F., Veldman, H., Schotman, P. and Gispen, W. (1979). Sequence of motor nerve terminal involvement in acrylamide neuropathy. *Acta neuropathol. (Berl).* **46**, 57-63.

Johnson, E. C. & Murphy, S. D. (1977). Effect of acrylamide intoxication on pyridine nucleotide concentrations and functions in rat cerebral cortex. *Biochem. Pharmacol.,* **26,** 2151-2155.

Kaplan, M. L. & Murphy, S. D. (1972). Effect of acrylamide intoxication on rotarod performance and sciatic nerve β-glucuronidase activity in rats. *Toxicol. Appld. Pharmacol.* **22,** 259.

Karnovsky, M. J. amd Roots, L. (1964). A 'direct colouring' thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12**, 219-221.

Katz, B. (1962). The transmission of impulses from nerve to muscle and the subcellular unit of synaptic action. *Proc. R. Soc. Lond. B.,* **155,** 455-477.

Kelly, S. S. & Ferry, C. B. (1994). The origin of the effects of an anticholinesterase on the latencies of action potentials in mouse skeletal muscles. *Br. J. Pharmacol.* **111,** 747-752.

Kelly, S. S., Ferry, C. B. & Bamforth, J. P. (1990). The effects of anticholinesterases on the latencies of action potentials in mouse skeletal muscles. *Br. J. Pharmacol.* **99,** 721-726.

Konishi, T. (1985). Electrical excitability of motor nerve terminals in the mouse. *J. Physiol.,* **366,** 411-421.

Kuperman, A. S. (1958). Effects of acrylamide on the central nervous system of the cat. *J. Pharm. Exp. Ther.*, **123,** 180-192.

Lapadula, D. M., Bowe, M., Carrington, C. D., Dulak, L., Freidman, M. & Abou-Donia, M. B. (1989). In vitro binding of acrylamide to neurofilament and microtubule proteins in rats. *Brain Res.*, **481,** 157-161.

Lapicque, L. (1907). Recherches quantitatives sur l'excitation électrique des nerfs traitée comme une polarisation. *J. Physiol., Paris,* **9,** 622-635.

Laskowski, M.and Dettbarn, W. (1975). Presynaptic effects of neuromuscular cholinesterase inhibition. *J. Pharmacol. Exp. Ther.* **194**, 351-361.

Lederer, W. J., Spindler, A. J. & Eisner, D. A. (1979). Thick slurry bevelling. A new technique for bevelling extremely fine microelectrodes and micropipettes. *Pflugers Arch. ges. Physiol.* **381,** 287-288.

Le Quesne, P. M. (1979). Acrylamide. In: Experimental and clinical neurotoxicology, eds, Spencer, P. & Schaumberg, H., Baltimore, Williams & Wilkins. pp309-325.

Leswing, R. J. & Ribelin, W. E. (1969). Physiologic and pathologic changes in acrylamide neuropathy. *Archs. Envir. Health.* **18,** 2-29

Liley, A. W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* **132**, 650-666.

Lloyd, D. P. C. (1942). Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.,* **5,** 153-165.

LoPachin, R., Castiglia, C., & Saubermann, A. (1992). Acrylamide disrupts elemental composition and water content of rat tibial nerve. I. Myelinated axons. *Toxicol. Appl. Pharmacol.,* **115,** 21-34.

LoPachin, R., Castiglia, C., Lehling, E. and Saubermann, A. (1993). Effects of acrylamide on subcellular distribution of elements in rat sciatic nerve myelinated axons and schwann cells. *Brain Res.* **608,** 238-246.

Lowndes, H. E. & Baker, T. (1975). Studies on drug-induced neuropathies. III. Motor nerve deficit in cats with experimental acrylamide neuropathy. *Eur. J. Pharmacol.,* **35,** 177-184.

Lowndes, H. E., Baker, T. & Riker, W. F. (1974). Motor nerve dysfunction in delayed DFP neuropathy. *Eur. J. Parmacol.,* **29,** 66-73.

Lowndes, H. E., Baker, T., Cho, E. & Jortner, B. S. (1978). Position sensitivity of de-afferented muscle spindles in experimental acrylamide neuropathy. *J. Pharmacol.,* **205,** 40.

MacWilliams, D. C. Rogers, J. H. & West, T. J. (1973). Water-soluble polymers in petroleum recovery. In: Water-soluble polymers, ed. N. M. Bikales, Polymer Science and Technology, 2, Plenum Press, New York.

