INVOLVEMENT OF CALCIUM IN ORGANOPHOSPHORUS-INDUCED DELAYED NEUROPATHY: A FUNCTIONAL, MORPHOLOGICAL, AND BIOCHEMICAL STUDY

by

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(ABSTRACT)

Organophosphorus compounds are widely used in agriculture as pesticides and in industry as petroleum additives and modifiers of plastics. Some of these compounds are capable of inducing an irreversible neuropathy developing weeks to months after exposure, yet there is no effective treatment. This may be due in part to the lack of knowledge of how this neuropathy develops.

In this dissertation it is proposed that as a consequence of a triggering event, peripheral nerves may be predisposed to an increase in calcium (Ca^{++}) mobilization and the neuronal accumulation of this cation. This increase in Ca^{++} could thereby initiate a cascade of events, in both nerve and muscle, that may account for some of the detrimental changes occurring during organophosphorus-induced delayed neuropathy (OPIDN).

The involvement of Ca^{++} in the pathogenesis of OPIDN was tested using functional, morphological and biochemical techniques in the domestic hen, the recognized animal model of OPIDN.

The isolated biventer cervicis nerve-muscle preparation was developed for quick assessment of the time course of OPIDN deficits and validated by comparison to in vivo preparations. This preparation proved more sensitive by functional and morphological evaluation indicating early damage at 4 days following exposure and before appearance of clinical signs. Regeneration was detected after 21 days.

OPIDN was modified by using Ca^{++} channel blockers, nifedipine and verapamil, in the presence of phenyl saligenin phosphate, an active neurotoxicant. Attenuation of OPIDN by these compounds was revealed by clinical assessment, by changes in nerve excitability denoted by strength-duration relationships in response to electrical stimulation, by denervation hypersensitivity to neurotransmitter and by morphology. These modifiers attenuated all degenerative responses.

Furthermore, it was revealed that the activity of Ca^{++}-activated neutral protease (CANP), an enzyme responsible for neurofilament degradation, was increased in OPIDN. Such increases were ameliorated by modifiers of Ca^{++} movement.

This study strongly suggests that Ca^{++}, possibly through activation of CANP, may contribute to functional and morphological deficits of OPIDN.

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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this dissertation was performed by me.

Determinations of plasma, brain and liver esterases were performed by (Toxicology Laboratory). Plasma CPK was assayed by the staff of Clinical Pathology Laboratories, VA-MD Regional College of Veterinary Medicine Teaching Hospital. Assay method for CANP was developed by (Toxicology Laboratory), and modified by myself. Atomic absorption determination of total nerve calcium was performed by (Toxicology Laboratory). Nerve samples for histological examination were collected by myself and processed by , , and (Ultrastructure Laboratory).

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ABBREVIATIONS

ACh : acetylcholine

AChE: acetylcholinesterase

BuChE: pseudocholinesterase

Ca^{++}: ionic calcium

CaM : calmodulin

CANP: calcium-activated neutral protease

CNPase: 2′,3′-cyclic nucleotide 3′-phosphohydrolase

CPK : creatine phosphokinase

DFP : diisopropylfluorophosphate

EMG : electromyography

NF: neurofilament triplet

NTE : neuropathy target esterase

OPIDN: organophosphorus (ester)-induced delayed neuropathy

PMSF: phenyl methyl sulfonyl fluoride

PSP : phenyl saligenin phosphate

PTP : post-tetanic potentiation

SDC : strength-duration curve

SR : sarcoplasmic reticulum

TOTP: tri-ortho-tolyl phosphate

PART I

HYPOTHESIS

Certain organophosphorus compounds may produce a delayed neuropathy that does not appear in humans and some animal species until weeks following exposure (Davis and Richardson, 1980). The hypothesis to be tested suggests that, as a consequence of an event triggered by organophosphorus compounds (possibly the inhibition and irreversible alteration of an axonal membrane bound protein such as neuropathy target esterase), peripheral nerves may be predisposed to an increase in calcium (Ca^{++}) mobilization and the intraneuronal accumulation of this cation. This increase in intracellular Ca^{++} could thereby initiate a cascade of events, in both nerve and muscle, that may account for some of the detrimental changes occuring during organophosphorus-induced delayed neuropathy (OPIDN) (Figure 1).

A. In Nerve

Intracellular Ca^{++} overload has been generally implicated in cell degeneration and death (Farber, 1981). Within the axoplasm itself, the activation of Ca^{++}-dependent enzymes, such as proteases and kinases, could lead to degradation and phosphorylation of axonal cytoskeletal elements (e.g. the neurofilament triplet, microtubules, and mitochondria). Indeed, many of the pathological changes seen during "dying-back", Wallerian degeneration of nerves correlate experimentally with these Ca^{++}-initiated events. "Dying-back", Wallerian degeneration is the primary lesion in OPIDN (Cavanagh, 1964).

Secondary to axonal degeneration, myelin degeneration occurs in OPIDN. This event, too, may be Ca^{++}-dependent. For example, Ca^{++}-activated proteolytic enzyme has been demonstrated to mediate breakdown of myelin basic protein (Berlet, 1987). Furthermore, free Ca^{++} could instigate swelling and expansion of myelin multilayer, as it does in trauma-induced nerve injury (Schlaepfer, 1977), and thus contribute to OPIDN-induced myelin breakdown.

Another anatomical location at which Ca^{++} may act is at the synapse. There Ca^{++}, through its second messenger calmodulin (CaM), is known to enhance neurotransmitter release by inducing exocytosis and the release of acetylcholine (ACh) into the neuromuscular junction (DeLorenzo, 1982). This could contribute to changes seen in muscle during OPIDN, as described below.

B. In Muscle

Enhanced ACh release caused indirectly by Ca^{++} may contribute to muscle degeneration seen in OPIDN. Even if acetylcholine inhibition is not pronounced, some excess ACh would be available to increase activation of nicotinic receptors. Activation of these receptors leads to accumulation of free Ca^{++} in the sarcoplasm and muscle fibers contract (Cavero and Spedding, 1983). The initial manifestation may be muscle fasciculation, but OPIDN ultimately leads to denervation fibrillations (Vasilescu and Florescu, 1980).

The denervation fibrillations that occur in OPIDN are believed to be due to the de novo synthesis and incorporation of ACh-receptors into the sarcolemma. This causes the muscle to become hypersensitive to the neurotransmitter ACh (Fambrough, 1979). Asynchronous muscle activity, particularly in slow postural muscle, results. The synthesis and incorporation of ACh-receptors is significantly enhanced by Ca^{++} (Metafora et al., 1980).

High Ca^{++} and enhanced muscle activity results in depletion of phosphogens, such as ATP and phosphocreatine (Gupta and Dettbarn, 1987). The activation of Ca^{++}-dependent proteases instigates degeneration of muscle elements (e.g. Z-discs, troponin, tropomyosin and mitochondria) and the loss of sarcomere integrity loss of sarcomere integrity with the result that critical enzymes, such as creatine phosphokinase (CPK) are lost (Baker and Margolis, 1987). Leakage of CPK has been noted in OPIDN (Cisson and Wilson, 1983).

Experimental evidence indicates that use of Ca^{++}-channel blockers and/or reduction of free intracellular Ca^{++} may modify activity of proteolytic enzymes in both nerve and muscle, decrease release of neurotransmitters, inhibit de novo synthesis of receptors, and reduce leakage of CPK. Therefore, it was proposed that the research for the dissertation would examine the effect of Ca^{++}-channel blockers on OPIDN. Effects were examined on nerve and muscle activity in the whole animal, using the tibial nerve-gastrocnemius

muscle preparation, and in the isolated biventer cervicis nerve-muscle preparation. In addition, the influence of Ca^{++}-channel blockers on nerve and muscle enzyme activities and CPK depletion were assessed, as was changes in peripheral nerve calcium concentrations.

