THE EFFECTS OF CYCLOLEUCINE ON NERVE, MUSCLE
AND NEUROMUSCULAR TRANSMISSION IN THE MOUSE:
AN ELECTROPHYSIOLOGICAL AND MORPHOLOGICAL STUDY

by

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ABSTRACT

Cycloleucine (CL) is a synthetic amino acid which inhibits the vitamin B₁₂/folate dependent methyl-transfer reaction.

A single dose of CL (2mg/g body weight) was administered intraperitoneally to 21-day-old and adult mice. Animals were allowed to survive for between 12 hours and 7 days. The 21-day-old mice showed paralysis of the hindlimbs within 24 hours, whereas this symptom became apparent in the adult mice at 48 hours. In the adult mice the twitch and tetanic responses of extensor digitorum longus (Edl) and soleus evoked by neural stimulation fell dramatically within 24 hours. In both the 21-day-old and the adult mice intracellular recordings made at 24 hours in soleus and Edl revealed that a significant number of end-plates were denervated, while other end-plates demonstrated intermittent failures in transmission and end-plate potentials (epps) with prolonged latencies. End-plates with abnormally high frequencies of miniature end-plate potentials (mepps) were commonly encountered in soleus and Edl, of 21-day-old mice at 12 hours, and in the adult mice at 24 hours. Morphological abnormalities in both the intramuscular nerves and the neuromuscular junctions of soleus and Edl were seen in both the young and adult mice at 24 hours. These abnormalities included areas of electron lucent axoplasm and swollen degenerative mitochondria and nerve terminals lacking synaptic vesicles. The innervation of proximal muscles was unaffected at this time. Over the next 2-3 days further reductions in the number of soleus and Edl fibres demonstrating mepps or epps occurred in both young and adult mice.

The muscle spindles in soleus were found to be both functionally and structurally intact.

At 7 days a limited recovery of function occurred in both soleus and Edl of young and adult mice. In biceps brachii of young mice, however, denervated end-plates and abnormally high mepp frequencies were found at 7 days.
It is suggested that the distal motor axonopathy induced by CL is caused by the failure of the methyl-transfer pathway which leads to abnormalities in the phospholipid composition of the axolemma at the neuromuscular junction. These changes are believed to cause an increase in microviscosity of the axolemma and hence a decrease in efficiency of ion channels/pumps responsible for maintaining electrochemical gradients essential for the structural and functional integrity of the neuromuscular junction.
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<td>Acetylcholine</td>
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<td>B₁₂</td>
<td>Vitamin B₁₂</td>
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<td>CSF</td>
<td>Cerebro spinal fluid</td>
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<td>CL</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>EdI</td>
<td>Extensor digitorum longus</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<td>Epp</td>
<td>End-plate potential</td>
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<td>Gmepps</td>
<td>Giant miniature end-plate potential</td>
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<td>HIV</td>
<td>Human immune deficiency virus</td>
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<td>HE</td>
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<td>HVG</td>
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<td>Light microscopy</td>
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<tr>
<td>5, 10CH₂THF</td>
<td>5, 10-methylenetetrahydrofolate</td>
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<td>MS</td>
<td>Multiple Sclerosis</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<td>MQC</td>
<td>Mean quantal content</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>SAH</td>
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<td>SAM</td>
<td>S-adenosylmethionine</td>
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<tr>
<td>SCD</td>
<td>Subacute combined degeneration of the spinal cord</td>
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<td>Tib ant</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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CHAPTER ONE: PART A.

GENERAL INTRODUCTION

1A1 Toxins in Neurobiology

The study of neurotoxins has frequently provided the neurobiologist with novel insights into the structure and function of the nervous system. In particular, naturally occurring toxins have been useful tools for localising and characterizing neuronal ion channels. Tetrodotoxin and saxitoxin, which bind to and block Na⁺ channels, have been used to estimate the numbers of Na⁺ channels at the node of Ranvier and in the internodal region of axonal membranes (Ritchie and Rogart, 1977). Dendrotoxin, which prolongs K⁺ currents, has been successfully employed to study K⁺ channels in a variety of neurons (Dolly, 1988).

A number of natural toxins which primarily affect either the pre- or post-synaptic areas of the neuromuscular junction have been purified. Toxins such as α-bungarotoxin, which selectively blocks neuromuscular transmission by irreversibly inactivating the nicotinic acetylcholine receptor on the post-synaptic membrane (Chang and Lee, 1963), have proved invaluable in both isolating and monitoring the distribution of the nicotinic acetylcholine receptor. Labelling the toxin with a radioisotope (Lee et al, 1967; Miledi and Potter, 1971) or a fluorochrome (Balice-Gordon and Lichtman, 1990) has allowed the distribution, density and movement of nicotinic acetylcholine (ACh) receptors to be determined. The stability of the receptor toxin complex has facilitated the isolation and hence the characterisation of the ACh receptors (Bowman, 1990).

Presynaptic toxins have been isolated from micro-organisms, arachnids and reptiles (reviewed by Ginsborg and Jenkinson, 1976; Howard and Gunderson, 1980; Bowman, 1990). Presynaptically active toxins have generated considerable interest, because presynaptic events such as the precise neurotransmitter release mechanism or the factors involved in the modulation of quantal release are not fully understood (Jones, 1987).
Insights into prejunctional mechanisms can be obtained by elucidating the mechanisms by which presynaptic toxins alter transmitter release.

The neurotoxins produced by the anaerobic bacterium *Clostridium botulinum* are among the most toxic substances known with a lethal dose estimated at 1ng/kg (Bowman, 1990). Botulinum toxin effects cholinergic neurons, by preventing both spontaneous and evoked quantal transmitter release (Brooks, 1956; Thesleff, 1960), thus paralysing skeletal muscle. The paralysis is long lasting, allowing the effect of functional denervation on the structure of the neuromuscular junction to be ascertained (Duchen and Strich, 1968). The long lasting paralysis induced by botulinum toxin has also been used to treat blepharospasm, strabismus, and other spastic muscle disorders (Gammon, 1984).

The venom of the *Latrodectus* genus of spiders causes a precipitous increase in spontaneous quantal transmitter release at a variety of synapses (Frontali et al, 1976). The mechanism of action of this venom has been studied using a combination of physiological, biochemical and morphological techniques, the results of which have strongly supported the vesicular hypothesis of quantal transmitter release (Ceccarelli and Hurlbut, 1980; Petrenko et al, 1991).

A variety of synthetic compounds have also been found to cause the degeneration of motor nerve terminals (see Cavanagh, 1985; and Jacobs and Le Quesne, 1992 for recent reviews). The fibres of the sciatic nerve which supply the muscles of the legs (or hindlimbs in animals) are often affected first (Schaumburg et al, 1983). Compounds such as Vincristine and 2-,5-hexanedione are thought to interrupt axonal transport and therefore starve the nerve terminal of proteins and cytoplasmic organelles vital for its function, whereas compounds such as mercury and adriamycin prevent protein synthesis in the perikaryon and hence prevent protein turnover or growth at the nerve terminal (Brimijoin, 1984). Neurotoxic chemicals with specific biochemical effects can be used to study the mechanisms responsible for maintaining the structural and functional integrity of axons and conversely, the degenerative changes when these mechanisms fail.
1A2 Animal models of neurological diseases

Animal models of human neurological diseases have frequently provided useful information regarding the pathogenesis and potential treatments of a variety of conditions (Rose and Behan, 1980). Animal models can either be inherited disorders or experimentally induced conditions. Inherited disorders resulting from spontaneous mutations have been found to occur in a wide range of species. Those detected in laboratory mice and rats have received most attention due to the low cost and ease with which a colony of these animals can be maintained. In a recent listing (Green, 1989) 97 neurological/neuromuscular mutants were catalogued. One such mutant, the progressive motor neuronopathy mouse, has recently been used in studies which led to the suggestion that ciliary neurotrophic factor might be a valuable treatment for human degenerative motor neuron disease (Sendtner et al, 1992).

Experimentally-induced neurological disorders have also provided a wealth of information regarding the pathogenesis of a variety of human diseases, i.e. experimental allergic neuritis (an animal model for acute inflammatory demyelinating polyneuropathy, Waksman and Adams, 1955), has provided valuable insights into the mechanisms involved in the pathogenesis of Guillain-Barré syndrome.

Animal models allow the earliest (possibly sub-clinical) stages of a disease to be studied, thus obtaining information that would be otherwise unavailable to the pathologist or neurobiologist.

1A3 Neurological disorders associated with vitamin B\textsubscript{12} deficiency

Leichtenstern (1884) was the first to link pernicious anaemia with a disease of the spinal cord. Later, the pathological lesions of the brainstem and spinal cord were extensively reviewed by Russell et al (1900) who named the condition ‘subacute combined degeneration of the spinal cord’. Both subacute combined degeneration (SCD) and pernicious anaemia are
now successfully treated with Vitamin B$_{12}$ (Healton et al, 1991). However, the pathogenesis of SCD is still poorly understood (Dinn et al, 1978).

Vitamin B$_{12}$ is a coenzyme for two important reactions (Stryer, 1981):

1). The regeneration of methionine from homocysteine in the methyl-transfer pathway.

2). The conversion of methylmalonyl-CoA to succinyl CoA using methylmalonyl-CoA mutase.

It is widely believed that the failure of the methyl-transfer pathway seen in Vitamin B$_{12}$ deficiency is the primary pathogenetic event in SCD (Lever et al, 1986). Failure of the methyl-transfer pathway leads to a fall in S-adenosylmethionine levels (see Fig. 1.1) which results in the inhibition of transmethylation reactions within the nervous system.

The methyl-transfer reaction and hence transmethylation within the nervous system can also be inhibited by the administration of cycloleucine (Lombardini et al, 1970) or nitrous oxide (Layzer et al, 1978). Cycloleucine (CL), is a synthetic amino acid which competitively inhibits the enzyme methionine adenosyltransferase reaction (Lombardini et al, 1970; Small and Carnegie, 1981) that converts methionine to S-adenosylmethionine in the methyl-transfer reaction. Oxidation of Vitamin B$_{12}$ by nitrous oxide prevents the use of Vitamin B$_{12}$ as a prosthetic group for the regeneration of methionine from homocysteine in the methyl-transfer reaction (Scott et al, 1981).

The administration of CL or nitrous oxide to experimental animals induces a progressive neurological condition resembling human SCD. Gandy et al (1973) were the first to demonstrate that CL can produce lesions in mice with a similar distribution to those seen in human SCD. Nitrous oxide has been used to produce a SCD like condition in monkeys (Scott et al, 1981), fruit bats (van der Westhuyzen et al, 1982) and pigs (Weir et al, 1988). Dinn et al (1978) however found that mice and rats failed to demonstrate the neurological symptoms of SCD when subjected to an atmosphere containing N$_2$O.

Recently there has been renewed interest in the neurological
complications associated with failures in the methyl-transfer pathway. This followed the suggestions that failures in this pathway might lead to depression (Reynolds et al, 1984) and also the vacuolar myelopathy seen in AIDS (Keating et al, 1991). Recent work on inborn errors of the methyl-transfer pathway has been undertaken by Dr Robert Surtees at the Institute of Child Health, (University of London). It was demonstrated, using micro assay techniques, that disturbances at any stage of the methyl-transfer pathway leads to a decrease in S-adenosylmethionine (SAM) concentrations in cerebro spinal fluid (CSF) and to neurological disease (Surtees et al, 1991). In the course of developing these assay techniques, Surtees administered CL to mice in order to inhibit the synthesis of SAM. It was found that the CSF SAM concentrations fell as the mice became progressively ataxic. A collaborative study was then initiated with the Department of Neuropathology, Institute of Neurology (University of London), to correlate the observed biochemical changes with morphological observations.

The results of this collaborative study were published by Lee (1991) and Lee, Surtees and Duchen (1992). A single dose of CL (2mg/g body weight) was found to induce intramyelinic vacuolation in the white matter of the brain and spinal cord of 21-day-old mice within 12 hours. The intramyelinic vacuolation progressed over the next 6 days, with secondary axonal degeneration also becoming evident. Myelin vacuolation was not present in the peripheral nerves. Axonal lesions were found, however, in the distal portions of motor nerves within 24 hours, progressing later to the degeneration of intramuscular nerve fibres and terminals. CL was also found to reduce brain SAM levels in 21-day-old mice, indicating the methyl-transfer pathway had been inhibited.

Adult mice treated with a single dose of CL (2mg/g) showed little or no myelin vacuolation. Distal axonal degeneration became apparent, however, within 48 hours of the administration of CL.
Aims of the Present Study

The observation that CL induces the degeneration of the motor nerve terminals and intramuscular nerves in mice made by Lee et al (1992) formed the starting point for this study. No reports concerning the effects of CL on the function of peripheral nerve, extrafusal muscle fibres, intrafusal muscle fibres, or neuromuscular junctions have been traced. The literature concerning the involvement of peripheral nerves in subacute combined degeneration (SCD) is also contradictory. Very little appears to be known about the distribution and pathogenesis of the lesions in the peripheral nervous system in Vitamin B₁₂ deficiency a factor noted by Schaumburg et al 1983, Victor 1984 and Duchen and Jacobs 1992. This study was therefore initiated to follow the development of the lesions induced by CL in the peripheral nervous system of mice. The information obtained is also likely to be relevant to other disorders caused by failures of the methyltransfer pathway (such as SCD) and other conditions were degeneration of the motor nerve terminals occurs.

The use of a combination of physiological and morphological techniques should hopefully allow a close understanding of the mechanisms underlying the pathology induced by CL to be obtained. The intracellular physiological techniques adopted for this study enabled a large number of end-plates (40) to be sampled from each muscle, which would not be possible using morphological techniques. Significant alterations in neuromuscular function may occur in the absence of morphological changes (Cangiano et al, 1980) therefore physiological observations, can permit the detection of abnormalities which might precede structural change.
CHAPTER 1: PART B.

LITERATURE REVIEW

This review is divided into three sections. The first section (1B1) relates to the physical and pharmacological effects of cycloleucine. The second section (1B2) is a review of the causes and effects of human neurological diseases, thought to be related to failures in the Vitamin $B_{12}$-dependent methyl-transfer reaction. In the third section (1B3) animal models of Vitamin $B_{12}$ deficiency are discussed.

1B1. CYCLOLEUCINE

1B1.1 Physical and chemical properties of cycloleucine

Aminocyclopentane-1-Carboxylic acid or cycloleucine (henceforth referred to as CL) is a synthetic amino acid first synthesized by Zelinski and Stainikov (1912) using the hydantoin synthesis method (cited by Ross et al., 1961).

In its pure form CL (Fig 1.1) is a white crystalline solid with a melting point of 328-329°C (Zelinski and Stainikov, 1912). It has a sweet taste and is soluble in water, approximately 5g can be dissolved in 0.1 litres of water (Aust and Roux, 1965). The chromatographic properties of CL are vastly different from naturally occurring amino acids. Leucine has the closest RF value but is still readily distinguishable from CL (Ross et al., 1961).

\[
\begin{align*}
\text{H} & - \text{C} - \text{COOH} \\
\text{H-C} & - \text{NH2} \\
\text{H} & - \text{C} - \text{NH2}
\end{align*}
\]

Fig. 1.1 The structure of cycloleucine (1-aminocyclopentane-1-carboxylic acid)
(i) The metabolism and tissue transport mechanisms of CL

CL was initially reported to be unmetabolisable by Christenson and Jones (1962). They found that CL had a very low urinary excretion rate, only 0.28% of the C\(^{14}\) labelled CL being excreted in the urine of mice during the first six hours following its administration. This result indicates that CL is reabsorbed against a concentration gradient in the kidney. CL was found to share the same tissue transport mechanism as the large neutral metabolisable amino acids such as isoleucine, leucine, valine, phenylalanine, tryptophan, methionine and tyrosine (Sterling and Henderson, 1963, Blasberg and Lajtha, 1965; Richelson, 1974). Reulius et al (1973) postulated that the renal reabsorption of CL in the proximal tubule also shares the same site and transport system used by the large neutral class of amino acids.

Nixon (1976a) examined the levels of CL accumulated in a variety of murine tissues, and attempted to correlate these findings with behavioural signs of neurotoxicity. It was found that plasma CL levels remained remarkably constant after a 0.4mg/g dose of CL and the plasma CL concentration did not significantly alter for 3 weeks after this single dose of CL. This demonstrated that CL was both reabsorbed against a concentration gradient in the kidney and was resistant to metabolic anabolism or catabolism. The renal reabsorption of CL, however, did appear to saturate when mice were given 2-3mg/g of CL. High peaks of urinary CL excretion were found at 0-6 and 9-20 hours post injection and occurred concomitantly with raised plasma CL concentrations. The pancreas showed by far the greatest accumulation of CL, a factor also shown by Berlinguet et al (1962). The brain and spinal cord were not found to accumulate CL, with the CL levels in the CNS being equivalent to that of the plasma. This is consistent with the finding of Richelson et al (1974) who demonstrated that large neutral amino acids enter the CNS by facilitated diffusion.

Perhaps the most interesting factor reported by Nixon (1976a) concerned CL concentrations in the liver. Hepatic CL concentrations
showed considerable variation with time, a factor not seen in other organs. Changes in hepatic CL levels were also found to correlate with behavioural signs of neurotoxicity. Hepatic toxicity was, however, believed to be secondary to the neurotoxicity. Variations in the hepatic CL concentrations were believed to be caused by the liver acting as a buffer, storing amino acids when plasma circulatory levels are high and releasing them when plasma amino acid concentrations fall.

(ii) The effects of other amino acids on the renal clearance of CL

It can be seen from the preceding section that CL shares a common tissue transport and renal reabsorption site with the large neutral class of amino acids. This factor is believed to account for some pharmacological affects of CL.

The circulatory levels of large neutral amino acids would therefore be expected to modulate the circulatory levels of CL and hence effect the pharmacological and neurotoxicological properties of CL. This was confirmed experimentally by Machlin et al (1963) who found that valine acted as a prophylactic to the neurotoxic effects of CL in developing chickens. The immunosuppressant action of CL was later demonstrated to be reversed by L-valine (Rosenthal et al, 1972).

Ruelius et al (1973) explored the basis of the antagonism between L-valine and CL. Large and frequent doses of valine (2mg/g/hour for 7 hours) were administered to mice pretreated with CL (2mg/g). The plasma and tissue concentrations, and the excretion rate of CL were then monitored for a 24 hour period. L-valine caused a marked increase in the excretion of CL in the urine with a concomitant fall in plasma and tissue CL concentrations. In addition to L-valine two other large branched neutral amino acids, L-leucine and L-isoleucine also increased the excretion of CL.

The precise mechanisms by which these large neutral amino acids increase the renal excretion of CL was not determined. Reulius et al (1973) believed that the most plausible explanation of their results was based on
a valine-CL interaction in the kidney tubule. High concentrations of valine such as those resulting from massive frequent doses would be expected to inhibit renal reabsorption of CL if these two amino acids interact at a common reabsorption site in the proximal tubule. Nixon (1974) found that the L-isomers of other large neutral amino acids such as tryptophan and phenylalanine also reverse the toxicity induced by CL. This effect was presumably also caused by these amino acids increasing the urinary excretion of CL. The excretion of CL was not monitored however.

Nixon et al (1973) suggested that valine could be used to adjust the CL plasma/tissue concentrations in experimental animals, as a way of reversing neurological symptoms. This should be possible at any stage of CL induced neurotoxicity and would yield valuable information concerning myelin and axonal regenerative processes.

(iii) CL ability to disrupt the Vitamin B₁₂ dependant methyl-transfer reaction

Lombardini et al (1970) published the results of a study aimed at developing selective and specific inhibitors of the methionine adenosyltransferase reaction. This was achieved by elucidating the steric, electrostatic and conformational features of methionine analogues necessary for their function as substrates or inhibitors. Partially purified transferase preparations obtained from bakers yeast *Escherichia coli* and rat liver were used in these tests. CL was found to be a powerful inhibitor of ATP:L-methionine adenosyltransferase. Lombardini et al (1970) expressed surprise at this finding since CL lacks a region of electronegativity and has no alkyl group corresponding in space to the S-methyl group of methionine. It was therefore postulated that the rigid cyclic structure of CL, enabling strong Van der Waals bonding to occur, compensates for this lack. This postulation is substantiated by the finding that ring size is critical to the inhibition of adenosyltransferase.
Fig. 1.2 The Vitamin $B_{12}$ /folate dependent methyl-transfer reaction. THF = tetrahydrafolate, $B_{12}$ = cobalamin. Cycloleucine prevents the formation of S-adenosylmethionine and hence methylation.

1B1.2 The use of CL to produce an animal model of subacute combined degeneration

(i) The similarity in the pathology induced by CL in animals and subacute combined degeneration in man

The finding that CL can produce a neuropathology in laboratory animals that is remarkably similar to that seen in subacute combined degeneration in man, was accidental. Jacobson et al (1973) were using CL (8-40mg per adult mouse) with the intention of disrupting the methylation
of phosphatidylethanolamine and hence the formation of phosphatidylethanolamine (lecithin), thus inhibiting the formation of pulmonary surfactant (dipalmitoyllecithin). It was hoped this would produce a model of hyaline lung disease in prematurely delivered young mice (Jacobson and Gandy, 1979). Instead, the experimental mice appeared to develop a neurological condition, becoming progressively less active with what was described as 'impaired proprioception', i.e. their toes failed to grip a wire grid when they were held on an inclined surface. Later the animals became ataxic and motor strength decreased. The hind limbs were affected before the fore limbs (Gandy et al, 1973). The condition was found to be dose dependent and to progress uniformly, irrespective of sex or strain. Death occurred in all animals between one and seven weeks after the administration of CL. The pathology exhibited by the CL treated mice was found to be similar to that seen in human subacute combined degeneration, for the following reasons.

1). The myelin sheaths of the thickly myelinated fibres in the spinal cord degenerate and the nerve fibres show axonal swelling.
2). Cellular and glial infiltration are not seen.
3). The cell bodies remain intact.
4). The spinal cord lesions show a patchy, though frequently symmetrical, distribution.
5). Lesions were found scattered throughout the medulla, cerebellum, brainstem and cerebral hemispheres.

Jacobson et al (1973) therefore proposed that a large single dose of CL could be used to produce an experimental animal model for subacute combined degeneration since the condition which they observed in their mice had both the characteristic symptoms and pathology of S.C.D. (see later section).

Jacobson et al (1973) believed that S.C.D. caused by Vitamin B_{12} deficiency and the CL induced condition shared the same causal mechanism. It was thought that these conditions both resulted from the inhibition of the methyl-transfer pathway (see Figure 1.2). Vitamin B_{12} is required for the
methylation of homocysteine to methionine whereas CL inhibits the formation of S-adenosylmethionine (SAM) which is a methyl donor for numerous vital biochemical reactions (see Baldessarini, 1987 for review).

(ii) Experiments aimed at determining the biochemical basis of the pathology induced by CL

Nixon, Jacobson and Sidman (1973) provided additional data on the biochemical changes induced by CL in mice showing signs of neurotoxicity. It was found that CL inhibited the methylation of ethanolamine to choline in the kidney, spleen and brain when either 14C-ethanolamine or 14C L-methionine were used as substrates. The brains of clinically affected CL treated mice were found to contain significantly reduced levels of phosphatidylcholine (PC) while phosphatidylethanolamine (PE), phosphatidylserine, inositol, sphingomyelin, total galactolipid and ganglioside levels were unchanged. It was suggested that transmethylation reactions such as the conversion of PE to PC are essential for the maintenance of myelin and hence the inhibition of transmethylation might lead to the breakdown of myelin.

Later, Nixon (1974) described a number of novel observations. He showed that mice treated with more than 1mg/g of CL demonstrated a dramatic fall in body temperature. Body temperatures of 28°C were recorded in mice 3 days post CL treatment whilst mice showing severe signs of toxicity eight days after the administration of CL had body temperatures as low as 26°C (control body temp = 37°C). When CL-treated mice were kept at a constant 35°C, a profound exacerbation of the neurological effects was seen. It was therefore concluded that reducing the body temperature was a protective mechanism. Exactly how reducing the body temperature might alleviate or delay the CL induced neurotoxicity was not discussed. Nixon was also the first to describe the degeneration of peripheral nerves in mice given 2mg/g of CL. This degeneration was most prominent in distal sections of the sciatic nerve and was not encountered in proximal regions.
of the sciatic nerve.

In a further study (Nixon, 1976b) it was demonstrated that mice treated with 2mg/g of CL showed the pathology outlined above and also a concomitant reduction in the protein content of the CNS, particularly at the cervical spinal cord and cerebellum (the two areas which showed the most advanced pathology). The protein levels in the sciatic nerve were also reduced, in the CL-treated mice. The most striking alteration, however, was in sulfatide concentrations. Reduced levels of sulfatide were found in the sciatic nerves and in the spinal cord but not in the brain. Sulfatide has been used as a marker for the myelin sheath (Matthieu et al, 1973) and therefore a reduction in its synthesis was suggested as a possible reason for myelin degradation.

The effect of CL on organotypic cerebellar cultures at various developmental stages were studied by Nixon, Suva and Wolf (1976). CL at concentrations comparable to those used to produce neurotoxicity in vivo, induced degeneration of myelinated axons but had little effect on the perikaryon in vitro. Mature cultures consisting mainly of unmyelinated fibres were less susceptible to the effects of CL than developing cultures in which newly myelinated/myelinating neurons were particularly adversely affected. It was the myelin sheath as opposed to the axons which appeared to be particularly vulnerable to the effects of CL. Nixon et al (1976) also demonstrated that raising the concentration of other amino acids in the culture medium antagonized the neurotoxic effects of CL. This finding therefore supports the proposition that CL competes with large neutral amino acids and in this way can inhibit protein synthesis.

The in vivo effects of CL on the developing nervous system were examined by Ramsey and Fischer (1978). CL (3-400mg/kg) was administered to 4, 7 and 22-day-old Wistar rats. Electron microscopic examination of the spinal cords of CL treated rats, revealed that animals receiving an initial dose of CL at 7 days showed a sporadic myelinopathy. This initially appeared as splitting of the myelin laminations at the intraperiod lines. The subsequent expansion of these lesions led to the formation of
large interaxonal pools. Animals given their initial dose of CL at 4 and 22
days of age did not show this pathology. This suggests that the age of the
experimental animal to which CL is administered is critical. CNS lesions only
appear to occur when the nervous system is rapidly undergoing
development. The phospholipid content and fatty acid composition of the
brain and spinal cord composition were also monitored in this study. A
decrease in plasmamylethanolamine (ethanolamide plasmagen) and its
derivative phosphatidylethanolamine (PE) was found in all the CL treated
animals. This decrease, however, was less pronounced in the older animals.
This finding contradicts that of Nixon et al (1973) who found PE levels were
unchanged by CL whereas phosphotidylcholine levels decreased.

Ramsey and Fischer (1978) also reported a considerable change in the
fatty acid composition of PE induced by CL. A general decrease in long
chain \((C_{20-22})\) fatty acid content and an increase in saturated fatty acids was
also described. A reduction in PE has also been detected in the brains of
both monkeys (Ansell and Spanner, 1968) and humans (Dayan and Ramsay,
1974) suffering from long term Vitamin \(B_{12}\) deficiency. Exactly how a PE
deficit might have lead to the ultrastructural alterations is not mentioned
however.

Crang and Jacobson (1980) put forward the idea that multiple, well
spaced, sub-lethal (0.3mg/g) doses of CL would produce a condition more
closely resembling human SCD than that seen when a single lethal dose (1-
2mg/g) was administered. Expression of signs of neurotoxicity occurred
more slowly with this multiple, sub-lethal, dose regime. The
histopathological lesions that eventually became evident were very similar,
however, to those they had previously reported in the acute studies (Gandy
et al, 1973 - see earlier section). As expected, the administration of folic
acid or Vitamin \(B_{12}\) failed to prevent the occurrence of neurological signs in
the CL treated animals. This is because CL blocks the conversion of
methionine to S-adenosylmethione, not homocysteine to methionine, the
latter being the reaction inhibited by reduced folate or Vitamin \(B_{12}\) (see
Figure 1.2).
Despite a number of studies being carried out in which biochemical and neuropathological changes induced by CL were characterised, the mechanisms of causation remained uncertain until the early 1980s when the results of two investigations provided experimental evidence in support of what was termed the ‘protein methylation hypothesis’.

1B1.3 The protein methylation hypothesis

The protein methylation hypothesis originated in 1971 when Baldwin and Carnegie found that myelin basic protein (MBP) was specifically methylated at just one of its 19 arginine residues to form firstly monomethyl arginine and then dimethylarginine. MBP makes up approximately 30% of the total myelin protein (Mehl and Harris, 1970) and it has attracted considerable research interest by virtue of its ability to produce experimental allergic encephalomyelitis, an experimental model of multiple sclerosis (MS) (Kies, Murphy and Alvord, 1960). MBP has also been proposed as important in the cross-linking of myelin lamellae, thus acting to stabilize the myelin sheath (Smith 1977).

Baldwin and Carnegie (1971) found that the methylation of MBP was accomplished using S-adenosyl methionine (SAM) as the methyl donor and was thus Vitamin B$_{12}$ dependent. It was proposed that the methylation of the arginine residue of MBP might aid the insertion of MBP into the non-polar hydrophobic myelin environment, the addition of methyl groups increasing the hydrophobicity of the arginine residue. It was speculated therefore, that incomplete methylation of the MBP, which might occur in Vitamin B$_{12}$ deficiency, could lead to ‘malformed myelin’. The ‘spongy myelin’ seen in SCD was predicted to be deficient in dimethylarginine.

This hypothesis was not referred to in any of the literature concerning the use of CL to produce an animal model for SCD published prior to 1980. This changed when Crang and Jacobson (1980) reported that CL suppresses the methylation of MBP *in vitro*. The incorporation of $^{14}$CH$_3$ from labelled methionine or SAM into MBP was monitored. CL was found to inhibit the
incorporation of $^{14}$CH$_3$ from methionine, but not from SAM, into MBP. The use of SAM by-passes the block in the methyl-transfer pathway induced by CL (see Fig. 1.2) and therefore acts as a control.

Further experimental evidence for this hypothesis was provided by Small et al (1981) and Small and Carnegie (1981) who administered CL to 2-day-old chickens. The birds developed considerable weakness and ataxia within 5 days. Ultra-structural changes, notably large vacuoles in the myelinated fibres of the brain were also seen. This pathology was found to correlate with a 50% reduction in brain MBP levels and a 50% reduction in the methylation of arginine in all myelin proteins in the CL treated chickens. In a review article Small and Carnegie (1981) summarised the experimental evidence implicating the protein methylation hypothesis. They suggested that the hypothesis might explain the difficulties that are frequently encountered inducing a neurological condition similar to SCO in rats, this being in direct contrast to the situation with non-human primates. A far smaller proportion of MBP contains dimethylarginine in rats than in humans (Martenson, 1980). Human/primate MBP therefore has a higher requirement for labile methyl groups and hence is affected to a greater extent by the inhibition of the methyl-transfer pathway. CL has, however, been shown to reduce the levels of methylated arginine from 8-9% to 5-7% in adult rats (Deacon et al, 1986). Unfortunately Deacon et al did not correlate this fall in methylation with clinical or pathological findings.

The reports concerning the neurochemical and morphological changes induced by CL were for the most part published more than ten years ago. Recently, however, there has been renewed interest in the neurological problems caused by failures in the methyl-transfer pathway. This renewed interest is exemplified by the work of Dr Robert Surtees at the Institute of Child Health, University of London. Surtees developed micro assay techniques for measuring the CSF concentrations of SAM and other metabolites of the methyl-transfer pathway. In the course of developing these assay techniques, Surtees administered CL to mice to inhibit the synthesis of SAM. The mice developed a progressive paralysis and ataxia
which was found to occur concomitantly with a reduction in the SAM content of the spinal cord. A collaborative study was then begun with the Department of Neuropathology, Institute of Neurology, where the structural and ultrastructural changes induced in both developing and adult mice were to be re-evaluated, hopefully permitting the biochemical and structural changes to be correlated.

The findings from these studies were initially reported by Lee (1991) and subsequently by Lee, Surtees and Duchen (1992). When 21-day-old mice were treated with a single dose of CL (2mg/g body weight) signs of toxicity developed within 24 hours and correlated with a 40% fall in the SAM content of the brain and spinal cord. Morphological abnormalities observed at this time consisted of intramyelinic vacuolation of the white matter of the brain and cord (see Fig. 1.4) and axonal lesions in the distal portions of the motor nerves of the tail and hind limbs (see Fig 1.6). Over the next 2-3 days the paralysis of the hind limbs progressed and the general condition of the mice worsened. The brain and spinal cord SAM concentration continued to fall but at a much slower rate. The inability of the CL-treated mice to convert methionine to S-adenosylmethionine (see Fig. 1.2) was further demonstrated by the finding that the methionine concentrations of the brains of the CL-treated mice had increased to almost 10 times the mean control level. The pathology had also progressed at this time. All the end-plates visualised in the more distal muscles of the hind limbs were denervated and degenerating fibres were occasionally observed in the tibial and sciatic nerves (see Fig. 1.6). In stark contrast to the motor innervation, the sensory fibres and muscle spindles appeared intact. The motor innervation of the forelimbs also appeared unaltered. The intramyelinic vacuolation of the white matter of the brain and spinal cord became both more widespread and severe.

The adult mice used in these experiments also received 2mg/g of CL. The signs of toxicity noted above, developed far more slowly in the adult mice with some surviving for up to 2 weeks. Differences in the pathology between the 21-day-old and adult mice were also seen. Notably, very little
intramyelinic vacuolation was seen in the white matter of the spinal cords and brains of adult mice. The predominantly motor distal axonopathy seen in the young mice was also found to occur in the adults. The axonopathy also developed more slowly in the adults. The vacuolar myelinopathy seen in the young mice was believed to occur in response to the inhibition of methylation of myelin basic protein (MBP), thus causing abnormalities in the formation and stabilization of the myelin sheath. The MBP found in the adult mice was believed to have been methylated prior to the administration of CL, therefore explaining the absence of a vacuolar myelinopathy in the adult mice.
Fig. 1.3 Electron micrograph of white matter of spinal cord from a normal 21-day-old mouse showing an oligodendrocyte (O) whose cytoplasm lies in close contact with many myelinated axons. There is some artefactual splitting of myelin because of the problems in obtaining perfect fixation of white matter in the CNS. (Neg. 90521 x 15,000. Bar = 2\(\mu\)m).
Fig. 1.4 EM of cerebellar white matter from a 21-day-old mouse 4 days post CL. There is severe vacuolation present, each vacuole being intramyelinic, the result of splitting at an intraperiod line. This vacuolation is typical of that seen throughout the CNS in young mice, but not in adult mice and not in peripheral nerve myelin. (Neg. 97601.4 x 6,000. Bar = 5µm).
Fig. 1.5 EM of transverse section of sciatic nerve from a normal 21-day-old mouse. Note the appearance of normal myelinated and non-myelinated axons (Neg. 97714.4 x 10,000. Bar = 3μm).
Fig. 1.6 EM of sciatic nerve at 4 days post CL. Several nerve fibres show degeneration of axon and collapse and fragmentation of myelin sheaths. This was a late finding in CL toxicity and may be due to several factors including the degeneration of some sensory ganglion cells as well as to an axonopathy of motor fibres. (Neg. 97537.4 x 6,000. Bar = 3\mu m).
**1B1.4 Pharmacological properties of CL**

CL has been demonstrated to possess a number of other interesting pharmacological properties. These include:

(i) Anti-tumoral effects;
(ii) Immunosuppressive properties;
(iii) Anti-malarial properties;
(iv) Antagonism of glycine at the N-methyl-D-aspartate receptor complex.

The reports concerning these effects are frequently very detailed and provide useful information regarding the biochemical properties of CL.

(i) CL use in chemotherapy

Interest in the pharmacological properties of cycloleucine dates back to the late 1950’s and early 1960’s when two independent groups simultaneously became interested in the possible anti-tumoral effects of cycloaliphatic amino acids such as CL. Noll (see Ross et al, 1961) submitted CL to the Cancer Chemotherapy National Service Center in the USA so that CL could be evaluated for possible anti-tumoral effects. CL was given the accession number NSC1026 (NSC referring to the National Service Centre). In the UK, Connors et al (1960) synthesized CL at the Chester Beatty Research Institute in London, where it was also to be tested as a potential anticancer agent. The Chester Beatty group gave CL the accession number CB1639 (Ross et al, 1961). In subsequent work CL was occasionally referred to by these accession numbers alone.

The Chester Beatty group evaluated the antitumoral activity of CL using the Walker rat carcinoma 256, the transplantable mouse sarcoma 180 and transplantable mammary tumours in C-Bagg albino mice. CL and its peptide and ester derivatives inhibited the growth of all of these tumours. Marked tumour inhibition, however, was seen only at CL doses which caused anorexia and a concomitant weight loss in the test animals. Connors
et al (1960) believed that the chemotherapeutic effects of CL could not be attributable solely to its toxicity and impaired food intake, since compounds such as cyclopentylanine were found to be considerably more toxic than CL but did not inhibit tumour growth.

The National Service Centre’s primary screening programme involved the use of the mouse sarcoma 180, carcinoma 755 and leukaemia 1210 tumours. CL was found to be most effective at inhibiting the growth of the carcinoma 755 without inducing weight loss (Ross et al, 1961).