Masland, R. L. & Wigton, R. S. (1940). Nerve activity in the phrenic nerve diaphragm preparation of rat. *J. Physiol.,* **256,** 130P.

McCollister, D., Oyen, F. and Rowe, V. (1964). Toxicology of acrylamide. *Toxicol. App. Pharmacol.* **6**. 172-181.

Miller, M. S., Carter, D. and Sipes, I. (1982). Pharmacokinetics of acrylamide in Fisher-334 rats. *Toxicology and applied pharmacology* **63**, 36-44.

Miller, M. S., Miller, M. J., Burks, T. F. & Sipes, I. G. (1983). Altered retrograde axonal transport of nerve growth factor after single and repeated doses of acrylamide in the rat. *Toxicol. Appld. Pharmacol.* **69,** 96-101.

Miller, M. S. & Spencer, P. S. (1985). The mechanisms of acrylamide axonopathy. *Ann. Rev. Pharmacol. Toxicol.,* **25,** 643-666.

Moretto, A. and Sabri, M. (1988). Progressive deficits in retrograde axon transport precede degeneration of motor axons in acrylamide neuropathy. *Brain Res.* **440**. 18-24.

Morrison, J. D. (1977). Thr generation of nerve and muscle repetitive activity in the rat phrenic nerve-diaphragm preparation following inhibition of cholinesterase by ecothiopate. *Br. J. Pharmacol.,* **60,** 45-53.

Moser, V., Anthony, D., Sette, W. and MacPhail, R. (1992). Comparison of subchronic neurotoxicity of 2-hydroxyethyl acrylate and acrylamide in rats. *Fundamental and applied toxicology* **18**, 343-352.

Munoz-Martinez, E. J. (1982). Axonal retention of transported material and the lability of the nerve terminals. In: Axoplasmic Transport, Ed: Weiss. Springer-Verlag. Berlin. pp 267-274.

Noble, D. & Stein, R. B., (1966). The threshold conditions for initiation of action potentials by excitable cells. *J. Physiol,* **187,** 129-162.

Ochs, S. & Smith, C. (1971). Fast axoplasmic transport in mammalian nerve *in vitro* after block of glycolysis with iodoacetic acid. *J. Neurochem.,* **18,** 833-843.

Ochs, S., Sabri, M. I., & Johnson, J. (1969). Fast transport system of materials in mammalian nerve fibres. *Science,* **163,** 686-687.

Pastoor, T. & Richardson, R. (1981). Blood dynamics of acrylamide in rats. *Toxicologist,* **1 (1),** 53.

Pastoor, T., Heydens, W. & Richardson, R. (1980). Time and dose-related excretion of acrylamide metabolites in the urine of Fisher 344 rats. In: 2nd international congress on toxicology, Brussels, Belgium, July 6-11.

Pestronk, A. and Drachman D. B. (1978). A new stain for quantitative measurement of sprouting at neuromuscular junctions. *Muscle and Nerve.* **1**, 70-74.

Pollard, S. L. (1984). Murine motor end plate disease and the mutant Jolting. **PhD Thesis**, University of Newcastle-upon-Tyne.

Pleasure, D. E., Mishner, K. C. & Engel, W. K. (1969). Axonal transport of proteins in experimental neuropathies. *Science,* **166,** 524.

Prineas, J. (1969). The pathogenesis of dying back polyneuropathies. Part II. An ultrastructural study of experimental intoxication in the cat. *J. Neuropath. Exp. Ther.* **28,** 598-621.

Pryor, G. T., Uyeno, E. T., Tilson, H. A. & Mitchell, C. L. (1983). Assessment of chemicals using a battery of neurobehavioural tests: A comparitive study. *Neurobehav. Toxicol. Teratol.* **5,** 91-117.

Randic, M. & Straughan, D. W. (1964). Antidromic activity in the rat phrenic nerve-diaphragm preparation. *J. Physiol.,* **173,** 130-148.

Rasool, C. G. & Bradley, W. G. (1978). Studies on axoplasmic transport of individual proteins. I. Acetylcholinesterase in acrylamide neuropathy. *J. Neurochem.* **31,** 419-425.

Riker, W. F. & Okamoto, M. (1969). Pharmacology of motor nerve terminals. *A. Rev. Pharmacol.,* **9,** 173-208.

Roots, B. I. (1983). Neurofilament accumulation induced in synapses by leupeptin. *Science,* **221,** 971-972.