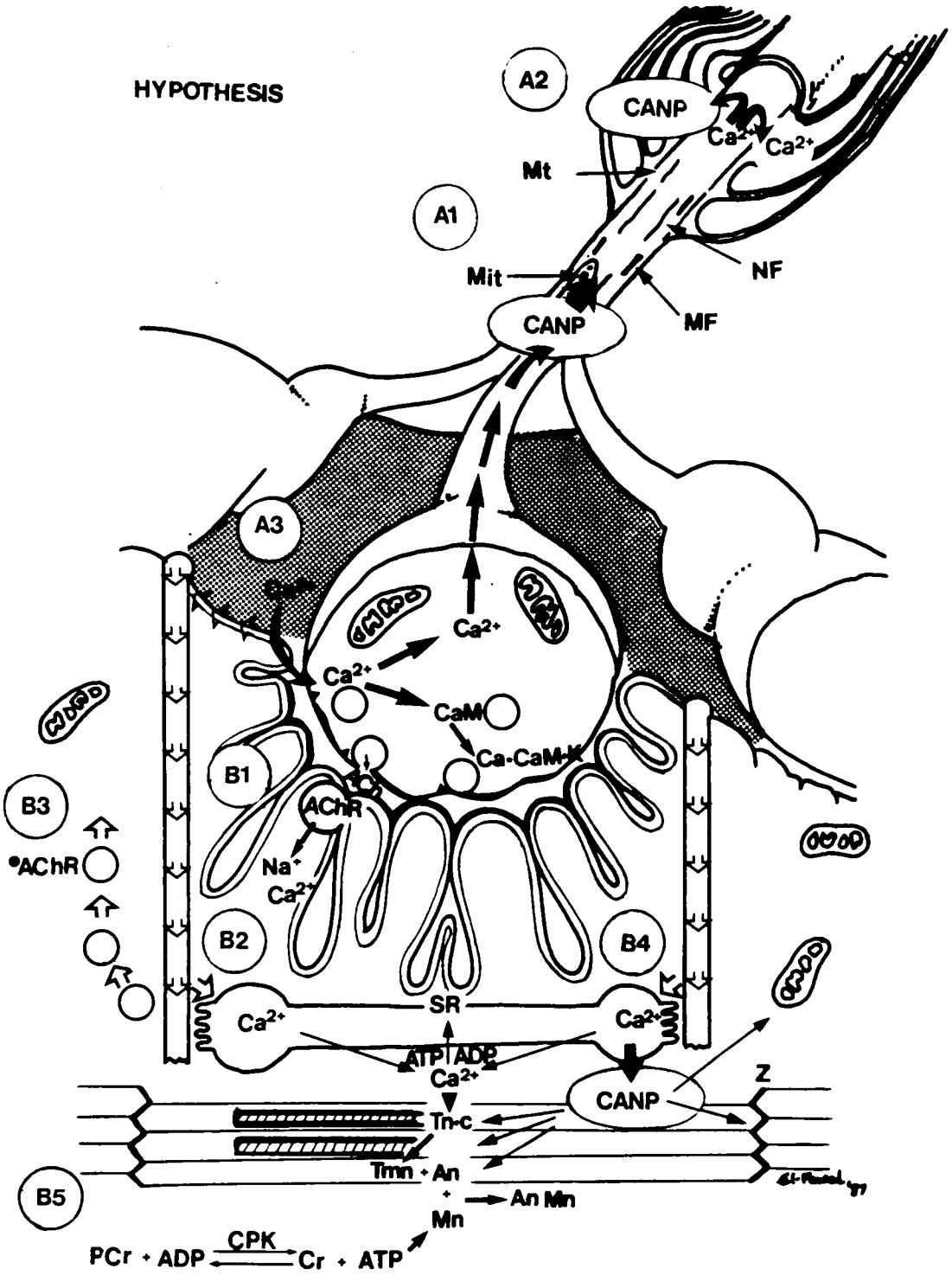

HYPOTHESIS
A2
CANP
Ca2+
Ca2+
Mt
A1
Mit
NF
CANP
MF
A3
Ca2+
Ca2+
CaM
Ca-CaM-K
B1
AChR
B3
•AChR
Na+
Ca2+
B2
B4
Ca2+
SR
Ca2+
ATP ADP
Ca2+
CANP
Z
Tn-c
Tmn + An
+
An Mn
Mn
B5
PCr + ADP
CPK
Cr + ATP

Figure 1. Schematic depicting the possible consequences of increases in intracellular Ca^{++} levels in OPIDN.

A: Events in Nerve

A1. Ca^{++}-mediated events in the axoplasm: activation of CANP and the subsequent proteolysis of cytoskeletal elements: neurofilaments (NF), microtubules (Mt), microfilaments (Mf), and mitochondria (Mit).

A2. Ca^{++}-mediated events in myelin: activation of myelin CANP, and Ca^{++}-mediated expansion of the myelin multilayer.

A3. Ca^{++}-mediated events at the nerve terminal: binding of Ca^{++} with CaM and the activation of Ca^{++}-CaM kinase to induce release of ACh at the neuromuscular junction.

B: Events in Muscle

B1 & B2. Ca^{++} influx and release in muscle: in response to ACh release and activation of nicotinic receptors (AChR), the muscle is depolarized and Ca^{++} is released from the sarcoplasmic reticulum (SR).

B3. Ca^{++}-mediated denervation hypersensitivity: by enhancement of AChR synthesis and their incorporation in the sarcolemma.

B4. Ca^{++}-mediated muscle degeneration: by activation of muscle CANP and the subsequent proteolysis of muscle structural elements: Z-discs (Z), troponin-C (Tn-c), actin (An), tropomyosin (Tmn), and mitochondria.

B5. Ca^{++}-mediated depletion of phosphogens and CPK: by enhancement of muscle contraction and attempts at decreasing sarcoplasmic Ca^{++} levels via the Ca^{++}-ATPase pump, ATP is consumed, phosphocreatine (PCr) is not regenerated from creatine (Cr). Muscle CPK levels decrease due to leakage from damaged muscle.

PART II

LITERATURE REVIEW

CHAPTER 1

BACKGROUND ON ORGANOPHOSPHORUS-INDUCED DELAYED NEUROPATHY (OPIDN)

Organophosphorus compounds (OPs) are widely used in agriculture as pesticides and in industry as petroleum additives and modifiers of plastics. Antidotes for acute poisoning by these compounds are available, yet no successful treatment exists for the irreversible neuropathy developing weeks to months after exposure to some OPs. This may be due in part to the lack of knowledge of how the neuropathy develops (Davis and Richardson, 1980).

1.1. History

The earliest record of neuropathy due to organophosphorus compound (OP) exposure was recognized by Lorot and occurred in 1899, more than 50 years before the insecticidal properties of other OPs were discovered (Metcalf, 1984; Cherniack, 1986). This was the result of using phosphocreosate (an undefined mixture of phosphoric acid esters) to treat tuberculosis (Davis and Richardson, 1980). It was an incident which occurred during the 1930's Prohibition Era, however, that attracted attention to the possible neurotoxic potential of these compounds. A massive outbreak of paralysis occurred in the United States due to the consumption of an alcohol substitute, Jamaica Ginger or Ginger Jake, adulterated with tri-ortho tolyl phosphate (TOTP) (Goldstein et al., 1988). The resulting neurological syndrome, Ginger Paralysis or Jake Leg, in reference to the "toe-drop" and hind limb involvement that characterized this syndrome, afflicted an estimated 4,000 to 20,000 persons (Davis and

Richardson, 1980; Cherniack, 1986).

a) Incidence

(1) Human Exposure: In addition to the incidences cited above, several outbreaks of neuropathy occurred during the 1930s and 1940s. Apiol, an abortifacient extracted from parsley seeds and containing TOTP, was responsible for 60 cases of paralysis in Europe (Beck et al., 1977). The use of cooking oil stored in drums contaminated with lubricating oil resulted in 60 cases of neuropathy in South Africa in 1937, and machine gun-contaminated cooking oil resulted in 80 cases of neuropathological disorders among a Swiss army unit in 1940 (Goldstein et al., 1988).

The second largest incident involving human exposure occurred in Morocco in 1959 and involved 10,000 persons who developed neuropathy following the use of imported cooking oil contaminated with jet-airplane lubricating oil (El-Sebae et al., 1977; Beck et al., 1977). In 1951, workers in British factories that produced the organophosphate pesticide mipafox developed paralysis typical of delayed neuropathy. In that same year workers in Texas factories manufacturing the experimental insecticide leptophos also developed delayed neuropathy (Abou-Donia, 1981). Other outbreaks of neuropathy have been ascribed to contaminated food and beverages. For example, contaminated flour in Fiji and Calcutta caused paralysis of several hundred people. Several other outbreaks, in Sri Lanka, Bombay and Durban, to name a

few, have occurred through the years. Most recently, workers employed in the production of synthetic shoes in Spain and Italy have developed neuropathy due to the exposure to TOTP, the plasticizer ingredient (Cavalleri and Cosi, 1980).