Encouraged by the initial success of these studies, the Cancer Chemotherapy National Service Centre instigated wide ranging toxicity tests on a variety of laboratory animals prior to the initiation of clinical trials. A variety of dosing regimes were used to determine the LD$_{50}$ (the dose required to kill 50% of test animals), MLD (minimum lethal dose) and long-term side effects of CL. The LD$_{50}$ was found to be 290mg/kg for rats, 309mg/kg for mice, 140mg/kg for guinea pigs, and 300mg/kg for dogs. The symptoms of CL toxicity were found to include anorexia, diarrhoea, severe malaise and death 7-21 days post treatment. Histopathological studies carried out on mice and rats receiving CL doses in excess of the LD$_{50}$ revealed myocardial degeneration, fatty degeneration of the liver, renal tubular dilatation, atrophy of the pancreas and bone marrow and slight demyelination of the nervous system. Repeated dose toxicity tests were undertaken using rats, monkeys and dogs. CL was found to have a cumulative effect. The dogs and rats eventually developed toxic reactions, and pathological changes identical to those outlined above. CL, however, was found to be well tolerated by rhesus monkeys *Macaco mulatto* with only one exception, monkeys receiving 120mg/kg of CL per day failed to exhibit toxic symptoms. It was not until this dose was doubled that monkeys showed toxic reactions and eventually died.

Following the initial success of the tumour screening experiments, and the finding that CL was well tolerated by non-human primates, clinical trials were deemed to be both worthwhile and relatively safe. The first clinical trial was organised by the Cancer Chemotherapy National Service Centre in
1958. Seventy one patients suffering from a range of far advanced malignancies were treated with CL at doses ranging from 10-150mg/kg/day for a 5-10 day period. The vast majority of patients showed no beneficial response to this treatment. In only one patient (1.4% of the group) suffering from a malignant melanoma was there both objective and subjective signs of improvement. Toxic side effects such as anorexia, nausea and vomiting were observed in some patients receiving doses of ≥ 60mg/kg/day.

Later, Benefiel et al (1960), using a cohort comprising of only 3 subjects, found that CL appeared to control the spread of multiple myelomas. This prompted Bergsagel (1962) to conduct a further trial employing eleven patients who were suffering from multiple myelomas. The patients were given CL at 80mg/kg/day for ten days. Improvement was not seen in any of the patients that received CL. The only beneficial effect was marked pain relief in 3 of the 5 patients who had described this symptom prior to the trial. CL toxicity was observed in 9 out of the 11 patients, again in the form of anorexia and weight loss, together with a fall in the level of serum proteins, leucopenia, thrombocytopenia and anaemia.

Aust and Roux (1965) published the results of a study aimed at determining an effective CL dose regime for treating cancer. Dosages ranging from 60-350 mg/kg/day for 10 days were used to treat 47 patients with cancer. Subjective improvement, i.e. weight gain or pain relief, were noted in 7 of the 47 patients with six of these seven having received more than 200mg/kg/day of CL. Gastrointestinal and haematological toxic effects identical to those previously noted were frequently observed. However, neurological side effects that had not previously been recorded were also encountered. These included vertigo, confusion and peripheral neuropathy. Aust and Roux considered that the subjective improvement observed in patients receiving large doses of CL warranted further investigations using patients suffering with measurable tumours despite the wide range of side effects observed.

The results of such a study were published by Aust et al in 1970.
One hundred and forty seven patients with tumours measurable with callipers or from X-rays were treated with 300mg/kg/day of CL for 8 days. Again, neurological symptoms of toxicity such as delirium, confusion, vertigo, numbness, shock, tingling and even convulsive seizures were noted in 57% of patients. The low response rate observed in this study led Aust et al to suggest that CL was not an effective chemotherapeutic agent for most forms of malignancy. The only malignancy demonstrated to show anything like a consistent response were leiomyosarcomas. No further reports on the use of CL to treat any form of malignancy have been traced. It seems, therefore, that the severity and high incidence of neurological side effects accompanied by a low response rate reported in this study led to the cessation of CL use in chemotherapy. CL is, however, still occasionally used to monitor the kinetics of the transport of amino acids into tumours (Fujiwara et al, 1989).

Studies aimed at determining the biochemical basis for the ability of CL to prevent the growth of certain tumours were initially undertaken by Berlinguet et al (1962). Using CL and valine labelled with C\(^{14}\) Berlinguet et al demonstrated that CL inhibited the incorporation of valine but not uridine into proteins in a concentration dependent manner. The largest inhibition was produced by injecting CL 48 hours prior to the C\(^{14}\)-valine. This effect was found to occur in the homogenate of liver from both control and tumour carrying rats and in the tumours themselves. It was therefore suggested that CL exerts its effect on tumours by preventing the incorporation of natural amino acids into proteins, without itself becoming incorporated, thereby suppressing protein synthesis.

Sterling and Henderson (1963) expanded these observations using ascites tumour cells in vitro. CL was again demonstrated to inhibit the incorporation of other amino acids into proteins. It was also found that CL entered, and was concentrated in, neoplastic cells by an active transport mechanism utilized by some naturally occurring amino acids. This led Sterling and Henderson to suggest that high concentrations of CL prevented other amino acids entering the neoplastic cells and thereby disrupting protein
synthesis by an indirect mechanism.

Lombardini and Talalay (1973) postulated another method by which CL might inhibit tumour growth. They suggested that CL might inhibit adenosyltransferase and hence the methyl-transfer pathway. Support for this theory was provided by two pieces of evidence. Firstly, the adenosyltransferase activity levels were low (5-10% of those found in the liver) in the Walker 256 and Lewis lung tumours and the B-16 melanoma. This suggests that the methyl-transfer pathway is likely to be disrupted in tumours at a lower CL concentration than would be the case in other tissues. Secondly, both the antitumour activity and the inhibition of adenosyltransferase were strictly dependent on the carbon ring size, compounds containing rings larger than CL having no effect on tumours (Ross et al, 1961) or adenosyltransferase (Lombardini et al, 1970).

(ii) Immunosuppression caused by CL

Frish (1969) was the first to demonstrate that CL could inhibit antibody synthesis. Swiss mice pretreated with CL showed reduced synthesis of haemolysins and of haemagglutonins in response to the administration of sheep erythrocytes. This immunosuppression was caused by CL reducing the number of plaque forming cells in the splenic pulp. Unlike other immunosuppressants such as S-mercaptopurine, thioguanine and methotrexate, which have the greatest effect when administered 18-48 hours after the antigen, CL induced maximal suppression when it was given prior to the antigen. Therefore, CL was believed to act on the preinduction phase in contrast to the aforementioned drugs which interfere with the induction phase of humoral antibody synthesis. CL had no effect on the secondary antibody response induced by a second injection of sheep erythrocytes fourteen days after the first.

Brambilla et al (1972) using a similar experimental protocol, again demonstrated that CL caused immunosuppression. Administration of CL at any time from 21 days before to 1 day after a treatment with the antigen
induced immunosuppression, with the greatest effect being achieved if mice received CL one day prior to receipt of the antigen. Further experiments using allografts demonstrated that CL could prolong the survival time of skin grafts. The greatest delay in rejection was also produced when CL was administered 1 day prior to the skin graft. A low dose of CL (0.14mg/kg) was used in these studies and hence neurotoxicity was not observed in the mice.

(iii) Anti-malarial properties of CL

The search for novel antimalarial compounds has many parallels with the search for compounds which inhibit or prevent tumour growth. A large variety of compounds have been evaluated for either or both of these activities. It is not surprising therefore that CL has been evaluated for antimalarial properties and given a further accession number WR14997 by the Walter Reed Army Institute for Research. Aviado and Reutter (1969) found that CL (10-100mg/kg) suppressed parasitemia and prolonged the survival time of mice infected with chloroquine-sensitive and, more importantly, chloroquine resistant *Plasmodium berghei*. At the time CL was one of the least toxic anti-malarial compounds under evaluation. CL was found not to cause cardiovascular depression in contrast to other antimalarial agents such as quinine, chloroquine and pyrimethane.

Chang et al (1990) found cycloleucine inhibited the formation of spermidine (which was used as a marker of polyamine synthesis) in red blood cells infected with both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium berghei*. These effects were reversed by the co-administration of methionine which implies that CL inhibits the methyl-transfer pathway in the sporozites within the red blood cells and hence prevents the synthesis of polyamines. The inhibition of polyamine synthesis in *Plasmodium berghei* might account for the suppression of parasitemia noted by Aviado and Reutter (1969).
(iv) The effects of CL on the N-methyl-D-aspartate receptor complex.

CL has been found to act as a competitive antagonist at the glycine modulatory site on the N-methyl-D-aspartate (NMDA) receptor complex (Hershkowitz and Rogawski, 1989). Glycine has previously been shown to potentiate the effects of NMDA (Johnson and Ascher, 1987). Therefore, CL causes a depression of the response of the L-glutamate receptor to NMDA in the presence of glycine. It is unlikely that this effect would lead to pathological changes as Watson and Lanthorn (1990) found CL to be a very weak glycine antagonist. Furthermore, CL was not found to have a prolonged effect on associative learning, a process believed to involve the NMDA receptor complex (Herberg and Rose, 1990).

1B2 HUMAN DISEASES CAUSED BY FAILURES IN THE METHYL-TRANSFER PATHWAY

The methyl-transfer pathway requires both Vitamin B<sub>12</sub> and folate. Failures in transmethylation can therefore occur as a result of Vitamin B<sub>12</sub> or folate deficiency. The second section of this review is concerned with the causation and pathophysiological consequences of such deficiencies.

1B2.1 Vitamin B<sub>12</sub> deficiency

(i) Neurological effects

Leichtenstern (1884) was first to associate a disease of the spinal cord with pernicious anaemia. Unfortunately, however, the anaemia was mistakenly interpreted as being secondary to tabes dorsalis, a parenchymatous syphilitic disease of the cord. Lichtheim (1887) later recognised that the pathology of the spinal cord associated with pernicious anaemia was in fact dissimilar to that associated with tabes dorsalis. The
pathology associated with tabes dorsalis is leptomeningeal thickening, degeneration of dorsal roots and posterior columns of the cord, especially in the lower thoracic and lumbosacral regions (Reid and Fallon, 1992). The lesions found in patients suffering from pernicious anaemia were different in the following respects.

1). Their development was sub-acute;
2). They involved the anterolateral and dorsal tracts of the cord and lipophages were frequently observed in the degenerating tracts;
3). The lumbosacral regions were only slightly affected.

One of Lichtheim's pupils, Minnich (1892), further demonstrated the link between pernicious anaemia and subacute lesions of the cord. Subclinical slight degeneration of posterior and lateral columns were found in five patients who had succumbed to pernicious anaemia.

Russell, Batten and Collier (1900) published a thorough clinico-pathological description of nine patients who were suffering from this condition. Designating it as a distinct morbid entity, they named the disease 'subacute combined degeneration of the spinal cord' for the following reasons. Firstly, combined degeneration of the spinal cord implies 'tracts of different function are affected concomitantly'. Secondly, the term 'subacute' was used to exclude other terms that were commonly in use at that time to describe other diseases affecting the cord, e.g. 'Friedreich's Ataxia', 'Ataxic paraplegia', 'Cerebellar Hereto-Ataxy' and 'General Paralysis of the Insane'. Russell et al were not completely satisfied with this name, however, for many different diseases are characterized by combined degeneration. The term 'subacute combined degeneration of the spinal cord' also does not indicate an association with pernicious anaemia nor the involvement of the brain and peripheral nerves (Duchen and Jacobs, 1992). Despite this it has remained in constant use and has not been superseded by a term which encompasses all aspects of the disease.

(ii) Clinical features

The following clinical features were outlined by Russell et al (1900)
following their examination of nine patients diagnosed as suffering from the condition. Correct diagnosis was subsequently confirmed by post mortem examination in seven of these patients. The course of the condition was clearly divisible into three stages which were separated by abrupt transitions and marked changes in symptoms. The stages were summarized as follows:

1). Spastic paraplegia with slight ataxia and marked paraparesis, e.g. numbness, tingling or stiffness, which may precede motor symptoms. The lower limbs usually being affected before the upper limbs.

2). Severe ataxic paraplegia which may develop very rapidly and can be associated with marked anaesthesia of the legs and trunk.

3). The terminal stage of complete flaccid paraplegia comprising: complete anaesthesia, absent knee jerks, rapid wasting and loss of faradic excitability of the muscles in the paraplegic region, increase of superficial reflex excitability and loss of sphincter control and oedema of the lower extremities and trunk.

Pernicious anaemia was found in some but not all cases. In some patients their ‘general health and anaemia’ showed considerable improvements. The neurological symptoms, however, did not show any signs of improvement or remission before death. The neurological symptoms of Vitamin B$_{12}$ deficiency, encountered in more recent times, are generally less severe and complete recovery is frequently seen following treatment with cyanocobalamin (Healton et al, 1991). Subsequent studies have also identified various mental changes associated with Vitamin B$_{12}$ deficiency ranging from depression to dementia (Duchen and Jacobs, 1992).

(iii) Pathology

a) The Central Nervous System

Following the post-mortem examination of 7 patients clinically diagnosed as suffering from SCD, Russell et al (1900) reported that the disease was characterised by the following pathology. The disease
particularly affected the mid-thoracic segments of the cord where destruction of the white matter might extend around the cord, affecting both the long tracts and exogenous and endogenous fibres. In advanced stages only the fibres adjacent to the grey matter survived. Degeneration was less severe at cervical levels of the cord, particularly in the anterolateral columns. In the upper cervical segments only the posterior columns, notably the funiculus gracilis and spinocerebellar tracts, were demyelinated.

The earliest lesions found by Russell were swellings of the myelin sheaths which initially did not appear to affect the axons. These early lesions usually occurred in the midthoracic region. The swollen myelin sheath became broken down and secondary axonal degeneration, followed by Wallerian degeneration affecting segments above and below the early thoracic lesions, occurred later.

In a review of the pathology subsequently reported to occur in S.C.D., Duchen and Jacobs (1992) described changes which show a remarkably constant distribution. Lesions first occur in the centre of the posterior columns and consist of oval-shaped areas of pallor, a factor first reported by Clarke (1904). Lateral column lesions at the surface of the cord occur concomitantly or slightly later. More frequently, however, small distinct foci of degeneration appear at the surface of the cord or in the lips of the anteromedian fissure. These early lesions of the posterior and anterolateral columns do not have the distribution of tract degeneration and the spinocerebellar and pyramidal tracts may become involved. The brain has also been found to show a characteristic pathology in some cases (Woltman, 1918; Adams and Kubik, 1944; Pant et al, 1968). This consists of small ill-defined, often perivascular, foci of demyelination in the cerebral white matter. The histology and progression of these lesions are similar to those described above for the cord.

b) Pathology of the peripheral nervous system and of muscle

Russell et al (1900) examined various peripheral nerves from patients
succumbing to SCD using either Marchi or Weigert-Pal methods. In most cases the peripheral nerves were normal in appearance or showed only slight insignificant changes. In one case, however, considerable degeneration affecting large diameter nerve fibres, fine intramuscular nerves and the fibres innervating muscle spindles was seen. This degeneration was considered to be a late manifestation of the disease as the patient demonstrating this pathology had a long history of SCD. Greenfield and Carmichael (1935) examined biopsy specimens from the most distal portions of the anterior tibial nerves from four patients suffering from pernicious anaemia. A reduction in the number of large fibres was observed in preparations stained for myelin with osmic acid. However, no pathology was seen when preparations were examined using the Gros-Bielschowsky method. Foster (1945) described a reduction in both the numbers of axons and myelin sheaths and an increase in the number of Schwann cells in the peripheral nerves of patients with pernicious anaemia.

Particularly relevant to the present study are the findings of Coërs and Wolf (1959). Muscle biopsies from 11 patients suffering from Vitamin B$_{12}$ deficiency were found to contain terminal axons with marked axonal swellings, frequent collateral sprouts and endplates with abnormally large and complex subneural apparatus. McCombe and McLeod (1984) studied the pathology of biopsied sural nerves obtained from 3 patients with Vitamin B$_{12}$ deficiency. Nerve conduction velocities were also measured in these patients. Material was studied both prior to and following treatment with Vitamin B$_{12}$. Prior to treatment extensive loss of large myelinated fibres was seen in the sural nerves of two of the three patients. Teased fibre and electron microscopy revealed clear evidence of axonal degeneration as did the electrophysiological studies. Following treatment with Vitamin B$_{12}$ the progression of the neuropathy was arrested. Residual neurological abnormalities, however, remained in all three patients.

The peripheral neuropathy associated with Vitamin B$_{12}$ deficiency is poorly understood. Duchen and Jacobs (1992) and Victor (1984) stated that detailed studies of the affects of Vitamin B$_{12}$ deficiency on the
peripheral nervous system have not been undertaken. This is despite the fact that Vitamin B\textsubscript{12} deficiency is commonly recognised as a cause of peripheral neuropathies (McComas, 1977; Shorvon et al, 1980; Newsholme and Leech, 1983). Shorvon et al (1980) considered peripheral neuropathy to be the most common neurological manifestation of B\textsubscript{12} deficiency. Further evidence for peripheral nerve involvement comes from electrophysiological studies of patients suffering from Vitamin B\textsubscript{12} deficiency (reviewed below) and from animal models of subacute combined degeneration of the cord. The condition of skeletal muscle can also provide information concerning the peripheral nervous system as muscle fibres undergo a series of well characterised structural and physical changes on denervation (reviewed by Gutmann and Zelena, 1962).

Russell et al (1900) found that the extent of muscle fibre pathology varied considerably between their seven post mortem cases although in all of the cases there was some degree of atrophy. In the most extreme cases muscle fibres were markedly atrophic, having lost their striations. Fatty degeneration was seldom seen although increasing amounts of connective tissue were frequently noted. In contrast to the extrafusal fibres the muscle spindles were found to be remarkably well preserved. Denervation atrophy of the leg muscles was reported in two of the 48 cases examined by Pant et al (1968) and was considered indicative of a peripheral neuropathy.

(iv) Neurophysiological effects

Neurophysiological studies have frequently provided evidence for a peripheral involvement in Vitamin B\textsubscript{12} deficiency. Gilliatt et al (1961) found that action potentials recorded from the lateral popliteal nerve were either abnormally small or absent in four patients suffering from SCD. Three patients with particularly small (\(<1\mu\text{V as compared with 5}\mu\text{V controls}) compound action potentials also showed impaired superficial sensation in the foot. In a later study Mayer (1965) attempted to correlate neurological symptoms with abnormal nerve conduction velocities in a group of 53
patients with a previous history of, or existing, Vitamin B\textsubscript{12} deficiency. Those patients who, following treatment, had recovered from Vitamin B\textsubscript{12} deficiency had normal nerve conduction velocities in the median, ulnar, peroneal and posterior tibial nerves. Patients with Vitamin B\textsubscript{12} deficiency occasionally showed slowing of conduction velocity in distal portions of sensory nerves but the proximal sections remained unaffected.

Cox-Klazinga and Endtz (1980) monitored neurological signs and nerve conduction velocities in 40 patients suffering from pernicious anaemia. Twenty had been treated with hydroxocobalamin, the remainder being untreated. Significantly reduced conduction velocities were seen in 13 (65%) of the untreated patients whereas only one (5%) treated patient was affected. It was therefore concluded that peripheral neuropathy in patients with pernicious anaemia was far more common than the literature prior to this study had suggested and that such abnormalities could be ameliorated by treatment with hydroxocobalamin.

A preliminary investigation of the neurophysiological changes in SCD was instigated by Fine and Hallet (1980) and later expanded by Fine et al (1990). The peripheral nerves were found to have normal or slightly reduced conduction velocities, indicating that demyelination was probably not occurring at the periphery. Absent or diminished sensory potentials from the sural and, to a lesser extent, median nerves were recorded in the first study. In the second study however, motor fibres were found to be affected and peroneal motor action potentials diminished. Electromyography (EMG) studies also highlighted fibrillation potentials in distal muscles and other indications of denervation. These results were considered to be characteristic of a primarily distal sensory-motor axonopathy.

Electroencephalograms were also recorded following auditory, visual and mechanical stimulation in patients suffering from SCD. Brain stem auditory evoked potentials (BAERs) were within normal limits, a factor anticipated from previous reports which described the absence of pathology in the pathways conducting auditory information (Pant et al, 1968). Visually evoked responses (VERs) were mildly prolonged, indicative of primary axonal
loss and secondary patchy demyelination in the optic nerves and pathways, which is consistent with the lesions reported by Bickel (1914) and Adams and Kubik (1944). Sensory evoked responses (SERs) were markedly reduced in the vast majority of patients, particularly when the distal extremities were stimulated. This finding is consistent with lesions in the posterior columns of the spinal cord and suggests that the legs and feet are affected before the hands. It was therefore concluded that SERs are the most sensitive neurophysiological index of SCD and thus provide a useful diagnostic aid.

Jones et al. (1987) monitored sensory evoked potentials (SEP) evoked by stimulation of the median nerve in 7 patients with Vitamin B\textsubscript{12} deficiency. In all 7 patients the SEP were abnormal. The abnormalities observed in 6 of the 7 patients were believed to be indicative of lesions within the CNS whereas the remaining patient showed SEP which was thought to be indicative of a distal peripheral neuropathy in the absence of CNS lesions. It should be noted, however, that the more distal portions of peripheral nerves were not examined so it is possible that some of the other patients may also have been suffering from a distal neuropathy affecting only the more distal areas.

1B2.2 Sources of Vitamin B\textsubscript{12}

Vitamin B\textsubscript{12} has not been found in plants, but is known to be synthesized by micro-organisms that occur in water, soil and in the intestines (Newsholme and Leech, 1983). Unfortunately, the Vitamin B\textsubscript{12} that is produced in human intestines occurs at a position that is too distal to permit for absorption to occur. Man is therefore entirely dependent on ingested meat, shell fish, fish, poultry and, to a lesser extent, milk and milk products as a source of Vitamin B\textsubscript{12}.
1B2.3 Causes of Vitamin B\textsubscript{12} deficiencies

(i). Inadequate dietary intake

Vitamin B\textsubscript{12} deficiency caused by inadequate dietary intake alone is very rare, as many foods contain Vitamin B\textsubscript{12} and only small amounts are required daily (Reisner, 1968; Duchen and Jacobs, 1992). Inadequate intake of B\textsubscript{12} has been reported occasionally in ‘vegans’ (Wokes et al, 1955) and also in developing countries where dietary sources of B\textsubscript{12} may be unavailable (Mechta et al, 1964). Jones et al (1987) found that dairy-produce eating vegetarians of Indian or Pakistani extraction, resident within the United Kingdom, also occasionally had abnormally low serum Vitamin B\textsubscript{12} levels.

(ii). Malabsorption

The vast majority of instances of Vitamin B\textsubscript{12} deficiency result from an inability to transfer Vitamin B\textsubscript{12} across the intestinal mucosa (Reisner, 1968; Victor, 1984). The absorption mechanism, unique to Vitamin B\textsubscript{12}, involves the formation of a four molecule complex, two molecules each of Vitamin B\textsubscript{12} and intrinsic factor. This complex is then carried to the ileum where it binds to the surface of the brush border (microvilli) of the absorptive cells. The Vitamin B\textsubscript{12} then breaks from the intrinsic factor and is absorbed. The intrinsic factor is then recycled to allow this process to be repeated (Newsholme and Leech, 1983). Failures in this system occur most frequently in autoimmune gastritis were the production of intrinsic factor is inhibited. Other conditions such as idiopathic and tropical sprue, regional ileitis, coeliac disease, and tuberculous ulceration, gastrointestinal tumours, infestation with the fish tapeworm Diphyllobothrium latum and total or subtotal gastrectomy may also affect the uptake of Vitamin B\textsubscript{12} (Pant et al, 1968; Victor, 1984).
(iii). **Inactivation of B$_{12}$**

A deficiency in active B$_{12}$ has been reported to occur in response to the repeated inhalation of Nitrous oxide (N$_2$O). Nitrous oxide causes the oxidation of reduced B$_{12}$ and renders it inactive (Deacon et al, 1986).

Layzer et al (1978) first described a peripheral neuropathy caused by N$_2$O inhalation. These observations were later extended Layzer (1978) who observed patients who had habitually abused N$_2$O, and had developed symptoms associated with myelopathy of the spinal cord. Layzer therefore concluded that N$_2$O could, by inactivating vitamin B$_{12}$, produce a condition remarkably similar to SCD.

(iv). **Inborn errors of B$_{12}$ metabolism**

Dayan and Ramsay (1974) described a patient with an inborn error of B$_{12}$ metabolism, with a history of megaloblastic anaemia, and with severe retardation of motor and intellectual skills. There was however, no obvious loss of sensation which would have been indicative of neuropathy. Post mortem examination revealed pathology similar to that seen in 'classical' Vitamin B$_{12}$ deficiency. A chemical analysis of the brain showed a relative loss of phosphatidylethanolamine and sphingomyelin and a generalised depletion in the unsaturated fatty acid content of all phospholipids.

Another patient with abnormal methylcobalamine metabolism was studied recently by Surtees et al (1991). The patient presented at 5 months of age with megaloblastic anaemia, developmental delay, and epilepsy. The anaemia responded to treatment with hydroxocobalamin and folinic acid, but the neurological symptoms did not. Magnetic resonance imaging showed areas of reduced signal intensity indicating that demyelination had taken place. The patient was subsequently diagnosed as suffering from methionine synthase deficiency, i.e. homocysteine could not be methylated to form methionine - see Figure 1.2. The patient was given oral L-methionine since methionine bypasses the inborn metabolic block. The
treatment resulted in a pronounced improvement in development, an abatement of the epilepsy and a substantial improvement of magnetic resonance imaging signal.

1B2.4 Biochemical reactions requiring Vitamin B$_{12}$

In order to understand the mechanisms responsible for inducing the pathology seen in Vitamin B$_{12}$ deficiency, a thorough understanding of the biochemical pathways requiring B$_{12}$ is needed. At present just two reactions requiring Vitamin B$_{12}$ have been identified (Stryer, 1981). They are:

1). Rearrangement reactions such as the conversion of L-methylmalonyl CoA into succinyl CoA. This reaction requires the deoxyadenosyl cobalamin form of B$_{12}$ (Newsholme and Leech, 1983).

2). Methylation reactions; methylcobalamin is a crucial coenzyme of the methyl-transfer pathway (see Fig. 1.2).

The pathology seen in Vitamin B$_{12}$ deficiency is likely therefore, to be a consequence of the inhibition of one or both of the above reactions.

Initially the inhibition of the conversion of L-methylmalonyl CoA to succinyl CoA was thought to cause the accumulation of methylmalonyl CoA and its precursor proprionyl-CoA (Cox and White, 1962) which might in turn lead to the incorporation of small amounts of branched and odd chain fatty acids into myelin (Barley et al, 1972).

The incorporation of phospholipids containing these abnormal fatty acids into myelin was suggested as a reason for the myelin degradation. Small and Carnegie (1981) believed that there was no convincing evidence for this hypothesis. Hyland et al (1988), following their study of inborn errors, stated that demyelinating conditions resembling SCD are not seen in patients with inborn errors that only affect the conversion of methylmalonyl CoA into Succinyl CoA.

The methyl-transfer pathway provides the methyl groups for a variety of reactions in the nervous system. It functions in the following manner. Single carbon groups join the pathway as formal groups (CHO) bound to
tetrahydrofolate (THF). They are subsequently reduced in a number of steps to methyl-THF, the methyl group is then transferred to homocysteine by the B₁₂ dependent enzyme methionine synthetase to form methionine. The methionine is then activated by S-adenosylmethionine transferase forming S-adenosylmethionine (SAM), the body's sole methyl donor (Baldessarini, 1987). SAM methylates a large variety of compounds in reactions catalysed by specific methyltransferases forming S-adenosyl homocysteine (SAH). SAH is converted into homocysteine by adenosylhomocysteinase. The homocysteine can then re-enter the cycle (Surtees et al, 1991).

SAM is required for the formation of purine and pyrimidine and hence is involved in DNA synthesis (Darnell et al, 1990). Highly proliferative tissues, such as haemopoietic tissue and the epithelial tissue of the tongue, and the gastrointestinal tract therefore have a large SAM requirement. This factor accounts for the megaloblastic anaemia, sore tongue and diarrhoea frequently observed in B₁₂ and hence SAM deficiency (Newsholme and Leech, 1983).

1B2.5 Methylation in the nervous system

Much recent interest has focused on SAM and the methyltransferase reactions it mediates in neural tissue (reviewed by Smythies, 1984 and Baldessarini, 1987) largely because SAM has been found to act as an antidepressant agent in certain forms of clinical depression (Reynolds et al, 1984; and Cantoni et al, 1989). Godfrey et al (1990) stated that about two thirds of patients with megaloblastic anaemia due to B₁₂ or folate deficiency also have neuropsychiatric complications. A fault in the transmethylation process would therefore appear to cause depression and psychiatric symptoms. Reynolds et al (1984) put forward the idea that faults in the methyl-transfer pathway might affect the turnover of monoamine neurotransmitters, notably dopamine and serotonin. This suggestion provides a link between the methylation and monoamine theories of affective disorders (Bottiglieri et al, 1984). Evidence for a link between
SAM and monoamine neurotransmitters is however somewhat contradictory and tenuous (Baldessarini, 1987). SAM has been found to have an excitatory effect on rat cerebral cortical neurons (Phillis, 1981). This affect might be responsible for the mood elevation caused by SAM.

The nervous system’s requirement for labile methyl groups in DNA and RNA synthesis will be markedly less than the requirement of proliferative mitotic tissues such as those previously discussed. However, at times of rapid growth or repair purines and pyrimidines derived from the methyltransfer pathway will be required by the nervous system.

One of the most interesting and important roles of SAM relates to phospholipid methylation. Methylation of phospholipids has important implications because changes in the properties of cell membranes caused by methylation can affect the functions of hormone or neurotransmitter receptors or ion transporters. Hirata and Axelrod (1980) found two enzymes located within the adrenal medulla that could convert phosphatidylethanolamine (PE) to phosphatidyl-N-monoethanolamine and by further methylation to phosphatidylcholine (PC), using SAM as the methyl donor. A further observation was that methylation begins on the cytoplasmic side of the membrane, the methylated phospholipid being then translocated to the exterior surface by an enzymatically facilitated flip-flop movement. Hirata et al (1979) demonstrated that the activation of the β-adrenergic system could stimulate the SAM mediated methylation of PE to phosphatidyl-N-monoethanolamine and PC. The methylation of PE has a number of interesting effects which are believed to be due to the decrease in microviscosity (increased fluidity) seen when the PC content of cell membranes is increased. (see below)

A reverse of this situation occurs with ageing. The cell membranes of laboratory animals and humans have been found to show increases in microviscosity with ageing. SAM was found to reverse these changes thereby restoring the fluidity of some brain regions in aged rats (Cimino et al, 1984). Cerebrospinal fluid SAM concentrations have also been found to decline with age in humans (Surtees and Hyland, 1990b).
The increase in fluidity of phospholipid membranes caused by methylation is thought to enable a greater ‘lateral movement’ of receptor proteins which might in turn lead to more efficient ‘receptor effector coupling’ (Hirata et al, 1979; Baldessarini, 1987). Another effect of physiological significance considered to be related to membrane methylation is the inhibition of sodium potassium ATPase mediated by SAM (Hattori and Kanfer, 1984).

The methyl-transfer pathway is required for the methylation of proteins found within the nervous system. Transmethylation of the arginine 107 residue of myelin basic protein occurs via this pathway and is thought to play an important role in the maintenance of the myelin sheath (Baldwin and Carnegie 1971: see earlier section for full discussion).

1B2.6 Folate deficiency

Folic acid, a water soluble vitamin, is also required for the methyl-transfer pathway. Folate in the methyltetrafolate form provides methyl groups to convert homocysteine to methionine in a complex reaction catalysed by cobalamin-dependent methionine synthase (Surtees et al, 1991) (see Figure 1.2).

Folate is found in most natural foods, notably yeast, liver, kidney, fresh green vegetables and some green fruits. The widespread occurrence of folate in foods means folate deficiency of dietary origin is rare (Newsholme and Leech, 1983). Folate deficiency normally results from malabsorption due to gastrointestinal conditions such as coeliac disease, tropical sprue, anticonvulsant drugs and alcohol which may affect absorption.

The effects of folate deficiency are remarkably similar to those associated with B12 deficiency, notably megaloblastic anaemia and other symptoms associated with reduced cell division in rapidly proliferating tissue. This provides further evidence for the idea that the inhibition of the methyl-transfer pathway is the cause of these effects, as it provides the
only link between Vitamin B₁₂ and folate (Newsholme and Leech, 1983). The psychiatric complications of B₁₂ deficiency have also been reported in folate deficiency and can be alleviated by methylfolate treatment (Godfrey et al, 1990). The occurrence of neuropathy or myelopathy in folate deficiency was at one time controversial (Manzoor and Runcie, 1976). A number of reports have however implied a strong causal link between folate deficiency and neurological disease.

Ahmed (1972) reported a patient who presented with megaloblastic anaemia, loss of vibration and position sensation from the feet. The patient had normal serum B₁₂ levels, but reduced serum folate. The anaemia and neurological symptoms responded to treatment with folic acid. Manzoor and Runcie (1976) reported 10 cases of folate responsive neuropathy. The neurological disease found in these patients was said to be indistinguishable from SCD. However, the patients had normal serum B₁₂ levels. Lever et al (1986) summarized the cases mentioned above and included a detailed case report of a further patient suffering from anaemia and SCD and who had a mild mixed, predominantly sensory, axonal polyneuropathy. The patient showed a marked and rapid improvement when treated with methyl folate.

(i) Inborn errors of folate metabolism

Clayton et al (1986) were the first to provide evidence suggesting that an inborn error of folate metabolism could cause SCD. A child with 5, 10-methylenetetrahydrofolate (5, 10CH₂THF) reductase deficiency was studied. This enzyme converts 5, 10CH₂THF to 5-methylenetetrahydrofolate which is subsequently used in the methyl-transfer pathway.

The patient showed normal development during the first year of life after which progress slowed. The patient became apathetic, ataxic and showed a Parkinsonian tremor of the arms. Fasciculations were seen in the muscles of the thigh and tongue and gross atrophy of the muscles of the limbs was also apparent. Neurophysiological assessment indicated that both the visual and auditory pathways were impaired. The former is frequently
affected in SCD, but not the latter (Fine et al, 1990). Electromyography of the legs revealed fibrillation potentials characteristic of denervation. The maximal motor conduction velocities of the nerves supplying these muscles were slightly reduced, indicative of the degeneration of large distal motor fibres. The neurological symptoms failed to respond to treatment with folic acid, methionine, Vitamin B₁₂ or carnitine.

Post mortem examination revealed numerous small, usually perivascular, foci of demyelination spread throughout the subcortical and central cerebral white matter, corpus callosum, internal capsule, fornix and optic nerves. Patchy demyelination was found in the brain stem and was particularly severe in the cerebellar white matter. The pathology of the spinal medulla was said to be typical of SCD with the most extensive lesions being found at the thoracic level. Coalescence of demyelinating patches in the anterior, lateral and posterior columns was seen. The dorsal root ganglia and peripheral nerves were unaffected. Muscles and end-plates were not examined, however.

Following the initial report described above, three further patients were diagnosed as being 5,10CH₂THF reductase deficient (Hyland et al, 1988). To assess the role of SAM, methionine, folate and neurotransmitter amine metabolism in the demyelinating process, the CSF metabolite concentrations from the previous terminal case were compared with three other cases. In the latter cases, demyelination was halted with the administration of betaine. Betaine provides an alternative route for the methylation of homocysteine to methionine using betaine as the methyl donor and requiring the enzyme betaine-homocysteine methyl-transferase. This process circumvents the block caused by 5,10MHF reductase deficiency (see Figure 1.2). Betaine therapy was found to halt the neurological deterioration and increase CSF levels of SAM. Folate and neurotransmitter amines were not normalized however. Hyland et al (1988) therefore concluded that the abatement of neurological symptoms produced by betaine was associated with the maintenance of SAM levels within the CNS. This again provided evidence for association between defects of the
methyl-transfer pathway and demyelination in humans. The abnormalities in neurotransmitter amine and protein metabolism were thought to be caused by folate deficiency and not as a consequence of SAM deficiency. This concurs with the findings of Botez et al (1979) who demonstrated that folate deficient rats showed decreased amine neurotransmitter synthesis.

The alleviation of neurological symptoms associated with increasing CSF SAM concentrations in previously deficient patients was found by Surtees et al (1991) to be due to remyelination. Surtees et al studied children with a variety of inborn errors of the methyl-transfer pathway, all of which errors caused a decrease in CSF SAM concentrations. Magnetic resonance imaging (MRI) techniques showed that the patients had abnormal myelination. The children were treated with either betaine, methionine or SAM, depending on the position of their inborn error. Treatment led to clinical improvement, apparent remyelination (viewed on MRI scans) and the correction of CSF SAM concentrations to within the normal range. MRI was also recently used by Tracey and Schiffman (1992) to demonstrate that the abatement of symptoms seen when SCD is treated with cobalamin is also due to remyelination.