Sabri, M. I. & Ochs, S. (1971). Inhibition of glyceraldehyde-3-phosphate dehydrogenase in mammalian nerve by iodoacetate. *J. Neurochem.,* **13,** 1509-1514.

Sabri, M. I. & Ochs, S. (1972). Relation of ATP and creatine phosphate to fast axoplasmic transport in mammalian nerve. *J. Neurochem.,* **19,** 2821-2828.

Sahenk, Z. & Mendell, J. R. (1981). Acrylamide and hexandione neuropathies: Abnormal bidirectional transport rate in distal axons. *Brain Res.* **219,** 397-405.

Schaumann, W. & Job, C. (1958). Differential effects of the quaternary cholinesterase inhibitor, phospholine and its tertiary analogue, compound 217-AO, on central control of respiration and on neuromuscular transmission. *J. Pharmacol. Exp. Ther.* **123,** 114-120.

Schaumberg, H., Wisniewski, H., & Spencer, P. (1974). Ultrastructural studies of the dying back process. I. Peripheral nerve terminal and axon degeneration in systemic acrylamide intoxification. *J. Neuropath. exp. Neurol.,* **33,** 260-284.

Schlaepfer, W. W. (1987). Neurofilaments: Structure, metabolism and implications in disease. *J. Neuropathol. Exp. Neurol.* **46,** 117-129.

Sickles, D. (1989). Toxic neurofilamentous axonopathies and fast anterograde axonal transport. 1. The effects of single doses of acrylamide on the rate and capacity of transport. *Neurotoxicology.* **10**. 91-102.

Sickles, D. (1991). Toxic neurofilamentous axonopathies and fast anterograde axonal transport. *Toxicol. and App. Pharmacol.* **108**, 390-396.

Sickles, D. & Pearson, J. K. (1987). ATP, CP and axoplasmic transport in sciatic nerves of acrylamide, 2,5-HD and DMHD exposed rats. *The Toxicologist,* **7,** 132.

Sidenius, P. & Jakobsen, J. (1983). Anterograde axonal transport in rats during intoxication with acrylamide. *J. Neurochem.* **40,** 697-704.

Souyri, F., Chretien, M.and Droz, B. (1981). Acrylamide-induced neuropathy and impairment of axonal transport of proteins. I. Multifocal retention of fast transported

proteins at the periphery of axons as revealed by light microscope radioautography. *Brain Res.* **205**, 1-13.

Spencer,P., S. and Schaumberg, H. H. (1974 Part I). A review of acrylamide neurotoxicity; I. Properties, uses and human exposure. *Can. J. Neurolog. Sci.,* **1,** 143.

Spencer,P., S. and Schaumberg, H. H. (1974 Part II). A review of acrylamide neurotoxicity; II. Experimental animal neurotoxicity and pathological mechanisms. *Can. J. Neurolog. Sci.,* **1,** 152.

Spencer, P., S. and Schaumberg, H. H. (1977). Ultrastructural studies of the dying back process. IV. Differential vulnerability of PNS and CNS fibres in experimental central-peripheral distal axonopathies. *J. Neuropathol. Exp. Neurol.,* **36,** 300-320.

Spencer, P. S., Sabri, M. I., Schaumberg, H. S. & Moore, C. L. (1979). Does a defect of energy metabolism in the nerve fibre underlie axonal degeneration in polyneuropathies? *Ann. Neurol.,* **5,** 501-507.

Stalberg, E. and Trontelj, J. (1979). Single fibre electromyography. Publisher: Miravelle Press, Old Woking, Surrey.

Sterman, A., Panasci, D. and Persons, W. (1983). Does pyruvate prevent acrylamide neurotoxicity? Implications for desease pathogenesis. *Experimental neurology.* **82**, 148-158.

Sumner, A. J. (1980). Axonal polyneuropathies. In: The physiology of peripheral nerve disease ed, Sumner, A. I., pp. 340-357. Saunders, Philadelphia.

Sumner, A. J. & Astbury, A. K. (1975). Physiological studies of the dying back phenomenom. Muscle stretch afferents in acrylamide neuropathy. *Brain,* **98,** 91-100.

Sumner, A. J., Pleasure, D. & Ciesielka, K. (1976). Slowing of fast axoplasmic transport in acrylamide neuropathy. *J. Neuropathol. Exp. Neurol.* **35,** 319.

Sussman, J., Harel, M. and Silman, I. (1993). Three-dimensional structure of acetylcholinesterase and of its complexes with anticholinesterase drugs. *Chem.-Biol. Interactions.* **87**.187-197.