(2) Animal Exposure: The occurrence and symptoms of neuropathy in humans have been paralleled by those in animals, though not in magnitude. In 1971 an outbreak of paralysis occurred in Egypt that resulted in the death of 1300 water buffalo after exposure to the OP insecticide leptophos (El-Sebae et al., 1977). A herd of cattle in British Columbia, Canada, was poisoned in 1975 by accidental spillage of TOTP intended for use in a compressor station (Julian et al., 1975; Beck et al., 1977). Also, several cases of delayed neuropathy were noted to appear among a flock of Suffolk sheep which had been exposed to transmission oil (Sanders et al., 1985). Case reports of delayed neuropathy have periodically appeared in the literature and all such cases have been attributed to exposures to OP (mostly TOTP)-containing products (Perdrizet et al., 1985; Sanders et al., 1985).

1.2.Chemistry and Metabolism of Organophosphate-Inducing Delayed Neuropathy.

The organophosphorus compounds with potential for producing delayed neurotoxicity are esters belonging to several chemical classes. They may be aliphatic phosphorus esters

(e.g. phosphonates, phosphorofluoridates, etc.), aliphatic aromatic esters, triaryl phosphate esters, and the saligenin cyclic phosphorus esters. The pyrophosphorus esters are not believed to be neurotoxic (Abou-Donia, 1981). For the purpose of the study, only the structure of the tri-aryl phosphate, TOTP, and its cyclic saligenin phosphate metabolite will be discussed.

Tri-ortho-tolyl (or cresyl) phosphate is metabolized by hydroxylation and cyclization of the hydroxymethyl derivatives (Eto et al., 1962). The initial side-chain oxidation is catalyzed by P-450 (Hansen, 1984) and is illustrated in Figure 1. The cyclic phosphate metabolite intermediate, 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphororan-2-one (or CBDP) is neurotoxic (Taylor, 1967). However, the primary neurotoxic metabolite is the cyclic o-tolyl saligenin phosphate (Baron et al., 1962). This saligenin cyclic-o-tolyl phosphate is believed to be at least five times as neurotoxic as TOTP after oral administration to chickens (Abou-Donia, 1984).

In the course of studying the structure-activity relationships, Aldridge et al., (1962) successfully predicted the neurotoxicity of a new series of compounds. This study resulted in the synthesis of phenyl saligenin phosphate (Figure, 2), which is considered the prototype of the highly active cyclic saligenin metabolites of TOTP (Eto et al., 1962). This compound was used extensively in the present study.

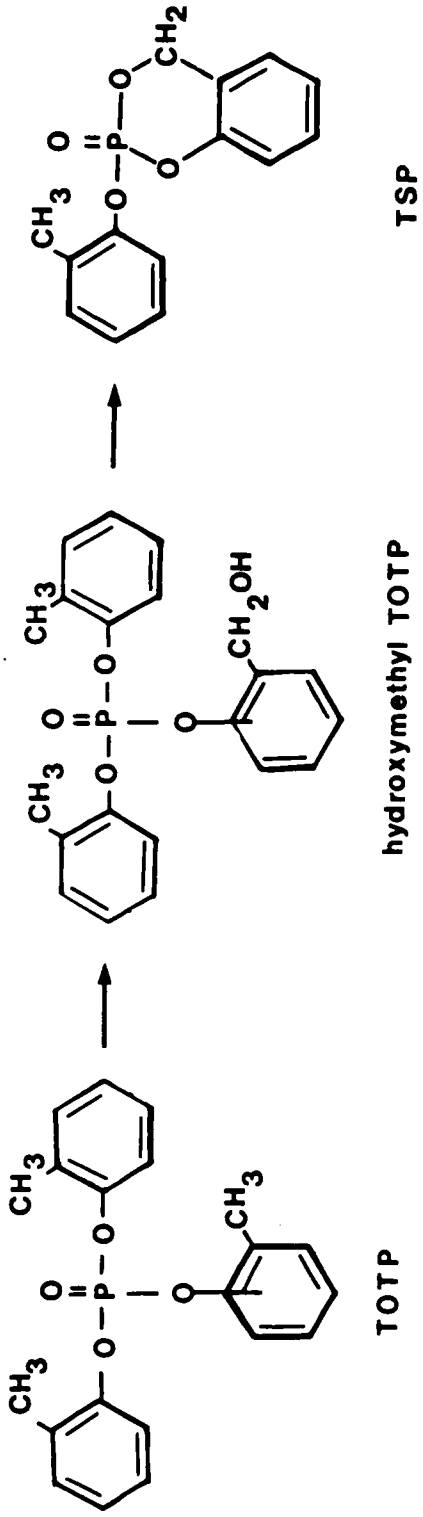
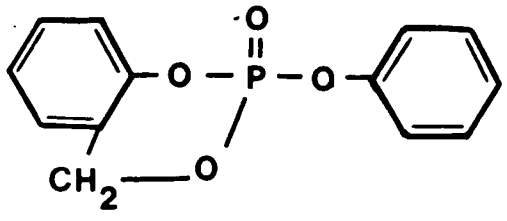

TOTP
hydroxymethyl TOTP
TSP

Figure 1. Hydroxylation and cyclization of tri-ortho-tolyl phosphate (TOTP) to its metabolite o-tolyl saligenin phosphate (TSP) (redrawn from Hansen, 1984).



Phenyl saligenin phosphate (PSP)

Figure 2. Phenyl saligenin phosphate (PSP), a congener of TOTP-toxic metabolites (redrawn from Aldridge et al., 1969).

1.3. Clinical Signs of OPIDN

Clinical signs of OPIDN differ from clinical signs associated with the acute inhibition of neural acetylcholinesterase (AChE) activity, which is also associated with this class of chemical agents. Only some OPs with potential for inducing delayed neuropathy are also inhibitors of acetylcholinesterase (AChE) (Abou-Donia, 1981), and in many cases this inhibition, when it does occur, is not significant. Initially, however, and especially in humans, any OP exposure may cause some gastrointestinal distress with nausea, vomiting and diarrhea, symptoms which may be due to AChE inhibition (Davis and Richardson, 1980). For development of neuropathy, however, there is a latent (delay) period of 6 to 21 days from time of exposure until onset of signs (Davis and Richardson, 1980; Abou-Donia, 1981; Stuart and Oehme, 1982). The route of administration and amount of exposure may alter both the onset and severity of clinical signs (Francis, 1983). The initial sign that appears following the delay period between exposure and appearance of OPIDN is a flaccid paralysis developing distally in the hind limbs. In severe cases the forelimbs are affected as well (Johnson, 1975; Davis and Richardson, 1980; Abou-Donia, 1981). Spasticity and ataxia (possibly due to spinal cord injury [Abou-Donia, 1981]) occur and are accompanied by a characteristic "toe drop" or high stepping gait (Johnson, 1975; Stuart and Oehme, 1982; Metcalf, 1984).

Sensitivity to OPIDN appears to be species as well as age dependent, since not all animals are susceptible to OPIDN and young animals are generally resistant. Among those sensitive to OPIDN are humans and some avian species. The adult chicken (>50 days), for example, is considered among the most sensitive of species and is generally recognized as providing the most reliable animal model for this chemically induced disease (Sprague et al., 1980; Abou-Donia, 1981; Bickford, 1984). In fact, the adult chicken is used as the test species for OPIDN by the United States Environmental Protection Agency (EPA) (Ananymous, 1985). Laboratory rodents have also been considered for studies of OPIDN (Francis et al., 1983). Even though the Long Evans hooded rat is considered to be sensitive to morphological damage after administration of neuropathy-inducing OPs, it is resistant to clinical manifestations of ataxia (Veronesi, 1984). The Fischer 344 rat was reported to be insensitive to both clinical and morphological changes following repeated administration of neuropathy-inducing OPs (Somkuti et al., 1988).