(ii) The methyl-folate trap

The relationship between Vitamin B₁₂ and folate deficiency was discussed at length by Scott and Weir (1981). It was suggested that in man the so called ‘methyl folate trap’ is the normal physiological response to imminent methyl group deficiency resulting from the insufficient availability of methionine. Reduced methionine levels cause a concomitant reduction of SAM, thereby putting at risk methylation reactions vital to the nervous system. SAM levels are maintained as a first priority using the following mechanisms.

1). Folate co-factors are directed away from cycles that produce purines and pyrimidines for DNA synthesis and are redirected towards the methyl-transfer pathway thus ensuring all the
available methionine is used for methylation reactions within the nervous system.

2). To retain 5-methyl-THF within a cell, the cell must contain adequate concentrations of homocysteine which is required for the conversion of 5-methyl-THF to polyglutamate which is stored within the cell. Since homocysteine is solely derived from methionine, methionine deficiency will prevent the conversion of 5-methyl-THF to polyglutamate thereby further reducing the rate of DNA synthesis in rapidly dividing cells.

Scott and Weir (1981) believed the two mechanisms outlined above evolved in response to methionine deficiencies. They also occur however in Vitamin B₁₂ deficiency which is misinterpreted as methionine deficiency. The redistribution of methionine during B₁₂ deficiency is inappropriate, leading to anaemia, and fails to prevent SCD. This hypothesis also explains why the administration of folic acid to B₁₂ deficient patients may lead to the progression or exacerbation of neurological symptoms, e.g. increased folate levels will induce cell division thus increasing the utilisation of methionine which further inhibits methylation reactions within the nervous system.

Folate deficiency also leads to a deficiency in the biosynthesis of methionine which the body responds to by directing folate away from DNA synthesis and redirecting it towards the methyl-transfer pathway. This selective use of the available folate, together with the ability of neural tissue to actively concentrate folate, explains why SCD is far more uncommon when folate deficiency occurs.

The ‘methyl folate trap’ does not occur in animals. This explains why megaloblastic anaemia is never observed in B₁₂ deficient animals (see later section). In animals it is not necessary to methylate 5-methyl-THF to allow its incorporation into cells. Therefore, folate levels are maintained within the bone marrow at levels which allow normal erythropoiesis throughout periods of B₁₂ deficiency.
Other factors causing failures in the methyl-transfer pathway

(i). HIV

A vacuolar myelopathy (VM) in patients with human immune deficiency virus (HIV) was first described by Goldstick et al (1985) and Petito et al (1985). Scaravilli (1992) considered VM to be the most frequent abnormality of the cord found in AIDS patients. This vacuolar myelopathy resembles SCD in that the lesions are symmetrical and most severe in the lateral and posterior columns of the thoracic cord. The vacuoles themselves are found within the myelin sheath or between the myelin and the axolemma. Ultrastructurally, the lesions are seen as an intramyelinic swelling with separation of lamellae. In areas of severe vacuolation the axons may also become affected, becoming swollen and undergoing Wallerian type degeneration (Petito et al, 1985).

The causes of this vacuolar myelopathy remain uncertain. HIV mRNA has been found in the macrophages and multinucleated giant cells in areas showing VM, suggesting a close link between HIV and VM (Eibott et al, 1989). Sharer et al (1986) believe however that opportunistic infections might cause VM.

Smith et al (1987) published the results of a study aimed at elucidating the mechanisms responsible for this pathology. Two children presenting with neurological symptoms associated with congenital HIV infection were found to have markedly reduced CSF folate levels, that were within the range previously demonstrated to cause progressive demyelination (Clayton et al, 1986). This finding therefore suggests folate deficiency may lead to failures in the methyl-transfer pathway and the SCD-like pathology seen in HIV. Further evidence for this hypothesis was presented by Surtees et al (1990) who found CSF SAM concentrations were
significantly reduced in six children with congenital HIV infection when compared to those of a control reference population. Boudes et al (1990) found that approximately 60% of adults infected with HIV were folate deficient but Vitamin B_{12} replete. This factor was thought to have important implications for the management of patients with HIV as many drugs used to treat opportunistic infections operate via an antifolate mechanism.

Smith et al (1987) proposed a mechanism by which HIV infection might lead directly to folate deficiency. This mechanism occurs in addition to folate deficiency caused by poor diet, malabsorption or the use of drugs with an antifolate action (see above). It was proposed that persistent activation of macrophages by γ-interferon would lead to macrophages synthesising increased amounts of dihydroneopterin from guanine triphosphate. This hypothesis was based on findings that both the children in their study had raised CSF dihydroneopterin concentrations, as did the patients of Surtees et al (1990). The reason for this increase in neopterin synthesis is not known but neopterin accumulation could inhibit folate metabolism (Surtees et al, 1990). Macrophages also produce oxygen free radicals which reduce the fluidity of cell membranes. The fluidity of these membranes can be restored by methylating phospholipids using SAM as the methyl donor. This process might lead to an increased utilisation of methyl groups in affected areas and hence might lead to demyelination (Surtees et al, 1990).

The results of a study conducted by Keating et al (1991) contradict this hypothesis. Keating et al monitored the SAM, S-adenosylhomocysteine (SAH), CSF and B_{12} and folate serum concentrations in 20 HIV-seropositive patients and 30 HIV-seronegative controls. The HIV-seropositive patients were found to have significantly reduced SAM and significantly increased SAH CSF concentrations when compared to controls. The ‘methylation ratio’ (SAM/SAH) was markedly reduced in the patients infected with HIV. All of the HIV-positive subjects were found to be Vitamin B_{12} and, more significantly, folate replete suggesting that a factor other than folate deficiency is responsible for inhibiting the methyl-transfer reaction. The
exact mechanism by which HIV-infection might prevent the conversion of homocysteine to methionine (see Fig 1.2), which must occur in order to account for the accumulation of SAH and depletion of SAH seen in these patients, is still uncertain. Keating et al (1991) put forward the following theories as to how this might occur:

1). Chronic viral infection might lead to the production of cobalamine analogues which would competitively inhibit methionine synthase and hence the conversion of methionine to homocysteine. This would also cause a rise in methylmalonic acid. This was not seen in the HIV-positive patients however.

2). HIV infection might induce a futile cycle causing the inappropriate use of SAM, an affect seen to occur after chronic alcohol consumption.

3). Interferon, which is produced in response to viral infections, has previously been reported to reduce the methylation ratio.

(ii) Drugs

Methotrexate is a folic acid agonist used as a chemotherapeutic agent (Jacobs and Le Quesne, 1992). It is administered intravenously or intrathecally and is frequently used in conjunction with cranial or craniospinal irradiation to treat acute leukaemia. Shapiro et al (1973) described neurological complications such as drowsiness, irritability, ataxia and confusion associated with the intraventricular administration of methotrexate. The subsequent pathology observed consisted of a leucoencephalopathy which was particularly prominent in the periventricular region. Coagulative necrosis or extensive demyelination, particularly in the centrum ovale, were also noted by Rubinstein et al (1975). Jacobs and Le Quesne (1992) state that the mechanisms of methotrexate toxicity is unknown and make no mention of a link between methotrexates antifolate action and the demyelinating pathology.

L-3,4-dihydroxyphenyalanine (levodopa), a compound used to treat Parkinsonism, dystonias and certain inborn errors of metabolism has been
found to reduce CSF SAM concentrations (Surtees and Hyland, 1990a). Administration of levodopa was found to cause a reduction of CSF SAM concentrations and a concomitant rise in 3-methoxytyrosine concentrations. The relationship between CSF SAM and 3-methoxytyrosine was found to be linear suggesting that the rate of levodopa methylation is a major factor governing CSF SAM concentration. No reports concerning neurological symptoms or pathology induced by levodopa are cited by Surtees and Hyland. It is suggested, however, that to alleviate the risk of neurological damage methyl-donors such as SAM, methionine or betaine should be administered as an adjunct to levodopa.

1B3 EXPERIMENTALLY INDUCED FAILURES OF THE METHYL-TRANSFER PATHWAY IN ANIMALS (ANIMAL MODELS OF $B_{12}$ DEFICIENCY)

A number of different methods have been used to disrupt the methyl-transfer pathway in laboratory animals and therefore produce an animal model for SCD.

1B3.1 Prolonged feeding of diets devoid of Vitamin $B_{12}$

Vitamin $B_{12}$ is found in a wide variety of animal foods and is produced as a result of microbial fermentation in all animals (Coates, 1968), coprophagy and from drinking contaminated water (Green et al, 1975). In addition, animals have also been found to carry large stores of $B_{12}$ which may amount to several years’ requirement (Chanarin, 1969). Vitamin $B_{12}$ deficiency is therefore difficult to induce in experimental animals, even when the animals become $B_{12}$ depleted haematological symptoms are never observed and neurological symptoms only occur infrequently (Green et al, 1975; Siddons et al, 1975; and Agamanolis et al, 1976). Despite this, however, a number of reports of a neurological involvement in animals with Vitamin $B_{12}$ deficiency of a dietary origin have been published.
Alexander (1957) was the first to described demyelination in association with prolonged feeding of Vitamin B\textsubscript{12} free diets. Demyelination of the sciatic nerves and spinal cord were ‘demonstrated histochemically’ (Coates, 1968) in chicks, rats and calves. Subsequent studies failed to show consistent neurological effects associated with the prolonged feeding of a B\textsubscript{12} free diet in these species (reviewed by Coates, 1968).

The most consistent neurological abnormalities have been produced in primates subjected to a prolonged (up to 4 years) B\textsubscript{12} free diet. It has been known since the turn of the century that captive monkeys occasionally develop a condition known as ‘cage paralysis’ which has both the clinical and pathological characteristics of human SCD (Brooks and Blair, 1904). Krohn and Oxnard (1963) was the first to demonstrate that the condition was associated with low serum B\textsubscript{12} levels and that it responded to parenteral treatment with Vitamin B\textsubscript{12}.

In a further study, Oxnard and Smith (1966) monitored both the serum B\textsubscript{12} concentrations and neurological symptoms (and subsequent morbid anatomy) of 15 rhesus monkeys *Macaca mulatto*. Five of these fifteen monkeys were found to have low serum B\textsubscript{12} levels and overt paralysis in the hind limbs and tail. The spinal cords of these animals showed spongiform demyelination of the posterior and, to a lesser extent, lateral columns of the cord. Demyelination of the peripheral nerves, which was most marked in distal portions, was found in all the animals examined. The other monkeys which had been captive for prolonged periods had reduced tendon and plantar responses and occasionally showed a milder form of the pathology noted above, without obvious signs of paralysis.

Torres, Smith and Oxnard (1971) provided additional information on the peripheral neuropathy associated with feeding a B\textsubscript{12} deficient diet to monkeys. Segmental demyelination was described as the main lesion was present in 80% of the nerves examined. A Wallerian-type axonal degeneration was also seen in severe cases.

Siddons et al (1975) describe an elaborate series of experiments undertaken to induce Vitamin B\textsubscript{12} deficiency in the baboon *Papio*
aynocephalus. In addition to feeding the animals a B<sub>12</sub> deficient diet, the following procedures were also used:

1). Additional sources of B<sub>12</sub>, obtained by coprophagy, eating insects or rodents, were excluded.

2). Body stores of cobalamine were reduced by subtotal hepatectomy.

3). Ampicillin at 50mg/kg/day was administered to reduce the amount of B<sub>12</sub> synthesized by intestinal flora.

4). The metabolic demand for B<sub>12</sub> was increased by feeding a low-fat diet with additional sodium propionate.

Despite the procedures noted above and after application of Vitamin B<sub>12</sub> deficient diets for up to four years all the baboons remained healthy and active. No haematological changes or clinical signs of neurological damage were seen despite serum Vitamin B<sub>12</sub> levels being reduced. The pathological examination of the nervous system and internal organs also failed to reveal any abnormalities.

It was then decided to administer Vitamin B<sub>12</sub> analogues to baboons depleted of B<sub>12</sub> in an attempt to inhibit the B<sub>12</sub>-dependent enzyme reactions and hence produce neurological and haematological symptoms. The compound 2-methyl-2-aminopropanol was found to cause paraparesis in three depleted of B<sub>12</sub> baboons 2-5 months after it was administered. Necropsy was carried out 9-12 months after the animals had been treated with 2-methyl-2-aminopropanol. Neuropathological examination of these three baboons revealed extensive scattered areas of demyelination in the cerebral hemispheres. These lesions were frequently observed to be centred around small blood vessels, a factor also reported in human SCD (see earlier section). No lesions were observed in the brainstem or basal ganglia. The spinal cords of the three animals examined showed lesions with an almost identical distribution. Demyelination and, to a lesser extent, axonal loss were seen in the posterior and lateral columns at all levels. Further demyelinating lesions and slight axonal degeneration were seen in the peripheral nerves of one of the two animals examined.

Agamanolis et al (1976) believed the studies of Oxnard and his group
were seriously flawed, firstly because they failed to provide sufficient descriptions or illustrations of the observed pathology and clinicopathologic correlations were inadequate or lacking and secondly because the animals diet was not precisely controlled. Agamanolis et al sought to rectify these faults and in the light of the Siddons et al (1975) findings, determine if Vitamin B₁₂ deficiency of purely dietary origin could induce neurological damage in monkeys.

After 5 years on a B₁₂ deficient diet haematological changes had not become apparent in any of the monkeys. However, five monkeys showed signs of visual impairment at between 33 and 45 months. Three of these five monkeys subsequently developed a progressive spastic paralysis of their hind limbs. The pathology of the B₁₂ deficient monkeys revealed degeneration of the visual pathway and a mild diffuse degeneration of cerebral white matter. The lesions found within the spinal cord were said to be indistinguishable from those seen in human SCD. Peripheral nerves were not affected.

The ultrastructural changes seen in these monkeys were reported by Agamanolis et al 1978. The pathological changes occurring in the posterior and lateral columns of the cervical, thoracic and lumbar cord were monitored. The areas which had in the previous study been shown to undergo ‘spongy’ changes. The ultrastructural pathology observed was again described as ‘indistinguishable topographically and histologically’ from human SCD. The spongy appearance of affected areas of the cord was found to be caused by the wide separation of the myelin lamellae forming extensive vacuoles and by the eventual destruction of the myelin. Most of the axons remained intact and few had undergone degenerative changes despite being denuded of myelin and frequently surrounded by astrocytic processes. At a later stage, however, axonal swelling and degeneration were observed.

The difficulty and length of time required to produce a condition resembling human SCD in monkeys led Green et al (1975) to induce Vitamin B₁₂ deficiencies in other animal species in studies aimed at producing a more
suitable model for human Vitamin B\textsubscript{12} deficiency. Neurological changes were found to occur in Egyptian fruit bats \textit{Rousellus aegypticus} maintained on a pest-free all fruit diet. Measures to prevent coprophagy were also taken. Two hundred days after the initiation of this maintenance regime the bats became progressively more B\textsubscript{12} deficient. Seven of the ten bats developed neurological symptoms consisting of an inability to disengage their claws when climbing and co-ordinational abnormalities during the downstroke while flying. The pathology observed at this time consisted of patchy spongiose changes restricted to the white matter of the lower cervical and upper thoracic cord. These changes mainly affected the dorsal and lateral columns. Bats treated with cyanocobalamin did not develop these neurological symptoms although cyanocobalamin failed to reverse the neurological symptoms once they had occurred. Green et al also noted that despite B\textsubscript{12} serum levels being reduced by approximately 20 times, haematological abnormalities were not observed.

Deacon et al (1986) found that the brains of Vitamin B\textsubscript{12} depleted fruit bats showing neurological symptoms did not demonstrate reduced methylation of myelin basic protein. They therefore contended that the observed pathology might not be caused by defective methylation of myelin basic protein.

1B3.2 Exposing animals to nitrous oxide

An alternative to producing Vitamin B\textsubscript{12} deficiency is the deactivation of Vitamin B\textsubscript{12} by preventing it acting as a co-enzyme and thus causing a functional deficiency.

Dinn et al (1978) were the first to suggest that prolonged exposure of animals to N\textsubscript{2}O might prove useful in the study of neurological damage related to Vitamin B\textsubscript{12} deficiency. It had previously been found that prolonged exposure to N\textsubscript{2}O can cause haematological changes (Amess et al, 1978) and neurological abnormalities in man (Layzer et al, 1978 - see earlier section). Dinn et al (1978) therefore exposed a variety of laboratory animals
to a 50% nitrous oxide and 50% oxygen mixture. Mice and rats quickly developed biochemical changes consistent with $B_{12}$ deficiency, e.g. decreased folate uptake and decreased polyglutamate biosynthesis, but, more importantly, no neurological or haematological changes after eight months.

A monkey subjected to a similar mixture showed both the biochemical and neurological symptoms characteristic of $B_{12}$ deficiency within two months. The monkey became uncoordinated and developed progressive ataxia. At necropsy, degeneration of both the myelin and axons of the posterior columns and lateral cortico spinal and spino-cerebellar tracts was seen. Spongy degeneration was also evident at the periphery of the anterior columns. Once again, haematological changes were not observed.

This initial observation was expanded upon by Scott et al (1981). In this study four pairs of monkeys were maintained in the $N_2O/O_2$ mixture noted above. The diet of four of these monkeys was supplemented with methionine. All the monkeys given unsupplemented diet developed ataxia at 10 weeks, with the disorder progressing over the next 2-3 weeks until the animals were moribund. The monkeys which received the methionine supplement had no detectable clinical changes. Spongy degeneration of the posterior columns and the lateral corticospinal, spinocerebellar and anterio-corticospinal tracts were observed in the monkeys which did not receive the methionine supplement. Severe demyelination of the peripheral nerves also occurred in these monkeys. Methionine supplementation appeared to prevent the development of severe neurological damage. Scott et al (1981) therefore suggested that the primary cause of the demyelination seen in $B_{12}$ deficiency is the inability to synthesize methionine, i.e. disruption of the methyl-transfer pathway. The rate at which demyelination occurs would therefore be dependent on dietary methionine intake.

Nitrous oxide was administered to fruit bats by van der Westhuysen et al (1982). This caused ataxia, paralysis and the eventual death of the experimental animals. Bats which had previously been subjected to $B_{12}$ deficiency developed neurological symptoms more quickly than $B_{12}$ replete
animals. Methionine was again found to protect the animals from neurological damage. The administration of folate, however, was found to exacerbate the neurological symptoms. This agonistic effect of folate was also prevented by providing a dietary methionine supplement. These findings lend further support to the idea that the neurological lesions seen in B\textsubscript{12} deficiency are caused by the inhibition of the methyl-transfer pathway, leading to a deficiency of methionine and hence SAM.

Three later reports using animal models cast some doubt on the methylation hypothesis. Van der Westhuysen and Metz (1983) found the SAM concentrations of fruit bats showing neurological symptoms were comparable to controls. Deacon et al (1986) administered nitrous oxide to rats for up to seven days and failed to observe a change in the methylation of myelin basic protein in the brains of these rats. This finding is somewhat misleading as the neurochemical results were not correlated with neurological or neuropathological findings. It is also unlikely that the seven day exposure period used by Deacon et al would lead to neurological damage as Dinn et al (1978) had already shown that rats exposed to nitrous oxide for up to eight months failed to exhibit neurological symptoms.

Weir et al (1988) defended the methylation hypothesis with further experimental evidence. Pigs treated with nitrous oxide developed progressive ataxia with a spinal neuropathy. The ataxia was completely, and the neuropathy partially, preventable by dietary methionine supplementation. Rats treated with nitrous oxide failed to develop neurological or pathological abnormalities. The affected pigs were found to have elevated levels of S-adenosylhomocysteine (SAH) and concomitantly low levels of SAM in their neural tissues. This situation was reversed, however, when the animals diet was supplemented with methionine. The methylation ratio (SAM/SAH) was affected to a far lesser extent in the rats than in the pigs. Raised neural S-adenosylhomocysteine levels would inhibit SAM-dependent methylation reactions. Weir et al suggested that it is this factor which is responsible for the neuropathy observed.
CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

More than 200 Balb C mice (3-25 weeks old) of both sexes were used, with 25 mice being designated as controls. Two adult sprawling mice were also used as negative controls in the muscle spindle experiments. The animals were housed in groups of 3-7, with food and water available ad-libitum. The animals were supplied with CRM-diet (Labsure Ltd) which contained 13.4mg/kg of Vitamin B$_{12}$ and 0.7mg/kg of folic acid. To facilitate the feeding of behaviourally affected mice, the food pellets were soaked in water and placed on the floor of the cage. The ambient temperature was maintained between 20 and 23°C with a 10 hour light, 14 hour dark, light cycle.

(i) The administration of Cycloleucine

CL (Sigma) was dissolved at a concentration of 500mg in 10mls of sterile saline (Antigen Pharmaceuticals Ltd). A single dose of CL (2mg/g) in this saline vehicle was injected intraperitoneally. The control mice received an equivalent volume of the saline alone.

(ii) Clinical examination of the mice

The mice were examined daily. Motor function was assessed by placing the mice on a wire grid and gently lifting the tail. Control mice gripped the grid firmly with the toes of both their fore and hind limbs. The mice were also encouraged to walk on the grid, to allow inspection of gait. Sensation was assessed by lightly pinching the following areas with small pointed forceps: the snout, fore limbs, hind limbs, trunk, hind feet and tail. This normally evoked a prompt strong reaction in control mice.
2.2 Preparation of tissues for physiological examination

(i). The dissection of the soleus and Edl

The mice were first stunned and then killed by cervical dislocation. The right hind limb was then amputated at the upper thigh level. After the skin was removed, the leg was placed in a dissecting bath containing cooled, oxygenated, mammalian Ringer solution. The floor of the bath was covered with a layer of silicone rubber (Sylgard 184, Dow Corning Ltd) allowing the leg to be firmly anchored and pinned in position. During the dissection the preparation was viewed using a Zeiss stereoscopic dissecting microscope (X60-120).

To remove the soleus muscle the gastrocnemius muscle was first gently reflected medially and then pinned, exposing the underlying soleus (see Figure 3.32). The upper tendon of the soleus was located and cut at as high a level as possible. The soleus was then carefully drawn away from the surrounding tissue. The nerve innervating the soleus was cleared and cut as high up as possible - approximately 5mm from the soleus. The Tendo Calcaneum was then severed, allowing the soleus with its accompanying nerve still attached to be rapidly transferred to a muscle chamber containing mammalian Ringer solution gassed with 95% O₂ and 5% CO₂ and maintained at room temperature.

The extensor digitorum longus (Edl) was then removed. The fascia overlying the tibialis anterior (tib ant) was removed allowing the margin of tib ant to be defined. This margin was then cut and tib ant was reflected medially to reveal the underlying Edl (see Fig. 3.32). The lower tendon of tib ant was located and cut. Tib ant was then drawn away from the leg, cut at its tendon of origin and removed. The lower tendon of Edl was then cleared and cut, and the muscle reflected upwards and pinned. The lower tendon of peroneus longus was identified and cut. Peroneus longus was then drawn upwards, cleared and cut at its origin. This allowed the underlying nerve, which innervates the Edl, to be cleared and cut at a point
approximately 5mm from the Edl. The lower tendon of the Edl was then grasped gently with a pair of watchmakers forceps and drawn out from the surrounding tissue, complete with its nerve supply. The tendon of origin was located and cut. The Edl was then placed in the organ bath with the soleus (see above).

During the dissection of the soleus and Edl, which took approximately 15 minutes, the Ringer solution was replenished several times.

(ii). The dissection of the biceps brachii

The biceps brachii were also removed from a number of 21-day-old mice. Freshly killed mice were placed ventral surface down on a cork dissecting board. The fore limbs were then pinned and the skin surrounding the right shoulder and upper arm removed. Cooled and oxygenated Ringer was dripped over the exposed upper arm and shoulder. Next, pectoralis major, pectoralis minor and cutaneous maximus were reflected away from the upper arm to reveal the biceps brachii. The tendons of both the long and short heads were identified and cleared and cut as far up as possible. Biceps brachii was then reflected laterally and cleared, the nerve innervating the short head was cut approximately 2mm from the muscle. The flexor carpi radialis, extensor carpi radialis longus and extensor carpi radialis brevis of the forearm were cut to allow the lacerta fibrosus of the biceps to be cut and the biceps brachii removed. The biceps brachii was then rapidly transferred to an organ bath.

2.3 Physiological Methods

(i) Organ bath and Ringers solution

The organ bath was made from clear perspex. Its floor was coated with a 5mm thick layer of transparent silicone resin (Sylgard 184, Dow Corning Ltd) which allowed the muscles to be pinned for intracellular
recordings. The preparation was illuminated with a quartz-halogen cold-light source (Barr and Stroud) and viewed at x20 - x150 through a Nikon stereoscopic dissecting microscope. Oxygenated mammalian Ringer solution flowed through the chamber at a rate of approximately 4ml/min.

Mammalian Ringer solution consisted of (mM): NaCl 115, KCl 3.5, MgSO\(_4\) 1.0, NaHCO\(_3\) 25, CaCl\(_2\) 2.0, KH\(_2\) PO\(_4\) 1.0, glucose 11.0, dissolved in glass distilled water. The Ringer solution was always equilibrated with 95% O\(_2\), 5% CO\(_2\).

(ii) Recording of the mechanical responses of mouse skeletal muscles

The mechanical responses of adult Edl and soleus muscles evoked by both direct and indirect stimulation were determined. The muscle was placed in the organ bath with its nerve lying uppermost. The tendon of insertion was then firmly pinned to the silicone resin coating the floor of the organ bath. The tendon of origin was tied using silk thread to the arm of a Life Bioscience UFI isometric tension transducer. The transducer was accurately positioned using a Narishige MD4 manipulator. The resting tension of the muscle was adjusted to produce the maximal twitch response.

Indirect stimulation (20V, for 0.05ms) was applied to the muscle using a suction electrode fashioned from a Pasteur pipette (Corning Ltd). Direct stimulation (90V for 0.5ms) was applied to either side of the mid-part of the muscle using a pair of silver wires (0.075mm diameter Johnson Matthey Metals Ltd). Square wave pulses were obtained from a Digitimer DS2 isolated stimulator. The timing and frequency of stimulation was controlled using a Digitimer D100 timer.

The signal produced by the transducer was amplified on a Bioscience A120 amplifier and displayed on a Tektronix 5113 oscilloscope. A permanent record was made using a Gould 2200S pen recorder.

Both the Edl and soleus muscles were stimulated at 0.5, 50Hz, and 100 Hz to allow both the maximal twitch and tetanic tension in response to
both direct and indirect stimulation to be ascertained, (to allow the comparison of maximal tensions produced by muscles of differing sizes the results are expressed herein as g/mg - of muscle weight). Therefore, following the completion of tension recordings the muscles were removed from the organ bath and their tendons removed. They were then blotted dry with tissue and their weight determined using Mettler AE50 scales.

(iii) Intracellular Recordings from Mouse Skeletal Muscle

With the nerve lying uppermost, the muscle was pinned through its tendons to the floor of the chamber at no more than its *in situ* length. Intracellular recordings were made from single muscle fibres using standard electrophysiological techniques (Fatt and Katz, 1951).

Microelectrodes were made from 4" lengths of single-barrel, filamented, borosilicate glass capillary tubing (Clark Electromedical Instruments, OD = 1mm and ID = 0.58mm). Pairs of electrodes were pulled on a moving-coil microelectrode puller (Camden Instruments Ltd, Model 753). Three molar KCL was introduced into the electrodes with a 1-ml plastic syringe which had been drawn out over a bunsen to form a fine canula. Only microelectrodes with a tip resistance between 10-20 megaohms were used. The microelectrodes were accurately positioned using a Prior micromanipulator.

Recorded potentials were amplified using a Neurolog NL102 DC preamplifier, NL 125 filters, and a NL106 variable gain AC-DC amplifier (Digitimer Research Instrumentation Ltd). A silver-silver chloride reference electrode (Clark Electromedical Ltd) was placed in the organ bath. Signals were displayed on a Tektronix 5113 storage oscilloscope. Permanent records were made using a Gould 2200s pen recorder. Signals were also digitized at 10Khz and stored on an IBM personal computer using a 1401 data acquisition and averaging system (Cambridge Electronic Design Ltd).

Single muscle fibres were impaled by slowly lowering the microelectrode until the tip passed cleanly through the plasmalemma. Since
impaling a muscle fibre could cause damage to the plasmalemma, altering the physiological characteristics of the muscle fibre, only fibres which were entered "cleanly" were used. The criteria used to define a clean entry were a rapid fall of the low gain DC trace of the oscilloscope and a stable resting membrane potential (RMP).

To allow the determination of the frequency and amplitude of miniature endplate potentials (mepps,) muscle fibres were impaled close to the endplate region (see Fig 3.34) and records were taken over one minute periods. The analysis of mepp amplitudes was restricted to focal recordings in which the mepp rise times were less than 1 ms. Mepp amplitudes were determined using a Kontron Mini-Mop image analyser interfaced with an IBM 8570 personal computer. The values obtained were standardized to correct for changes in RMP (Katz and Thesleff, 1957). The formula used for this correction are given in a later section.

End-plate potentials (epps) were recorded and analysed. The nerve innervating the muscle was stimulated (10 volts for 0.05ms) at 5Hz using a suction electrode fashioned from a Pasteur pipette (Corning Ltd). The muscles were bathed in Ringer solution containing d-tubocurarine, the concentration of which was altered as required to ensure epp amplitudes did not exceed 5-10mV. This procedure avoided the need to correct epp amplitudes for non-linear summation (Martin, 1955). The digitized epp waveform was analysed using the following method. The first twenty epps observed during analysis were discarded due to the characteristic fall in amplitude observed (Hubbard et al, 1969). The next fifty epps were used to create an average waveform, which was then plotted using a Hewlett Packard Colorpro X-Y plotter. The epp latency and time to peak signal were then ascertained. Only focal epps with a rise time of less than 1.5ms were used for analysis.

The mean quantal content of neuromuscular transmitter release was estimated in the following manner; the first twenty epps which demonstrated a characteristic fall in amplitude were discarded, the following 50 epps were analysed for their variance of amplitude using the image
analysis equipment described above. The mean quantal content was calculated using the variance method described by Boyd and Martin (1956) and Elmqvist and Quastel (1965) (see later section).

(iv) Extracellular Recordings from muscle Spindles of the soleus

These experiments were undertaken to determine the effect of CL on the functioning of muscle spindles.

The soleus was removed from adult mice (see earlier section) and placed in an organ bath containing oxygenated mammalian Ringer. The nerve innervating the soleus was raised above the Ringer solution with a pair of silver wire hook-electrodes (0.075mm Johnson Matthey Metals Ltd). The position of the electrode was controlled with micromanipulators. Compound action potentials in response to the muscle stretch were recorded between the two silver wire hook electrodes. An identical reference electrode was also placed in the organ bath. The signal was amplified using a NL104 AC preamplifier, NL125 filters and a NL106 variable gain AC-DC amplifier (Digitimer Research Instrumentation Ltd). The signal was also amplified with an NL120 audio amp linked to a loudspeaker to produce an audio signal. The signal was simultaneously displayed on a Tektronix 5113 oscilloscope and a Gould OS1420 digital storage oscilloscope. The signal was digitized at 10KHz and stored using a CED1401 and IBM personal computer. A permanent record was made using a Gould 2200S pen recorder.

2.4 Morphological Methods

(i) Demonstration of Acetycholinesterase in whole muscles

Knowledge of the distribution of endplates enables the physiologist to produce focal recordings of neuromuscular events. To develop such a knowledge whole muscles were stained for cholinesterase allowing the endplates to be visualized (see Fig 3.34). Muscles were dissected as
described in earlier section and stained by the method of Lewis (1958). Muscles were incubated with alpha-naphthyl acetate in the presence of Fast Red TR (a diazonium salt), for 1 hour. Esterase hydrolysis causes the release of free alpha-naphthyl residue, which combines with the diazo salt producing a dark precipitate at the site of esterase activity. The muscles were then fixed in formal calcium, mounted, and photographed.

(ii) Perfusion technique

Under ether anaesthesia the mice were pinned to a cork board in the supine position. The hind feet were secured at 90° to the lower legs, to slightly stretch the calf muscles, the thorax opened and the right atrium cut. The heart was then gently held in place with blunt forceps and a shortened and resharpened 21 gauge needle (Gillette Ltd) was passed into the left ventricle. Approximately 40mls of fixative were administered using 20ml syringes (Gillette Surgical Ltd.). The time taken between opening the thorax and the end of the perfusion was usually 15-30 seconds. A successful perfusion was characterised by muscle contractions causing a rapid stiffening of the body and tail with the viscera turning a yellowish colour.

(iii) Fixation

a). For light microscopy

Tissue to be embedded in paraffin wax was fixed by perfusion with formal-calcium. Occasionally limbs were amputated from mice which were also used in the physiological study. The limbs were rapidly skinned and placed in Formal-Calcium which was prepared as described below:

Formaldehyde 40% 10ml
Calcium Acetate 1g
Distilled water 90ml

Following perfusion, the skin and viscera were removed, and the
mouse was cut into three sections consisting of; the head, the trunk and fore limbs, and the hind limbs and tail. The tissue was then left in Formal-acetic-methanol (FAM) for twelve hours. This fixative was used as it rapidly penetrates tissue and protects against shrinkage during dehydration and embedding. FAM was prepared from:

Formaldehyde 40%  
Glacial acetic acid  
Absolute methanol  

b). For electron microscopy

Tissue to be viewed with the electron microscope was fixed by perfusion with freshly prepared fixative prepared as described below. The fluid is a modified form of the fixative described by Karnovsky (1965):

Glutaraldehyde 25%  
Paraformaldehyde 10%  
Sodium Cacodylate  
Buffer (pH 7.3-7.5)  
Distilled water  
Anhydrous calcium chloride  

(iv) Paraffin section histology

Following perfusion and post-fixation tissues were decalcified in a Formic Citrate solution (98% Formic acid 35ml, 20% Trisodium Citrate 65ml). The tissue was then washed for approximately 1 hour in running tap water prior to being stored in a 10% Formal-Saline solution (40% Formaldehyde 10ml, 0.9% Saline 19ml). The limbs were separated from the trunk and dehydrated in ascending alcohol concentrations, cleared in chloroform and impregnated with paraffin wax.

The right limbs were then cut into 2-3mm slices at standardised
anatomical levels and sequentially embedded in a single paraffin block. The left limbs were bisected longitudinally and the two halves separately embedded. The head and trunk were cut into transverse sections 3-5mm thick using the serial block technique described by Beesley and Daniel (1956). The segments were dehydrated in ascending alcohols, cleared in chloroform and embedded in paraffin in anterior to posterior or proximal to distal order, allowing many levels of the head and trunk to be examined in a single block.

Previous studies conducted in this laboratory have shown the serial block technique to be extremely useful in evaluating both the pathology caused by neurotoxins, and the abnormalities seen in mutant mice. The blocks containing the head were cut in the coronal plane, from the anterior most region (snout) to the posterior region (the neck) in seven sections. This allows the nerve endings of the scalp muscle fibres and nerves of the tongue, retina, optic nerves, inner ear, and all regions of the brain to be systematically examined.

Transverse and longitudinal blocks of the limbs and feet were made to demonstrate the condition of muscle fibres, motor endplates, nerve bundles, muscle spindles and to allow comparisons between proximal muscles, i.e. rectus femoris, biceps brachii, and distal muscles such as extensor digitorum longus and the plantar muscles of the hindfoot.

Sections cut at 5µm were stained with Haematoxylin eosin (HE) and Haematoxylin Van-Gieson (HVG). Those cut at 14µm were stained for axons using the silver impregnation method of Glees (1946), modified by Marsland et al (1954). Luxol-fast-blue combined with Cresyl Violet was used to stain myelin (Kluver and Barrera, 1953) in other sections.

(v) Electron microscopy

Mice which were to be processed for electron microscopy were perfused with 40mls of freshly prepared, modified Karnovsky’s solution at 4°C. Following the perfusion, 0.3-0.5mls of Karnovsky’s solution was
Injected intramuscularly into the right thigh, calf muscles and plantar muscles of the foot ensuring fixation of the nerves and muscles in these areas.

Using a dissecting microscope the skin and viscera were removed and transverse cuts in cervical and lumbar spine were made. The spinal cord was exposed allowing further penetration of fixative. The mice were then fully immersed in fixative and left overnight in a cold room at 4°C.

Blocks were then taken from the cerebrum, midbrain, cerebellum, spinal cord, dorsal root ganglia, optic nerve, tibial nerve, sciatic nerve, and the soleus, extensor digitorum longus and plantar muscles. The blocks were then carefully labelled and transferred to 1% aqueous osmium tetroxide for approximately 3 hours at 4°C and then twice subjected to dehydration in ascending grades of ethanol prior to being placed in propylene oxide for 15 minutes. They were then transferred to a 1:1 mixture of propylene oxide and resin mixture consisting of; Araldite 20ml, DDSA 25ml and DMP30 0.8ml and placed on a rotator for between 30 to 60 minutes. The blocks were then placed in a pure resin mixture and placed on a rotator overnight. The following morning the blocks were embedded in a fresh resin mixture and polymerised for between 15 and 40 hours at 60°C.

Semithin (1μm) sections were cut using a glass knife and stained with 1% toluidine blue in 1% aqueous borax, prior to being examined under the light microscope. Fields were then selected and the blocks were trimmed with a razor-blade for ultrathin sectioning.