Suzuki, K. & Pfaff, L. D. (1973). Acrylamide neuropathy in rats - an electron microscopic study of degeneration and regeneration. *Acta. Neuropath.* **24,** 197-213.

Takahashi, M., Ohara, T., & Hashimoto, K. (1971). Electrophysiological study of nerve injuries in workers handling acrylamide. *Int. Arch. Arbeitsmed.,* **28,** 1-11.

Tammelin, L. E. (1958). Organophosphoryl cholines and cholinesterase. *Arrkiv. Kemi.,* **12,** 287-298.

Tanii, H., Hayashi, M. & Hashimoto, K. (1988). Neurofilament degradation in the nervous system of rats intoxicated with acrylamide, related compounds or 2,5-hexanedione. *Arch. Toxicol.,* **62,** 70-75.

Tattersall, J. E. H. (1990). Effects of organophosphorus anticholinesterases on nicotinic receptor ion channels at adult mouse muscle endplates. *Br. J. Pharmacol.* **101,** 349-357.

Teal, J. J. & Evans, H. L. (1982). Behavioural effects of acrylamide in the mouse. *Toxicol. Appld. Pharmacol.* **63,**470-480.

Thomann, P., Koella, W. P., Krinke, G., Peterman, H., Zak, F., & Hess, R. (1974). The assessment of neurotoxicity in dogs - comparative studies with acrylamide and clioquinol. *Agents Actions,* **4,** 47-53.

Tilson, H. A. & Cabe, P. A. The effects of acrylamide given acutely or in repeated doses on fore- and hindlimb function in rats. *Toxicol. Appl. Pharmac.* **47,** 253-260.

Tsujihata, M., Engel, A. and Lamabert, E. (1974). Motor end plate fine structure in acrylamide dying-back neuropathy: a sequential morphometric study. *Neurology.* **24,** 849-856.

Webb, S. N. & Bowman, W. C. (1974). The role of pre-and post-junctional cholinoceptors in the action of neostigmine at the neuromuscular junction. *Clin. Exp. Pharmacol. Physiol.,* **1,** 123-134.

Weir, R. L., Glaubiger, G. & Chase, T. N. (1978). Inhibition of fast axoplasmic transport by acrylamide. *Environ. Res.* **17,** 251-255.

Weiss, G. (1901). Sur la possibilité de rendre comparables entre eux les appareils servant à l'excitation electrique. *Archs. ital. Biol.,* **35,** 413-446.

Werner, G. & Kuperman, A. S. (1963). Actions at the neuromuscular junction, in: Handbuch der experimentellan pharmakologie, Vol 15, ed. Koelle, G (Springer-Verlag, Berlin).

Younkin, S., Rosenstein, C., Collins, P. and Rosenberry, T. (1982). Cellular localisation of the molecular forms of acetylcholinesterase in rat diaphragm. *J. Biol. Chem.* **257.** 13630-13637.

APPENDIX II:

Composition of solutions:

COMPOSITION OF PHYSIOLOGICAL SALINE: (pH 7.4 as modified by Krnjevic and Miledi; 1958 from that proposed by Liley; 1956):

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

1.0 M volumetric $CaCl_2$ solution (BDH) was added to saline to make up the $CaCl_2$ component as required (2 ml per L). The saline was gassed with 95% oxygen / 5% carbon dioxide prior to and following the addition of the $CaCl_2$.

COMPOSITION OF SOLUTIONS USED IN CHOLINESTERASE ASSAYS (Ellman *et al.*, 1961; Younkin *et al.*, 1982):

Colour reagent: 5-5 dithiobis (2 nitro-benzoic) acid (**DTNB**):
This solution was stored as a 0.01M stock solution at 4°C for approx. 1 week.
Stock solution:

	AMOUNT
DTNB	39.6 mg
$NaHCO_3$	15.0 mg
0.1 M pH 7.0 phosphate buffer	10.0 ml

This stock solution was diluted 1:40 with distilled water for use in the assay.

0.1 M pH 7.0 Phosphate Buffer:

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

Substrate: Acetylthiocholine iodide:

	AMOUNT
Acetylthiocholine iodide	21.7 mg
Distilled water	50.0 ml

Substrate was prepared to give a final cuvette concentration of 0.5 mM.

Phosphate buffer 0.1 M pH 8.0:

	AMOUNT
0.2 M Na_2HPO_4	47.4 ml
0.2 M NaH_2PO_4	2.6 ml
Distilled water	50.0 ml

Low ionic strength buffer:

	AMOUNT
Phosphate buffer 10 mM pH 7.0	---
Triton X100	1%

High ionic strength buffer:

High ionic strength buffer was low ionic strength buffer supplemented with 1.0 M sodium chloride.