Clinical signs of OPIDN can be graded using one of several systems, such as the one described by Sprague and coworkers for use in the chicken (1980). This system uses blind assessment to score signs as 0 for normal, 1 for altered gait, 2 for difficulty in walking and standing, 3 for moderate to severe ataxia, 4 for paralysis of one or both legs and 5 for paralysis of both legs and wings. Other

systems use grades for individual endpoints, such as locomotion, posture, equilibrium, and walking strength (Sprague et al., 1980), and yet others use a grading scale from 0-8 and include criteria such as wing droop, tail and leg reflexes, and stumbling (Roberts et al., 1984). Cavanagh (1964), utilized a system consisting of "+" and "-", with ranges from indefinite change (±) to gross weakness in hind limbs (+++).

1.4. Biochemistry of OPIDN

The activities of several enzymes have been examined for their correlation with OPIDN, including neuropathy target esterase, acetylcholinesterase, pseudocholinesterase, creatine phosphokinase, and 2',3'-cyclic nucleotide 3'-phosphohydrolase. In addition, OPIDN has been proposed to involve protein phosphorylation.

a) Esterases

(1) Neuropathy Target Esterase (NTE): NTE, also known as neurotoxic esterase, has been proposed as the initial molecular site of action of neurotoxic OPs (Abou-Donia, 1981; 1985; Richardson, 1984; Davis et al., 1985), yet this membrane bound enzyme (receptor) can also be inhibited by other compounds (e.g., carbamates, paraoxon) which did not induce OPIDN (Richardson, 1984; Davis et al., 1985). In fact, non-OPIDN-inducing compounds offered protection from OPIDN, if they were administered prior to a neurotoxic OP (Abou-Donia, 1981; Richardson, 1984; Davis et al., 1985). This paradoxical observation was resolved by the discovery

that the enzyme-receptor (NTE) must undergo a process of "aging" (Richardson, 1984; Davis et al., 1985; Johnson, 1987), during which the OP becomes covalently and irreversibly bound to the enzyme. Binding of non-OPIDN-inducing compounds is reversible.

The interaction of NTE with OPs has been extensively studied by Johnson (1975, 1980, 1987). He suggested that the enzyme (receptor) exists in three forms, as expressed by the following equation:

$$\text{(T-NTE)} = \text{(CA-NTE)} + \text{(UI-NTE)} + \text{(MI-NTE)}$$

where T-NTE is the total NTE, CA-NTE is the catalytically active form of the enzyme, UI-NTE is the unmodified enzyme that has been inhibited by the OP, and MI-NTE is the modified form of the enzyme that has been inhibited by the OP. It is the capability to irreversibly phosphorylate the MI-NTE (the process called "aging") that identifies compounds which are capable of inducing OPIDN. Compounds like carbamates or sulfonates, although interacting with NTE, do not modify the enzyme irreversibly, or else the enzyme-compound product is unstable and decomposes to yield free enzyme (Johnson, 1980; Richardson, 1984; Davis et al., 1985). Johnson, who elucidated this process and confirmed its relevance to OPIDN, proposed that nerve degeneration results from an upset of normal physiological control process(es) by MI-NTE (Johnson, 1980). On this basis, assays of NTE activity have been suggested to serve as biomonitors of OP-toxicity (Lotti, 1987), and a 70-80% reduction of NTE

activity considered predictive of OPIDN (Davis et al., 1985).

Predictive ability of NTE inhibition has not received universal acceptance, since although NTE activity is initially inhibited, this inhibition is not long-lasting, and enzyme activity may be comparable to those in controls 7 or more days prior to onset of ataxia (Abou-Donia, 1981; Davis et al., 1985). A physiological role for NTE has yet to be defined and, furthermore, NTE activity can be measured in extraneural tissues (Lotti, 1987; Richardson, 1984). Despite this, monitoring activity of NTE 1-48 hours following OP exposure has proven to be a strong indicator that OPIDN will occur (Davis et al., 1985; Zech and Chemnitius, 1987). Recently, the inhibition and "aging" of NTE has been correlated with a 70% reduction of retrograde axonal transport (Moretto et al., 1987). This transport system is thought to play a role in recycling endogenous materials for degradation by perikaryal enzymes, as well as being part of a communication network within the axon (e.g. signal of distal nerve injury and need for regeneration [Price and Griffin, 1980]).

(2) Pseudocholinesterase (BuChE) and Acetylcholinesterase (AChE): OPs which induce delayed neuropathy may also inhibit cholinesterases (BuChE, AChE) as well as carboxylesterases other than NTE (Abou-Donia, 1981; Ehrich and Gross, 1982). All esterases, but especially carboxylesterases, are

key enzymes in OP detoxification (Cohen and Ehrich, 1976). Esterase inhibition was initially proposed as the primary step in OPIDN (Abou-Donia, 1981; 1985). Although BuChE, AChE and carboxylesterases may be inhibited early after exposure to some neuropathy-inducing OPs, the correlation is tenuous at best. These enzymes are more likely to serve as temporary storage depots for OPs (Abou-Donia, 1981).

b) Protein Phosphorylation

Recent studies by Abou-Donia's group at Duke University suggest a role for calcium (Ca^{++}) and calmodulin (CaM)-enhanced protein phosphorylation during the course of OPIDN (Abou-Donia et al., 1984; Patton et al., 1985; 1986; Suwita et al., 1986a; 1986b). Administration of tri-ortho-tolyl phosphate (TOTP) was demonstrated to increase the phosphorylation of tubulin (α and β), the neurofilament triplet (Suwita et al., 1986a), and microtubule-associated protein-2 (MAP-2) (Suwita et al., 1986b). All of the proteins participate in slow anterograde axonal transport. Although other OPs can also phosphorylate proteins, the enhanced phosphorylation of tubulin was suggested as being specific for OPs which cause OPIDN (Abou-Donia et al., 1984). The above studies were conducted in avian brain and spinal cord, and catalyzed by calcium-calmodulin dependent kinase.

c) Creatine Phosphokinase (CPK)

As a consequence of OPIDN, a chemical denervation occurs, which results in muscle necrosis and atrophy (Cisson and Wilson, 1982). This was shown to be accompanied by an increase in extrajunctional AChE and BuChE even though AChE was reduced by 43% at the endplate itself (Cisson and Wilson, 1982). These investigators also reported a fourfold increase in plasma CPK activity and a decrease in muscle CPK (Cisson and Wilson, 1982; 1983; Wilson et al., 1984). This change in CPK activity, the enzyme responsible for ATP generation, was analogous to that observed both in dystrophic muscle and as a consequence of muscle damage resulting from physical denervation (Linkhart and Wilson, 1975).

d) 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase (CNPase)

This enzyme, also known as 2', 3'-cyclic nucleotide phosphodiesterase, is an unequivocal component of myelin, and has been widely used as a marker for the presence of myelin in the brain (Sims and Carnegie, 1978). A physiological role for this enzyme has not, however, been defined (Olajos, 1987). Studies of experimentally-induced Wallerian degeneration in rat and hen sciatic nerve following nerve transection indicated a significant decrease in CNPase 7, 14 and 21 days later (Mezei et al., 1974). Significant decreases in CNPase have also been reported in brain, spinal cord and sciatic nerve during the course of OPIDN in white leghorn hens and in rats (Olajos et al., 1982; Schlager et

al., 1982; Mansour et al., 1986). In contrast, another OP that causes neurotoxicity without typical OPIDN (S,S,S-tri-n-butyl phosphorotrithioate, DEF), increased CNPase activity in hen brain (Abou-Donia et al., 1986b). Assay of CNPase has been suggested to be an indicator of effects on myelin during OP-induced Wallerian degeneration (Olajos, 1987).

1.5. Electrophysiology and Pharmacology of OPIDN

a) Neurophysiology

Neurophysiological parameters of OPIDN have been studied in cats and chickens. For example Lowndes et al., (1974) and Baker et al., (1977) found that intra-arterial injection of one OP, diisopropylfluorophosphate (DFP), in cats produced a localized neuropathy without affecting nerve conduction velocities. DFP did, however, reduce indirectly evoked contractile strength in soleus muscle, and reduced neurogenic post-tetanic potentiation (PTP). Similar observations were seen by Lapadula et al. (1982) in spinal cord reflexes of cats treated with TOTP. Furthermore, these investigators noted a significant decrease in dorsal and ventral root compound action potentials.