Ultrathin sections were cut at the thickness of silver-greyish reflection using a diamond knife. The sections were collected on bare copper grids and stained with saturated methanoic uranyl acetate and lead citrate (Reynolds, 1963). The grids were subsequently examined and photographed in a JEOL 100CX electron microscope at an accelerating voltage of 80KV.
2.5 **Statistical methods and formulae**

The amplitude of recorded miniature endplate potentials and endplate potentials were corrected for differences in resting membrane potential by using the equation described by Katz and Thesleff (1957),

$$V_{corr} = \frac{V_{uncorr} (V_{stand} - V_{eq})}{(V_{obs} - V_{eq})}$$

Where

- $V_{corr}$ = corrected end-plate or miniature end-plate potential
- $V_{uncorr}$ = Uncorrected end-plate or miniature end-plate potential
- $V_{stand}$ = Standard value chosen for resting membrane potential
- $V_{obs}$ = Observed resting membrane potential
- $V_{eq}$ = Equilibrium potential for acetylcholine (assumed to be $-15\text{mV}$)

The variance method was used to estimate the mean quantal content (mqc) of neuromuscular transmission. This method depends on the assumption that the quantal content of a train of epps has a Poisson distribution, i.e. the observed variance is due to the statistical nature of the release process. Therefore, only trains of epps which had reached plateau conditions were analysed, the first twenty epps (which showed regression) were ignored (Boyd and Martin, 1956; Elmqvist and Quastel, 1965). The following formula was used to calculate the mqc:

$$mqc = \frac{V^2}{VarV}$$

Where

- $V$ = mean corrected epp amplitude
- $VarV$ = variance of corrected epp amplitude

Student’s t-test was used to evaluate the statistical significance of differences between normally distributed populations, with similar standard
deviations. Differences of $P < 0.05$ were considered significant. Using Graphpad software and an IBM personal computer, the line of best fit was also calculated using regression analysis, for some of the data. The relationship between $X$ and $Y$ was then further analysed by calculating the Pearson's correlation coefficient ($r_p$) of the data. A $t$-test was then carried out on both the slope of the regression line and the $r_p$ value.
CHAPTER 3

TWENTY-ONE-DAY-OLD MICE RESULTS

3.1 Clinical observations

(i) Control mice

At regular intervals following the injection of saline the control mice were examined and their condition noted. At all times the mice appeared active and demonstrated no signs of muscle weakness, or paralysis. When placed on an inclined wire grid (with 2cm x 2cm grid squares) the mice climbed without losing their grip using all four limbs (see Fig 3.1). When picked up by the tail the hindlimbs were fully extended, as were the toes of both the forepaws and hindpaws (see Fig 3.2). When the animals were lifted by the tail from the wire grid their response was to grip the grid very firmly with toes of all four paws (see Fig 3.3). Pinching of the snout, forelimbs, hindlimbs, trunk and tail, with forceps caused the mice to squeak and to try to withdraw that part of the body pinched, or alternatively caused them to bite the forceps.

(ii) Cycloleucine-treated mice

Immediately following the administration of cycloleucine the mice appeared normal and were indistinguishable from the controls. At 12 hours the mice demonstrated a slight difficulty in gripping the wire grid with the toes of the hindpaws. No further ill-effects were observed at this time. One day after the administration of cycloleucine the mice began to show further signs of toxicity such as a ruffled coat, indicative of an inability to groom. The mice also appeared reluctant to move when disturbed. Weakness of the hindlimbs became more apparent and was in marked contrast to the forelimbs which remained unaffected. Sensation in all the areas tested appeared normal at this time.

At 36 hours the mice were ataxic and tended to huddle together in the corner of the cage. When lifted by the tail the hindlimbs were not
extended and only the toes of the forepaws were outstretched (see Fig 3.2).

Two days after the administration of cycloleucine the mice showed signs of weight loss and frequently a thick, cathar-like, substance was seen exuding from their eyes. They responded sluggishly to being touched and could not be coerced into walking more than a few steps, this being accomplished with a wobbling gait. When placed on a wire grid the mice climbed using just their fore limbs (see Fig 3.1) and when lifted by the tail only the toes of the forepaws were used to grip the grid (Fig 3.3).

Over the next 24 hour period some of the mice died. The surviving mice showed almost complete paralysis of the hindlimbs and they could only move using their forelimbs to drag themselves along. When the mice were placed on a wire grid the hindlimbs frequently fell through the spaces in the grid. The forelimbs, however, could still be used to grip the grid. The abdominal and intercostal muscles appeared paralysed, which lead to costal recession. Sensation appeared to remain intact at this stage.

Four days after the administration of CL the surviving mice looked severely ill. Their coats were ruffled and costal recession, loss of body weight and severe paralysis of the hindlimbs were apparent. The forelimbs still did not show signs of paralysis.

Over the next three days the mice either died or their condition appeared to stabilise slightly, with some surviving for a further 3-4 days. However, all the CL-treated twenty-one-day old mice died within 8 days.

The general clinical impression after treatment with CL was that of increasing muscle weakness, leading to paralysis, which particularly affected the hindlimbs whilst sparing the forelimbs. In addition, investigations of the morphological abnormalities induced by CL in young mice carried out by Dr C C Lee indicated pathological changes in motor nerve fibres and vacuolation of myelin in the white matter of the brain and spinal cord. In view of the clinical and structural observations, it was decided to concentrate the physiological study on the abnormalities of function caused by CL at the peripheral nerve, muscle and neuromuscular junction.
Fig. 3.1  A) a control 21-day-old mouse climbing an inclined wire grid. The grid is gripped firmly with the toes of all four limbs. B) a 21-day-old mouse, 2 days after the administration of CL (2mg/g). Climbing is achieved using only the forelimbs. The toes of the hindlimbs appear unable to grasp the grid.
Fig. 3.2 A) a control 21-day-old mouse lifted by the tail. The limbs and toes are fully extended. B) a 21-day-old mouse, 2 days after the administration of CL (2mg/g) lifted by the tail. The limbs and toes are not extended, a sign indicative of neurological impairment.
Fig. 3.3 A) a control 21-day-old mouse being lifted by the tail from a wire grid. The grid is grasped firmly with the toes of all four limbs. B) a 21-day-old mouse 2 days after the administration of CL (2mg/mg) being lifted from a wire grid by the tail. The grid is gripped with the toes of the forelimbs alone, the mouse appeared to be unable to grasp the grid with its hindlimbs.
3.2 The effects of CL on skeletal muscle

(i) Muscle fibre resting membrane potentials

The mean control RMP of biceps brachii fibres was $-72.76 \pm 0.57\text{mV}$ whilst that of Edl fibres was $-70.35 \pm 0.53\text{mV}$. For soleus fibres the mean control RMP was $-66.76 \pm 0.49\text{mV}$ (mean $\pm$ SEM $n=200$ from 5 muscles). The mean RMP of control biceps brachii fibres was significantly higher than that of both Edl ($P = 0.0021$) and soleus ($P < 0.0001$) fibres. The mean RMP of control Edl fibres was also significantly lower ($P < 0.0001$) than that of soleus fibres.

Previous studies have also found that the mean RMP of EDL fibres is higher than that of the soleus fibres (Harris, 1971; Banker et al, 1983; Brook, 1986; Brook & Duchen, 1990).

The RMP of a muscle fibre is thought to be related to its diameter, the larger the diameter the higher the RMP (Katz and Thesleff, 1957). Therefore this data suggests that biceps brachii fibres are larger than those of the Edl and that Edl fibres are in turn larger than those of the soleus. This has previously been demonstrated by Rowe and Goldspink (1969) and Harris and Ribchester (1979).

Within a given muscle, variations in RMP existed between fibres in control preparations. The values for the fibres of the soleus ranged from $-57$ to $-87\text{mV}$, for those of the Edl from $-58$ to $-90\text{mV}$, and for those of the biceps brachii from $-58$ to $-92\text{mV}$.

Following the administration of CL the mean RMP of the soleus, Edl and biceps brachii fibres showed a steady decrease over the seven day period during which recordings were made (see Fig 3.4). From between 5 and 7 days after the administration of CL, Edl and soleus fibres with RMP's in the range $-50$ to $55\text{mV}$, i.e. below the normal range, were occasionally seen.
THE EFFECTS OF CL ON 21-DAY-OLD MUSCLE RMP

Fig. 3.4 The effects of CL on the mean resting membrane potential of soleus (○), biceps brachii (♦), and EDL (■) fibres in 21-day-old mice. Each point represents the mean±SEM of 200 observations made from five muscles at each time interval. The results were compared with their respective controls, and the statistical significance of any such difference was calculated using Student's t-test: $P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$. 

A line of best fit was calculated using regression analysis for each of the muscle RMP data series shown in Fig 3.4. The line produced using the mean EDL RMP data was found to have a slope of $-0.67 ± 0.19 \text{mV/day}$ ($\overline{x} ± \text{Sd}$) indicating the mean EDL RMP repolarises at a mean rate of 0.67mv/day following the administration of CL. A hypothesis test was carried out on the slope of this regression line, the slope was found to be significant ($P = 0.0008$). Pearson's correlation co-efficient ($r_p$) was also calculated to further ascertain the correlation between mean EDL RMP and time after the administration of CL. The EDL RMP data had an $r_p$ value of $-0.8$, which is significant for $n = 8$ at $P = <0.01$, which again demonstrates that the mean EDL RMP is highly dependent upon time after the
administration of CL, i.e. following the administration of CL the mean EdI RMP repolarises with time in a linear manner.

A similar trend was seen with a line of best fit was calculated using the mean RMPs of the biceps brachii against time after the administration of CL. This line had a slope of \( -0.76 \pm 0.17 \text{mV/day} \) (\( \bar{x} \pm \text{SD} \)) which is significant (\( P = 0.0043 \)). The rp value was -0.88 which is also significant for \( n = 8 \) (\( P < 0.005 \)). The mean biceps brachii RMP can therefore be seen to fall in a linear manner following the administration of CL, the mean RMP of biceps brachii fibres being significantly dependent upon time after the administration of CL.

The slope of the regression line and the rp value produced using the soleus RMP data were not significant. The soleus mean RMP, therefore, would not appear to depolarize in a linear fashion following the administration of CL, unlike the biceps and Edl. It can be seen from fig 3.4, however, that at 7 days the mean soleus RMP showed a large increase. If this point is excluded the line of best fit has a slope of \( -0.68 \pm 0.14 \text{mV/day} \) which is significant (\( P = 0.0031 \)), indicating that up until six days the mean soleus RMP (as in the case of the biceps and Edl) repolarises linearly after the administration of CL. This is substantiated further by the use of Pearsons correlation co-efficient on the soleus RMP data from 0 to 6 days. The rp value of -0.89 produced is highly significant (\( P < 0.005 \)), demonstrating that the mean soleus RMP is significantly correlated with time for the first 6 days following the administration of CL.
(ii) Fibrillation potentials

Fibrillation was first observed in the soleus and Edl 6 days after the administration of CL. It consisted of spontaneous, rhythmical contractions confined to the surface of the muscle. Only a few fibres from each muscle were affected, which did not prevent intracellular recordings at these muscles. Sharp angular fibrillation potentials were also recorded from some of these muscles, (see Fig 3.5).

Fig.3.5 Fibrillation potentials recorded from a soleus removed from a 21-day-old mouse 7 days after the administration of CL. Recorded with an extracellular electrode placed on the surface of the muscle. Vertical bar 20mV, horizontal bar 1s.
3.3 The effects of CL on the neuromuscular junction

(i) Spontaneous neurotransmitter release

Miniature end-plate potentials (mepps) were recorded in more than 90% of control soleus, EdI and biceps brachii fibres.

It can be seen from Fig 3.6 that the mean number of EdI and soleus fibres showing mepps fell dramatically within the first 24 hours following the administration of CL. The soleus was more severely affected than the EdI at 24 hours, this difference between the EdI and soleus was found to be significant (P = 0.0185). In marked contrast to the EdI and soleus, the number of biceps brachii fibres showing mepps remained well within normal limits over the first 24 hours.

Over the subsequent 24 hour period the number of EdI and soleus fibres demonstrating mepps fell still further. No mepps could be recorded in two of the five soleus and one of the five EdI preparations tested at this time, despite 40 fibres from each preparation being sampled (see Fig 3.7).

Between two and six days after the administration of CL the mean number of EdI and soleus fibres showing mepps remained at very low levels - see Fig 3.6. Occasionally at these times, however, a soleus or EdI was encountered from which up to 50% of fibres demonstrated mepps (see Fig. 3.7). A slight recovery in the mean number of soleus and EdI fibres showing mepps was seen seven days after the administration of CL. The numbers of EdI and soleus fibres showing mepps at this time varied considerably from one animal to another. Those muscles removed from 3 animals which appeared severely ill had very few fibres (<5) from which mepps could be recorded, while those removed from 2 mice demonstrating limited neurological symptoms showed mepps from more than 10 of the 40 fibres sampled (see Fig 3.7). In contrast to the slight recovery observed at the EdI and soleus, a significant reduction (P = 0.0094) in the mean number of biceps brachii fibres showing mepps was observed at this time (see Fig 3.7).
Unfortunately the twenty-one-day-old mice did not survive beyond seven days after the administration of CL, so it was not possible to ascertain if the recovery in the Edl and soleus continued or, alternatively, if further reductions in the numbers of biceps brachii fibres showing mepps occurred.
The effects of CL on the mean number of 21-day-old biceps soleus and EDL fibres showing MEPPS.

**Fig. 3.6** The effects of CL on the mean number of biceps brachii (♦), EDL (■) and soleus (○) fibres showing spontaneous MEPPs in 21-day-old mice. Each point represents the mean ± SEM of 5 observations with 40 fibres being tested in each muscle. Student's t-test was used to compare the results with their respective controls and to ascertain the statistical significance of any such differences: $P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$.

The effects of CL on the number of EDL and soleus fibres showing MEPPs in 21-day-old mice.

**Fig. 3.7** The effects of CL on the number of soleus (○) and EDL (■) end-plates at which MEPPs could be demonstrated in 21-day-old mice. Forty fibres from each muscle were tested in each of five animals at each time interval.
**Mepp frequency**

In control animals mepp frequencies were $0.34 \pm 0.05$ Hz for the soleus, compared to $0.79 \pm 0.09$ Hz for the EdI and $0.68 \pm 0.08$ Hz for the biceps brachii ($x \pm$ SEM). The mean control mepp frequencies of the EdI and biceps brachii were not significantly different. The mean mepp frequency of control soleus preparations however was significantly smaller than that of the EdI ($P = <0.0001$) and the biceps brachii ($P = 0.0004$).

Prior studies have also found that the mean mepp frequency in the soleus is lower than that of the EdI (Cangiano et al, 1980; Banker et al, 1983; Kim et al, 1984; Brook and Duchen, 1990). In contrast to the soleus and EdI, the biceps brachii is rarely used for *in vitro* physiological recordings. Duchen and Stefani (1971) reported a mean mepp frequency of $0.74 \pm 0.27$ Hz ($x \pm$ SEM) recorded from biceps brachii of control 21-day-old mice. This figure is remarkably similar to that reported in the present study.

Mepp frequency has been shown to increase with end-plate area (Kuno et al, 1971) and end-plate area has been shown to be proportional to muscle fibre diameter (Nystrom, 1968; Balice-Gordon and Lichtman, 1990). The present findings are consistent with the above reports, as type I fibres such as those found in the soleus are smaller than the type II fibres which predominate in the EdI and biceps brachii (Harris and Ribchester, 1979; Torres, 1986).

The effects of CL on the mean mepp frequencies of the soleus, EdI and biceps are shown in Figs 3.8, 3.10 and 3.12. Within 12 hours of the administration of CL there was an increase in the mean mepp frequency in both the soleus and the EdI (see Figs 3.8 and 3.10). This increase in the mean mepp frequency was caused by a small number of fibres, 14.3% of soleus and 8% of EdI fibres, which had abnormally high (<5Hz) mepp frequencies (see Figs. 3.9 and 3.11). No significant changes in the mean mepp frequency in the biceps brachii occurred at this time.

Only low mepp frequencies were recorded from the soleus and EdI four days after CL, whereas at the biceps brachii, mepp frequencies
were still within normal limits. A greater variation in mepp frequencies was seen in both the soleus and Edl at five days - see Figs 3.9 and 3.11.

Seven days after the administration of CL the mean mepp frequency in the soleus was slightly less than twice that of the control levels and the vast majority of the mepp frequencies recorded fell within the normal control range (see Figures 3.8 and 3.9). With one exception, all the mepp frequencies recorded from the Edl at this time, however, were below the mean control mepp frequency (see Fig 3.11). The mean mepp frequency of the biceps brachii at this time was more than twice that of the controls. This rise in mean mepp frequency was again caused by abnormally high mepp frequencies being recorded from a small proportion (i.e. 10.4%) of the biceps brachii fibres sampled at this time. Therefore, in terms of mepp frequencies, the biceps brachii at seven days appears similar to the soleus at 12 hours and the Edl at 12 and 24 hours post CL treatment (see above and Figs 3.13 and 3.14).
Fig. 3.8 The effects of CL on the mean mepp frequency in the soleus
of 21-day-old mice. Each point represents the mean ± SEM. The number of
fibres used to calculate this figure is shown in parenthesis alongside the
point. Five preparations were tested at each time interval. The control
value was 0.34 ± 0.048 Hz (X ± SEM).

Fig. 3.9 The effects of CL on mepp frequencies in the soleus of 21-
day-old mice. The mean control mepp frequency (●) was 0.34 ± 0.625 Hz
(X ± SD), calculated from 182 fibres sampled from 5 preparations.
Subsequent points (○) are the mepp frequencies of individual soleus fibres.
Fig. 3.10 The effects of CL on the mean mepp frequency in the Edl of 21-day-old mice. Each point represents the mean ± SEM. The number of fibres used to calculate this figure is shown in parenthesis alongside the point. Five preparations were tested at each time interval. The control value was 0.79 ± 0.087 Hz (X ± SEM).

Fig. 3.11 The effects of CL on mepp frequencies in the Edl of 21-day-old mice. The mean control mepp frequency (■) was 0.79 ± 1.137 Hz (X ± SD) calculated from 171 fibres sampled from 5 preparations. Subsequent points (□) are the mepp frequencies recorded from individual fibres.
Fig. 3.12 The effects of CL on the mean mepp frequency in the biceps brachii of 21-day-old mice. Each point represents the mean ± SEM. The number of fibres used to calculate this figure is shown in parenthesis alongside the point. Five preparations were tested at each time interval. The control value was 0.682 ± 0.81 Hz (X ± SEM).
Fig. 3.13 Intracellular recordings of mepps made from a control 21-day-old mouse. A) mepps recorded in the soleus. B) in the biceps brachii. C) in the EdI. The larger amplitude and lower frequency of the soleus mepps is clearly discernible. Vertical bar 1mV, horizontal bar 1 second.

Fig. 3.14 Intracellular recordings of mepps made from a 21-day-old mouse 12 hours after the administration of CL. A) mepps recorded in the soleus. B) mepps recorded in the EdI. Trace C) shows mepps recorded in the biceps brachii of a 21-day-old mouse 7 days after the administration of CL. Note the high frequency and large amplitudes of these mepps. Vertical bar 1mV, horizontal bar 1 second.
Mepp amplitudes

The mean mepp amplitude in the soleus of the control mice was $1.015 \pm 0.009\text{mV} \ (\bar{x} \pm \text{SEM}, n = 2509 \text{ from 5 preparations})$. For the Edl it was $0.9 \pm 0.0038\text{mV} \ (\bar{x} \pm \text{SEM} \ n = 5748 \text{ from 5 preparations})$ and for the biceps brachii $0.865 \pm 0.0075\text{mV} \ (\bar{x} \pm \text{SEM} \ n = 4668 \text{ from 5 preparations})$. The mean control mepp amplitude of the soleus was significantly ($P = <0.0001$) larger than that of the Edl and biceps brachii, and the mean Edl control mepp amplitude was significantly ($P = 0.0007$) larger than that of the biceps brachii.

Banker et al (1983) and Brook (1986) also reported that the mepps recorded from the murine soleus were significantly larger than those recorded in the Edl. Mepp amplitude has been found to be inversely related to muscle fibre diameter (Katz and Thesleff, 1957). Therefore, all other factors being equal (i.e. quantal size and muscle fibre input resistance per unit area), the smaller fibres of the soleus would be expected to have larger mepps than those of the Edl and biceps brachii. (For discussion see Ginsborg and Jenkinson, 1976.)

The effects of CL on mepp amplitudes can be seen from Figs 3.15, 3.16, 3.17, 3.18, 3.19, and 3.20. A significant increase in the mean amplitude of mepps sampled from the soleus and Edl was seen within 12 hours of the administration of CL. This effect was most pronounced in the soleus. The increase in the mean mepp amplitude was caused by an increase in the occurrence of mepps larger than $1.5\text{mV}$ skewing the mepp amplitude distribution to the right.

The mean mepp amplitude of the soleus and Edl had increased further by day one, again caused by an increase in the proportion of mepps with amplitudes greater than $1.5\text{mV}$ (see Figs 3.18 and 3.19). A smaller increase (although still significant) in the mean mepp amplitude also occurred in the biceps although a far smaller proportion of mepps with amplitudes above $1.5\text{mV}$, however, occurred after one day (see Fig 3.20) compared with the Edl and soleus.

Over the next 24 hour period the mean mepp amplitude of the soleus
and EdI fell back towards control levels, while in the biceps a further slight rise was seen.

Three days after the administration of CL few functioning EdI or soleus end-plates remained (see Fig 3.6). Many of the mepps recorded from the remaining end-plates were abnormally large (see Figs 3.18 and 3.19) resulting in mean mepp amplitudes significantly larger than the respective controls. The mepps recorded from the biceps at this time were also frequently larger than those seen in control preparations (see Fig 3.20).

At four days few mepps were recorded from either the EdI or the soleus. The mean amplitude of the mepps recorded from the soleus at this time was significantly smaller than the control value while the mean mepp amplitude of the EdI was not significantly different from its control.

Five days after the administration of CL the mean mepp amplitudes of the soleus, EdI and biceps were significantly larger than the respective controls. In the biceps, mepps with amplitudes between 1.25 and 2.5mV became increasingly evident (see Fig 3.20), while in the EdI a large proportion of the mepps observed had amplitudes between 1 and 2mV (see Fig 3.19).

At six days the mean mepp amplitude of the soleus and EdI were significantly smaller than their controls. The mepp amplitude distributions of the soleus (Fig 3.18) and EdI (not shown) were skewed to the left at this time. The mean mepp amplitude of the biceps fell slightly but remained significantly ($P = 0.0012$) higher than that of the control.

The mean mepp amplitude of the soleus rose to above control levels at seven days, a large proportion of mepps with large amplitudes being recorded (Fig 3.15). The mean mepp amplitude at the EdI rose slightly but was still significantly smaller ($P = 0.0026$) than the control value. A large proportion of the mepps recorded from the EdI at this time had amplitudes below 0.5mV. The biceps’ mean mepp amplitude was again significantly larger than its control. The biceps’ mepp amplitude distribution seen at this time was also very different from the control, i.e. a large proportion of the mepps recorded at 7 days had amplitudes below 0.5mV, and large mepps
were also more evident.

**The Effects of CL on MePP Amplitudes in the Soleus of 21-Day-Old Mice**

Fig. 3.15 The effects of CL on the mean amplitude of spontaneous mepps recorded in the soleus of 21-day-old mice. Each point represents the mean ± SEM of the pooled results obtained from 5 muscles, the figures in parenthesis indicate the number of mepps used to calculate the mean. The mepp amplitudes were corrected to a standard RMP of -75mV using the method of Katz and Thesleff (1957). The results were compared with the control value of 1.015 ± 0.009mV (x ± SEM) using Student's t-test: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.

**The Effects of CL on MePP Amplitudes in the EDL of 21-Day-Old Mice**

Fig. 3.16 The effects of CL on the mean amplitude of spontaneous mepps recorded in the EDL of 21-day-old mice. Each point represents the mean ± SEM of the pooled results obtained from five muscles, the figures in parenthesis indicate the number of mepps used to calculate the mean. The amplitudes were corrected to a standard RMP of -80mV using the method of Katz and Thesleff (1957). The results were compared to the control value of 0.9 ± 0.0038mV (x ± SEM) using Student's t-test: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.
**Fig.3.17** The effects of CL on the mean amplitude of spontaneous mepps, recorded in the biceps brachii of 21-day-old mice. The points represent the mean ± SEM of the pooled results obtained from 5 muscles, the figures in parenthesis indicate the number of mepps used to calculate the mean. The mepp amplitudes were corrected to a standard RMP of -80mV using the method of Katz and Thesleff (1957). The results were compared with the control value of 0.865 ± 0.0075mV using Student’s t-test: P<0.05 = *; P<0.01 = **; P<0.001 = ***.
Fig. 3.18 Amplitude distributions of spontaneous mepps recorded in the soleus of 21-day-old mice at the following times: A) control-hatched columns; B) 12 hours; C) 1 day; D) 4 days; E) 6 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from five preparations. The mepp amplitudes were corrected to a standard RMP of -75mV using the method of Katz and Thesleff (1957). Note the differences in the scales of the ordinate in each of the histograms.
Fig. 3.19 Amplitude distributions of spontaneous mepps recorded in the Edl of 21-day-old mice at the following times: A) control-hatched columns; B) 12 hours; C) 1 day; D) 3 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The mepp amplitudes were corrected to a standard RMP of -80mV using the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
Fig. 3.20 Amplitude distributions of spontaneous mepps recorded in the biceps brachii of 21-day-old mice at the following times: A) control-hatched columns; B) 1 day; C) 3 days; D) 4 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The mepp amplitudes were corrected to a standard RMP of -80mV using the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
(ii) The effects of CL on neurally evoked transmitter release - end-plate potentials (epps)

Epps were recorded from $35.8 \pm 0.2$ of the 40 21-day-old control soleus preparation fibres sampled compared to $35.2 \pm 0.37$ of the 40 Edl fibres (Mean ± SEM, n=5 for both muscles) tested. A variety of factors may have been responsible for the failure to record epps from all forty of the fibres sampled from each of the control preparations. Occasionally a partially curarized fibre would contract and break the tip of the recording electrode possible damaging the end-plate and rendering it unresponsive to further neural stimulation. The nerve supplying the muscle may also have been damaged during the dissection or when it was being drawn into the stimulating electrode.

The effects of CL on the number of Edl and soleus fibres showing epps are shown in Figs 3.21 and 3.22. One day after the administration of CL the mean number of both Edl and soleus fibres showing epps fell to less than 25% of the control levels. Some variation in the proportion of fibres showing epps was seen between preparations at this time (see Fig 3.22). Failures in transmission in response to successive stimuli were seen in some end-plates at this time (see Figs 3.25 and 3.31). The mean number of Edl fibres responding to neural stimulation with an epp decreased further over the next 24 hours, while the mean number of soleus fibres exhibiting an epp rose slightly. Three days after CL-treatment only $0.4 \pm 0.25$ of Edl and $0.4 \pm 0.4$ (Mean ± SEM) of soleus fibres exhibited epps. At four days the mean number of soleus fibres showing an epp rose to 7/40, which is significantly higher ($P=0.0162$) than the Edl at this time. This rise was not sustained, however, for five days after CL treatment the mean number of both soleus and Edl fibres showing an epp fell. Seven days after the administration of CL the mean number of fibres showing epps was at its highest since the administration of CL (see Fig 3.21). In the soleus the mean number of fibres exhibiting epps increased significantly ($P=0.0023$) between five and seven days. Unfortunately, no 21-day-old animals
survived for more than seven days after the administration of CL, so it was not possible to see if this increase continued. The epp waveforms recorded at this time, however, were frequently abnormal (see Figs 3.25 and 3.26). Successive stimuli frequently failed to evoke a response which was consistent in latency or amplitude. At some end-plates two distinct populations of epps were seen (Fig 3.26B), which might indicate some muscle fibres were multiply innervated.

![Graph showing the effects of CL on the mean number of 21-day-old soleus and EDL fibres showing EPPs](image)

Fig.3.21 The effects of CL on the mean number of EDL (■) and soleus (○) fibres in 21-day-old mice responding to neural stimulation with a discernible epp. Each point represents the mean ± SEM of 5 observations, with 40 fibres being tested on each muscle. Student’s t-test was used to ascertain the statistical significance of the difference between the results and their respective controls: P<0.05 = *; P<0.01 = **; P<0.001 = ***.
Fig. 3.22 The effects of CL on the number of Edl (■) and soleus (○) fibres responding to neural stimulation with a discernible epp. Forty fibres from each preparation were tested, and five soleus and Edl preparations were sampled at each time interval.

**Epp latency**

The epps recorded from control soleus preparations had a mean latency of 1.76±0.02ms. \(n=158\) from 5 preparations) which was significantly greater \(P < 0.0001\) than those recorded from control Edl preparations which had a mean latency of 1.56±0.02ms. \(N=155\) from 5 preparations). This difference might represent a longer length of nerve stimulating the soleus. Efforts were made to standardise the length of nerve used to stimulate the muscle, however so this explanation is unlikely. Brook and Duchen (1990), using similar equipment, also noted that the latency of the end-plate potentials recorded at the soleus were greater than those recorded at the Edl. The effects of CL on the mean epp latency recorded in the soleus and Edl are shown in Fig 3.23.

Within two days of the administration of the mean latency of the epps recorded from the Edl was significantly prolonged \(P = 0.0083\) while those
recorded from the soleus remained within normal limits. The few epps recorded from the soleus and Edl at 3 and 4 days post CL treatment showed a large range of mean latencies, some being prolonged and others being within normal limits (this factor is responsible for the large error bars seen at these times on Fig 3.23). At seven days post CL treatment the mean epp latencies recorded at both the soleus and Edl were significantly (P < 0.0001 and P < 0.0001 respectively) prolonged compared to those of the controls (see Fig 3.23). Lines of best fit were calculated from both of the lines shown in Fig 3.23. The line produced using the soleus mean epp latency data had a slope which was not significant. The Pearsons correlation coefficient calculated for this data was also insignificant. The mean soleus epp latency, therefore, is not linearly related to time after the administration of CL. In contrast the epp latencies recorded in the Edl do appear to increase in a linear fashion with time after the administration of CL as the line of best fit produced using the Edl data has a slope of 0.0036 ± 0.0012 ms/day, which is significant (P = 0.032) and the Pearsons correlation coefficient produced using this data, was r_p = 0.8, which is also significant (P < 0.01). Epp waveforms recorded at seven days are shown in Fig 3.26. The prolonged latency of this response can be clearly seen. At most control endplates, successive epps recorded from a single endplate showed reasonably constant latencies as can be seen from Figure 3.25 At the latter stages of CL toxicity successive epps did not show this uniformity in latency.

**Time to peak epp**

The effects of CL on the time to peak epp at the Edl and soleus is shown in Fig 3.24. This graph is very similar to that in Fig 3.23. The time to peak epp however is frequently easier to ascertain than the latency of the response and therefore the time to peak is probably a better indicator of changes in the epp characteristics than the latency.

The mean control time to peak epp of the soleus was significantly (P = < 0.0001) slower than that of the Edl (soleus - 2.37 ± 0.02, Edl =
CL caused a steady increase in the mean time to peak epp at the Edl (see Fig 3.24). A line of best fit was calculated using this data. The resulting regression line has a slope of $0.0069 \pm 0.0026$ ms/day which is significant ($P = 0.065$) indicating that the mean time to peak epp at Edl end-plates increases in a linear fashion following the administration of CL. The Pearson's correlation co-efficient calculated for this data was $r_p = 0.76$ which is also significant for $n = 6$ at $P < 0.025$), further demonstrating the correlation between the mean time to peak epp at the Edl and time following the administration of CL. The mean time to peak epp at the soleus, however, did not increase in a linear fashion following treatment with CL. The mean time to peak epp at soleus end-plates was found to be within normal limits at 1, 4, and 5 days. At 7 days however the soleus mean time to peak epp was significantly ($P < 0.001$) larger than the control value. Both the slope of the regression line and the $r_p$ value produced using the soleus data were not significant, demonstrating that although epps with abnormally large times to peak were frequently observed, following the administration of CL the incidence of such epps did not increase in a linear fashion.
Fig. 3.23 The effects of CL on the mean epp latency in the soleus (○) and Edl (■) of 21-day-old mice. Each point represents the mean ± SEM of the results pooled from five preparations. The figures in parenthesis indicate the number of readings used to calculate the mean at each time interval. The results were compared with their respective controls: Edl 1.56 ± 0.02 ms; soleus 1.76 ± 0.02 ms (X ± SEM) using Student's t-test to ascertain the statistical significance of any differences: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.

Fig. 3.24 The effects of CL on the mean time to peak epp in the soleus (○) and Edl (■) of 21-day-old mice. Each point represents the mean ± SEM of the results pooled from 5 preparations. The figures in parenthesis indicate the number of readings used to calculate the mean at each time interval. The results obtained from Edl preparations were compared with the Edl control value of 2.12 ± 0.2 ms while the soleus results were compared to the soleus control value of 2.37 ± 0.02 ms (X ± SEM), using Student's t-test to ascertain the statistical significance of any such differences: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.
Fig. 3.25 Successive epp waveforms recorded at single endplate's of 21-day-old mice, at the following times: A) Control soleus, each stimulation evoked a clearly discernible epp. A slight variation in the size and latency of the responses can be seen however. B) Soleus 1 day after the administration of CL. A number of failures can be seen at this time, a failure being a stimulus which fails to evoke an epp, seen as a line across the base of the other epps. A marked variation in the latency of successive waveforms is seen at this time. C) EDL 2 days after the administration of CL. The response of this end-plate is indistinguishable from that of control end-plates indicating that some terminals are still functionally intact at this time. Vertical bar 1 mV, horizontal bar 1 ms.
Fig. 3.26 Successive epp waveforms recorded at single end-plates of the soleus and Edl of 21-day-old mice 7 days after the administration of CL.

A) Edl, epps; a large variation in the amplitude of epps can be seen, and some failures in transmission are also apparent. B) Edl epps; two distinct epps with a large amplitude and normal latency are seen alongside 20 further epps which show an abnormally prolonged latency and smaller amplitude. This muscle fibre might be innervated by two separate neuromuscular junctions. C) Soleus epps, which show an enormous variation in both amplitude and latency. Vertical bar 1mV, horizontal bar 1ms.
Quantal content

The mean quantal content (mqc) of control soleus end-plates was 38.81 ± 2.59 (x ± S.E.M. n = 188) which is significantly larger (P = 0.045) than that of control EdI end-plates which had a mqc of 31.71 ± 2.29 (x ± S.E.M. n = 155). This finding was unexpected and contrasts with earlier findings of Tonge (1973), Banker et al (1983) and Brook (1986) who observed that the mqc of the EdI was greater than that of the soleus. These reports, however, concerned adult muscles and far fewer observations were made. Tonge (1973) and Brook (1986) used higher rates of stimulation (i.e. 50Hz) to evoke trains of epps which might also account for this difference as quantal release might be more efficient in the soleus than the EdI at lower stimulation frequencies.

The effects of CL on quantal content is shown in Figs 3.27, 3.28, 3.29 and 3.30. Twenty four hours after the administration of CL the mean quantal content of the EdI fell to almost half its control level, only quantal contents below 40 were recorded (see Fig 3.30). The mean quantal content calculated from the remaining soleus end-plates was within normal limits. It can be seen from Fig 3.29 that the distribution of quantal content recorded from the soleus is markedly different from that of the controls in that the proportion of quantal contents of between 30 and 60 is much reduced at 24 hours. One end-plate with a quantal content of ≥190 was observed.

Two days after the administration of CL few end-plates responded to neural stimulation. At both the EdI and soleus a few of the surviving end-plates were found to have large quantal contents whilst the majority had quantal contents below 40 (see Figs 3.29 and 3.30).

Between three and five days after the administration of CL the few end-plates found that responded to neural stimulation all had mean quantal contents below 50 (see Figs 3.29 and 3.30).

Seven days after the administration of CL more than 50% of the quantal contents recorded from the EdI were below 10 and all were below
At the soleus a similar trend was seen, i.e. more than 60% of the quantal contents recorded at this time were below 20.

**Fig. 3.27** The effects of CL on the mean quantal content of neurally evoked transmitter release in the soleus of 21-day-old mice. Each point represents the mean ± SEM of the results pooled from 5 preparations. The figures in parenthesis indicate the number of observations used to calculate the mean at each time interval. The quantal contents were corrected to a standard RMP of -75mV according to the method of Katz and Thesleff (1957). The results were compared with the control value of 38.81 ± 2.59 (X ± SEM), using Student's t-test to ascertain statistical significance: P<0.05 = *; P<0.01 = **; P<0.001 = ***.
Fig. 3.28 The effects of CL on the mean quantal content of neurally evoked transmitter release in the EdI of 21-day-old mice. Each point represents the mean ± SEM of the results pooled from 5 preparations. The figures in parenthesis indicate the number of observations used to calculate the mean at each time interval. The quantal contents were corrected to a standard RMP of -80mV according to the method of Katz and Thesleff (1957). The results were compared to the control value of 31.71 ± 2.29 (X ± SEM), using Student’s t-test to ascertain statistical significance: P<0.05 = *; P<0.01 = **; P<0.001 = ***.
Fig. 3.29 Quantal content distributions recorded in the soleus of 21-day-old mice at the following times: A) control-hatched columns; B) 1 day; C) 3 days; D) 4 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The quantal contents were corrected to a standard RMP of -75mV according to the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
Fig. 3.30 Quantal content distributions recorded in the Edl of 21-day-old mice at the following times: A) control-hatched columns; B) 1 day; C) 2 days; D) 4 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The quantal contents were corrected to a standard RMP of -80mV according to the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
Fig. 3.31 Intracellular recordings of epps made from the soleus and Edl of 21-day-old mice at the following times: A) Control soleus; B) Control Edl; C) Edl 2 days after CL; D) Soleus 2 days after CL; E) Edl 7 days after CL; F) Soleus 7 days after CL. The mean quantal contents are shown on each trace. Vertical bar 5mV, horizontal bar 1 Second.
(iii) Morphological observations

The position of the Edl and soleus within the mouse hind limb is shown in Figure 3.32. The Edl is one of the anterior crural muscles, a group which also includes the tibialis anterior, tibialis posterior and the extensor hallucis proprius. The soleus however is located amongst the posterior crural muscles, namely the gastrocnemius and plantaris. The musculature at this level is completed by the lateral crural muscles which are the peroneus brevis and peroneus longus.