COMPOSITION OF CHOLINESTERASE STAINING MEDIUM (pH 5.0) (Karnovsky and Roots, 1964):

	AMOUNT
0.06 N sodium acetate	11.6 ml
0.1 N acetic acid	4.6 ml
0.1 M sodium citrate	4.8 ml
Acetylthiocholine iodide	12.5 mg
30 mM copper sulphate	2.5 ml
5 mM potassium ferricyanide	2.5 ml

The reagents were added together in the order listed and the final solution was a clear apple green colour. The solution was always made immediately prior to staining.

COMPOSITION OF SOLUTIONS USED IN SILVER/CHOLINESTERASE STAINING (modified from Pestronk and Drachman, 1978):

<u>Acetylcholinesterase incubating medium</u>:

	AMOUNT
Acetylcholine iodide	5 mg
Sodium acetate buffer pH 5	6.5 ml
0.1 M potassium citrate	1.5 ml
0.03 M copper sulphate	1.0 ml
0.0005 M potassium ferricyanide	1.0 ml

<u>Sodium acetate buffer pH 5</u>:

		AMOUNT
SOL A	0.2 M sodium acetate	70 ml
SOL B	0.4 M acetic acid	15 ml approx.

Solution B was added to solution A until the pH was correct and then the solution was diluted to 200 ml with distilled water.

<u>Silver incubating medium</u>:

	AMOUNT
Silver nitrate	2.0 g
Copper sulphate.5H_2O	0.01 g
Calcium chloride	0.02 g
Distilled water	20 ml

The copper sulphate was dissolved first followed by the silver nitrate. The calcium chloride was then suspended and the solution was agitated with a pasteur pipette for 15 seconds and then allowed to settle. this was repeated 3 times. The solution was then centrifuged and about 2 ml of the supernatent used.

<u>Reducing solution</u>:

	AMOUNT
Hydroquinone	1 g
Sodium sulphite (anhydrous)	10 g
Distilled water	100 ml

APPENDIX III:

OUTLINE OF FUNCTIONAL OBSERVATIONAL BATTERY:

This appendix is intended to provide a brief overview of the function observational battery used in this thesis in conjunction with the climbing performance test.

INTRODUCTION:

The functional observational battery or FOB comprises a routine clinical examination (including locomotion and behaviour), reflex testing, an assessment of sensory function and an assessment of muscle weakness.

The objective is to examine animals for signs of systemic toxicity (with a particular emphasis on neurotoxicity) following the administration of the test substance.

The FOB supplied by Zeneca plc was slightly simplified in that the testing of muscle weakness specified was substituted for the climbing performance test Coughenour *et al.* (1976). The methodology and results of this aspect of the test are reported separately in this thesis.

PROCEDURE:

Before detailed examination commenced, the animals were observed through the cage in order that any behavioural effects such as circling or excessive grooming could be observed. The cage was then opened and the animal examined. The examination included but was not limited to the following:

1) Appearance and general signs of toxicity

2) The control of muscle activity and locomotion

3) Autonomic mechanisms

4) CNS effects and behaviour

Detailed listings of observations to be made are included in the full FOB but below is a summary of the main focus of the categories described above.

1) **Appearance and general signs of toxicity:**

The animal was examined for its general appearance with particular attention being paid to the state of its coat, the areas around the nose, eyes, mouth, anus and penis. On being placed on the bench any abnormalities in general posture or respiration were noted.

2) **The control of muscle activity and locomotion:**

The animal was placed on flat surface and its locomotor activity assessed. During this period, the animal's ability to move, its gait and muscular control were examined. Reflex responses (i.e. responses to sound, righting reflex, splay reflex, pinna reflex, foot-withdrawal, visual placing response and corneal reflex) were all routinely checked at this point. Abnormalities of muscular control such as fasciculations, tremors, shaking, twitching or convulsions were also noted at this point. Alterations to abdominal tone was also assessed.

3) **Autonomic mechanisms:**

Each animal was examined for obvious changes in urine flow/frequency, defecation, lachrymation, salivation and pupil size.

4) **CNS effects and behaviour:**

The animals overall behaviour pattern was assessed. Any evidence of CNS depression such as sedation, comatose or hypothermia were noted. The same was true of evidence of CNS excitation such as hyperactivity, aggression or bizarre behaviour.