Durham and Ecobichon (1984), using the adult chicken as their test animal, also reported a reduction in PTP as ataxia followed TOTP administration, but this reduction did not correlate with the severity of the ataxia. Studies in another laboratory noted that action potentials of sciatic and tibial nerves from chickens were altered, onset of con-

duction velocity in the sciatic nerve was reduced, and the action potential in the tibial nerves was prolonged after administration of TOTP (Robertson et al., 1987). In addition, these investigators reported that the sciatic nerve became more refractory, while the tibial nerve became less refractory; that is, the ability to carry high frequency impulses was impaired in the sciatic and enhanced in the tibial nerve. Both nerves in TOTP-treated chickens exhibited an increase in threshold excitability, as indicated by the upward shift of strength-duration curves (Robertson et al., 1987). This latter observation has also been recently confirmed for these two nerves following DFP-administration (Anderson et al., 1988). The decrease in excitability thresholds, as indicated by strength-duration curves, appeared to be detectable as early as 24 hours following OP-treatment, but improved 7-14 days later (Robertson et al., 1988).

The above results appear to closely parallel studies conducted in humans with OPIDN (Vasilescu and Florescu, 1980; Vasilescu et al., 1984). In these two studies, both motor conductance velocity (MCV) and sensory conductance velocity (SCV) did not deviate from normal. However, there was a decrease in the amplitude of evoked muscle action potentials (EMAP) and of sensory evoked potentials (SEP). Nerve excitability thresholds were increased and PTP was decreased.

b) Electromyography (EMG)

EMG is used clinically to monitor progressive deficits and/or recovery of muscle function following nerve trauma or muscle damage. Screening of human subjects working with OPs indicated a reduction in EMG voltage (Roberts, 1977), a marked distal denervation of lower limbs (Vasilescu et al., 1984) and muscular fibrillations at rest (Vasilescu and Florescu, 1980). In cats exposed to a single arterial injection of DFP, muscle spindle function was compromised, discharge frequencies in response to various stretch lengths were depressed and thresholds were slightly elevated (Baker and Lowndes, 1980). Also in the cat, following treatment with TOTP, there was abnormal spontaneous activity, identified as fibrillation and presence of positive waves. This activity is a manifestation of denervation (Abou-Donia et al., 1986a).

In the single EMG study conducted in the usual animal model of OPIDN, the chicken, needle EMG did not demonstrate any distinguishable differences between control and OP-treated birds based on spontaneous insertional activities or on denervation potentials (Shell et al., 1988).

1.6. Morphology of OPIDN

a) Nerve

The essential lesion in OPIDN is a "dying back" process or Wallerian-like degeneration of the distal region of the longer, larger nerve fibers, particularly in the hindlimbs

(Cavanagh, 1964; 1985; Bouldin and Cavanagh, 1979; Krinke et al., 1979; Itoh et al., 1984; 1985; Jortner, 1984a; 1984b), and the axons of ascending and descending fiber tracts of the spinal cord (Davis and Richardson, 1980; Stuart and Oehme, 1982; Jortner, 1984a). The primary insult apparently occurs in the axon, preferentially in the distal regions, followed by fiber breakdown below this site (Jortner, 1984a), which leaves the proximal portion of the nerve intact (Bouldin and Cavanagh, 1979; Davis and Richardson, 1980; Jortner, 1984a; Cavanagh, 1985). Peripheral nerve lesions detected by light microscopy are seen to begin about the time of onset of clinical signs. This is depicted as swelling and associated degeneration of the myelin sheath (Jortner 1984a; 1984b), with progression to breakdown of the affected fiber (Davis and Richardson, 1980; Jortner, 1984a; 1984b; Jortner and Ehrich, 1987). In later stages there is complete breakdown and phagocytosis of the nerve fiber (Jortner, 1984a; 1984b) associated with Schwann cell and macrophage proliferation (Stuart and Oehme, 1982; Jortner, 1984a; Jortner and Ehrich, 1987).

Ultrastructurally, focal intra-axonal aggregates of mitochondria and dense bodies are apparent (Jortner, 1984a). Proliferation of axonal agranular endoplasmic reticulum (SER) (Davis and Richardson, 1980; Abou-Donia, 1981), or SER-like tubules are sometimes present (Jortner, 1984a; 1984b; Jortner and Ehrich, 1987). An advanced lesion noted was the breakdown of axonal cytoskeletal elements (Jortner

1984b), with aggregation and accumulation of neurofilaments and neurotubules (Abou-Donia, 1981; Stuart and Oehme, 1982). Later stages showed accumulation of fine granular debris and degenerating mitochondria as well as dense bodies (Jortner, 1984a). Subsequent degeneration of the nerve fiber is known to lead to the presence of axonal and myelin debris undergoing phagocytosis by Schwann cells (Jortner, 1984a; 1984b), and the formation of digestive chambers, with phagocytes containing cytoplasmic myelin and axonal debris (Jortner and Ehrich, 1987).

b) Regeneration of Nerve Fibers

Improvement in clinical signs of OPIDN after a period of time has been reported, suggesting the possibility of peripheral nerve regeneration (Cavanagh, 1964; Glazer et al., 1978; Abou-Donia et al., 1983). Glazer and co-workers (1978) demonstrated sprouting and reinnervation of the neuromuscular junction 1-2 weeks following DFP administration to cats. In Long Evans hooded rats, regeneration initially appeared to accompany degeneration, but since multiple doses were required to induce morphological changes in this specie, frequency of regeneration was diminished with continued dosing (Veronesi, 1984). In the adult hen, the animal of choice for the study of OPIDN, regeneration accompanied by incomplete clinical recovery has been reported at 120 days following exposure to TOTP (Sprague et al., 1984). Durham and Echobichon (1986) reported signs of regeneration 6 weeks

after exposure to the same agent. A more complete description of regeneration in adult chickens with OPIDN has been recently provided by Jortner et al., (1989). This study followed the course of degeneration and regeneration over a 64 day period after administration of TOTP and phenyl saligenin phosphate (PSP). Peripheral nerve regeneration was evident as early as 16 days following exposure, as indicated by axonal sprouts within the basal lamina border of bands of Bungner. Regeneration was well established on days 23-28, and by day 64 nerves were qualitatively indistinguishable from control nerves by light microscopy. Although regeneration is accompanied by significant improvement in clinical signs of OPIDN in both chickens and cats, recovery from ataxia is incomplete (Cavanagh, 1964; Jortner et al., 1989).

c) The Neuromuscular Junction and Muscle

Neuropathy associated with OP exposure can also lead to changes at the neuromuscular junction and in muscle (Prineas, 1969). For example, muscle of hens with OPIDN showed abnormal morphology and a significant reduction in gastrocnemius muscle fiber diameter. This was paralleled by a proportional loss in muscle weight (Cisson and Wilson, 1982). In addition, scattered loss of muscle fibers and their replacement with connective tissue and fat have been reported due to TOTP exposure in humans (Abou-Donia, 1981). Ultrastructural examination of muscle tissue from both humans and animals with OPIDN demonstrated that osmiophilic

bodies containing degenerating mitochondria, synaptic vesicles, and small electron dense granules were present in the neuromuscular junction (Abou-Donia, 1981).

1.7. Experimental Modification of OPIDN

Attempts to modify the clinical outcome of OPIDN first concentrated efforts on the use of compounds that inhibited NTE without "aging" (see Section 1.3:a:1). These compounds, such as phenyl methyl sulfonyl fluoride (PMSF), when administered 24 hours before a neuropathy-inducing OP, prevented the development of OPIDN (Carrington and Abou-Donia, 1983; Caroldi et al., 1984; Veronesi and Padilla, 1985). This was indicated by absence of developing clinical signs and the lack of changes in electrophysiological parameters (Baker et al., 1980; Drakontides and Baker, 1983). Such compounds, however, are themselves intrinsically toxic and have not been considered to be of possible therapeutic benefit. PMSF, for example, is a serine protease inhibitor, capable of inhibiting most proteases (Carrington and Abou-Donia, 1983; Drakontides and Baker, 1983).