The position of both the long and short heads of biceps brachii in the upper arm is shown in Figure 3.33. At this level the medial, lateral and long heads of the triceps brachii, the dorso-epithrochlearis and the coracobrachialis muscles can also be seen.

a) Motor innervation of muscles

The precise distribution of the points of contact between motor axons and muscle fibres (i.e. the motor points) varies from one muscle to another (see Bowden and Duchen, 1976). The knowledge of the pattern of motor innervation of a muscle is important, for it facilitates focal electrophysiological recording of neuromuscular events. The end-plate regions of the soleus and Edl are shown in Figure 3.34, where the whole muscles have been stained for acetylcholinesterase activity, which is confined to the end-plates. The end-plates of the Edl can be seen to occur in a V-shaped band towards the middle of the muscle. The soleus end-plates are distributed in a disc like pattern in the mid portion of the muscle.

The pattern of innervation seen in mouse skeletal muscle is very similar to that observed in other species of mammals (Bowden and Duchen, 1976), in that both motor and sensory nerves enter the muscle at the neurovascular hilum and undergo repeated branching (Duchen and Gale, 1985). The groups of motor axons eventually break into a spray of simple myelinated preterminal axons each of which eventually forms a single end-
plate on one muscle fibre. Prior to entering the end-plate the axon loses its
myelin sheath and forms a delicate terminal arborization (see Fig 3.35)
which enters grooves on the muscle surface.

Electron microscopy permits the structure of the neuromuscular
junction to be viewed in far greater detail - see Fig 3.36. The ultrastructure
of normal mammalian neuromuscular junctions has been the subject of a
number of reviews, notably Couteaux (1972), Bowden and Duchen (1976),
Duchen and Gale (1985), and Salpeter (1987). The neuromuscular junction
consists of four distinct compartments (Salpeter, 1987):
1). The presynaptic compartment, consisting of the axon terminal and
associated structures.

2). An extracellular compartment between the axon and other pre and
post synaptic structures, i.e. the synaptic cleft.

3). A distinctive post synaptic membrane compartment (the junctional
folds).

4). A post synaptic muscle compartment known as the sole plate.

The axon terminal lies below the Schwann cell and contains
mitochondria and abundant clear vesicles, but lacks the neurofilaments and
tubules seen in the preterminal axon (Duchen and Gale, 1985).

The extracellular compartment is made up by both the primary and
secondary clefts and extends around the junction, both below the axon
terminal (between the pre and post synaptic membranes) and between the
terminal axon and Schwann cell.

The postsynaptic membrane of the neuromuscular junction in both 21-
day-old and adult junctions undergoes a series of irregular invaginations
forming the junctional folds. The folds in adult mice tend to be more
complex and deeper than those in the 21-day-old mice. The depth and
complexity of these folds also varies from one species to another (Coërs and
Woolf, 1959; Bruchnal and Schmalbruch, 1980; Salpeter, 1987). In the
mouse the junctional folds of fast twitch (type II) muscle such as the Edl
tend to be deeper and more numerous than those of slow twitch (type I)
muscles such as the soleus (Duchen, 1971).
The structure and contents of the sole-plate compartment differ from other areas of the muscle fibre in that the sarcoplasm at the sole-plate contains abundant mitochondria, vesicles, and a complex network of microtubules and filaments (Hirokawa and Heuser, 1982). The muscle nuclei seen within the sole-plate are known as sole-plate nuclei and tend to be larger and have more prominent nucleoli than the nuclei seen at other areas of the muscle. The structure and ultrastructure of the muscle spindles of control mice is discussed in the "Adult mice" results section.
Fig. 3.32 Transverse section through the middle third of the crural muscles of the right leg of an adult control mouse. The positions of tibialis anterior (TA), extensor digitorum longus (EDL), soleus (S), plantaris (P), peroneus longus (PL) and gastrocnemius (G) are indicated. HvG x 40.
Fig. 3.33 Transverse section through the middle of the upper right arm muscles of an adult control mouse. The positions of long head of the biceps brachii (Bbl), short head of the biceps brachii (Bbs), triceps brachii (Tb), dorso-epitrochlearis brachii (Deb) and coracobrachialis (Cb). HvG x 40.
Fig. 3.34 Whole muscle preparations stained for cholinesterase activity, to demonstrate the distribution of end-plates using the method of Lewis (1958). The end-plates are seen as dark spots which lie in a band across the soleus (A). The end-plates of the EdI (B) are distributed in a V-shaped pattern. The tendons of origin (O) and insertion (I) are also shown.
Fig. 3.35 Longitudinal paraffin sections of calf muscle, from a normal adult mouse silver impregnated by the method of Glees (1946), modified by Marsland et al (1954) was used. The axons in the intramuscular nerve, and the unbranched preterminal fibres, each innervating one motor end-plate with a terminal arborization (arrows) can be seen. X 400.

Fig. 3.36 Motor end-plate of flexor muscle from a normal 21-day-old mouse. The axonal terminal (A) contain abundant clear synaptic vesicles and mitochondria. The presynaptic axonemal membrane lies close to the postsynaptic membrane of the muscle fibre. The basal lamina separates the axoneme from the muscle fibre, and extends down into the postsynaptic folds of the sarcolemmal membrane. Some Schwann cell cytoplasm (S) is seen. [Neg. 90534 x 30,000. Bar = 1 µm.]
Fig. 3.36 Motor end-plate of foot muscle from a normal 21-day-old mouse. The axonal terminal (A) contain abundant clear synaptic vesicles and mitochondria. The presynaptic axolemmal membrane lies close to the postsynaptic membrane of the muscle fibre. The basal lamina separates the axolemma from the muscle fibre, and extends down into the postsynaptic folds of the sarcolemmal membrane. Some Schwann cell cytoplasm (S) is seen. (Neg. 90534 x 30,000. Bar = 1μm).
b) Morphological abnormalities induced by CL in twenty-one-day old mice

The effects of CL on the peripheral nervous system and muscle of 21-day-old mice were studied using a combination of light and electron microscopy in a collaborative study with Dr C C Lee. Observations were made between 12 hours and 7 days after the administration of CL.

(i) 12 hours

No abnormalities were observed in the peripheral nerves. In endplates the plasmalemma of the axon terminals appeared intact and the terminal itself was normally positioned between the Schwann cell and post synaptic junctional folds. The pre-synaptic terminals contained abundant clear and coated vesicles (see Fig 3.37). Electron lucent areas of rarefied axoplasm (see Fig 3.37) observed in two end-plates might have been due to preparational artifacts but, in view of the later physiological and morphological changes, they may in fact represent the beginnings of CL-induced pathology.

(ii) One day

The silver impregnation technique used (a modification of the Glees method) allowed intramuscular nerves and the terminal arborizations of motor nerves to be observed. Large nerves of the hind limbs, such as the posterior tibial and sciatic and certain intramuscular nerve bundles, appeared unaffected. Argyrophilic granules which were considered to be evidence of axonal degeneration were seen within some intramuscular nerve sheaths or surrounding end-plates.

Only one of 16 end-plates identified at this time appeared normal, while a proportion of the others appeared slightly abnormal, containing electron lucent areas of axoplasm and dilated membrane bound cisternae
(see Fig 3.38). The remaining end-plates were clearly abnormal. In some end-plates the whole terminal was electron lucent and contained few synaptic vesicles. The mitochondria found within these terminals were frequently rounded and swollen in appearance. Schwann cell processes were occasionally found to interpose between the axon terminal and the post-synaptic junctional folds.

No completely denervated end-plates were seen at this time. The sciatic and posterior tibial nerves appeared normal. In the smaller distal nerves of the hind limbs, however, axons demonstrating degenerative changes were observed. These changes included rarefaction of the axoplasm, fragmentation of the axolemma and development of large membrane bound vacuoles. Axons demonstrating these changes were seen in the intramuscular nerve bundles and preterminal myelinated axons of the soleus and foot muscles.

(ii) Two days

The end-plates identified were severely abnormal. The axon terminal was frequently found to be replaced by the Schwann cell which now covered the post-synaptic membrane (see Fig 3.39). The sciatic and tibial nerves again rarely showed ultrastructural abnormalities. Both of these nerves contained axons with normal myelin sheaths that showed no signs of vacuolation. The only abnormality seen was an occasional nerve fibre showing axon degeneration (the myelin sheath being intact) in the tibial nerve. In contrast to the sciatic and tibial nerves abundant evidence of degeneration was seen in the intramuscular nerves of the hind limbs.
Fig. 3.37 Motor end-plate of hind limb muscle from a 21-day-old mouse 12 hours after CL. This end-plate appears within normal limits; the axolemma, vesicles and mitochondria appear normal. Areas of rarefied axoplasm (*) might be artefactual, or may represent early abnormalities in view of the latter changes. (Neg. 91464 x 30,000. Bar = 1μm).
Fig. 3.38 Soleus end-plate from a 21-day-old mouse 24 hours after CL. Some of the axon terminals are rarefied and contain few vesicles (arrows) while others contain dense axoplasm with clumps of vesicles (arrowheads). The mitochondria contained within the axonal terminals are rounded and swollen in appearance. (Neg. 98413.4 x 20,000. Bar = 1μm).
Fig. 3.39 Motor end-plate of hind limb muscle from a 21-day-old mouse 2 days after CL. No intact axons remain. The Schwann cell (SC) now lies directly above the post synaptic membrane (arrow). Debris (possibly from the nerve terminal) can be seen within the cytoplasm of the Schwann cell (*). (Neg. 99621 x 30,000. Bar = 1μm).
(iv) Three days

Paraffin sections of both fore and hind limbs were prepared from mice used in the physiological study. No indications of axonal degeneration were seen in the fore limbs, i.e. end-plate terminal arborizations were numerous, and the small intramuscular nerves appeared intact. In contrast to the fore limbs, in the hind limbs only large nerve bundles contained intact axons and the terminal arborizations of axons rarely entered end-plate regions (end-plates were identified by the presence of sole plate nuclei) (see Fig 3.40). The small intramuscular nerves also showed extensive axonal fragmentation.

All the end-plates seen with EM were denervated, the terminal axon having been replaced by the Schwann cell which now covered the postsynaptic junctional folds. The Schwann cells were frequently seen to contain cellular debris.

The small intramuscular nerves fibres of the soleus, Edl and hind foot muscles were severely degenerated. Frequently, all that remained was axonal debris and myelin ovoids. A few degenerating fibres were also seen in the large nerves (notably the plantar).

(v) Four days

Large myelinated axons at various stages of degeneration were seen within the sciatic and posterior tibial nerves. The dissolution of the axoplasmic organelles appeared to precede the disruption and eventual disintegration of the myelin indicating a primary axonopathy. The Schwann cells surrounding the degenerating axons were frequently seen to contain debris.

A striking contrast was apparent between the muscles of the fore limbs (e.g. the biceps brachii) and the muscles of the hind limbs (such as the Edl, soleus). Intramuscular nerve bundles and end-plates appeared normal within the biceps brachii whereas in the Edl and soleus marked degeneration of the fibres within the intramuscular nerves and the axons innervating the
end-plates was seen. These findings were confirmed when the muscles were viewed with the electron microscope. The end-plates of the biceps brachii were normal in appearance (see Fig 3.41), while those of the Edl and soleus were denervated.

(vi) Five days

Paraffin section histology alone was used to study the pathology. The innervation of the fore limbs was again found to be intact. Examination of sections of the proximal muscles of the hind limbs (at the pelvic and thigh level) revealed that the innervation at this level was preserved. At lower levels, i.e. the crural muscles of the lower leg, intramuscular nerve bundles containing degenerating fibres and denervated end-plates were seen. The muscles of the hind feet appeared to be particularly severely affected. Very few axons remained in the intramuscular nerves. The sensory innervation of the skin of the feet however appeared to be spared, numerous intact axons being seen in the skin.

(vii) Seven days

As few 21-day-old mice survived beyond 5 days only a limited amount of material was available for light or electron microscopy. Abnormalities in the innervation of the fore limbs were observed for the first time at 7 days, when silver stained paraffin sections were viewed under LM. Terminal arborizations were conspicuously absent from some end-plates.

In the hind limbs marked degeneration of the intramuscular nerve bundles was seen in both resin and paraffin sections. Almost all of the end-plates observed were denervated. An abundance of intact small (probably sensory) nerve fibres were seen in sections of skin. Under EM most end-plates were found to be denervated with Schwann cells overlying the post synaptic junctional folds. There was some evidence, however, suggesting
that reinnervation might have been taking place in the form of tiny axonal profiles lying just above the post-synaptic junctional folds and small unmyelinated fibres, possibly regeneration sprouts, were found amongst degenerating fibres of an intramuscular nerve.

Fig.3.40 Sections of calf muscle from a 21-day-old mouse 3 days after CL, showing the area of innervation. In the nerve bundle there are granules of argyrophilic debris indicating axonal degeneration. These granules were also seen (arrows) in the remains of preterminal axons and terminals, none of which remain intact. Silver impregnation x 400.
Fig. 3.41 EM of end-plate in biceps brachii from a 21-day-old mouse 4 days after CL. The axon terminal (A) is intact containing abundant vesicles and normal mitochondria. The muscle fibre also shows no abnormalities. (Neg. 99565.4 x 30,000. Bar = 1μm).
3.4 Summary of the effects of CL on 21-day-old mice

Cycloleucine was found to induce paralysis of the toes of the hind limbs within 24 hours of administration. Physiological recordings made from the soleus and the Edl at 24 hours revealed that some end-plates failed to respond to neural stimulation while others failed to show spontaneous mepps. At further soleus and Edl end-plates failures in neuromuscular transmission and epps with prolonged latencies were seen. Abnormally high mepp frequencies were frequently encountered in the soleus and Edl within 12 hours of the administration of CL.

Morphological abnormalities were seen in both the intramuscular nerves and the neuromuscular junctions of the distal muscles at 24 hours. These abnormalities consisted of areas of electron lucent axoplasm, swollen degenerative mitochondria with degenerative cristae and nerve terminals lacking synaptic vesicles. The proximal muscles were unaffected at this time.

The clinical condition of the mice worsened over the next 2-3 days correlating with further reductions in the number of soleus and Edl fibres which demonstrated either epps or mepps. At four days very few soleus or Edl fibres exhibiting either mepps or epps were seen, almost all the 400 fibres sampled appeared denervated. A concomitant increase in the number of denervated end-plates where the axon terminal had been replaced by Schwann cell processes were seen. The biceps brachii however remained intact.

In mice surviving beyond 6 days an increase in the number of soleus and Edl fibres exhibiting both mepps and epps was found. Morphological indications of reinnervation such as tiny axonal sprouts at some soleus end-plates were seen at 7 days. In the fore limbs, however, at 7 days degeneration of the terminal arborizations of motor fibres was observed for the first time. High mepp frequencies were recorded in some bicep brachii fibres while others appeared denervated.
4.1 Clinical observations

The adult mice treated with CL showed neurological and behavioral symptoms of toxicity which were very similar to those previously outlined for the 21-day-old mice. The development and time course of these changes was far slower however in the adult mice. A considerable amount of variation in both the time course of the development, and the severity of symptoms was seen between individual mice.

Immediately following the administration of CL the mice appeared alert and active and were indistinguishable from the controls.

At 48 hours, signs of neurotoxicity became apparent. Many of the mice had ruffled coats with particles of food and sawdust adhering to them, indicating that they had stopped grooming. Slight weakness of the hindlimbs, particularly the toes, became evident when the mice were encouraged to climb on a grid.

By 3 days weakness in the hindlimbs had become more apparent. Sensation, however, still appeared to be intact. The mice showed the same responses as control mice when the fore and hindlimbs, trunk, snout and tail were pinched with forceps.

Four days after the administration of CL the mice tended to huddle together in the corner of the cage and moved only when touched. Loss of body weight was also apparent at this time.

By 5 days some of mice had died. Those which remained were frequently observed to drag their hind limbs when crawling. The abdominal and intercostal muscles of some mice also appeared to be paralysed, leading to costal recession.

At seven days the condition of the remaining mice appeared to stabilise somewhat. Despite the loss of body weight and paralysis of the hindlimbs the mice appeared slightly more active and lively.
4.2 The effects of CL on skeletal muscle in adult mice

(i) The effects of CL on the weight of the Edl and soleus in adult mice

In adult control mice the Edl weighed $12.58 \pm 0.675$mg ($\bar{x} \pm$SEM $n = 5$) whereas the soleus weighed $7.64 \pm 0.46$mg ($\bar{x} \pm$SEM $n = 5$). The Edl is significantly larger ($P < 0.003$) than the soleus, a finding previously reported by Stephens (1985) and Brook and Duchen (1990).

The effects of CL on the weight of the soleus and Edl is shown in Fig 4.1. Within 2 days of the administration of CL the weight of the Edl was below the control range. Over the next 5 days it fell still further. This trend was not repeated for the soleus where the administration of CL did not appear to have a consistent effect. A line of best fit was calculated for the Edl data. The resulting line had a slope of $-0.559 \pm 0.008$ mg/day which is significant at $P < 0.001$. The correlation coefficient for this data was $r_p = -0.9035$, which is also significant ($P < 0.005$). The weight of the Edl appears to decrease in a linear manner following the administration of CL.

![Graph showing the effects of CL on the weight of the EDL and soleus of adult mice](image)

Fig. 4.1 The effects of CL on the weight of the soleus ($\circ$) and Edl ($\square$) of adult mice. The weight of the soleus of control mice was $7.64 \pm 1.03$mg ($\bar{x} \pm$SD $n = 5$) whereas the weight of the Edls removed from the same mice was $12.58 \pm 1.51$mg ($\bar{x} \pm$SD $n = 5$).
(ii) The effects of CL on the mechanical response of the soleus and EdI to direct and indirect stimulation

a) The response to direct stimulation

_Twitch tension_

The twitch tension produced by control soleus preparations in response to direct stimulation was $0.296 \pm 0.0085$ g/mg ($\bar{x} \pm$ SEM $n = 5$) compared to $0.31 \pm 0.027$ g/mg ($\bar{x} \pm$ SEM $n = 5$) for control EdI preparations (see Fig 4.8 for tracings of twitches). The difference between these figures is not significant. This is somewhat surprising for Brook and Duchen (1990) found the twitch tension produced by the EdI was 0.44 g/mg greater than that produced by the soleus. The reasons for this apparent anomaly are uncertain. It is possible that the EdI was fatigued prior to the determination of the maximum twitch tension in response to direct stimulation, the determination of the maximum twitch and tetanic tension in response to indirect stimulation always being carried out first. The soleus being more fatigue resistant would be less severely affected than the EdI by this procedure.

The effects of CL on the maximum twitch tension produced in response to direct stimulation is shown in Fig 4.2. CL does not have a clearly discernible affect on the maximum twitch tension of the soleus or EdI produced in response to direct stimulation. Over the first two days following the administration of CL the twitch tensions of both the soleus and EdI fell slightly below their respective control values. Beyond 2 days, however, soleus and EdI preparations with maximum twitch tensions within or above the control range were seen.
**Twitch rise time**

Control adult Edl muscles had a twitch rise time of 22±0.84ms (X±SEM n=5) compared to 63.6±79ms (X±SEM n=5) for control adult soleus muscles. The twitch rise time of the Edl was significantly faster (P<0.0001) than that of the soleus. This finding is consistent with the reports of Duchen and Tonge (1973); Lewis and Ridge (1981) and Brook and Duchen (1990), who all found that fast twitch muscles such as the Edl contract approximately three times faster than slow twitch muscles such as the soleus. The speed of contraction has been found to correlate with myosin ATPase activity (which is higher in fast twitch muscles such as the Edl) which is integrally involved in the isometric shortening of the sarcomere (Mastaglia, 1985).

The effects of CL on the twitch rise time are shown on Fig 4.3. CL caused a steady increase in the twitch rise time of both the soleus and Edl. A line of best fit was calculated using regression analysis for both of the sets of data shown in Fig 4.3. The line produced using the Edl data had a slope of 0.605±0.263 ms/day which is significant (P<0.05). The correlation coefficient for this data was rp = 0.5876 which is also significant (P<0.025). The soleus data produced a line with a slope of 2.54±0.704 ms/day which is significant (P<0.048) and a correlation coefficient of rp =0.7522 which is also significant (P<0.001). It should be noted, however, that few muscles were used in this study and therefore its validity is open to question.
THE EFFECTS OF CL ON THE MAXIMUM EDL AND SOLEUS TWITCH TENSION PRODUCED IN RESPONSE TO DIRECT STIMULATION

Fig. 4.2 The effects of CL on the maximum Edl (□) and soleus (○) twitch tensions produced in response to direct stimulation. The control Edl value (■) was 0.31 ± 0.061 g/mg (X ± SD n = 5) and the control soleus (●) value was 0.296 ± 0.019 g/mg (X ± SD n = 5).

Fig. 4.3 The effects of CL on the twitch rise time of the soleus (○) and Edl (□) of adult mice muscle twitches were evoked by direct stimulation. The control soleus value (●) was 63.6 ± 1.52 MS (X ± SD n = 5) and the control Edl value was 22 ± 1.87 MS (X ± SD n = 5).
Time to 50% fall of the adult soleus and EdI twitch

The half relaxation time (or time to 50% fall of the twitch), $t_{50}$, of control adult EdI preparations was $26.4 \pm 0.683$ ms ($\bar{x} \pm \text{SEM } n=5$), compared to $81.6 \pm 0.40$ ms ($\bar{x} \pm \text{SEM } n=5$) for adult control soleus preparations. The $t_{50}$ of the EdI is similar to the twitch rise time in that it is more than 3 times faster than that of the soleus, a finding previously reported by Brook (1986) and Brook and Duchen (1990).

The speed of relaxation (and hence $t_{50}$ fall) is determined by the rate at which calcium is sequestered by the sarcoplasmic reticulum. This occurs more rapidly in fast twitch muscles such as the EdI. The calcium-binding protein parvalbumin is thought to facilitate the uptake of $\text{Ca}^{2+}$ in fast twitch muscles by shuttling $\text{Ca}^{2+}$ away from the myofibrils and towards the sarcoplasmic reticulum (Mastaglia, 1985).

The effects of CL on $t_{50}$ of the adult soleus and EdI twitch is shown in Fig 4.4. CL did not appear to have a consistent effect on the $t_{50}$ fall of either the soleus or EdI in adult mice. Lines of best fit were calculated for the two sets of data shown in Fig 4.4 using regression analysis. The slopes of these lines and the correlation coefficients were not significant, indicating CL does not cause the $t_{50}$ of the twitch of the adult soleus or EdI to increase or decrease in a linear fashion.

Tetanic tension

The mean maximum tetanic tension produced by adult control EdI preparations was $2.2 \pm 0.19$ g/mg ($\bar{x} \pm \text{SEM } n=5$) compared to $1.89 \pm 0.16$ g/mg ($\bar{x} \pm \text{SEM } n=5$) for adult control soleus preparations. These results are similar to those obtained by Brook and Duchen (1990), who found the mean tetanic response of the soleus and EdI to be $1.66 \pm 0.18$ g/mg and $2.1 \pm 0.2$ g/mg respectively. The tension developed by a muscle is largely dependent on the number of myofilaments per cross-sectional area. Myofilaments are responsible for the generation of tension and are present in a higher density.
in fast-twitch muscles such as the Edl (Brook, 1986).

The effects of CL on the tetanic response of the adult Edl and soleus to direct stimulation is shown in Fig 4.5. No clearly discernible trends are shown by either the soleus or Edl results. Regression and correlation analysis also failed to demonstrate any significant trends in these results. It would therefore appear that CL causes little or no effect on the tetanic response of the adult Edl and soleus to direct stimulation. In all the muscles tested, tension was well maintained in response to direct stimulation. The traces produced after the administration of CL were indistinguishable from the controls.
Fig. 4.4 The effects of CL on the twitch half relaxation time at the soleus (○) and Edl (□) of adult mice. The twitch was evoked by direct stimulation. The control soleus value (●) was 81.6 ± 0.89 ms (X ± SD n = 5) whereas the control Edl value (■) was 26.4 ± 1.52 ms (X ± SD n = 5).

Fig. 4.5 The effects of CL on the maximum tetanic tension produced by the Edl (□) and soleus (○) of adult mice and response to direct stimulation. The control soleus value (●) was 1.92 ± 0.36 g/mg (X ± SD n = 5) and the control Edl (■) value was 2.2 ± 0.42 mg (X ± SD n = 5).
b) The response to indirect or neural stimulation

*Twitch tension*

In adult control soleus and Edl preparations the amplitude of the twitch response evoked by neural stimulation was very similar to that evoked by direct stimulation. In Fig 4.6 the twitch response of the Edl and soleus to indirect or neural stimulation is expressed as a percentage of the same muscle response to direct stimulation.

It can be seen from Fig 4.6 that CL causes a precipitous fall in the response at both the Edl and soleus within 24 hours. At 2 days the response of the Edl and soleus to neural stimulation remained at below 10% of their responses to direct stimulation. A slight increase in the response to neural stimulation occurred at 3 days, particularly in one soleus muscle at which the response to neural stimulation (NS) was over 50% of its response to direct stimulation. At days 4 and 6 the response to neural stimulation was very low, being less than 10% of the response of these muscles to direct stimulation. The response of both the Edl and soleus to neural stimulation appeared to recover slightly at 7 days. All of the four muscles tested at 7 days showed a clearly discernible twitch, in response to neural stimulation. This twitch had an amplitude of > 20% of the amplitude of the same muscle’s twitch response to direct stimulation (DS).

*Tetanic stimulation*

The amplitude of the NS/MS tetanic response of adult control Edl preparations was 96.46 ± 1.47% (x ± SEM n = 5) compared to 96.87 ± 1.70% (x ± SEM n = 5) for adult control soleus preparations. The similarity of the responses of control Edl and soleus muscles to NS versus DS is shown in Fig 4.8.

The effects of CL on this parameter are shown in Fig 4.7. Within 24 hours of the administration of CL the response of both the Edl and soleus fell
to below 10% of control levels. At 2 days the two soleus muscles tested failed to respond to tetanic neural stimulation. A slight recovery in the tetanic tension produced by NS was seen at 3 days. This was particularly evident in the soleus and Edl of one mouse which both developed and maintained a tetanic tension in response to NS which was more than half of their respective responses to DS. This recovery did not continue, however, for at 4 days the response of the two muscles tested to NS fell back to below 25% of that produced by DS. The Edl tested at this time was unable to maintain tension in response to NS at either 50 or 100Hz (see Fig 4.9). At 6 days the four muscles tested were either completely unresponsive to NS or showed a response of less than 5% of their respective responses to DS. A recovery, similar to that seen in fig 4.6, was observed at 7 days. All of the four muscles tested were able to develop and maintain tetanic tension in response to NS at 50Hz. However, the tension produced was still markedly smaller than that produced by DS.
Fig. 4.6 The effects of CL on the maximal twitch tension of the adult Edl (□) and soleus (○) evoked by indirect or neural stimulation. The results are expressed as a percentage of the response to direct stimulation. The control soleus (●) value was 95.07 ± 4.96% (X ± SD N = 5) and the control Edl value (■) was 95.91 ± 2.77% (X ± SD n = 5).

Fig. 4.7 The effects of CL on the maximal tetanic tension of the adult Edl (□) and soleus (○) evoked by indirect or neural stimulation. The results are expressed as a percentage of the response to direct stimulation. The control soleus value was 96.46 ± 3.3% (X ± SD N = 5) and the control Edl value was 96.87 ± 3.81% (X ± SD n = 5).
Fig. 4.8 Isometric twitch and tetanic tension produced by adult soleus and Edl muscles in response to both direct and indirect stimulation. A) Control Edl twitch in response to direct stimulation. B) Control soleus twitch in response to direct stimulation. C) Control Edl tetanic tension in response to i) Direct stimulation at 50 Hz ii) Indirect stimulation at 50 Hz iii) Direct stimulation at 100 Hz. D) Control soleus tetanic tension in response to i) Direct stimulation at 50 Hz ii) Indirect stimulation at 50 Hz iii) Indirect stimulation at 100 Hz.
Fig. 4.9 Isometric twitch and tensions produced by adult soleus and EdI muscles in response to indirect stimulation at the following times after CL. A) EdI at 4 days, stimulated at i) 50 Hz ii) 100 Hz. Note that the muscle was unable to maintain a steady tension. The response of this muscle to direct stimulation was indistinguishable from that of a control. B) Soleus at 4 days, stimulated at 100 Hz. This muscle is able to maintain a very low tetanic tension in response to indirect stimulation. The response to direct stimulation however, was unaffected.
(iii) Muscle fibre resting membrane potentials

The mean control resting membrane potential (RMP) of the adult soleus was $-67.14 \pm 0.43 \text{mV}$ and that of the EdI $-73.25 \pm 0.54 \text{mV}$ ($x \pm \text{SEM} \ n = 200$ fibres sampled from five muscles). The mean control RMP of adult soleus fibres was not significantly different from that of the soleus 21-day-old mice, although it was slightly higher in the adult mice. The mean RMP of control adult EdI fibres, however, was significantly ($P = <0.0005$) higher than that of equivalent 21-day-old controls. Kelly (1978) also found that the mean RMP of rat hemidiaphragm fibres increased by $-7 \text{mV}$ between the ages of 11 and 30 days. The increase observed in both the soleus and EdI might be related to an increase in the fibre diameter of both of these muscles as the animal grows, for muscle fibre RMP has been shown to be related to fibre diameter (Katz and Thesleff, 1957).

The effects of CL on the mean RMP of the adult EdI and soleus is shown in Fig 4.10. By two days after the administration of CL the mean RMP of the soleus had fallen to $-64.77 \pm 0.38 \text{mV}$ which is significantly lower ($P = <0.0001$) than that of the control levels. The mean RMP of the EdI also fell, but to a far lesser extent (see Fig 4.10). At four days the mean RMP of the EdI fell sharply and was significantly ($P = 0.001$) smaller than that of the controls. At seven days the mean RMP of both the soleus and EdI reached their lowest levels, which were significantly lower ($P = <0.0001$ for the soleus and $P = <0.001$ for the EdI than their respective controls. A line of best fit was calculated using regression analysis for both the EdI and soleus RMP data. The line produced by the soleus data had a slope which was not significant and a Pearsons correlation co-efficient below 0.5 which is also not significant at $n = 8$. The line of best fit calculated for the EdI data had a slope of $-0.71 \pm 0.009 \text{mV/day}$ which is significant ($P = 0.0002$). The Pearsons correlation co-efficient of for the mean EdI RMP data is $r_p = -0.96$ which is very significant ($P < 0.0005$). Therefore, both the regression and correlation analysis show that the mean EdI RMP depolarizes in a linear manner following the administration of CL. This finding is in accordance with the effects of CL previously observed for the 21-day-old biceps brachii
and Edl.

Fig. 4.10 The effects of CL on the mean resting membrane potential of soleus (○) and Edl (■) fibres in adult mice. Each point represents the mean ± SEM of 200 observations made from 5 muscles at each time interval. The results were compared with their respective controls and the statistical significance of any such difference was calculated using Student's t-test: $P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***$

(iv) Fibrillation potentials

Fibrillation was first seen in both the Edl and soleus five days after the administration of CL. Very few fibres were affected at this time and beyond. Less than 5% of the fibres sampled at seven days showed fibrillations and therefore intracellular recordings were possible from the muscles examined. Sharp, angular fibrillation potentials (similar to those shown in Figure 3.5) were occasionally recorded.

(v) The structure of extrafusal fibres

The morphology of extrafusal fibres in adult muscles is very similar to that seen in the 21-day-old mice, the fibres however tend to have a larger diameter. Necrotic fibres or regenerating fibres with central nuclei were not seen.
The effects of CL on muscle spindles

Physiological observations

a). Control observations

Five control soleus preparations were examined. In each of these preparations bursts of compound action potentials were seen when the muscle was gently stretched in the absence of fusimotor stimulation (see Fig 4.11). When the stretch was maintained the rate of firing decreased slightly but remained above the rate recorded prior to the stretch (see Fig. 4.11). B.H.C. Matthews (1933) was the first to demonstrate that muscle spindles respond to stretch, whereas tendon organs respond to tension. The responses recorded from the mouse soleus were initiated by stretching the muscle, a procedure which should stimulate both the primary and secondary sensory nerve endings (Matthews, 1972; Boyd, 1985b) of the 8-12 spindles found in the soleus of the mouse (Stephens, 1985). The initial increase in the rate of the compound action potentials is likely to represent the rapid increase in the firing rate of the primary sensory endings of both the nuclear bag and the nuclear chain fibres which occurs during the dynamic phase of the stretch (Kuffler and Nicholls, 1976; Boyd, 1985b). The firing observed when the stretch was maintained is likely to represent the firing of mainly the secondary sensory nerve terminals but also, to a lesser extent, the primary terminals where the rate of firing decreases during maintained stretch (Kuffler and Nichols, 1976; Boyd, 1985b). Two adult Sprawling mice were also included in this study. A deficiency in the sensory ganglion cells of this mutant leads to failure in the development of muscle spindles and tendon organs resulting in a complete lack of these structures in the muscles of the hind limbs in the adult mouse (Brook and Duchen, 1990). The bursts of action potentials seen when control soleus preparations were stretched were not observed when Sprawling soleus preparations were used (see Fig 4.11). Some tonic firing, however, was
observed prior to the muscle being stretched and a slight increase in the frequency of this firing was occasionally observed when the muscle was stretched.

b). The effects of CL

Two days after the administration of CL muscles from four mice beginning to show behavioral signs of neurotoxicity were removed and examined. The responses recorded from these muscles were indistinguishable from those of the controls (see Fig 4.12). Muscles from five more mice were examined at four and seven days after the administration of CL. The responses observed from each muscle were again indistinguishable from those of controls in terms of both the amplitude and frequency of firing (see Fig 4.12). It was not possible to accurately measure the latency of the response, and therefore to ascertain the effect of CL on the conduction velocity of sensory fibres.

It should be noted that these recordings were of a qualitative nature and no attempt was made to differentiate between the responses of primary or secondary endings or the exact frequency of firing induced by stretching the muscles. It is believed, however, that if the spindles were affected by CL to the same degree as motor innervation, the recorded responses would be very much attenuated, as Stephens (1985) found there are only 8-12 spindles in the murine soleus.
Fig. 4.11 Extracellular recordings of muscle spindle activity in response to the soleus being manually stretched. A) Control soleus i) prior to stretch; ii) at the beginning of the stretch; iii) during maintained stretch. B) Sprawling soleus during maintained stretch. Vertical bar 100 µV, horizontal bar 100 mS.
Fig. 4.12 Extracellular recordings of muscle spindle activity in response to the soleus being manually stretched. A) Soleus from an adult mouse 2 days after CL during maintained stretch. B) Soleus from an adult mouse 4 days after CL during maintained stretch. C) Soleus from an adult mouse 7 days after CL during maintained stretch. Vertical bar 100 μV, horizontal bar 100 mS.
Morphological observations

a). In control animals

Muscle spindles were observed using both light and electron microscopy. The morphology of the murine muscle spindle has previously been described by Barker (1974), Edwards (1975); Queiroz and Duchen (1982) and Stephens (1985).

Mouse spindles were found to consist of between 2 and 6 fibres (the normal number was 4) which were surrounded for most of their length by a laminated capsule (see Fig 4.17). In the centre of the spindle the capsule was seen to enclose a fusiform fluid-filled periaxial space. In Fig 4.13 a thick annulospiral ending can be seen with its branches encircling the intrafusal fibre. Thin spirals on nuclear chain fibres were occasionally observed and may represent secondary endings. Queiroz and Duchen (1982) found using electron microscopy that primary and secondary sensory endings were indistinguishable from one another in the mouse spindle. Sensory terminals were found to occur within indentations of the surface of the muscle fibre, and were therefore partially surrounded by sarcoplasm. Basal lamina was not found to be present between the sensory terminal and the intrafusal muscle fibres. The external surface of the sensory terminal, however, was enclosed within a basal lamina. The sensory nerve endings were seen to contain varying sized vesicles, tubules, filaments and numerous small elongated mitochondria (see Fig 4.15).