Experiments performed here in the Toxicology Laboratory, as well as at Cornell University Medical College, have demonstrated a capability of adrenocorticosteroids to modify OPIDN in chickens (Ehrich and Gross, 1982; 1983; 1986; Ehrich et al., 1985; 1986a; 1986b; 1988) and in cats (Baker et al., 1982; Drakontides et al., 1982; Baker and Stanec, 1985). These studies fall within two categories:

a) Electrophysiological and Morphological

b) Clinical and Biochemical

a) Electrophysiological Studies

Treatment of cats with an i.v. injection of methylprednisolone following intra-arterial injection of DFP and seven doses of triamcinolone (once every three days) prevented the appearance of clinical signs for up to 70 days after administration of the OP. The incidence of stimulus-evoked repetitive discharge (i.e. tetanic stimulation) was normal in cats treated with DFP and glucocorticoid, but not in cats treated with DFP alone (Baker et al., 1982; Baker and Stanec, 1985). Electron microscopy showed the ultrastructure of the neuromuscular junction to be normal in cats given DFP and glucocorticoid (Baker et al., 1982).

In another study, chickens treated with TOTP or phenyl saligenin phosphate (PSP) were found to exhibit abnormal action potentials, whether or not they had been treated with an OP-corticosterone combination. However, action potentials in hens given the combination were comparable to controls (Lidsky et al., 1988).

b) Clinical and Biochemical Studies

In studies performed in chickens, treatments that ameliorated clinical signs of OPIDN, such as low social stress environments and low dietary concentrations of adrenocorticoids, protected from ataxia without altering OP-induced NTE

inhibition (Ehrich and Gross, 1982; 1983; Ehrich et al., 1985; 1986a; 1986b; 1988). However, a low stress environment apparently may have provided some biochemical protection, as chickens tested under these conditions had the lowest microsomal enzyme activity, (possibly reducing activation of protoxicant TOTP) (Ehrich and Gross, 1983). The low-dose (30-50 ppm) clinical benefit of corticosterone was evident after TOTP but not after DFP was administered to chickens (Ehrich et al., 1985; 1986a). High doses of this steroid (>200 ppm) exacerbated clinical signs of neuropathy after administration of TOTP, DFP and PSP (Ehrich et al., 1986a). Because corticosterone has both gluco-and mineralocorticoid properties (Haynes and Murad, 1985), a study using a compound that is exclusively glucocorticoid (triamcinolone) and exclusively mineralocorticoid (deoxycorticosterone) was conducted. Results indicated that low doses of both could partially alleviate ataxia induced by TOTP, DFP and PSP, but high concentrations of the glucocorticoid exacerbated clinical signs (Ehrich et al., 1988). Modulation of OPIDN appeared more dependent on glucocorticoid than on mineralocorticoid properties of the steroids.

The above studies indicate, therefore, that benefits of corticoids may be dependent on the specific corticoid, the dose of the corticoid, the particular OP examined, and the specie of animal to which these compounds are administered.

CHAPTER 2

THE ROLE OF Ca^{++} IN NERVE AND MUSCLE FUNCTION AND THE RATIONALE FOR ITS IMPLICATION IN OPIDN

The hypothesis to be tested suggests that organophosphorus compounds that cause neuropathy predispose peripheral nerves to intracellular increases in Ca^{++} mobilization and that this may precipitate detrimental events characteristic of delayed neuropathy. Ca^{++} is implicated because it contributes to neurotransmitter release, degradation of neuronal cytoskeletal structures, myelin integrity, excitation-contraction coupling, and degradation of muscle. All of these features are altered as an animal develops OPIDN.

2.1. The Role of Ca^{++} and Calmodulin (CaM) in Nerve Function

a) Ca^{++} and Neurotransmitter Release

The work of Katz and Miledi (cited in Kandel, 1985a) established that Ca^{++} influx is essential for exocytosis and neurotransmitter release. More recently it has been demonstrated that the heat stable Ca^{++}-binding protein CaM also plays a role in exocytosis and the release of neurotransmitters (DeLorenzo, 1982; Cavero and Spedding, 1983; Miller, 1987). CaM in the nerve terminal is found intimately associated neurotransmitter-containing synaptic vesicles, as well as in the synaptic cytosol. Inhibition of CaM, both *in vivo* and *in vitro*, caused a significant reduction in neurotransmitter release, despite the presence of Ca^{++} (DeLorenzo, 1982).

CaM also mediates protein phosphorylation, which is necessary for neurotransmitter release. For example, inhibition of Ca^{++}-CaM kinase, the phosphorylating enzyme, with

phenytoin or diazepam inhibited neurotransmitter release. The effect was the same as when CaM was directly inhibited by trifluoperazine (DeLorenzo, 1982). Ca^{++} and CaM are both likely to be intimately associated in facilitating transmitter release since fusion of the synaptic vesicle with the synaptic membrane during exocytosis was markedly enhanced in the presence of both Ca^{++} and CaM. Inhibition of both Ca^{++}-CaM kinase protein phosphorylation and direct inhibition of CaM abolished the interaction of synaptic vesicles and membrane (DeLorenzo, 1982).

In addition to Ca^{++} and CaM effects noted above, Ca^{++} is also implicated in neurotransmitter release because depolarization-induced Ca^{++} uptake and neurotransmitter release from synaptasomal fractions and cultured neuronal cells was inhibited by Ca^{++}-channel blockers, such as verapamil, nifedipine and nitrendipine (Godfraind et al., 1986; Miller, 1987). Subsequent studies using a radioligand demonstrated that specific netrendipine and nifedipine (dihydropyridines) receptors were functional Ca^{++}-channels (Miller, 1987).

An enhancement of neurotransmitter release mediated by an increase in intra-synaptic Ca^{++} may initiate some of the detrimental events observed in muscle during OPIDN (see below, sections 2.2 and 2.3a).

b) <u>Ca^{++}, CaM and Neuronal Structural Proteins</u>

Neurofilaments (NF), along with other cytoskeletal proteins (tubulin and actin), play an important function in

regulating the caliber and configuration of neurons, particularly the larger neurons with long axonal processes (Schlaepfer, 1987). NF are thus found in abundance in large myelinated fibers, where they stabilize the axonal cytoskeleton and determine axonal size. An advanced lesion, noted following axotomy and during OPIDN, is the breakdown, aggregation and accumulation of NF and microtubules (Abou-Donia, 1981; Stuart and Oehme, 1982; Jortner, 1984b; Hoffman, 1986).

The turnover and degradation of NF has been shown to be dependent on the activity of Ca^{++}-activated (or Ca^{++}-dependent) proteases (Schlaepfer and Hasler, 1979; Schlaepfer, 1977). In the absence of Ca^{++}, NF remain intact, while degradation is triggered and may be accelerated by increasing Ca^{++} directly or indirectly by use of ionophores. The Ca^{++}-activated neutral proteases (CANP) are enzymes with two isomers, one requiring micromolar levels of Ca^{++} (μCANP), and the other requiring macromolar levels of Ca^{++} (mCANP). The first of these is named calpain I and the latter calpain II. These isozymes coexist with an endogenous enzyme inhibitor calpastatin (Kamakura et al., 1985; Schlaepfer, 1987). CANPs have been identified and localized in peripheral nerve (Kamakura et al., 1982; 1985b), and are believed to be associated with the NF triplet. The initiating events occuring during Wallerian degeneration (e.g. granular disintegration of NF and axoplasmic microtubules) implicate Ca^{++}, through CANP, in this process (Schlaepfer,

1971; Kamakura et al., 1983; Schlaepfer and Zimmerman, 1984). It has been suggested that specific inhibitors of CANP (e.g. leupeptin) would alleviate clinical signs of Wallerian degeneration (Badalamente et al., 1987), although studies with this agent in OPIDN have not yet been attempted.

Disintegration of the axoplasmic cytoskeleton by CANP may lead to liquefication of axoplasm with the collapse and fragmentation of myelin (Schlaepfer and Zimmerman, 1984), events which do occur in OPIDN (Jortner, 1984a). When axotomized nerves are left to degenerate in vitro or in vivo, breakdown of the axonal cytoskeleton has been prevented by the removal of Ca^{++} (Schlaepfer, 1971; Schlaepfer and Bunge, 1973; Schlaepfer and Hasler, 1979; Schlaepfer and Zimmerman, 1984). Elevated intra-axonal Ca^{++} has been suggested to contribute to the development of neuropathies (Nixon et al., 1983; Jansco et al., 1984; Komulainen and Bondy, 1987; 1988; Ochs, 1987; Watson and Griffin, 1987), including those following nerve crush (Mata et al., 1986; Balantine, 1988), and possibly OPIDN (Seifert and Casida, 1982; Moretto et al., 1987). Indeed, increases in cations, including Ca^{++}, in nerve following transection have recently been demonstrated by microprobe analysis (Randall et al., 1988). This technique also demonstrated Ca^{++} increases in nerves of hens with OPIDN (LoPachin et al., 1988).

Another role for Ca^{++} and CaM in nerve function may be ascribed to a Ca^{++}-CaM kinase that phosphorylates alpha and

beta tubulin (DeLorenzo, 1982), additional structural elements in the axonal cytoskeleton (Schlaepfer, 1987). Ca^{++}-CaM kinase appears to have special importance in the turnover of axonal cytoskeletal elements during OPIDN. This enzyme has been implicated in phosphorylation of the NF triplet and both tubulin peptides during the course of TOTP-induced neuropathy (Abou-Donia et al., 1984; Patton et al., 1985; 1986; Suwita et al., 1986a; 1986b).

CaM has been found associated with a third structural protein, actin (Brady et al., 1980), and the rapid axonal transport of membrane associated proteins (Iqbal and Ochs, 1980; Chan et al., 1984). Elevated intra-axonal Ca^{++}, possibly through CaM, has been reported to inhibit axonal transport of key elements essential for nerve regeneration (Ochs and Iqbal, 1982). The direct infusion of the CaM-inhibitor chlorpromazine could completely prevent the Wallerian degeneration that followed axotomy of rat sciatic nerve (Medinacelli and Church, 1984). Studies on the interaction of CaM-inhibitors and OPIDN have not appeared in the literature.

c) $\underline{Ca^{++}}$ and myelin

As in the axon, studies have revealed the presence of a neutral protease in myelin that depends on Ca^{++} for its activation (Banik et al., 1985; Berlet et al., 1984; Berlet, 1987). This protease may be the same as the CANP found associated with NF in the axon (Banik et al., 1985; Berlet,

1987). The CANP isolated and intimately associated with purified myelin induced the degradation of myelin protein with progressive increases in Ca^{++} concentrations (Banik et al., 1984).

Ca^{++} itself has been reported to induce swelling of myelin multilayers from a compact to an expanded form. This was subsequently abolished by placement of nerve in a Ca^{++}-free medium. These authors suggested that wholesale expansion of this sort may be an early step in myelin breakdown in vivo (Blaurock, et al., 1986; Blaurock and Yale, 1987). In another study, rat sciatic nerve segments showed extensive disruption of myelin in presence of Ca^{++} and Ca^{++}-ionophore A 23187 (Schlaepfer, 1977), without death of Schwann cells (Smith et al., 1985).

The breakdown and degeneration of myelin, secondary to axonal degeneration, is known to occur during OPIDN (Bouldin and Cavanagh, 1979; Jortner, 1984a), and this may be facilitated by Ca^{++}-activated processes in the myelin sheath.

2.2. Ca^{++} and Skeletal Muscle Function

a) Excitation-Contraction Coupling and Ca^{++} Homeostasis in Muscle

As a consequence of ACh release from the axonal terminal into the synaptic cleft of the neuromuscular junction, two molecules of ACh bind to the nicotinic receptor, opening an ionic channel (6.5 A) (Ritchie, 1986). Within 1 msec of its release ACh diffuses out the synaptic gutter and no longer

acts on the muscle fiber. Normally, almost all ACh remaining is hydrolysed by AChE in the basil lamina between the nerve terminal and subneural clefts. However, this interval between release and degradation is sufficient to excite the muscle fiber (Guyton, 1986). The ACh-operated channel, although relatively nonspecific, allows only the passage of cations, preferentially Na^{+} and Ca^{++} (Ritchie, 1986; Guyton, 1986). Na^{+} insurgence into the muscle fiber depolarizes the fiber to produce an endplate potential (e.p.p.). A strong e.p.p. will usually initiate an action potential, since those produced are usually 3-4 times that required for stimulation of the muscle fiber (Guyton, 1986). These ACh-operated channels become highly sensitive to activation during denervation (such as in OPIDN), facilitating the generation of action potentials (Stefani and Chiarandini, 1982; Tanaka and Barchi, 1987).

The generated action potential penetrates to the vicinity of the myofibrils by transmission along transverse tubules (T-tubules) running all the way through the muscle fiber. The T-tubule action potentials induce the sarcoplasmic reticulum to release Ca^{++} in the vicinity of the myofibrils. It is these Ca^{++} ions which trigger contraction (Guyton, 1986). The T-tubule-sarcoplasmic reticulum system (SR) surrounding and interlaced among all individual myofibrils is actually an internal extension of the sarcolemma. Therefore, an action potential spreading along the membrane is transmitted inwards (Guyton, 1986). The mechanism of

Ca^{++} release is not known, although it is thought to occur through specific channels. This release raises cytosolic Ca^{++} levels above 10^{-7} M, the concentration necessary for contraction (Cavero and Spedding, 1983; Guyton, 1986). Four Ca^{++} ions bind to troponin C, bringing about a conformational change, shifting troponin I (I: inhibitory) and moving the tropomyosin bound to the actin filament along its groove to reveal the actin-myosin binding site (Cavero and Spedding, 1983; Guyton, 1986). The myosin head of the thick filament binds ATP, which is cleaved by ATPase into its products ADP and Pi, which remain bound to the head (McGilvery, 1983; Guyton, 1986). This ATP-activation relaxes the head perpendicular to actin. The myosin head binds to the actin active sites, causing the head to move in a power stroke (ratchet effect) and move the actin filament. As a consequence of the myelin head tilting, ADP and Pi are released, providing a free site for subsequent ATP binding and myosin activation. This sequence of events persists as long as cytosolic Ca^{++} levels are high (McGilvery, 1983). The potentiation of action potential generation and the resulting enhanced release of Ca^{++}, with its accumulation in the sarcoplasm, significantly enhances muscle contraction (Gupta and Dettbarn, 1987), particularly in the case of denervated muscle (Stefani and Chiarandini, 1982; Salpeter, 1987).

To reduce cytosolic Ca^{++} levels, Ca^{++} is resequestered by the Ca^{++}-ATPase pump. This pump has the capability of

concentrating Ca^{++} ions up to 10,000 times in the SR (Cavero and Spedding, 1983; Guyton, 1986). Furthermore, Ca^{++} is reversibly bound to a specific acid protein, "calsequestrin" (Cavero and Spedding, 1983). Ca^{++} may also be extruded extracellularly by exchange with Na^{+}, or by an active Ca^{++}-Mg^{++} ATPase pump (Cavero and Spedding, 1986; Hoyle, 1983). Under situations of Ca^{++} overload, the mitochondria can take up Ca^{++} rather than phosphorylating ADP for ATP generation. Large amounts of Ca^{++} may be deposited in these organelles in the form of a Ca^{++}-phosphate complex (Cavero and Spedding, 1983; Hoyle, 1983; McGilvery, 1983).

It should be noted that both muscle contraction and maintenance of Ca^{++} homeostasis are energy consuming processes. Critical increases in Ca^{++} levels are thereby associated with inhibition of ATP synthesis, ultrastructural lesions and cell death (Wrogeman and Pena, 1976; Schanne et al., 1979; Farber, 1981). During enhanced muscle activity, due to ACh-activated accumulation of Ca^{++}, the muscle cannot cope with both the heightened activity and the resequestering of Ca^{++}. Therefore, phosphogens are depleted during muscle contraction or in an effort to regulate extra free Ca^{++}, and the muscle is rendered incapable of regenerating phosphocreatine (Gupta and Dettbarn, 1987). Eventually lesions occur and the muscle atrophies. CPK is also depleted from muscle and an increase in plasma CPK occurs. Increases in plasma CPK have been demonstrated to occur during OPIDN (Cisson and Wilson, 1982; Wilson et al., 1984).

b) Ca^{++} and CANP in Skeletal Muscle Degeneration

Skeletal muscle degeneration occurs in OPIDN (Cisson and Wilson, 1982; Wilson et al., 1984). As in the axon and myelin there is a CANP in skeletal muscle. The enzyme has been purified and characterized in both rabbit and chicken muscle (Reddy et al., 1975; Ishiura, 1981). Muscle CANP is believed responsible for the turnover and degradation of myofibril proteins (Z-discs and actin) and other components of the contractile apparatus, particularly troponin-T. CANP's role in degeneration and atrophy of muscle has been demonstrated in muscle dystrophy (Baker and Margolis, 1987) and cardiomyopathy (Spalla et al., 1987). Experimentally, muscle degeneration has been induced relying on Ca^{++} ionophore-induced activation of CANP (Duncan, 1978; Llados, 1985).