The motor terminals of mouse intrafusal fibres are situated in the polar regions of the spindles. The basal lamina is found to separate the motor terminals from the intrafusal fibres. The motor terminals are covered by Schwann cell processes and contained numerous mitochondria and synaptic vesicles. Postsynaptic folds are usually absent (Queiroz and Duchen, 1982) but may occasionally be found (Edwards, 1975) in the sarcolemma. The end-plates without post synaptic specialisations probably represent "trail endings" and those with postsynaptic folds "plate endings" (Barker, 1974).

b). The effects of CL

Muscle spindles were examined from a variety of muscles at many anatomical levels in both 21-day-old and adult CL-treated mice. Observations were made from 1 to 7 days after the administration of CL using both light and electron microscopy.

i). Light microscopy

In silver stained paraffin sections the annulospiral nerve terminals of the nuclear bag fibres were found to be intact in all the many spindles identified from 1 to 7 days after the administration of CL in both adult and 21-day-old mice (see Figs 4.13 and 4.14). Even in areas where the motor innervation of the extrafusal fibres was fragmented or had completely degenerated, i.e. the foot or calf muscles of the hindlimbs, the sensory innervation of the spindles still appeared intact (Fig 4.13). Using light microscopy the motor innervation of the spindles was not studied in detail in either the control or the CL treated mice. To do this adequately it would have been necessary to examine serial sections using a combined cholinesterase-axon stain. Time did not permit this.

ii). Electron microscopy

In all the spindles visualised with EM annulospiral endings also appeared intact at all times after CL in both 21-day-old and adult mice. The abnormalities observed in the intramuscular motor nerves of the hind limbs, such as rarefaction of the axoplasm, fragmentation of the axolemma and the development of membrane bound vacuoles, were not seen in the sensory
nerve terminals of the spindles (Figs 4.15, 4.16 and 4.17).

The motor innervation of the intrafusal fibres also appeared to be unaffected by the administration of CL, but very few intrafusal end-plates were found with EM, this aspect of the research could be expanded by more extensive sampling and serial sections. It seems likely that differences between intrafusal and extrafusal motor endings will be found. The degenerative changes of the spindle seen following nerve section or the administration of neurotoxins have been reviewed by Swash (1982) and Boyd and Smith (1984). The morphological changes described in these reviews were not observed following the administration of CL.
Fig. 4.13  Section of foot muscle from a 21-day-old mouse at 3 days post CL, showing a muscle spindle cut longitudinally. An intact annulo-spiral ending is wrapped around an intrafusal fibre. In this muscle the innervation of extrafusal end-plates was totally degenerated. Silver impregnation x 400.

Fig. 4.14  Sections of foot muscle from adult mice 7 days after CL. Intact annulo-spiral endings can be seen on both spindles. The innervation of the surrounding extrafusal fibres had degenerated in these muscles. Silver impregnation x 400.
Fig. 4.15 EM of muscle spindle from a foot muscle from a 21-day-old mouse 2 days after CL. The annulo-spiral nerve (AS) is intact and contains abundant mitochondria and other organelles. (Neg. 99616.4 × 15,00. Bar = 2 μm).
Fig. 4.16 EM of intrafusal muscle fibre (? nuclear chain fibre) in the soleus, from an adult mouse 2 days after CL. The nerve terminal lies beneath the basal lamina, is seen in two places (arrows) and shows no significant abnormality. (Neg. 91684 x 7,800. Bar = 5μm).
Fig. 4.17 EM of soleus from a 21-day-old mouse 4 days after CL, showing part of a muscle spindle and an extrafusal nerve fibre. Within the spindle the myelinated fibres and part of the annulo-spiral endings (AS) are intact. The extrafusal nerve fibre (arrow) is severely degenerated. The spindle capsule (SC) is clearly visible. (Neg. 99615 x 7,800. Bar = 5μm).
4.3 The effects of CL on the neuromuscular junctions of adult mice

(i) Spontaneous transmitter release

In adult control soleus muscles, $37.6 \pm 0.55$ of the 40 fibres sampled exhibited spontaneous mepps while in the Edl $36.6 \pm 0.81$ of the 40 fibres exhibited mepps (both figures $= \bar{x} \pm S.E.M \ n=5$). These results are not significantly different from those recorded for the 21-day-old control soleus and Edl. There are a number of reasons that could contribute to the failure to record mepps from every fibre sampled. End-plates may have become damaged during the dissection or, alternatively, the muscle fibre may have been sampled in a region away from the end-plate where the mepps were not discernible above the background noise.

The administration of CL caused a dramatic fall in the number of both soleus and Edl fibres at which mepps could be recorded - see Figs 4.18 and 4.19. Within 24 hours the mean number of both Edl and soleus fibres showing mepps had fallen to less than 16. A considerable amount of variation between preparations was seen at this time particularly at the soleus - see Fig 4.19. At one soleus, 30 of the fibres sampled exhibited mepps while with another preparation all of the fibres sampled failed to show mepps. There is no significant difference between the mean proportion of adult soleus and Edl fibres showing mepps and their 21-day-old counterparts at 24 hours.

Over the next four days the mean proportion of Edl and soleus fibres showing mepps appears to stabilise (see Fig 4.18). This stabilisation observed at 3 to 5 days in the adult muscles is very different from results recorded from the soleus and Edl of 21-day-old mice at these times. The extremely low mean proportion of fibres (<5%) showing mepps frequently observed at 21-day-old muscles at these times are only seen on three occasions with adult muscles. The mean number of adult soleus fibres showing mepps was significantly higher than the equivalent 21-day-old muscles at 3 ($P=0.029$) and 4 ($P=0.0005$) days after the administration of
Similarly the mean number of adult Edl fibres showing mepps was significantly higher than the equivalent 21-day-old muscles at three (P = 0.011) and four (P = 0.014) days.

At six and seven days a slight increase in the mean proportion of both soleus and Edl fibres demonstrating mepps was seen. All the muscles tested at 7 days exhibited mepps at more than 20% of the fibres tested. The mean proportion of adult Edl fibres showing mepps was again significantly larger than the equivalent 21-day-old muscles at both 6 (P = 0.002) and 7 (P = 0.016) days while the soleus results were significantly (P = 0.05) higher at 6 days.
Fig. 4.18 The effects of CL on the mean number of soleus (○) and Edl (■) fibres showing spontaneous mepps in adult mice. Each point represents the mean ± SEM of 5 observations with 40 fibres being tested in each muscle. Student’s t-test was used to compare the results with their respective controls and ascertain the statistical significance of any such difference. All the points were significantly different from their respective controls at P < 0.001.

Fig. 4.19 The effects of CL on the number of soleus (○) and Edl (■) endplates at which mepps could be demonstrated in adult mice. Forty fibres from each muscle were tested in each of five animals at each time interval.
Mepp frequency

The mean mepp frequency of adult control EdI preparation was 1.71 ± 0.19Hz (x ± S.E.M. n-183 from 5 muscles) which is significantly (P<0.0001) higher than the mean mepp frequency of control adult soleus muscles which was 0.38 ± 0.04Hz (x ± S.E.M n = 186 from 5 muscles). The mean mepp frequency of adult EdI was significantly higher (P<0.0001) than that of the 21-day-old control EdI where the mean mepp frequency was less than half that of the adults. In contrast to this the mean mepp frequency of the adult control and 21-day-old control soleus were not significantly different, there being only 0.04Hz difference.

This increase in mepp frequency as the animal grows has previously been documented. Duchen and Stefani (1971) found the mepp frequency in the short head of biceps increased four times between 14 and 21 days of age in control mice. Kelly (1978) demonstrated that the mepp frequency of the rat hemidiaphragm increased considerably between 20 and 100 days. The lower mepp frequency in the end-plates of younger mice is thought to reflect the smaller area of synaptic contact which is in turn related to the muscle fibre diameter. The present data therefore suggests the soleus end-plates undergo little or no increase in size between 21-days and 6-10 weeks of age whereas the Edl fibres, and hence end-plates, grow considerably.

The administration of CL to adult mice caused a dramatic increase in the mean mepp frequency of both the soleus and the Edl within 24 hours (see Fig 4.20). At 24 hours the mean mepp frequency of the soleus was more than 5 times that of the controls, while at the Edl the mean mepp frequency was more than 4 times the control value. These increases in the mean mepp frequency are caused by the appearance of abnormally high (>10Hz) mepp frequencies. (see Fig 4.22 and 4.23) At this time 45% of Edl and 12% of the soleus mepp frequencies were above 10Hz. These results are very similar to those seen in the 21-day-old soleus and Edl at 12 hours, and the biceps brachii at seven days, post CL treatment.

By two days the mepp frequencies recorded from the remaining end-
plates of the EdI and soleus were frequently within control limits (see Figs 4.22 and 4.23). At three days end-plates with abnormally high mepp frequencies became more evident causing an increase in the mean mepp frequency of both the EdI and soleus. At four days only one soleus end-plate with a mepp frequency outside the control range was observed (see Fig 4.22). Very few soleus end-plates with mepp frequencies outside the control range were seen subsequently, the mean mepp frequency therefore stabilised within the control range. The mean mepp frequency of the EdI at four days, however, was significantly higher ($P < 0.0001$) than the controls, resulting from a large number of the sampled end-plates having mepp frequencies between 5 and 15 Hz. Subsequently, fewer end-plates with mepp frequencies outside the control range were observed and the mean mepp frequency fell accordingly.

**THE EFFECTS OF CL ON THE MEAN MEPP FREQUENCY AT THE SOLEUS AND EDL OF ADULT MICE**

![Graph](image)

Fig.4.20 The effects of CL on the mean mepp frequency in the soleus ($\circ$) and Edl ($\blacksquare$) of adult mice. Each point represents the mean $\pm$ SEM. The number of fibres used to calculate this figure is shown in parenthesis alongside the point. Five preparations were tested at each time interval. The soleus control value was $0.38 \pm 0.04$ Hz ($\bar{x} \pm$ SEM) and the Edl control value was $1.71 \pm 0.19$ Hz ($\bar{x} \pm$ SEM).
Fig. 4.21. Intracellular recordings of mepps made from adult mice at the following times. A) Control soleus. B) Control Edl, note the higher frequency, but lower amplitude of these mepps compared with those recorded at the soleus. C) Soleus 1 day after the administration of CL. D) Edl 1 day after the administration of CL. Note the abnormally high mepp frequencies shown in traces C) and D). Vertical bar 1mV, horizontal bar 1 second.
Fig. 4.22 The effects of CL on mepp frequencies in the soleus of adult mice. The mean control mepp frequency (●) was $0.38 \pm 0.6$ Hz ($x \pm SD$), calculated from 186 fibres sampled from 5 preparations. Subsequent points (○) are the mepp frequencies of individual soleus fibres.

Fig. 4.23 The effects of CL on mepp frequencies in the Edl of adult mice. The mean control mepp frequency (■) was $1.71 \pm 2.58$ Hz ($x \pm SEM$) calculated from 183 fibres sampled from 5 preparations. Subsequent points (□) are the mepp frequencies of individual Edl fibres.
The mean amplitude of the mepps recorded at control soleus end-plates was $1.025 \pm 0.0059\, \text{mV}$ ($x \pm \text{SEM} \, n = 3783$ from 5 muscles) while the mean amplitude of mepps observed in control EdI preparations was $0.895 \pm 0.0038\, \text{mV}$ ($n = 9974$ from 5 muscles), significantly smaller ($P < 0.0001$). This is likely to be due to the soleus fibres having a smaller diameter than the EdI (see below). The mean amplitude of the mepps recorded from both the adult control EdI and soleus were not significantly different from those seen in the 21-day-old controls.

Although there was no significant difference between the 21-day-old and adult mepp amplitudes, the mean mepp amplitude at the adult EdI was slightly smaller than that of the corresponding 21-day-old mean mepp amplitude. A fall in the mean mepp amplitude has frequently been reported to occur as the animal ages. Kelly (1978) found that the mepps recorded at the rat hemidiaphragm had mean amplitude of $1.1 \pm 0.07\, \text{mV}$ ($x \pm \text{SE}$) at 11 days of age, falling to $0.405 \pm 0.03\, \text{mV}$ at 75 days. Nagel et al (1990) reported a fall of $0.27\, \text{mV}$ in the amplitude of mepps recorded in the mouse hemidiaphragm between 2½ and 8½ weeks of age. Nagel et al (1990) suggested that this fall was due to an increase in the muscle fibre diameter as mepp amplitude has been shown to be proportional to the diameter of the muscle fibre (Katz and Thesleff, 1957).

The effects of CL on the mean mepp amplitude of the soleus and EdI is shown in Fig 4.24, while CL's influence on the amplitude distribution of soleus mepps is shown in Fig 4.25 and on EdI mepps in Fig 4.26. The mean mepp amplitudes of the soleus and EdI remained well above their respective controls at days two and three. The mepp amplitude distributions continued to be skewed to the right at these times (particularly the soleus at day 3 - see Fig 4.25).

Four days after the administration of CL the mean mepp amplitude of the soleus and EdI fell but remained significantly higher than the control values (Fig 4.24). At five days this fall had continued, the mean mepp
amplitude at the EdI being within the control range, while at the soleus the mean mepp amplitude was lower than that of the controls. The soleus mepps appeared to be normally distributed at this time, the distribution being similar, though not identical, to that seen for the control soleus mepps. The distribution of the EdI mepp amplitudes at five days was less skewed than the distributions seen at 1, 2 and 3 days. The proportion of mepps with amplitudes of between 0.75 and 1mV was also noticeably smaller.

Six days after the administration of CL, the mean mepp amplitudes of both the soleus and EdI were significantly ($P < 0.001$) smaller than the controls. This situation was reversed at seven days when the mean mepp amplitude of both soleus and EdI mepps was found to be significantly larger than the controls. The mepp amplitude distributions produced at this time were skewed to the right (see Figs 4.25 and 4.26).

**THE EFFECTS OF CL ON MEPP AMPLITUDES IN ADULT MICE**

![Graph](image)

Fig.4.24 The effects of CL on the mean amplitude of spontaneous mepps recorded in the EdI (■) and soleus (○) of adult mice. Each point represents the mean ± SEM of more than 500 observations made from five preparations at each time interval. The mepp amplitudes were corrected to a standard RMP of -75mV for soleus mepps and -80mV for EdI mepps using the method of Katz and Thesleff (1957). The results were compared with their respective control values using Student’s t-test to ascertain the statistical significance of any such differences. $P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***$
Fig. 4.25 Amplitude distributions of spontaneous mepps recorded in soleus preparations of adult mice at the following times: A) Control-hatched columns. B) 1 day. C) 2 days. D) 3 days. E) 5 days. F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The mepp amplitudes were corrected to a standard RMP of -75mV using the method of Katz and Thesleff (1957). Note the difference in the scale of the ordinate in each of the histograms.
Fig. 4.26 Amplitude distributions of spontaneous mepps recorded in the Edl of adult mice at the following times: A) Control-hatched columns; B) 1 day; C) 2 days; D) 3 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. Mepp amplitudes were corrected to a standard RMP of -80mV using the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
(ii) The effects of CL on neurally evoked transmitter release at the adult neuromuscular junction

In control adult soleus muscles $36.2 \pm 1.79$ of the 40 fibres sampled from each preparation responded to neural stimulation with a discernible epp compared to $36.6 \pm 0.75$ of the 40 Edl fibres sampled per preparation ($\bar{x} \pm \text{SEM}, \ n = 5$ for both muscles).

CL caused a significant decrease in the mean proportion of both Edl and soleus fibres responding to neural stimulation within 24 hours, see Fig. 4.27. A considerable degree of variation in the number of fibres per muscle responding to neural stimulation was observed at this time, however, particularly amongst soleus preparations (see Fig 4.28). The decline in the mean proportion of fibres demonstrating epps was not nearly as pronounced as that seen in the 21-day-old mice over the same period (compare Fig 4.27 with Fig 3.21). The mean proportion of adult soleus fibres showing epps at 24 hours was significantly higher ($P = 0.003$) than in the comparable 21-day-old muscles.

Two days after the administration of CL the mean proportion of Edl and soleus fibres demonstrating mepps fell further to below 12. Slightly less variation between individual muscles was seen (see Fig 4.28). At this time some end-plates were found which showed spontaneous mepps but failed to respond to neural stimulation with an epp (Fig 4.29).

A slight increase in the mean number of soleus fibres demonstrating epps was seen at three days (see Fig 4.27). Considerable variation in the numbers of fibres responding to neural stimulation was again noted between muscles. At one soleus preparation 75% of the fibres sampled demonstrated epps while at another soleus preparation only 1 (2.5%) of the fibres sampled showed epps.

After four days, the mean number of soleus and Edl fibres responding to neural stimulation fell to below five. Little variation between muscles was observed at this time. In 3 of the 5 soleus and 1 of the 5 Edl preparations tested it was not possible to locate a single fibre which would respond to
neural stimulation despite sampling 40 fibres.

The mean number of both EdI and soleus fibres responding to neural stimulation recovered slightly between four and seven days. At five days the number of end-plates showing epps was found to vary considerably between preparations. It was also noted at this and subsequent days the muscles which contained few fibres exhibiting epps were often those which were removed from severely ill mice, e.g. mouse CYC5DAL was noted to be ataxic and severely paralysed. When the soleus and EdI of CYC5DAL were examined only 2 out of 40 EdI and none out of 40 soleus fibres tested responded to neural stimulation. Conversely, the muscles removed from mice showing limited neurological symptoms frequently contained a large number of end-plates still responsive to neural stimulation, e.g. mouse CYC7DE was noted to show only a slight weakness in the hind limbs, and 32 of the 40 EdI end-plates tested were found to show epps.
Fig. 4.27 The effects of CL on the mean number of EDL (■) and soleus (○) fibres in adult mice responding to neural stimulation with a discernible epp. Each point represents the mean ± SEM of 5 observations, with 40 fibres being tested in each muscle. Student’s t-test was used to ascertain the statistical significance of any differences between the results and their respective controls: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.

Fig. 4.28 The effects of CL on the number of EDL (■) and soleus (○) fibres responding to neural stimulation with a discernible epp. Forty fibres from each preparation were tested and five soleus and EDL preparations were sampled at each time interval.
**Epp latency**

The mean latency of the epps recorded from control adult soleus preparations was $1.53 \pm 0.013\text{ms}$ ($\bar{x} \pm \text{SEM} n = 171$ from 5 muscles) while at comparable EdI preparations epps had a mean latency of $1.52 \pm 0.013\text{ms}$ ($\bar{x} \pm \text{SEM} n = 161$ from 5 muscles). In contrast to the epp latencies recorded from the 21-day-old control soleus and EdI there was no significant difference between the adult muscles mean epp latencies. The mean latency of the epps recorded from adult control EdI preparations was remarkably similar to that recorded from comparable 21-day-old controls. The mean epp latency recorded from the adult soleus, however, was significantly ($P < 0.0001$) lower than that recorded from the 21-day-old control soleus preparations. This indicates that either the conduction velocity of the fibres supplying the soleus neuromuscular junction has increased or, alternatively, stimulus-secretion coupling is more efficient in the larger neuromuscular junctions of the adult soleus. Successive epp waveforms recorded at single end-plates in adult mice were found to be far more uniform in their latency and amplitude than the epps recorded from 21-day-old controls.

The effects of CL on the mean epp latency of adult soleus and EdI fibres is shown in Fig 4.30. It can be seen that CL caused the mean latency of both soleus and EdI epps to be significantly prolonged. Within 24 hours the epps recorded from the soleus and EdI had latencies very significantly ($P < 0.001$) different from their respective controls. A further rise occurred at both of these muscles over the next 24 hour period. At 3 days a slight reduction in the mean epp latency was seen at EdI end-plates. The mean epp latency of soleus end-plates continued to rise however.

Four days after the administration of CL, very few EdI or soleus fibres responded to neural stimulation. The small number of epps observed at this time had widely differing latencies, some being within normal limits, while others were severely prolonged. This difference being responsible for the large error bars on Fig 4.30.
The mean EdI epp latency fell at five days but remained significantly higher than the control. The mean soleus epp latency continued to rise however. This situation was reversed at six days when the mean soleus epp latency fell slightly, with the mean EdI latency rising. At seven days, the mean epp latency of both the soleus and the EdI reached its highest level. An averaged epp waveform recorded at 7 days after CL is shown in Fig 4.31, the prolonged latency is apparent.

A line of best fit was calculated for both the soleus and EdI mean epp latencies. The line produced by the EdI data had a slope which was not significant indicating that the increase in epp latency seen at the EdI was not linear. The soleus data, however, produced a line with a slope of $0.005 \pm 0.001$ms/day which is significant ($P = 0.0029$). The mean latency of soleus epps was therefore shown to increase in a linear fashion after the administration of CL. The Pearson correlation co-efficient for the soleus data was ($r_p = 0.8921$) which is significant at $P = <0.005$, showing there is a significant positive correlation between time after the administration of CL and the mean soleus epp latency.
Fig. 4.29 Intracellular recordings made in muscle fibres which showed spontaneous mepps, but failed to respond to neural stimulation with a discernible epp. Trace A) was made from an adult soleus 2 days after the administration of CL. B) was made from an adult soleus 7 days after the administration of CL. Vertical bar 1mV, horizontal bar 1 second.

THE EFFECTS OF CL ON EPP LATENCY IN ADULT MICE

Fig. 4.30 The effects of CL on the mean epp latency in the soleus (○) and Edl (■) of adult mice. Each point represents the mean ± SEM of the results pooled from five preparations. The figures in parenthesis indicate the number of readings used to calculate the mean at each time interval. The data points were compared with their respective control values using Student’s t-test to ascertain the statistical significance of any such differences. P<0.05 = *; P<0.01 = **; P<0.001 = ***
Fig. 4.31 An epp waveform created by averaging 50 successive epps recorded intracellularly in the presence of d-tubocurarine from the soleus of A) A control adult mouse, B) An adult mouse 7 days after the administration of CL. The averaged waveform shown in B) has a far greater latency than the waveform shown in A). Vertical bar 1mV, horizontal bar 1 ms.
**Time to peak epp**

The mean time to peak epp from adult control Edl end-plates was $1.93 \pm 0.01$ ms (mean ± SEM n = 161 from 5 muscles) compared to $2.07 \pm 0.02$ ms (mean ± SEM n = 167 from 5 muscles) for adult soleus controls. The mean time to peak epp recorded from adult Edl muscles is significantly smaller ($P < 0.001$) than that from adult soleus controls. The mean time to peak epp of both the adult Edl and soleus end-plates was significantly smaller ($P < 0.001$) than the comparative 21-day-old controls. As there was no discernable difference between the shape of the epp recorded in the adult and young muscles it is most likely that this difference represents a difference in the latency of the response. A decrease in the latency and time to peak epp would be expected as the mouse matures because the motor axons which supply the neuromuscular junctions of the soleus and Edl are larger in the adult mice and therefore have more rapid conduction velocities.

The effects of CL on the mean Edl and soleus time to peak epp is shown in Fig 4.32. The mean time to peak was significantly prolonged ($P < 0.001$) for both the Edl and soleus at all the time intervals sampled. At twenty four hours, the mean time to peak epp for both the soleus and Edl was approximately 0.3 ms longer than the respective controls. A further slight increase was seen at two days. At three days the mean time to peak epp at the soleus increased slightly whereas at the Edl a slight fall was seen. Epps with a wide range of times to peak epp were recorded at 4-days, hence the large error bars seen at this time. At five days a large fall in the mean time to peak epp occurred at both the soleus and the Edl, reaching its lowest level following the addition of CL.

This fall did not continue beyond five days. At subsequent days the mean time to peak epp at the soleus and Edl increasing. The increase was most noticeable at the soleus where the mean time to peak epp reached its highest level at seven days.
Fig. 4.32 The effects of CL on the mean time to peak epp in the soleus (○) and Edl (■) of adult mice. Each point represents the mean ± SEM of the results pooled from 5 preparations. The figures in parenthesis indicate the number of readings used to calculate the mean at each time interval. The results obtained from Edl preparations were compared with the Edl control value of 1.93 ± 0.015 ms (X ± SEM) while the soleus results were compared to the soleus control value of 2.07 ± 0.021 ms (X ± SEM), using Student's t-test to ascertain the statistical significance of these differences. It was found that all the data points were significantly different from their respective controls at P < 0.001.
Fig. 4.33 Successive epp waveforms recorded at single end-plates of adult mice, in the presence of d-tubocurarine, at the following times:

A) Control Edl, note the uniformity of the latency of successive epps and the absence of failures.  
B) Soleus 2 days after the administration of CL. The epps show a large variation in latency and many failures are seen.  
C) Soleus 4 days after the administration of CL. No failures in transmission are seen, the amplitude of successive epps however is abnormally varied.  
Vertical bar 1 mV, horizontal bar 1 ms.
Fig. 4.34 Successive epp waveforms recorded at single end-plates of adult mice, in the presence of d-tubocurarine, 7 days after the administration of CL. A) Soleus epps which have an abnormally large latency and also show considerable variations in amplitude. B) Soleus epps recorded from the same muscle as A). These epps have a normal latency and show a far less pronounced variation in amplitude than the epps shown in A). This figure therefore demonstrates that end-plates which appear functionally intact are found alongside those which are clearly abnormal. Vertical bar 1 mv, horizontal bar 1 ms.
The mean control quanta! content of adult soleus end-plates was 54.22 ± 3.2 (X±SEM n = 158 from 5 muscles) compared to 49.83 ± 2.99 (X±SEM n = 162 from 5 muscles) for adult Edl end-plates stimulated at 5Hz. These figures are not significantly different from one another. They are, however, both are significantly (P < 0.001) larger than the mean quanta! contents obtained from the comparable 21-day-old controls.

It is difficult and misleading to compare the mqc values obtained in this study with those published elsewhere because a variety of techniques can be used to calculate the mqc, all of which give differing results (Lyons and Slater, 1991). Therefore, only mqc obtained using the variance method of calculation are compared with the results obtained in the present study. Quanta! content (calculated using the variance technique) has been reported to increase with age. Kelly (1978) found the mqc of plateau epps elicited at a stimulation frequency of 10Hz increased from 20.5 to 169.9 between 11 and 175 days of age in the rat hemidiaphragm. Nagel et al (1990) demonstrated that the mqc recorded in the murine hemidiaphragm also increases with age, at 2½ weeks the mqc was 56.9 which increased to 106.8 at 8½ weeks of age.

The effects of CL on the mean quanta! content of adult soleus and Edl end-plates is shown in Fig 4.35. It can be seen that the administration of CL did not cause a clearly discernible linear decrease or increase in the mqc of either soleus or Edl end-plates.

At 24 hours, the mqc of both the soleus and Edl was significantly larger (P < 0.05) than their respective controls. This increase was caused by an increase in the proportion of soleus and Edl end-plates with quanta! contents above 80 (see Figs 4.36 and 4.37). Two days after the administration of CL, the mqc at the soleus had increased to almost twice control levels. This was caused by a marked increase in the incidence of soleus end-plates with quanta! contents of between 90 and 160. The results obtained from the Edl at this time were markedly different. The mean quanta! content was slightly smaller than the control, caused by a
decrease in the proportion of Edl end-plates found to have quantal contents of above 40.

At four days the mqc of the soleus and Edl showed a marked fall (see Fig 4.35). The mean quantal contents of both of these muscles were now significantly ($P < 0.05$) smaller than their controls. The majority of functioning Edl and soleus end-plates were found to have quantal contents of between 10 and 20 and no end-plates with quantal contents above 100 were located at this time.

At five days a slight increase in the mqc was seen at the soleus and Edl. Despite this increase however the mqc’s of both muscles remained below their respective controls.

The Edl mqc continued to rise slightly at six days while the soleus mqc fell slightly. The quantal content amplitude distributions prepared for both the soleus and Edl end-plates at six days show an increase in the proportion of end-plates with quantal contents above 40 when compared to the distributions produced at 4 days. The quantal content distributions produced at 6 days are still very different from the controls however, being skewed to the left relative to the controls.

At seven days the Edl mqc remained significantly ($P < 0.01$) smaller than the control. At the Edl at this time, quantal contents of below 50 predominated (see Fig 4.37). Few (5) end-plates with quantal contents above 90 were found at this time however. The soleus mqc had increased to a point slightly above the control value at 7 days. The amplitude distribution of the soleus quantal contents recorded at this time, however, was still very different from the soleus control distribution. The distribution seen at seven days differed from the control distribution in the following ways:

1). Quantal contents of between 40 and 50 were not observed at seven days.

2). A far higher proportion of the end-plates had quantal contents below 10 at seven days.

3). Quantal contents above 140 were not recorded at seven days.
Fig. 4.35 The effects of CL on the mean quantal content of neurally evoked transmitter release in the soleus (○) and Edl (■) of adult mice. Each point represents the mean ± SEM of the results pooled from 5 preparations. The figures in parenthesis indicate the number of observations used to calculate the mean at each time interval. The Edl quantal contents were corrected to a standard RMP of -80mV whereas the soleus quantal contents were corrected to -75mV according to the method of Katz and Thesleff (1957). The Edl quantal contents were compared with their control value of 49.83 ± 2.99 (x ± SEM) whereas the soleus results were compared with a control value of 54.22 ± 3.2 (x ± SEM), using Student's t-test to ascertain the statistical significance of any such differences. P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***
Fig. 4.36 Quantal content distributions recorded in the soleus of adult mice at the following times: A) Control-hatched columns; B) 1 day; C) 2 days; D) 3 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The quantal contents were corrected to a standard RMP of -75mV according to the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
Fig. 4.37 Quantal content distributions recorded in the Edl of adult mice at the following times: A) control-hatched columns; B) 1 day; C) 2 days; D) 3 days; E) 5 days; F) 7 days after the administration of CL. Each histogram represents the results pooled from 5 muscles. The quantal contents were corrected to a standard RMP of -80mV according to the method of Katz and Thesleff (1957). Note the differences in the scale of the ordinate in each of the histograms.
Fig. 4.38 Intracellular recordings of epps made from the muscles of adult mice in the presence of D-tubocurarine at the following times: A) Control EDL. B) Control soleus. C) EDL 7 days after the administration of CL, note the irregularity in the size of successive epps. D) Soleus 7 days after the administration of CL, note the large number of failures. E) Soleus 7 days after the administration of CL, recorded from the same muscle as D). The mean quantal contents are shown on each trace. Vertical bar 5mV, horizontal bar 1 second.
(iii) Morphological observations

a). In control mice

The innervation of muscles in control mice has been reviewed above and illustrations of both the structure and ultrastructure of the neuromuscular junctions of 21-day-old mice are shown in Figs 3.35 and 3.36. The neuromuscular junctions in adult mice were found to be larger than those seen in the 21-day-old mice. The junctional folds were also found to be more complex in the adult mice. An increase in the complexity and depth of the junctional folds has previously been reported by Matthews-Belinger and Salpeter (1983).

b). In CL-treated mice

A combination of both light and electron microscopy was used to study the morphological abnormalities induced by CL in adult mice.

(i) 24 hours

When paraffin sections were examined using light microscopy the intramuscular nerves and terminal arborizations in the foot and calf muscles of the hind limbs appeared to be within normal limits. Ultrastructural abnormalities, however, were seen in these areas at this time. Intramuscular nerves with rarefied areas of axoplasm, swollen mitochondria and membrane bound vacuoles in the axoplasm were occasionally seen in the soleus and foot muscles (see Fig 4.39). The neuromuscular junctions visualised at this time showed considerable variation in both the severity and nature of abnormalities affecting them, e.g. in Fig 4.40 the neuromuscular junction appears normal, the only slight abnormality being a small area of rarefied axoplasm which might represent an early morphological change. In Fig 4.41, which is taken from the same muscle as Fig 4.40, the axonal
terminals appear to be devoid of vesicles and contain membrane bound electron lucent areas. In Fig 4.42 (which is also from the same muscle) the whole presynaptic area appears electron lucent, and very few vesicles are present. The neuromuscular junction shown in Fig 4.43 is different from the junctions described above. Rather than being electron lucent, this neuromuscular junction contains abnormally dense aggregations of synaptic vesicles and electron dense accumulations. The cisternae of the mitochondria also appear fragmented and disrupted.

(ii) 2 days

Degeneration of the intramuscular nerves and terminal arborizations of the distal muscles of the hind limbs was apparent in paraffin sections. Electron microscopy revealed further degeneration of the distal intramuscular nerves. The axoplasm of some of these nerves appeared abnormally dense whereas in others electron-lucent areas of axoplasm were seen, similar to that illustrated in Fig 4.39. Some of the end-plates found appeared denervated with Schwann cell processes lying directly above the junctional folds (Fig 4.44).

(iii) 3 days

Four mice were examined in serial paraffin sections at this time. It was found that the innervation of the muscles of the forelimb, head and trunk appeared intact. Intramuscular nerves and terminal arborizations were found to be abundant in these areas. The innervation of the distal areas of the hind limbs, however, appeared severely abnormal. Degenerating intramuscular nerves, and denervated end-plates were seen in the calf.
Fig. 4.39 A single myelinated axon, probably a preterminal motor fibre in the soleus of an adult mouse 24 hours after CL. The axoplasm lacks tubules and filaments and is composed largely of granular amorphous material. Mitochondria are rounded and degenerating and several irregularly shaped membrane bound vacuoles are present. The myelin sheath however is intact. These early axonal abnormalities might account for the prolonged latencies or failures of evoked transmission found at this time. (Neg. 91666 x 30,000. Bar = 1μm).
Fig. 4.40 Motor end-plate from an adult soleus 24 hours after CL. The end-plate is within normal limits, the axolemmal membrane appears intact, vesicles are abundant and the mitochondria are normal. The only slight abnormality is a membrane bound area of rarefied axoplasm (arrow). This might be artefactual or alternatively might represent a very early abnormality. (Neg. 91673 x 30,000. Bar = 1μm).
Fig. 4.41 EM of adult soleus at 24 hours post CL showing part of a motor end-plate. The axonal terminal (A) seems swollen and rarefied and contains few vesicles. Mitochondria are swollen, rounded and their cristae are disorganised. The presynaptic membrane may be breaking down (arrows) though this may be artefactual. Schwann cell cytoplasm (S) lies between the axon and the postsynaptic membrane. The muscle fibre looks normal. (Neg. 91985 x 30,000. Bar = 1μm).
Fig. 4.42 Adult soleus motor end-plate 24 hours after CL. The nerve terminal is clearly abnormal. The axolemmal membrane is disrupted, few vesicles can be seen and mitochondria are absent. Two large areas of rarefied axoplasm are also present. Processes of Schwann cell cytoplasm (SC) are surrounding the terminal. (Neg. 91682 x 30,000. Bar = 1μm).
Fig. 4.43 Preterminal myelinated axon and its motor end-plate in adult soleus 24 hours post CL. The axoplasm is swollen and electron lucent in both preterminal and terminal axons. Mitochondria are rounded and have disorganised cristae. In one part of the terminal (arrow) there are dense clumps of vesicles. End-plates and axons with abnormalities such as shown in this micrograph may represent the end-plates which failed to respond to successive neural stimulation, or alternatively showed responses with a prolonged latency. (Neg. 91977 x 7,500. Bar = 5μm).
Fig. 4.44 Soleus motor end-plate from an adult mouse 2 days after CL. The axon terminal has been replaced by Schwann cell cytoplasm (SC) which now covers the junctional folds of the postsynaptic membrane. (Neg. 91689 x 25,000. Bar = 1 µm).
(iv) Four days

The degeneration of the intramuscular nerves in the distal portions of the hind limbs was found to have progressed further at this time (Fig 4.45). The innervations of the fore limb muscles still appeared intact however (Figs 4.45).

The end-plates observed with EM were frequently denervated, the axon terminal being replaced by Schwann cell processes which covered the post-synaptic membrane. The small intramuscular nerves found in the distal portions of the hind limbs were severely abnormal. Frequently, all that remained was a myelin ovoid surrounding axonal debris or rarefied areas of axoplasm (Fig 4.47, compare Fig 4.46 with 4.47). In some nerves the myelin itself also appeared to be degenerating. This abnormality always occurred in association with the severe degenerative changes of the axon noted above. It appeared that the degeneration of the axon occurred prior to the degeneration of its myelin sheath.

(v) Six days

In some of the paraffin sections of the soleus and hind feet muscles viewed at this time, intact terminal arborizations innervating end-plates could occasionally be seen (Fig 4.48). The majority of the muscle fibres, however, in these muscles still appeared denervated and the degeneration of intramuscular nerves was still widespread.

(vi) Seven days

Some degeneration of the intramuscular nerves supplying the muscles of the upper thigh and fore limbs was observed for the first time at 7 days (Fig 4.50). In the distal portions of the hind limbs degenerating intramuscular nerves were frequently seen. In sections of skin, however, small nerve fibres were found to be abundant and appeared intact (Fig 4.49).
Fig. 4.45 Sections of muscle from an adult mouse 4 days after CL, taken from the following regions: A - muscle from the hindfoot, B - calf muscle, C - proximal forelimb muscle. In A and B no intact preterminal axons or terminal arborizations remain, and granules of argyrophilic debris indicating degeneration are widespread. In C however the preterminal axons, terminal arborizations (arrows), and intramuscular nerves appear intact. Silver impregnation x 400.
Fig. 4.46 Myelinated intramuscular nerve fibres in the foot muscle of a normal adult mouse. The axons contain well-organised neurofilaments and microtubules, smooth endoplasmic reticulum and mitochondria. The myelin lamellae are compact. Each fibre lies within Schwann cell cytoplasm and there is a delicate perineurial sheath enclosing all the fibres. (Neg. 99865 x 15,000. Bar = 2μm).
Fig. 4.47 Intramuscular nerve of a foot muscle from an adult mouse 4 days after CL. All the axons have degenerated and the myelin is beginning to break down. (Neg. 97651 x 10,000. Bar = 3μm).
Fig. 4.48 Section of calf muscle obtained from an adult mouse 6 days after CL. Some agyrophilic debris and preterminal axons without terminal arborizations can be seen. At one end-plate however both the preterminal axon and terminal arborization appear intact (arrow). Silver impregnation x 400.

Fig. 4.49 Section of skin from the hind foot of an adult mouse 7 days after CL. Small intact (probably sensory) nerves are shown (arrows). There are no signs of axonal degeneration such as agyrophilic debris. Silver impregnation x 400.
Fig. 4.50 Sections of muscle from an adult mouse 7 days after CL. A - proximal forelimb muscle, B - proximal thigh muscle. In both of these sections some preterminal nerves and terminal arborizations appear intact (arrows) while other preterminal nerves end abruptly indicating degeneration of the terminal arborization (arrow heads). Silver impregnation x 400.
4.4 Summary of the effects of CL on adult mice

The adult mice were also found to develop paralysis of the hind limbs following the administration of CL. This paralysis became evident and progressed more slowly in the adult mice, however. The weight of the soleus and Edl were found to decrease after the administration of CL, as did the mean RMP.

Both the twitch and tetanic responses of the Edl and soleus to neural stimulation fell dramatically within 24 hours of the administration of CL, whereas the responses to direct stimulation was unaffected. Physiological abnormalities such as end-plates with high mepp frequencies and epps with prolonged latencies were seen at 24 hours in the soleus and Edl. Areas of electron lucent axoplasm and swollen degenerative mitochondria were seen in the intramuscular nerves and end-plates in the distal muscles at 24 hours.

Between 2 and 6 days after CL, the mean number of soleus and Edl end-plates exhibiting spontaneous mepps stabilised at around 30% of the control levels. Further reductions in the number of end-plates responding to neural stimulation were found, and end-plates which showed spontaneous mepps but failed to respond to neural stimulation were also found.

At 7 days signs of functional recovery were seen in the Edl and soleus, i.e. the twitch and tetanic responses evoked by neural stimulation increased as did the mean number of fibres exhibiting epps and mepps.

The innervation of the intrafusal fibres was found to be unaffected by CL. This is in marked contrast to the degenerative changes observed in the innervation of the extrafusal fibres.
CHAPTER 5.

DISCUSSION

The ability of CL to induce neurological abnormalities was first reported by Aust and Roux (1965). Neurological side effects such as vertigo, confusion and peripheral neuropathy were noted after CL was administered to patients suffering from cancer. Later, Gandy et al (1973) found by chance that mice treated with a single dose of CL developed a pathological condition similar to human subacute combined degeneration of the spinal cord (SCD). This led to the suggestion that the administration of CL to mice could provide an animal model for SCD in man. Further morphological studies followed using a variety of laboratory animals. These studies were aimed at characterising the lesions produced by CL and determining the pathogenetic mechanisms (Nixon, 1974; Nixon 1976a; Nixon, 1976b; Nixon et al, 1976; Ramsey and Fischer, 1978; Crang and Jacobson, 1980; Small et al, 1981). Little or no mention of peripheral nerve abnormalities was made in any of these studies. This is somewhat surprising since symptoms of distal axonopathy such as atrophy of distal muscles together with slowed conduction velocities in the distal portions of nerves, had been described in Vitamin B₁₂ deficiency (Gilliatt et al, 1961; Mayer, 1965; Pant et al, 1968). "Impaired proprioception" has also been observed in CL-treated mice (Gandy et al, 1973). The results from such a study would be of considerable value as it is generally acknowledged that, compared with the extensive studies of the CNS lesions in vitamin B₁₂ deficiency (e.g. Pant et al, 1968), very little work has been done to record the distribution and pathogenesis of lesions of the peripheral nervous system in Vitamin B₁₂ deficiency (a factor noted by Schaumberg et al, 1983; Victor, 1984; Duchen and Jacobs, 1992).

The work described in this thesis was aimed at elucidating the causative mechanisms and the progression of the distal axonopathy induced by CL initially reported by Nixon (1974) and later in more detail by Lee (1991). These observations may also provide useful insights into the
pathogenesis of other distal axonopathies, particularly those thought to be associated with failure in the methyl-transfer pathway such as in Vitamin B₁₂ or folate deficiency.

5.1 Clinical effects of CL

Paralysis of the hindlimbs was the most consistent and obvious clinical symptom to affect both the 21-day-old and adult mice. Hindlimb paralysis was readily demonstrated by the animals failure to use their hindlimbs when climbing an inclined grid, and in their inability to extend the hindlimbs and toes when lifted by the tail. These symptoms, along with other indications of toxicity such as ataxia and weight loss, became evident and progressed more rapidly in the younger mice. Gandy et al (1973) also found that CL intoxicated mice demonstrated an inability to use their toes when climbing on an inclined surface. This symptom was believed to be indicative of 'impaired proprioception' although in the latter stages of toxicity motor strength was deemed to 'decrease without paralysis'. Hindlimb paralysis has been described previously in monkeys subjected to Vitamin B₁₂ deficient diets (Oxnard and Smith, 1966) and also in monkeys and pigs treated with N₂O to deactivate Vitamin B₁₂ (Scott et al, 1981; Weir et al, 1988).

Gandy et al’s (1973) interpretation of the inability of CL-treated mice to use their toes when climbing as indicative of 'impaired proprioception' might be flawed for the following reasons. Firstly, in the present experiments sensation was found to be intact in the toes and hind limbs long after the animals began to show impaired movement of the hind limbs. Secondly, the Edl and soleus muscles of CL-intoxicated mice showed considerably reduced or negligible response to neural stimulation at a time when the muscle spindles of the soleus were found to be functionally intact. Thirdly, sensory organs and their innervation (e.g. muscle spindles and the sensory fibres of the skin) were found to be far more resistant to CL than the motor innervation of the extrafusal fibres. It is possible, however, that
the myelin vacuolation of the CNS reported by Gandy et al (1973) and Lee (1991) might cause proprioception to be impaired.

The finding that the 21-day-old mice were considerably more susceptible to CL than the adult mice is in agreement with previous studies. Prior work has suggested that developing neurones (both in culture and in vivo) are considerably more susceptible to CL than mature neurones. Nixon et al (1976) demonstrated that organotypic cultures of immature cerebella were more adversely affected by CL than mature cultures. Ramsey and Fischer (1978) found that a vacuolar myelinopathy was only seen in rats if the administration of CL preceded a period of rapid development in the nervous system. Similarly, Lee (1991) found pronounced vacuolar myelinopathy of the white matter in the spinal cord and brain of 21-day-old CL treated mice. This rarely occurred in CL-treated adults. The present study also demonstrated that distal neuromuscular junctions and intramuscular nerves of young mice also appear more susceptible to CL than those of the adult mice. Very high mepp frequencies, failures in neurally evoked transmitter release and degenerating intramuscular nerve fibres and terminals were more frequent in the 21-day-old mice. The greater susceptibility of distal neuromuscular junctions and the additional CNS pathology seen in the young mice is therefore likely to be responsible for the more rapid onset of clinical symptoms and the premature deaths of the younger mice when compared to the adults.

Another factor which might help to explain the delayed onset of clinical symptoms in, and the later deaths of, CL-treated adult mice is the anorexia induced by CL (Ross et al, 1961) and the difficulty paralysed animals have in feeding. Frequently, large amounts of adipose tissue were found during the dissection of the adult mice, whereas it was not present in younger animals. Anorexia or any other symptom causing weight loss would therefore be better tolerated in the adult mice.

Considerable variation in both the rate of the development and in the severity of the symptoms in different mice of the same age was frequently encountered. One possible explanation for this apparent anomaly is related
to the route of administration and the eventual absorption of CL. Despite attempts to standardise the exact point of administration, it remains a possibility that the CL/saline mixture was occasionally introduced into the intestine or bladder, in which case it would be excreted more rapidly. Factors such as the general health of the animals prior to the administration of CL would also have played a role in determining the resistance of the animal to CL.

Occasionally, the 21-day-old and adult mice died within 3-4 days of the administration of CL. Prior to death these animals were severely paralysed. Costal recession due to paralysis of the abdominal and respiratory muscles was frequently apparent. Paralysis of the fore limbs was occasionally evident. Physiological and morphological evidence of pathology in the motor innervation of proximal muscles, such as the biceps brachii, was seen in the later stages of the toxicity in the 21-day-old mice. The appearance of costal recession and the demonstrable changes in the motor innervation of proximal muscles suggests that the phrenic nerve may also have been affected. This would have lead to paralysis of the diaphragm and hence the cessation of the respiration. Respiratory paralysis has frequently been implicated as the cause of death in laboratory animals treated with natural neurotoxins (Strong, 1987) and in naturally occurring mutant animals suffering from the progressive degeneration of motor neurones such as the progressive motor neuronopathy or PMN mouse (Sendtner et al, 1992).

It is possible that some of the mice died from opportunistic infections as CL has previously been found to be an immunosuppressant (Frish 1969; Brambilla et al 1972).

5.2 The effects of CL on skeletal muscle

(i) Is CL myotoxic?

The weight of both the soleus and EdI of adult mice was decreased after the administration of CL. Measurements of the weight of the soleus and EdI of the CL-treated 21-day-old mice were not made, but these
muscles frequently appeared to be much smaller than the muscles of controls. Muscle fibres make up approximately 80-90% of the muscle’s total mass and therefore any appreciable change in the weight mass of the muscle is likely to be caused by changes in the diameter of the fibres. Such a fall in the diameter of the soleus and Edl fibres could occur in response to any of the following mechanisms, either alone or in combination:

1). Following denervation both type I and type II fibres become atrophied, although the effect is more pronounced in type II fibres (McComas, 1977; Mastagia, 1985).

2). The ability of CL to cause anorexia (see above section) combined with the feeding difficulties of behaviourally affected mice causes the animals to lose weight (Nixon, 1974).

3). Proteolysis of muscle proteins takes place in early starvation (Stryer, 1981) which also leads to muscle fibre atrophy (McComas, 1977).

A thorough morphological investigation of the effects of CL on the extrafusal fibres of skeletal muscle was not done. However, indications of myotoxicity such as necrosis or ultrastructural changes such as Z-line streaming (Torres, 1986) were not observed in any of the animals examined. The observation that CL does not cause a significant change in the response of the adult Edl or soleus to direct stimulation does not concur with a direct myotoxic effect.

(ii) Denervative changes induced by CL

A number of interesting changes were observed in the muscles of CL-treated mice however. Certain changes were caused by the degeneration of the neuromuscular junctions and intramuscular nerves supplying the muscles. In particular the fall in both the twitch and tetanic response of the adult Edl and soleus to indirect stimulation can undoubtedly be attributed to the degeneration of the motor fibres innervating the extrafusal fibres of these muscles (see below). The appearance of fibrillation potentials is also likely to have been caused by the degeneration of motor nerves leaving
muscle fibres denervated. Fibrillation is not seen in muscle atrophy due to other causes (Hnik and Skorpil, 1962). The occurrence of fibrillation potentials is therefore taken to indicate pathology in the peripheral motor neurones (Hnik and Skorpil, 1962; McComas and Johns, 1981).

Hnik et al (1958) found that fibrillation first appeared 40 hours after nerve section in mice. In the present study, fibrillation become evident 6 days after the administration of CL to 21-day-old mice whereas denervated end-plates were first seen 2 days after the administration of CL. Therefore the findings presented here confirm the observations of Hnik et al (1958) in that fibrillation occurs approximately 4 days after denervation in mice. Fibrillation potentials developed over the same time course in both the soleus and the Edl.

The exact physiological mechanisms causing fibrillations in denervated muscles remain elusive. Belmar and Eyzaguirre (1966) studied the fibrillation potentials at various points along the length of denervated muscle fibres and found that fibrillation potentials appeared to originate at the site of the original end-plate. The frequency of fibrillation potentials could be altered by passing a current through the end-plate region, at all other points along the fibre this procedure had no effect. Initiation of fibrillation potentials is not likely to be caused by the increased sensitivity of the fibre to circulating acetylcholine as was suggested by Denny-Brown and Pennybacker (1938), as fibrillation potentials are resistant to D-tubocurarine (Purves and Sakmann, 1974).

Purves and Sakmann (1974) studied the fibrillation in rat diaphragm explants in vitro. The diaphragmatic muscle fibres could be divided into 2 groups on the basis of the rhythm and pattern of their fibrillation potentials. One group consisted of fibres with a regular pattern or rhythm of fibrillation activity, whereas another group consisted of fibres with erratic fibrillation potentials. In the present study only fibres of the first type, i.e. showing a regular periodicity of fibrillation activity, were found.

Different events were thought to be responsible for initiating the two types of fibrillations. In irregularly discharging fibres discrete depolarizations
or fibrillary origin potentials (fops), which had an amplitude of up to 15mV, occurred over a small area of the fibre. The amplitude and frequency of the fops was dependent on the RMP. In regularly discharging fibres the initiating event was an oscillation of the membrane potential which was also found to be sensitive to changes in the RMP of the fibre. Both the fops and the oscillations in the membrane potentials were reversibly abolished by tetrodotoxin or low Na⁺-Ringer solution. This indicated that fibrillation potentials commence with local variation in the Na⁺-conductance of the muscle membrane.

The slight slowing of the twitch rise time in response to direct stimulation observed in both the soleus and the Edl following the administration of CL could also be attributable to post-denervative changes of the electrical properties of the muscle. Slowing of the rise time of muscle action potentials has previously been found to occur following denervation (Harris and Thesleff, 1972). Both a slowing in the rise time and the propagation of the action potential along the muscle fibre, would lead to an increase in the latency of calcium release from the sarcoplasmic reticulum and hence prolong the time to maximum twitch tension. Furthermore, the reduction of fibre diameter in the muscle fibres of CL treated mice would also cause a slowing in the propagation of action potentials along the muscle, leading to a slowing in the twitch rise time.

The fall in the mean RMP of both the 21-day-old and adult soleus and Edl muscles could result from post denervative changes. Albuquerque et al (1971) described a fall in the RMP of muscles within 2 hours of denervation, which began at the end-plate and gradually spread outwards. This fall was believed to be due to inhibition of the sodium pump (McComas, 1977) and may amount to a fall of as much as 20mV occurring over the first 9-10 days following denervation. In the present study a mean fall of between 5-7mV occurred in both the soleus and Edl after treatment with CL.

It can be seen from Figs 4.19 and 4.28 that some muscle fibres were not denervated, or alternatively quickly became reinnervated following treatment with CL. This would prevent the mean RMP falling to the levels
described by McArdle and Albuquerque (1973). Difficulties arise when the results of the present study are compared with those obtained when denervation is achieved by crushing or cutting the motor nerve. When a nerve is transected or crushed, all the fibres degenerate and eventually regenerate at approximately the same time. This does not occur in CL-intoxication, where normally innervated muscle fibres can be found alongside fibres which have been denervated for a number of days.

It is probable that the fall in mean RMP seen in the muscles of CL treated mice was not related to post-denervative changes. The evidence for this statement is based on the following findings:

1). A significant fall in the mean RMP of the soleus and biceps brachii in 21-day-old mice occurred within 24 hours of the administration of CL. At this time the neuromuscular junctions in the biceps brachii were functionally and morphologically indistinguishable from controls, and completely denervated end-plates were not seen in the soleus or the Edl.

2). The fall in the mean RMP of the biceps brachii fibres continued linearly until 6 days after the administration of CL, while the motor innervation of this muscle remained intact. At 7 days, when some of the bicep brachii fibres were found to be denervated, the mean RMP actually increased slightly.

3). A continuation in the fall of mean RMP in the 21-day-old Edl and in both the adult soleus and Edl was seen 7 days after the administration of CL, despite indications of reinnervation such as an increase in the number of fibres showing mepps or epps.

4) Fibres with very low RMP (-50mV) were only occasionally seen.

It appears therefore that mechanism (or mechanisms) aside from post denervative changes are responsible for causing the linear fall in the mean muscle fibre RMP after the administration of CL. It is possible that this generalised fall in the RMP of muscle fibres is related to the ability of CL to prevent the methylation of membrane phospholipids, such as the conversion of phosphatidyl ethanolamine to phosphatidylcholine (Nixon et al, 1973).
Alterations in the relative proportions of these and other phospholipids in the sarcolemma could alter the electrical properties of the fibre. CL may also effect the synthesis of membrane proteins (such as ion channels), as failures in the methyl-transfer pathway frequently cause disruptions in protein synthesis (Scott and Weir, 1981). A decrease in the turnover of the proteins which make up the sodium pump would lead to a fall in the RMP of affected fibres (McComas, 1977).

Perhaps the most likely explanation for the linear fall in the RMP of the muscles in CL treated mice is related to the fibre atrophy. The weight of adult soleus and EdI decreased after the administration of CL. This reduction is also likely to have occurred in the 21-day-old mice. A fall in the weight of a muscle is likely to lead to a concomitant fall in the mean fibre diameter (see above). The RMP of muscle fibres has been found to be consistently higher in the larger type II fibres of the EdI compared to the smaller type I fibres of the soleus (Harris, 1971; Banker et al, 1983; Brook and Duchen, 1990). The RMP of muscle fibres has also been shown to correlate with fibre diameter (Kelly, 1978). A reduction in the diameter of the muscle fibres might therefore lead to a concomitant diminution in mean RMP. Further work will be required properly to ascertain the mechanisms responsible for causing the observed fall in the RMP of muscle fibres following the administration of CL. Morphometric analysis of the effects of CL on muscle fibre diameter might substantiate the idea that the decrease in the mean RMP is correlated with a reduction in fibre diameter. Alternatively the effects of CL on the electrical properties of muscle fibres could be determined using voltage or patch clamp techniques.

(iii) The effects of CL on intrafusal fibres

Lee (1991) noted that muscle spindles appeared to be particularly resistant to the effects of CL. In areas of the hind limbs, where the motor innervation of the extrafusal fibres had undergone marked degeneration, the sensory and motor innervation of the muscle spindles appeared intact. This
finding is of considerable interest in the context of Gandy’s et al (1973) description of "impaired proprioception" in CL-treated mice, which may have resulted from impairment of muscle spindles and other related sensory structures. Absent tendon reflexes or impaired vibration sensation has frequently been described in Vitamin B_{12} deficient patients (Foster, 1945; Fine and Hallet, 1980; Cox-Klazinga and Endtz, 1980; Lever et al, 1986; Fine et al, 1990; and Healton et al, 1991) which has frequently been considered to be indicative of lesions in the distal reaches of sensory nerves (Cox-Klazinga and Endtz, 1980; Fine et al, 1990; and Healton et al, 1991). Lowndes et al (1978) believed that a major contributor to the monosynaptic tendon reflex response was the discharge from the primary endings of muscle spindles (see also Boyd, 1984b). Muscle spindles signal both the length of a muscle and its rate of movement. Lowndes et al (1978) therefore suggested that the attenuation of tendon reflexes might be caused by abnormalities in the function of muscle spindles (also see Boyd, 1985b).

Despite neurological symptoms indicative of the impaired function of muscle spindles frequently being described in Vitamin B_{12} deficient patients (see above), the morphological or physiological effects of Vitamin B_{12} deficiency on the mammalian spindle has not been ascertained. Russell et al’s study of 1900 is the only that could be found in which muscle spindles were mentioned. Russell et al (1900) described degenerative changes in the spindles of one patient, while in another patient the spindles were found to be ‘well preserved’.

The combined physiological and morphological approach adopted for this study confirmed the preliminary findings of Lee (1991), it being shown that the muscle spindles of the soleus and lumbrical muscles of the foot were unaffected by the administration of CL. This was despite the fact that the motor innervation of the extrafusal fibres is severely affected in these muscles. Physiological abnormalities can occur in the absence of, or prior to, demonstrable morphological changes in the sensory structures of animals with experimentally induced distal axonopathies (Sumner and Asbury, 1975;
Spencer and Schaumburg, 1984). Therefore the application of physiological techniques may provide a more sensitive measure of the abnormalities induced by CL at the muscle spindle.

In other experimental distal axonopathies, most notably those produced by the administration of acrylamide and pyridoxine, the onset and progression of ataxia has been found to correlate with functional abnormalities and with the eventual degeneration of the sensory terminals of muscle spindles in the affected limbs (Boyd and Smith, 1984). Sumner and Asbury (1975) found that the clinical condition of cats treated with acrylamide correlated with the number of unresponsive muscle spindles or tendon organs in the medial gastrocnemius muscles. As the animals clinical condition worsened the number of unresponsive spindles and tendon organs increased. Lowndes et al (1978) demonstrated that the attenuation of achilles tendon reflexes in cats intoxicated with acrylamide was caused by failure of the primary sensory endings of soleus spindles.

Krinke et al (1978) found that the primary sensory nerve endings of the plantar lumbrical muscle spindles in ‘lame’ rats treated with multiple doses of pyridoxol hydrochloride undergo degenerative changes such as floccular disintegration of the axoplasm, loss of neurofilaments and microtubules and a decrease in the number of mitochondria. The molecular basis for the sensitivity of the primary nerve ending of the distal muscle spindles to either of these compounds was not discussed and remains uncertain.

Other toxins which cause the parallel degeneration of the innervation of extrafusal and intrafusal fibres have also been described. Queiroz and Duchen (1982) found that the venom obtained from the Latrodectus genus of spiders caused progressive degeneration of both the sensory and motor innervation of murine spindles. Duchen et al (1981) had previously described similar degenerative changes occurring over an identical time course at the motor terminals of extrafusal fibres.

The reason for the apparent resistance of both the motor and sensory innervation of the intrafusal fibres, compared to the sensitivity of the motor
innervation of the distal extrafusal fibres, to CL intoxication is uncertain. It is unlikely that the differing sensitivities of these classes of axons can be attributed to differences in the diameter and hence metabolic demands of these classes of axons. In mammalian muscles the fibres terminating in primary sensory terminals, the plate motor end-plates of intrafusal fibres, and the motor end-plates of the extrafusal muscle fibres all have similar diameters (Barker, 1974). This situation is further complicated by Lee et al’s (1992) description of widespread necrosis of dorsal root ganglion cells in CL treated mice, which would be expected to lead to the denervation of sensory structures.

Further work is required to ascertain the biochemical basis of the resistance of sensory fibres and the structures they innervate to CL intoxication and why the fibres innervating extrafusal fibres do not share this resistance. The use of a larger experimental animal (e.g. a cat) might enable the functional capabilities of individual sensory structures to be ascertained. The recording of the responses of single active fibres from dorsal root filaments (see Sumner and Asbury, 1975) would allow an accurate interpretation of the number of responsive versus non responsive muscle spindles/tendon organs in early and late CL intoxication. This method could also be modified slightly to allow the functional capabilities of sensory structures responsive to other stimulatory modalities to be measured (e.g. Pacinian corpuscles and free nerve endings sensitive to temperature/pain). It would also be very interesting to examine the structure and function of muscle spindles in animals subjected to other procedures (such as the administration of nitrous oxide) which cause failures in the methyl-transfer pathway and hence neurological symptoms such as the attenuation of tendon reflexes and paralysis of the hind limbs. Likewise a study of the morphology of both the muscle spindles and the motor innervation of the extrafusal fibres in Vitamin B₁₂ deficient patients (from biopsy or autopsy specimens), particularly those in which tendon reflexes are absent or attenuated, would also be of interest. The information obtained from such a study would help in deciding the controversy concerning the primary site
of the lesions causing the neurological abnormalities seen in some Vitamin B$_{12}$ deficient patients and how these lesions might be related to abnormalities in the CNS.

5.3 The effects of CL on the murine neuromuscular junction and its innervation

The work presented here primarily concerns the effects of CL on the structure and function of the neuromuscular junction, and the intramuscular nerve fibres innervating these structures, in a variety of murine muscles. Prior to discussing the effects of CL on the structure and function of the neuromuscular junction, the events thought to be responsible for normal neuromuscular transmission and the role each of the structures found within the motor nerve terminal is thought to play in these events will be briefly discussed.

Presynaptic neuromuscular events, particularly those associated with the storage and release of acetylcholine (ACh) are not fully understood (see reviews by Ginsborg and Jenkinson, 1976; Jones, 1987; Bowman, 1990).

It is generally accepted that ACH is released from the motor nerve terminal by two separate processes (Torri-Tarelli et al, 1990), namely:

1). Quantal release, i.e. the release of multimolecular packages or quanta of ACh;

2). Molecular leakage from the presynaptic terminal, often referred to as non-quantal release.

Quantal release was initially described by Fatt and Katz (1952). It was demonstrated that in the absence of nerve impulses, small spontaneous potentials named miniature end-plate potentials (mepps) could be recorded intracellularly from the post-junctional membrane below the motor nerve terminal. These mepps were thought to be the result of the synchronous release of many molecules of ACh in the form of a multimolecular packet or quanta from the nerve terminal. The neurally evoked end-plate potential (epp) was found to result from the synchronous release of several hundred
of these multimolecular packets or quanta of ACh (del Castillo and Katz, 1954). The recording and subsequent analysis of both spontaneous and evoked quantal release from the neuromuscular junction has proved to be an immensely powerful tool for studying both neuromuscular disease and the action of neurotoxins which selectively block neuromuscular transmission (see Thesleff et al, 1990). The recording of both spontaneous mepps and neurally evoked epps permitting neuromuscular transmission to be quantified in a number of ways which allows the functional correlates of morphological or biochemical changes to be accurately obtained.

Mepps can be quantified in terms of their frequency, amplitude, and rise times. The recording and quantification of neurally evoked quantal release (epps) is frequently more complex than the recording of sub-threshold spontaneous events (mepps). Under normal conditions an epp causes a large depolarization of the post-synaptic membrane which initiates an action potential and the contraction of the muscle fibre. The contraction of the muscle would either break or dislodge the intracellular electrode and hence prevent the epp from being recorded. The epp amplitude is normally reduced therefore by adding a reversible non-depolarising ACh agonist such as d-tubocurarine or alternatively by increasing the Mg$^{2+}$ and decreasing the Ca$^{2+}$ content of the bathing media. These procedures prevent muscle contraction following neural stimulation and hence facilitate the recording of epps. Under such conditions epps can be evoked at a variety of stimulation frequencies.

Leakage of neurotransmitter across the presynaptic membrane (or non quantal release) was first described by Katz and Miledi (1977). This leakage may represent the predominant form of transmitter release from the neuromuscular junction under resting conditions (Torri-Tarelli et al, 1990). Edwards et al (1985) attributed this form of release to the presence of vesicular membrane transport systems in the membrane of the pre-synaptic terminal. The inclusion of these transport systems into the pre-synaptic membranes was believed to occur during exocytosis, when the vesicular membrane combines with the membrane of the pre-synaptic terminal.
(Zemkova et al, 1990). The physiological significance of this form of release and the factors regulating it have yet to be defined (Torri-Tarelli et al, 1990).

There has been much debate and controversy concerning the functional significance of the ‘vesicular’ and ‘axoplasmic’ storing of ACh in the motor nerve terminal. The roles of each of these stores in the quantal release of ACh from the neuromuscular junction have been extensively reviewed by Israel et al (1979) and Ceccarelli and Hurlbut (1980). Israel et al (1979) believed that the experiments they reviewed for the most part failed to substantiate the ‘vesicular hypothesis’ (see below). An alternative hypothesis which encompassed the findings of their studies was therefore proposed, known as the ‘operator hypothesis’. It was proposed that specialised structures in the axoplasm bound newly synthesised axoplasmic ACh in a saturable and reversible manner. An increase in intracellular free Ca^{2+} would cause these structures to release ACh into the synaptic cleft, when the nerve terminal was depolarized. This operation would preferentially release newly synthesised cytoplasmic ACh, whereas the vesicular ACh would act as a reserve supply.

The conceptual basis for the vesicular hypothesis, i.e. that ACh is preferentially stored in vesicles prior to release, and that vesicles are the anatomical correlates of quanta was proposed by del Castillo and Katz in 1955. This hypothesis has been challenged by some workers, however, (see above). Recent electrophysiological, biochemical, and ultrastructural studies have provided additional evidence to substantiate this hypothesis, including micrographs which appear to show vesicles fusing with the presynaptic membrane (Torri-Tarelli et al, 1990) and the implication that vesicle-associated proteins play a role in the final stages of neurotransmitter release (Valtorta et al, 1988; Petrenko et al, 1991). The most persuasive evidence in support of the vesicle hypothesis comes from the electrophysiological studies of Lim et al (1990). Lim et al used ‘whole terminal’ patch clamp and circuit analysis techniques to measure membrane capacitance in individual nerve terminals isolated from the mammalian neurohypophysis. Depolarizing these fibres with a small pulse was found to
cause the entry of \( \text{Ca}^{2+} \) through voltage gated channels. An increase in the nerve terminal capacitance then rapidly followed. This increase in capacitance was believed to be caused by an increase in the area of nerve terminal, resulting from the fusion of synaptic vesicles with the membrane of the nerve terminal and hence the incorporation of vesicle membranes into the axolemma of the cell.

The fundamental proposition of the vesicle hypothesis is that the ACh available for immediate release is stored within the vesicles lying nearest to the release sites at the nerve terminal. Transmitter release is thought to be triggered by an increase in intracellular \( \text{Ca}^{2+} \) levels. Such an increase occurs when the nerve terminal is depolarised by the nerve action potential, as this depolarization opens voltage dependent \( \text{Ca}^{2+} \) channels and hence permits the entry of \( \text{Ca}^{2+} \) into the terminal. Calcium ions then permit the ACh containing vesicles to fuse with specialist active zones on the axolemmal membrane, causing the release of ACh into the synaptic cleft.

Exactly how a rise in intracellular \( \text{Ca}^{2+} \) concentrations causes vesicles to fuse with the axolemmal membrane remains uncertain (Trimble and Scheller, 1988). Recent evidence, however, suggests that synaptic vesicle-associated proteins might play an important role in regulating the fusion of synaptic vesicles with the axolemmal membrane (Valtorta et al, 1988; Petrenko et al, 1991). The massive increase in transmitter release following the administration of \( \alpha \)-latrotoxin (a purified constituent of black widow spider venom) has recently been attributed to the high affinity binding of \( \alpha \)-latrotoxin to a presynaptic receptor located on the external face of the presynaptic terminal. This receptor then binds to a synaptic vesicle-associated protein known as synaptogamin in the axoplasm of the terminal, modulating its phosphorylation (Petrenko et al, 1991). The structure and properties of synaptogamin previously led Perin et al (1990) to suggest it might play a role in mediating membrane interactions during synaptic vesicle exocytosis.

Following the release of the vesicular contents into the synaptic cleft, the vesicle membrane is incorporated into the axolemmal membrane. The
vesicular membrane is subsequently recycled (a process called endocytosis). Vesicle recycling is necessary to maintain a constant number of vesicles within the terminal, as the synthesis of new vesicular membranes occurs relatively slowly and would be insufficient to maintain a consistency of vesicle numbers (Jones, 1987).

Vesicle membranes are thought to be retrieved by two different processes. Firstly retrieval may occur close to the sites of vesicle fusion (the active zone), where uncoated cisternae are formed. Secondly, retrieval may occur at sites more distal to the active zones, where coated vesicles are formed (Miller and Heuser, 1984). Once internalised these vesicles are loaded with ACh from the axoplasm. The loading of the ACh into the vesicles is energy dependent and is driven by creating a proton gradient within the vesicle (see Bowman, 1990).

The amount of free Ca²⁺ inside the neuromuscular junction is maintained at a low level by intracellular organelles such as the mitochondria, synaptic vesicles and sarcoplasmic reticulum which sequester free Ca²⁺ and therefore act as buffers (Alnaes and Rahamimoff, 1975; McGraw et al, 1980), prior to the removal of Ca²⁺ from the neuromuscular junction, possibly by a Na⁺/Ca²⁺ exchange mechanism or an ATP-dependent Ca²⁺ pump (Jones, 1987). The maintenance of low intraterminal free Ca²⁺ levels is vital to maintain the structural (Ng and Howard, 1980) and functional (Alnaes and Rahamimoff, 1975) integrity of the neuromuscular junction.

(i) The quantitative analysis of the CL-induced degeneration of distal neuromuscular junctions.

Lee (1991) was the first to demonstrate that CL induced structural abnormalities and the eventual degeneration of distal motor nerve terminals, while the motor innervation of proximal muscles remained intact. The labour intensive nature of the morphological studies undertaken by Lee (1991) meant that few end-plates from each animal could be sampled and hence
the effects of CL on the motor innervation of muscles could not be properly quantified. Lee’s findings have been confirmed and extended by the present study.

The paralysis of the hind limbs in CL-treated mice previously reported by Gandy et al (1973), Nixon (1974), and Lee (1991) was found in this study to be caused by a dramatic fall in the response of the muscles in the paralysed region (the EdI and soleus) to neural stimulation. The sensory innervation of these muscles was found to be unaffected by CL. These findings therefore contradict the suggestion that the hind limb paralysis in CL-treated mice is caused by impaired proprioception made by Gandy et al 1973.

The effects of CL on the motor innervation of distal muscles in adult mice and in both proximal and distal muscles in young mice, was then examined using intracellular techniques. These intracellular techniques enabled a large number of muscle fibres (40) to be sampled from each preparation. This permits the effects of CL on the motor innervation of a muscle to be accurately quantified.

In both the adult and the 21-day-old mice, CL caused a dramatic fall in the number of EdI and soleus fibres which demonstrated mepps or alternatively responded to neural stimulation with an epp within 24 hours. The number of fibres showing mepps or epps was found to fall further over the following 3 days. Structural abnormalities were also seen at 24 hours. From 2 days onwards denervated end-plates were seen where Schwann cell cytoplasm was seen directly above the post synaptic junctional folds. The number of fibres per preparation showing epps is probably a better indicator of the number of end-plates where the nerve terminal is still in close apposition and hence in functional contact, with the muscle fibre. Monitoring the number of fibres demonstrating mepps alone could be misleading (Dennis and Miledi, 1974a), as Schwann cells have been shown to release small amounts of ACh into the synaptic cleft in denervated muscle, which causes small mepp-like potentials (Birks et al, 1960; Dennis and Miledi, 1974a). This might explain why a larger proportion of the adult
soleus and EdI fibres tested were found to show mepps rather than epps four days after the administration of CL (compare figs 4.18 with 4.27).

It is inadvisable, however, to reach definite conclusions by comparing the mepp and the epp data as these two parameters were measured in different groups of animals and a large amount of variability was encountered between individual animals in both the mepp and the epp groups. Further experiments could be undertaken to ascertain if and at what point following the administration of CL Schwann mepps (Dennis and Miledi, 1974a) occur. For Birks et al. (1960) found that when the K⁺ concentration or the tonicity of the bathing fluid is increased the frequency of mepps at normal end-plates increases, whereas these procedures have little or no effect on the frequency of Schwann mepps.

In the latter stages of CL intoxication (i.e. at 6-7 days post-administration) signs of a functional recovery such as an increase in the number of soleus and EdI fibres showing epps and a concomitant recovery in both the twitch and tetanic tension produced in response to neural stimulation, were observed. In direct contrast to this recovery of function seen in the distal calf muscles of both 21-day-old and adult mice, early degenerative changes were observed in the biceps brachii where a significant number of fibres were found to be denervated.

There are two possible reasons for the recovery of function observed at the EdI and soleus. Firstly it is possible that this apparent recovery may have been artefactual. It is possible that the mice which survived for 7 days were either abnormally resistant to CL, or that they were able to excrete the CL due to inadequacies in the administration procedure (see earlier section of discussion). This might prevent large scale degenerative changes in these mice and hence aided their chances of survival, leading to the ‘recovery’ seen at seven days. Secondly, the recovery observed may have been caused by the reinnervation of previously denervated soleus/Edl end-plates. It seems unlikely that the observed ‘recovery’ was in fact artefactual and caused by the measurements made in the latter stages of CL-intoxication being biased towards abnormally resistant or incorrectly
injected animals. The use of a large number of animals (i.e. >10, 21-day-old and >15 adult mice were used at 7 days) and the selective use of animals manifesting, or which previously had demonstrated clinical symptoms should help to prevent this form of experimental bias. The demonstration of functional and structural abnormalities in the biceps brachii of 21-day-old mice in the latter stages of CL intoxication also contradicts such a bias.

It is therefore most likely that the functional recovery observed is indeed due to reinnervation. Previously Lee (1991) found small axonal profiles at previously denervated distal end-plates in the soleus of a 21-day-old mouse. Reinnervation of previously denervated end-plates has been characterised in a variety of animals following a number of experimental procedures. In most of these experiments the motor nerve has been either cut or crushed (Miledi 1960; Saito and Zacks, 1969; Dennis and Miledi, 1974a; Dennis and Miledi, 1974b; Tonge, 1974). Following a sciatic nerve lesion at mid thigh level in the mouse, axonal sprouts were observed in the denervated mouse foot muscles, three weeks after the implementation of the lesion (Saito and Zacks, 1969). The difference in the timescale of the reinnervation noted by Saito and Zacks (1969) and that proposed here is due to the difference in the site of the primary lesion. The time course of degenerative changes is prolonged by increasing the length of the nerve stump i.e. sectioning the nerve at a position more proximal towards the cord, (Miledi and Slater, 1970) and the time course of reinnervation is dependent on the distance the fibres have to regrow (Max and Mayer, 1984).

Studies where toxins which specifically affect the neuromuscular junction, causing the eventual degeneration of the presynaptic nerve terminal, are more applicable to the present study. The venom of the *Latrodectus* genus of spiders was found by Duchen et al (1981) to cause the degeneration of all motor end-plates within 24-hours. Similarly, Abe et al (1976) found that ß-bungarotoxin, a presynaptically active neurotoxin obtained from the snake *Bungarus multicinctus*, also caused the rapid
destruction of motor nerve terminals. Following the destruction of the neuromuscular junctions by these toxins re-innervation followed remarkably rapidly. Duchen et al (1981) first noted small vesicles containing axonal profiles lying in contact with the post synaptic sarcolemma 2 days after the administration of Latrodectus venom. Two factors were thought to underlie this rapid reinnervation. Firstly, only a short length of axonal regeneration was required and secondly the sprouts are likely to reinnervate the sites of former synaptic contact (Bennett et al, 1973), thus preventing the need for the synthesis of new postsynaptic specialisations.

The pathology observed following the administration of CL is essentially similar to that seen following the administration of Latrodectus venom, in that the Schwann cell tube and post-synaptic specialisations remain intact. Reinnervation could occur very rapidly in CL-intoxicated mice. In the later stages of CL-toxicity a paradoxical situation arises, of reinnervation and functional recovery taking place in distal muscles while degeneration of the motor end-plates is beginning in the proximal muscles.

It is somewhat surprising that reinnervation occurs at all, for CL is unlike the other toxins noted above in that it is unmetabolisable (Christenson and Jones, 1962), does not invoke the production of antibodies, and is actively reabsorbed in the kidney (Reulius et al, 1973). Toxic levels of CL are still likely to be present while reinnervation is taking place. Inhibitors of phospholipid methylation (such as CL) have however been found to promote neurite extension, however, in cultured neuroblastoma cells (Smythies, 1984). Regeneration has also been described in other experimentally induced distal axonopathies (Spencer and Schaumburg, 1984).

Due to the limited number of animals which survived beyond 5 days (notably amongst the 21-day-old mice) and the limited number of end-plates that can be viewed under EM from one animal, it was not possible to properly substantiate the physiological findings with detailed morphological observations. The results obtained in the latter stages of the CL intoxication were particularly difficult to interpret, it being very difficult to distinguish between newly formed nerve terminals and those which may have been
altered by CL but still retained some residual functional capabilities. This factor also makes it difficult to compare the present results with prior degeneration/reinnervation studies. As the procedures most frequently used to cause degeneration, i.e. nerve cut or crush or the local administration of natural toxins which specifically affect nerve terminals (see above), cause the simultaneous degeneration of all the nerve terminals in the affected muscle at approximately the same time. This was not found to occur following the administration of CL where a considerable amount of variation was encountered both between animals and between individual end-plates within the same muscle.

(ii) The effects of CL on mepp frequencies.

a). In early CL intoxication

Abnormally high mepp frequencies were recorded from between 10-45% of the fibres sampled in the soleus and Edl during the early stages of CL intoxication, i.e. at 12 hours in the 21-day-old mice and at 24 hours in the adult mice. Abnormally high mepp frequencies were found to occur in approximately 10% of biceps brachii fibres much later in the 21-day-old mice. At the early stages of nerve damage the number of muscle fibres showing mepps or alternatively epps had begun to fall, indicating that the motor innervation of these muscles had begun to degenerate leaving some fibres denervated. However, fibres showing normal mepp frequencies and quantal contents were also found in these muscles. Therefore, in the early stages of CL induced motor nerve degeneration, neuromuscular junctions can be divided into three classes in terms of their mepp frequencies:

1). Those which showed mepp frequencies within the normal range;
2). Those which had abnormally high mepp frequencies; and
3). Fibres which failed to demonstrate mepps indicating the absence of motor innervation.

It is possible that the end-plates with very high mepp frequencies
might represent an intermediary stage between ‘normal’ and ‘denervated’ end plates. Previously it has been demonstrated that abnormally high mepp frequencies followed by the disappearance of mepps has been found to occur following hypoxia (Hubbard and Løyning, 1966), the treatment of the nerve terminal with inhibitors of mitochondrial electron transport and oxidative phosphorylation (Alnaes and Rahamimoff, 1975), surgical denervation (Winlow and Usherwood, 1976), treatment with 2,5-hexanedione (Cangiano et al, 1980), the administration of black widow spider venom (Longenecker et al, 1970) and following the administration of certain phospholipase A₂ neurotoxins such as crotoxin (Hawgood and Santana De Sa, 1979).

The complete degeneration of the affected neuromuscular junctions (and subsequent denervative changes in muscles) has been found to follow some of the above treatments. The effects of surgical denervation and the time scale of the development of both structural and functional abnormalities at the neuromuscular junction has been studied in the frog (Birks et al, 1960), in the rat (Miledi and Slater, 1970) and in the mouse (Winlow and Usherwood, 1975; 1976). Cangiano et al (1980) found that in rats treated with 2,5-hexanedione (a compound which causes a distal axonopathy) for two weeks, some soleus and Edl fibres were denervated (i.e. they failed to show mepps or epps and had reduced RMP) whereas other fibres showed abnormally high mepp frequencies.

Duchen et al (1981) found that within 24 hours of the administration of black widow spider venom to the calf muscles of mice all the soleus end-plates visualised were denervated. Gopalakrishnakone and Hawgood (1984) described the degeneration of the neuromuscular junctions in mice subjected to an intramuscular injection of crotoxin. It can be seen from the above that abnormally high mepp frequencies may precede the degeneration of the neuromuscular junction in a variety of experimental conditions.

If abnormally high mepp frequencies are taken as an indicator of the beginnings of degeneration it would appear that most of the early degenerative change occurred within 1 day of the administration of CL in the
21-day-old mice. From two days onwards few end-plates showing high mepp frequencies, and hence, degenerative changes were seen in the young mice. The situation was somewhat different in the adult mice. Abnormally high mepp frequencies were most apparent at 24 hours at both the soleus and the Edl. However, a large number of end-plates (particularly those found in the Edl) continued to show high mepp frequencies beyond two days. It would appear therefore that a significant number of the adult soleus and Edl end-plates survived the first few days following the administration of CL only to degenerate later. Correlating the morphological and physiological results in early CL intoxication is difficult. During early CL intoxication the end-plates could be roughly divided into three groups in terms of their mepp frequencies (see earlier section for mepp groups). In terms of their structure end-plates could be divided into the following groups:

1). End-plates which appeared normal;
2). End-plates which appeared slightly abnormal, i.e. they had small electron lucent vacuoles but otherwise appeared normal;
3). End-plates which appeared severely abnormal, i.e. being devoid of vesicles with electron lucent axoplasm and severely swollen mitochondria; and
4). End-plates with dense accumulations of vesicles and mitochondria with fragmented cisternae.

It is tempting to suggest that the end-plates demonstrating high mepp frequencies are those with dense accumulations of vesicles whereas, the end-plates which failed to show mepps might correlate with the swollen electron lucent terminals devoid of vesicles. Following nerve section Winlow and Usherwood (1976) were unable to correlate abnormally high mepp frequencies with structural abnormalities or changes in the spatial arrangement of synaptic vesicles in terminals undergoing early degenerative changes. However, vesicle aggregations have been correlated with high mepp frequencies at the frog neuromuscular junction in high Ca$^{2+}$ ringer (Heuser et al, 1971).
The validity of any conclusions made by correlating the functional changes with morphological abnormalities in the present study is questionable due to the wide variation of structural and functional changes seen during early CL intoxication. This is an area worthy of future research, particularly if methods which allow a particular end-plate to be visualised under TEM after its physiological characteristics have been ascertained are used. Such methods would allow a direct correlation of physiological and structural abnormalities, and would thus prove to be a very powerful tool for studying this and other neuromuscular disorders.

Jones (1987) stated that the use of changes in mepp frequency to define presynaptic changes must be undertaken carefully, as mepp frequencies are notoriously unstable and inadvertent damage to the nerve terminal can produce an impressive barrage of mepps. In the present study the increase in mepp frequency seen at some end-plates is believed to characterise early CL induced changes.

Abnormally high mepp frequencies were very occasionally recorded in 21-day-old control biceps brachii and Edl preparations and in 6% of adult control Edl preparations. However, fibres showing abnormally high mepp frequencies were never encountered in 21-day-old or adult control soleus preparations. The fact that the incidence of abnormally high mepp frequencies increased in control Edl preparations with age and that abnormally high mepp frequencies were not seen in control soleus preparations suggests that their occurrence is related to the size of the nerve terminal. This seems a viable idea, for as the size of the terminal increases so does the likelihood of inadvertent damage to the terminal as the muscle fibre is impaled with a microelectrode.

It might appear therefore that the high mepp frequencies recorded in early CL intoxication might occur as a result of the nerve terminals becoming swollen, which would increase the likelihood of damage during intracellular recordings and hence the occurrence of abnormally high mepp frequencies. In both paraffin sections and EM nerve terminals (LM) and neuromuscular junctions (EM) with a swollen appearance were observed in early CL
intoxication. To test this idea, recordings of non focal mepps (distinguished by their large rise time) were made. These recordings were made a small distance away from the areas where cholinesterase staining had previously been demonstrated. High mepp frequencies were occasionally observed with such recordings suggesting the high mepp frequencies were not caused by mechanical damage to the nerve terminals during recordings.

Mepp frequencies are governed by the concentration of free Ca$^{2+}$ within the nerve terminal (Ginsborg and Jenkinson, 1976; Bowman, 1990). Procedures which increase intracellular free Ca$^{2+}$ cause an increase in the frequency of mepps, e.g. the administration of calcium ionophores, high K$^+$, or any other procedure which depolarises the nerve terminal causing the prolonged opening of voltage sensitive Ca$^{2+}$ channels cause increases in mepp frequency. Alternatively, procedures which cause the release of Ca$^{2+}$ from intraterminal Ca$^{2+}$ stores as the endoplasmic reticulum or mitochondria, e.g. the administration of inhibitors of the electron transport chain, oxidative phosphorylation and specific inhibitors of mitochondrial Ca$^{2+}$ uptake such as ruthenium red, all increase mepp frequencies (Alnaes and Rahamimoff, 1975).

In the early stages of CL intoxication therefore certain distal neuromuscular junctions appear to show an increase in intracellular free Ca$^{2+}$. Large increases in the intracellular Ca$^{2+}$ might eventually lead to the autolysis of the nerve terminal, as the hydrolytic enzymes involved in cell death are thought to be activated by high cytosolic free Ca$^{2+}$ concentrations (Sher, 1985). The mechanisms believed to be responsible for increasing the free Ca$^{2+}$ in affected nerve terminals will be discussed in a later section.

It is also possible that CL might induce changes in the production or structure of synaptic vesicle associated proteins (such as synapsin I, synaptophysin, and synaptogamin) or the enzymes which modulate their phosphorylation and in this way increase spontaneous quantal release, i.e. the phosphorylation of synapsin frees vesicles from their association with the cytosteleton and hence increases their availability for release.
b). In late CL intoxication

In the latter stages of CL intoxication (beyond 4 days) in the 21-day-old mice the majority of the soleus and Edl end-plates which demonstrated mepps showed mepp frequencies below their respective controls. It is possible that the mepps recorded at this time originated from Schwann cells (see earlier section), as Dennis and Miledi (1974b) state that Schwann mepps usually have a frequency of 0.0042-0.17Hz. Alternatively, the nerve terminals which have survived may be much reduced in size or the small vesicle containing axonal sprouts seen re-innervating some previously denervated end-plates might be responsible for these low mepp frequencies. Kuno et al (1971) found that mepp frequency increase with the size of the end-plate. It would have been interesting to determine the origin of the mepps recorded at this time. This would have been possible by altering the K⁺ concentration or the tonicity of the bathing media as these treatments increase the frequency of mepps released from nerve terminals without affecting the frequency of Schwann mepps. Increasing the K⁺ concentration or the tonicity of the Ringer solution to determine the origin of the mepps recorded at a given end-plate is likely to alter the functional characteristics (notably the mepp frequency and RMP) of all the other end-plates in the muscle. Further end-plates could therefore not be sampled following changes in the Ringer solution. Experiments aimed at determining the exact origin of mepps recorded in the later stages of CL-intoxication would therefore be very labour intensive and involve the use of many animals.

(iii) Mepp amplitudes

Mepp amplitude is largely dependent on the diameter of the muscle fibre (Katz and Thesleff, 1957). In an earlier section of the discussion it was suggested that the diameter of muscle fibres in CL treated mice is likely to decrease. Therefore in the absence of presynaptic changes an overall increase in mepp frequencies would be expected. In the biceps brachii of
the CL-treated 21-day-old mice presynaptic changes did not occur until 6-7 days after the administration of CL. An increase in the mean mepp amplitude and a generalised shift of the mepp amplitude distributions to the right was observed in the biceps brachii, which further substantiates the idea that the diameter of the muscle fibres decreases in the early stages of CL intoxication and that these changes are not related to denervation. An increase in mepp amplitude, which was believed to be caused by muscle fibre atrophy caused by inactivity and intoxication, has previously been reported in rats treated with 2,5-hexanedione (Cangiano et al, 1980).

In the soleus and Edl of both the adult and the 21-day-old CL-treated mice, very large increases in the mean mepp amplitude occurred within 24 hours of the administration of CL. These changes in the mean mepp amplitude were caused by a large increase in the proportion of mepps with amplitudes above 1.5mV. The size and early development of these changes suggests that they are not related to muscle fibre atrophy alone. It was noted that the mepps recorded from the end-plates showing abnormally high mepp frequencies were often much larger than those recorded elsewhere (see Fig 3.14). The increase in the occurrence of end-plates with abnormally high mepp frequencies might therefore be responsible for this increase in the occurrence of large mepps and hence the increase in the mean mepp amplitude. Increases in mepp amplitude have previously been described in other situations where mepp frequencies have been increased, such as following the administration of crotoxin (Hawgood and Santana De Sa, 1979), and incubating muscles in ringer containing high K⁺ concentrations (Molenaar et al, 1987).

Increased transmitter release and hence increased exocytosis is believed to cause abnormalities in the endocytotic retrieval of vesicular membranes which leads to the formation of abnormally large vesicles (Heuser, 1974), giving rise to large mepps when such vesicles combine with the presynaptic axolemma during exocytosis (Heuser, 1974; Molenaar et al, 1987).

The changes in the mepp amplitude distributions reported by Heuser
(1974), Hawgood and Santana De Sa (1979), and Molenaar et al (1987) are primarily due to the increased incidence of giant mepps which have amplitudes ≥ 2 times the modal mepp amplitude at a given end-plate. Such mepps were occasionally observed in the soleus and Edl of the 21-day-old mice in early CL intoxication, but were rarely seen in the adult mice. In the adult mice the main change in early CL intoxication was an increase in the occurrence of mepps with amplitudes of between 1.25 to 1.5 times the mean control mepp amplitude. Alternatively, the large mepps seen during high frequency mepp release may represent the synchronous release of more than one quantum, or the overlap of successive quanta.

A morphometric analysis of vesicle diameters and a detailed study of the occurrence of gmepps in nerve terminals during early CL induced changes might reveal abnormalities in endocytotic vesicular membrane retrieval.

The changes in mepp amplitude seen in the later stages of CL intoxication are far more difficult to characterise and explain in terms of presynaptic events for the following reasons:

1). Some of the ‘mepps’ recorded may originate from the release of ACh from the Schwann cells.

2). The input impedance of the muscle fibres is likely to vary significantly from that of the muscle fibres in control preparations.

3). Very few mepps are available for analysis due to the small number of fibres still showing mepps, particularly in the 21-day-old mice.

4). Uncertainties concerning whether the neuromuscular junctions giving rise to mepps at six and seven days are reinnervative sprouts or the original junctions which have retained their ability to release ACh.

Further work is necessary to ascertain the significance of the low mepp amplitudes seen in the later stages of CL intoxication. It is possible they relate to abnormalities in vesicular membrane retrieval or the incomplete filling of vesicles with ACh.
(iv) The effects of CL on neurally evoked transmitter release from the neuromuscular junction

A dramatic reduction in the number of soleus and Edl end-plates showing an epp in response to neural stimulation occurred in both the adult and the 21-day-old mice within 24 hours of the administration of CL.

Occasionally, end-plates which failed to respond to neural stimulation yet continued to show high frequencies of spontaneous mepps were seen. It would seem therefore that evoked transmitter release fails prior to spontaneous release in CL intoxication. Unfortunately it is not possible to further substantiate this idea by a direct comparison of the mepp and epp results, as these results were obtained from different groups of animals which showed considerable variation in their resistance to CL. The persistence of mepps following the failure of evoked transmission has previously been found to occur in degenerating nerve terminals following nerve section (Miledi and Slater, 1970), in hereditary 'motor end-plate disease' of the mouse (Duchen and Stefani, 1971), and following the application of crototoxin to the frog neuromuscular junction (Hawgood and Santana De Sa, 1979).

This therefore suggests that a failure in excitation-secretion coupling or a failure of the action potential to invade the motor nerve terminal prevents the evoked release of ACh in early CL intoxication rather than a shortage of available, releasable, ACh, as the spontaneous release of ACh continues after evoked release has failed. Successive epps recorded from some end-plates during early CL intoxication showed a marked variation in their latencies and some failures in transmission. These changes might represent early failures in the excitation-secretion coupling mechanism or alternatively might represent changes in the electrical properties of the terminal which either prevent or prolong the depolarization of the nerve terminal following the arrival of an action potential at the terminal node of Ranvier. It is unlikely that propagation of the action potential fails proximally in the motor nerve as the sciatic and posterior tibial nerve appear
structurally intact during early CL intoxication.

At the mouse neuromuscular junction, depolarization of the nerve terminal is thought to be brought about by the passive spread of depolarization from the last few nodes of Ranvier (Bowman, 1990), despite the fact that Na$^+$ channels are thought to present in the nerve terminal itself (Konishi, 1985). Changes at the nerve terminal which might lead to an increase in the resistance or capacitance of the terminal which could effect the rate of depolarization of the nerve terminal and hence the latency of the epp or, alternatively the ability of the terminal to respond to successive stimuli. Morphological abnormalities such as dense accumulations of vesicles, or intra axonal/terminal vacuoles of rarefied axoplasm might represent terminals where the action potential is blocked or only occasionally invades the terminal, due to an increase in the resistance of the terminal brought about by these abnormalities. Prolonged epp latencies have previously been described following the intra neural injection of *Phoneutria nigriventer* spider venom (Cruz-Höfling et al, 1985). The prolongation of epp latency, seen following the administration of the venom, was considered to be associated with swelling of the nodal and paranodal axoplasm and the accumulation of electron lucent areas in the periaxial space of affected nerves.

Alternatively, the synthesis of the structures and compounds required for maintaining the electrochemical gradients at the neuromuscular junction might be affected directly by CL. Both the phospholipid and protein constituents of the nerve terminal might be affected in this way. A breakdown in electrochemical gradients would lead to the failure of evoked release.

In the later stages of CL-intoxication the prolonged epp latencies might be attributable to the axonal sprouts seen reinervating some end-plates, as the epps recorded early in reinnervation frequently have a prolonged latency (Miledi 1960; Duchen et al, 1981). The prolonged latency of the epp seen in axonal sprouts is thought to be related to the small diameter and hence slow conduction velocity of the axonal sprout and
the immaturity of the release mechanism.

The evoked release of neurotransmitter was quantified by calculating the mean quantal content at each end-plate using the variance technique (Boyd and Martin, 1956; Elmqvist and Quastel, 1965). Essentially, it is the degree of variation between successive epsps that is measured using this technique; the smaller the variation the larger the mean quantal content (mqc). The end-plates which showed failures of transmission or a wide degree of variation in the amplitude of successive epsps will therefore have small mqc's. Quantal content can be estimated using a variety of other techniques (see Hubbard et al 1969 for discussion).

A wide range of factors have previously been found to alter mqc (see Ginsborg and Jenkinson 1976 and Bowman 1990 for reviews). Some of these factors may be relevant to the changes seen in CL-intoxication, notably end-plate size and the concentrations of free Ca^{2+} within the nerve terminal. Kuno et al (1971) showed that mqc is correlated with end-plate area, whereas Alnaes and Rahamimoff (1975) found that increasing the concentrations of free Ca^{2+} within the terminal also increases mqc. The latter is likely to be particularly relevant as high mepp frequencies which are indicative of high Ca^{2+} within the nerve terminal (Alnaes and Rahamimoff, 1975) were found in early CL-intoxication.

The CL-induced changes in both the mean quantal content and the quantal content distributions are extremely complex and thus difficult to interpret. The changes seen in early CL-intoxication are likely to represent very early abnormalities as the failure of evoked release is thought to occur prior to the cessation of spontaneous release (see earlier section of discussion). The increase in the mean quantal content seen in the adult soleus and Edl within 24 hours of the administration of CL to adult mice are of particular interest. This indicates that CL either causes an increase in the quantal content of the remaining nerve terminals, possibly by increasing the free Ca^{2+} within the terminal, or that smaller nerve terminals with smaller quantal contents degenerate first. The latter hypothesis seems unlikely as the large end-plates (with large mqc) degenerate first in the CL-treated 21-
day-old mice and the Edl end-plates, which are thought to be larger than those of the soleus in both 21-day-old and adult mice (see Results section for discussion), were not found to be more resistant than those of the soleus. This suggests therefore that the observed increase in mqc is likely to be caused by CL increasing the quantal content at a given end-plate rather than by selectively affecting a particular size class of terminals.

In the latter stages of CL-intoxication in the 21-day-old mice the vast majority of end-plates which responded to neural stimulation had quantal contents below 20. This is likely to be due to the appearance of reinnervating axonal sprouts in previously denervated end-plates. Reinnervating sprouts in the murine soleus have previously been seen initially to have a low quantal content (Duchen et al, 1981). The low quantal content of reinnervative sprouts was believed by Duchen et al (1981) to be related to the small size of the terminal (Kuno et al, 1971) and the limited area of synaptic contact. In the adult mice, complete denervation of the soleus and Edl is unlikely to have taken place. Reinnervative sprouts alone therefore cannot account for the low mqc seen at 6 and 7 days in the soleus and Edl in adult mice. The adult soleus and Edl nerve terminals which have resisted degeneration and retained the ability to respond to neural stimulation are likely to have atrophied because the diameter of the muscle fibres of the soleus and Edl is believed to have fallen following the administration of CL (see earlier section of discussion), and Balice-Gordon et al (1990) have shown that the area of the pre-synaptic nerve terminal decreases with the muscle fibre diameter as the muscle fibre atrophies. The low mean quantal content and absence of quantal contents above 140 might therefore be due to a generalised reduction in the size of the nerve terminal brought about by muscle fibre atrophy.
5.4 The mechanisms responsible for causing the distal-motor-axonopathy seen following the administration of CL

Lee (1991) was the first to describe a distal motor axonopathy in mice following the administration of CL. No other reports concerning the pathogenesis of this condition have been traced. Information obtained from the studies aimed at elucidating the mechanisms underlying CLs other pharmacological properties will therefore have to be used. The pathogenetic mechanisms thought to cause other toxin induced distal axonopathies will also be discussed to generate ideas relating to the pathogenesis of the distal axonopathy induced by CL in mice. Comparing and contrasting the clinical, pathological, and physiological characteristics of the distal axonopathy seen in CL-intoxication with the abnormalities that characterise other distal axonopathies where the pathogenetic mechanisms are better understood can provide the basis for the formation of hypotheses concerning the pathogenesis of the distal axonopathy induced by CL.

Distal axonopathies have been described in a variety of metabolic, genetic, and malignancy associated diseases, in a variety of nutritional deficiencies, and following exposure to toxins (see Schaumburg et al, 1983; and Cavanagh, 1985 for reviews). In all of these conditions the large diameter, long, axons are affected early in the condition with the fibres of the sciatic nerve (which innervate the Edl and soleus in the mouse) being particularly vulnerable (Schaumburg et al, 1983).

Spencer and Schaumburg (1976) reviewed the literature concerning the 'dying back' hypothesis of distal axonal degeneration. In most studies it was assumed that toxins which produce distal axonopathies primarily effect the metabolic machinery within the nerve cell perikaryon. The nerve cells which support the greatest volume of axoplasm would therefore be most severely affected and hence degeneration would occur first in the region furthest removed from the nerve cell, i.e. the nerve terminal. If the toxicity persisted, degeneration would also occur in regions more proximal
to the cell body and in smaller fibres. Initially this hypothesis appears to explain the progressive distal to proximal degeneration seen in CL-treated mice. Unfortunately, this hypothesis does not explain the resistance of sensory fibres, or the rapid onset of degeneration, which indicates a direct effect on the nerve terminal.

In a later review Cavanagh (1985) attempted to classify distal axonopathies in terms of the mechanisms thought to be responsible for causing the 'dying back phenomenon'. These conditions were divided into two broad categories:

1). Substances which cause, or conditions which lead to, cellular energy production being compromised, e.g. thiamine deficiency, the administration of thiamine analogues and toxins such as arsenic, thallium, nitrofurans.

2). Substances or conditions which affect specific cell constituents, e.g. adriamycin and mercury which effect nuclear functions, vincristine and vinblastine which effect microtubules and transport functions, hexacarbons which effect neurofilaments and structural elements, and acrylamide which effects smooth endoplasmic reticulum and axonal functions.

It can be seen from the above that a wide range of unrelated mechanisms are now believed to cause the 'dying back' of distal axons, thus demonstrating the susceptibility of distal axons to a wide variety of pathogenetic mechanisms. This complicates the formation of hypotheses concerning the mechanisms responsible for the distal axonopathy induced by CL. Lee et al (1992) believed that the axonopathy seen in CL-intoxicated mice most closely resembled the distal axonopathy seen in organophosphate intoxication. Schaumburg et al (1983) reviewed the causes and effects of organophosphate induced axonopathy, with particular reference to triorthocresyl phosphate (TOCP). TOCP primarily affects the large diameter motor fibres of the peripheral nervous system, which causes paralysis of the feet. In the later stages proximal regions may also be affected. The axonopathy induced by organophosphates is thought to be caused by the
phosphorylation of esterase enzymes within the nervous system as organophosphates are good phosphorylating agents and numerous esterases are found within the nervous system (Schaumburg et al, 1983).

A major difference between the neuropathy seen following a single exposure to organophosphates and that seen following the administration of a single dose of CL is the interval between exposure and the onset of the signs of a neuropathy. The onset of these signs occurs within 24-48 hours of the administration of CL to mice whereas distal neurological impairment becomes apparent between one to three weeks after exposure to organophosphates (Schaumburg et al, 1983). Another difference between CL and TOCP axonopathy relates to the ultrastructural changes seen prior to Wallerian-type degeneration. Membranous accumulations have been described in affected axons in organophosphate intoxicated animals; an abnormality not seen in CL-treated animals. These two major differences indicate that the axonopathy induced by CL is unlikely to share the same pathogenetic mechanism as the neuropathy induced by organophosphates.

The interruption of axonal transport has been implicated as a possible pathogenetic mechanism in a number of distal axonopathies (see review by Brimijoin, 1984). It is now firmly established that axons are capable of transmitting material both towards the terminal from the perikaryon (anterograde transport) and from the terminal towards the perikaryon (retrograde transport). Both anterograde and retrograde transport are believed to be vital for maintaining the structural integrity of the neuron (see reviews by Ochs, 1984 and Schnapp and Reese, 1986).

It is possible that the pathogenesis of the axonopathy induced by CL is related to the inhibition of axoplasmic transport. The axonopathies induced by hexacarbons (such as n-hexane, methyl n-butyl and 2,5-hexanediione) are thought to be caused by abnormalities in axoplasmic transport. Dense accumulations of neurofilaments at the proximal sides of nodes of Ranvier are believed to block axoplasmic transport and thus cause the portions of the nerve fibre distal to the block to degenerate (Spencer and Schaumburg, 1978; Cavanagh, 1985). Dense accumulations of
neurofilaments were not observed in the CL-treated mice however.

Brimijoin (1984) outlines the following structures and reactions essential for rapid anterograde axonal transport, which are thought to be susceptible to attack by toxins known to cause distal axonopathies:

1). Protein transcription and translation.
2). The packing of proteins by the Golgi apparatus.
3). Direct effects on oxidative phosphorylation which powers axonal transport.
4). The structure and function of microtubules and neurofilaments (see above).

It has previously been suggested that CL suppresses protein synthesis (Berlinguet et al, 1962) and it has been found that the protein content of mouse sciatic nerve is reduced by CL (Nixon, 1976b). Such an effect might inhibit rapid axonal transport (see above) which may in turn lead to the degeneration of distal nerve terminals. The extremely rapid degeneration of distal motor nerve terminals seen in CL-treated mice (particularly the 21-day-old mice) indicates that the primary disturbance inducing axonal degeneration is unlikely to occur in the nerve cell perikaryon (where protein synthesis occurs) as there is likely to be a considerable delay between failures in the perikaryon and deficiencies in the nerve terminal.

Interruptions in axoplasmic flow can occur at the nerve terminal. The motor axonopathy produced by zinc pyridinethionine (ZPT) (Sahenk and Mendell, 1979) is thought to be caused by ZPT preventing the anterograde-retrograde conversion of transport vesicles at the axon tip (Sahenk and Lasenk, 1988). The tubulo-vesicular profiles found in ZPT axonopathy (Sahenk and Mendell, 1979) were not observed in CL-treated mice, so it seems unlikely that the axonal degeneration seen in CL-toxicity is related to abnormalities in anterograde-retrograde conversion at the axon tip.

The effects of CL on axoplasmic transport could be ascertained using radioisotopic tracers, using techniques similar to those described by Pleasure et al (1969). The results from such experiments might help reveal, if, and at which point abnormalities in cellular metabolism and hence axoplasmic...
transport occur.

The rapid degeneration of distal nerve terminals, seen in CL-treated mice, indicates a direct toxic action on the motor nerve terminal. Why the motor nerve terminals in the distal muscles are affected prior to the motor terminals in the proximal muscles and why sensory fibres/terminals appear resistant to CL is uncertain and are topics worthy of future research.

The physiological findings reported here indicate that CL causes a gradual breakdown of the nerve terminal. Abnormalities in, and the eventual cessation of, evoked release are believed to occur prior to abnormally high mepp frequencies. These changes might indicate a gradual depolarization of the nerve terminal which would lead to an increase in the free Ca\(^{2+}\) within the nerve terminal due to Ca\(^{2+}\) entering the nerve terminal through voltage dependent channels or the release of Ca\(^{2+}\) from intracellular organelles such as the mitochondria and endoplasmic reticulum. Depolarization of the nerve terminal indicates a breakdown of the structures and mechanisms responsible for maintaining electrochemical gradients at the nerve terminal.

Abnormalities in the structure and function of the axolemma at the presynaptic terminal would lead to such a breakdown. A reduction in the synthesis of proteins which make up ion channels or ion pumps might conceivably lead to the depolarization of the nerve terminal. Protein synthesis occurs at the perikaryon (Brimijoin, 1984) and therefore the effects of the inhibition of protein synthesis are unlikely to be felt at the nerve terminal immediately. Proteins synthesised prior to the administration of CL would continue to be supplied to the nerve terminal by anterograde transport after the synthesis of proteins had ceased at the perikaryon. The rapid degeneration of distal motor nerve terminals is therefore unlikely to be directly attributable to CL compromising or preventing protein synthesis.

Changes in the phospholipid composition of the axolemma might also lead to the depolarization of the nerve terminal. A decrease in the levels of phosphatidylcholine (PC) has previously been described in the CNS of CL-treated mice (Nixon, 1976b). This decrease in the concentration of PC is thought to be caused by a deficiency in SAM (caused by CL see Fig. 1.2,)
which is required for the conversion of phosphatidylethanolamine (PE) to PC (Hirata and Axelrod, 1980). In animal cells, phospholipid synthesis is associated with the smooth endoplasmic reticulum (Darnell et al, 1990) as is the enzyme phosphatidylethanolamine-N-methyltransferase, which converts PE to PC (Zubay, 1984). Smooth endoplasmic reticulum is frequently found within the axoplasm (Landon, 1985), therefore it is possible that both phospholipid synthesis and the conversion of PE to PC occurs, at or, near the nerve terminal. Decreasing the levels of PC within a membrane causes an increase in the microviscosity of the membrane, i.e. a decrease in its fluidity (Baldessarini, 1987). Increasing the microviscosity of a membrane leads to a decrease in the lateral mobility of membrane proteins (Hirata and Axelrod, 1980) and hence to a loss of efficiency in receptor-effector coupling and other processes mediated by membrane proteins.

Membrane viscosity has previously been found to affect Ca\(^{2+}\) - ATPase activity (Hirata and Axelrod, 1980). Reducing membrane fluidity (increasing viscosity) decreases the Ca\(^{2+}\) - ATPase activity of sarcoplasmic reticulum (Hidalgo et al 1978) and SAM has been found to increase the active efflux of Ca\(^{2+}\) from erythrocyte ghosts (Hirata and Axelrod, 1980).

The SAM deficiency seen in CL-treated mice (Lee et al 1992) might lead to a decrease in the fluidity of membranes due to the inability of SAM deficient animals to convert PE to PC. A decrease in the fluidity of the membranes of presynaptic motor terminals (and the organelles within the terminals) might lead to an increase in the intracellular free Ca\(^{2+}\) within the terminal. This is due to a decrease in the efficiency of both the organelles such as the endoplasmic reticulum to sequest Ca\(^{2+}\) and the nerve terminals ability to export Ca\(^{2+}\) using an ATP dependent pump (Jones, 1987). The effects of increased free cytosolic Ca\(^{2+}\) on both evoked and spontaneous quantal release has been discussed in earlier sections. The hypothesis that CL causes an increase in free Ca\(^{2+}\) within distal neuromuscular junctions prior to degeneration could be tested using a combination of the \textit{in vivo} visualization techniques for mouse neuromuscular junctions (Lichtman et al, 1987) and calcium sensitive fluorescent dyes such as Flura 2 which signal
differences in the concentration of free Ca\(^{2+}\) within a neurone (Silver et al, 1990).

The axolemma of the neuromuscular junction is likely to be particularly vulnerable to changes in fluidity because the membranes of axon terminals are in a state of constant flux (Sahenk and Lasenk, 1988), with vesicular membrane being constantly added to and retrieved from the axolemma by exo- and endocytosis (Heuser and Reese, 1973). A decrease in the PC content of the axolemma in cholinergic nerve terminal may lead to deficiencies in ACh as it has been suggested that PC may be quickly degraded at cholinergic nerve terminals to provide choline for the synthesis of ACh (Blusztajn et al, 1982). Alternatively, the consumption of PC to provide choline for the synthesis of ACh might lead to further abnormalities in the membranes of cholinergic nerve terminals in CL-treated mice, in which the membranes are already PC deplete as a result of CL-induced SAM deficiency.

The differing sensitivities of proximal, distal, and intrafusal neuromuscular junctions might therefore be related to either differences in the phospholipid composition of the axolemma at these nerve terminal or alternatively to differences in the rate of phospholipid turnover. This again is an area worthy of future research which might reveal information relevant to the role of phospholipid derived second messenger systems (e.g. Arachidonic acid metabolism) in neuromuscular modulation, or the biochemical basis of the neurological damage seen in failures of the methyltransfer pathway.
CHAPTER 6

CONCLUSIONS

The experiments described within this thesis have shown that the ataxia and paralysis of the hind limbs seen in CL-treated animals is caused by the degeneration of the motor fibres innervating the muscles of the hind limbs.

The motor nerves innervating the distal muscles of the hind limbs were found to be particularly susceptible to CL, physiological abnormalities were found in the soleus and EDI of 21-day-old mice within 12 hours of the administration of CL. The degeneration of motor fibres gradually spread to more proximal regions, i.e. denervated biceps brachii fibres were found for the first time 7 days after the administration of CL.

The physiological observations indicate that CL induces a gradual loss of function at the nerve terminal, with the end-plate becoming unresponsive to neural stimulation prior to the cessation of spontaneous quantal release.

The pathogenetic mechanisms responsible for the degeneration of motor nerve terminals induced by CL remain unknown. The speed at which degeneration occurs (in the distal muscles) indicates that the primary lesion occurs at, or very close to, the neuromuscular junction rather than at the perikaryon or along the length of the axon. The fact that some regeneration occurs in the animals which survived beyond 5 days also indicates that metabolism at the perikaryon is unlikely to seriously impaired.

CL has previously been found to cause changes in the phospholipid composition of the nervous system, notably a decrease in phosphatidylcholine and increase in phosphatidylethanolamine levels. Such a change causes an increase in the microviscosity of membranes, which in turn decreases the efficiency of processes mediated by membrane proteins. A number of ion pumps and channels are responsible for maintaining the electrochemical gradients vital for the normal function of the nervous system. A reduction in the efficiency of these pumps/channels is suggested as a possible cause of the functional and morphological abnormalities and the eventual degeneration of the motor nerve terminals induced by CL. It
is also suggested that the differing sensitivity of sensory and motor fibres is related to differences in either the phospholipid composition or rate of phospholipid turnover at the terminals of these fibres.
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