Functional ACh-receptors are required for instigation of myopathy, including that produced by acute OP administration (Duncan, 1978; Fenichel et al., 1972; Laskowski et al., 1975; Wecker et al., 1978a; 1978b; Leonard and Salpeter, 1979). A direct association between OPs and CANP was established by Toth and co-workers (Toth et al., 1981; 1983) during acute and subacute intoxication with DFP and methylparathion. Ultrastructural studies revealed extensive muscle damage, with swollen mitochondria and dilation of sarcoplasmic reticulum due to Ca^{++} overload and the ensuing activation of CANP. This may partially explain the usefulness of a Ca^{++}-channel blocker (verapamil) in protecting against the acute effects of DFP (Dretchen et al., 1986), and the atte-

nuation of denervation-induced muscle degeneration with the use of the CANP-inhibitor leupeptin (Badalamente et al., 1987).

Surgical or chemically-induced denervation, including TOTP-induced delayed neuropathy, has been shown to result in the degeneration of chicken muscle (Linkhart and Wilson, 1975; Cisson and Wilson, 1982; Wilson et al., 1984). During this process plasma levels of CPK were significantly elevated, particularly after onset of clinical signs. This elevation of plasma CPK was associated with a concurrent reduction (43-50%) of CPK activity in the gastrocnemius muscle (Cisson and Wilson, 1982; Wilson et al., 1984). Increases in plasma levels of CPK and the depletion of phosphogens has been shown to be a general consequence of cytosolic and mitochondrial Ca^{++} overload (Wrogeman and Pena, 1976), including myopathy following acute OP administration (Gupta and Dettbarn, 1987). In addition, the elevation of plasma CPK may be directly attributed to CANP-induced degeneration of muscle membrane, rendering it "leaky" (Ebashi and Sugita, 1979; Clow and Boegman, 1987). These observations further support the interaction of OPs, Ca^{++}, and CANP in muscle degeneration that occurs during OPIDN.

In addition to effects mediated by CANP, increases in Ca^{++} in mammalian skeletal muscle have been demonstrated to cause mitochondrial abnormalities and to compromise the sarcolemma's ability to control influx of ions, including Ca^{++} (Soza et al., 1986). Muscle damage induced by Ca^{++} was pre-

vented by the use of Ca^{++}-channel blockers (verapamil, nitrendipine, and diltiazem). These compounds also prevented CPK efflux, in vivo and in vitro (Anand and Emery, 1982; Bhattacharya et al., 1982; Soza et al., 1986). The effect of Ca^{++}-channel blockers on muscle damage that occurs during OPIDN has not been reported.

2.3. Other Mechanisms by Which Ca^{++} May Contribute to Changes in Neuromuscular Function During OPIDN

a) Contribution of Ca^{++} to Denervation Supersensitivity

As stated above, in addition to neural damage, OPIDN appears to cause muscle degeneration and atrophy. These changes are characteristic of those that occur following denervation (Cisson and Wilson, 1982). Denervation of skeletal muscle, whether by nerve transection or chemically as in OPIDN, has been shown to be accompanied by a rise in extrajunctional AChE, a reduction in endplate AChE and a rise in plasma CPK (Cisson and Wilson, 1982; Wilson et al., 1984). The reduction in endplate AChE following denervation was attenuated or abolished by the use of protease inhibitors (Fernandez and Duell, 1980), and was apparently Ca^{++}-dependent (Rubin, 1985).

Skeletal muscle denervation results in hypersensitivity of muscle to ACh (Brown, 1937; Axelsson and Thesleff, 1959; Miledi, 1960a). The ensuing hypersensitivity has been suggested as being due to the de novo synthesis of ACh-receptors (Gramp et al., 1972; Chang and Tung, 1974; Fam-

brough, 1970; 1979; Tipnis and Malhotra, 1979; Anderson, 1987), and an enhanced cell division (Blunt et al., 1975). The ACh-receptors can be differentiated into "intrinsic" (junctional), which respond to nerve stimulation, and "extrinsic" (extrajunctional), which respond to exogenous ACh (DelCastillo and Katz; 1957; Miledi, 1960b; Marshall, 1971; Hartzell and Fambrough; Chang and Tang, 1974; Gordon et al., 1976). The new ACh-receptors appear to have a higher affinity for binding α-bungarotoxin, and possibly ACh (Almon et al., 1974). This would increase the activation of muscle, as discussed above (Section 2.2:a). Proliferation of these newly synthesized receptors has been demonstrated to occur 3 days to weeks after denervation (Almon and Appel, 1976; Lindsley and Holmes, 1984), a time course consistent with the development of OPIDN.

The de novo synthesis of ACh-receptors in denervated muscle has been suggested to be influenced by ACh itself and products of neural tissue degeneration (Lomo and Rosenthal, 1972; Jones and Vrbova, 1974; Jolesz and Sreter, 1981; McArdle, 1983; Kandel, 1985b). As discussed above, Ca^{++} contributes to nerve degeneration (Schlaepfer, 1987) that, subsequently, may increase ACh-receptor synthesis in muscle. In fact, alterations in denervated muscle have been attenuated by use of protein extract from normal nerve (Davis, 1983; Otto et al., 1987). ACh-receptor synthesis has also been demonstrated to be enhanced by ionophorically increasing Ca^{++} concentrations intracellularly (Metafora et al.,

1980), or suppressed by regulation of Ca^{++} concentrations in incubation mediums (Birnbaum et al., 1980; McManaman et al., 1981). The increase in ACh-receptors of denervated muscle strips could also be eliminated by D-600, the methoxy derivative of the Ca^{++}-channel blocker verapamil (Metafora et al., 1980; Kallo and Steinhardt, 1983).

In addition to increased muscle sensitivity to ACh due to increased receptor synthesis, it has been suggested that increases in ACh sensitivity following denervation may in fact be mediated by the activity of a proteolytic enzyme (and CANP is a protease) (McLaughlin et al., 1974; Harborne et al., 1978) or by Ca^{++}-induced regulation of cyclic nucleotides (Betz and Changeux, 1979). In these studies muscle activity suppressed or reduced the appearance of hypersensitivity and/or ACh-receptor synthesis (Lomo and Rosenthal, 1972; Jones and Vrbova, 1974; Birnbaum et al., 1980; McManaman et al., 1981).

The ACh-receptor from denervated muscle has recently been characterized as being composed of four homologous subunits, similar to ACh-receptors from other tissues (Raftery, 1986). However, muscle receptors during denervation differ by having a lower single channel conductance and a longer open lifetime (Ritchie, 1986). This would contribute to enhanced activation of muscle, and the accumulation of Ca^{++} from both extracellular and intracellular sources. ACh-receptors in denervated muscle also lack any associated AChE (Bowman and Marshall, 1971; Brimijoin, 1983; Salpeter, 1987;

Rotundo, 1987; Dettbarn et al., 1987). The absence of this hydrolytic enzyme, the enhanced Ca^{++}-mediated ACh release, and the increase in highly sensitive receptors would greatly exaggerate the response of muscle to stimulus. This may contribute to the denervation fibrillation seen in OPIDN.

Chronic denervation of chick leg muscle as a result of sciatic nerve transection has revealed a twofold increase in functional voltage-sensitive Ca^{++}-channels which serve as receptors for ^{3H}nitrendipine and other dihydropyridines (Schmid et al., 1984; Miller, 1987). These dihydropyridine-receptors are inhibited by low concentrations of the dihydropyridine Ca^{++}-channel blockers (Rios and Brum, 1987). The increase in receptors appears three days following denervation, and is believed to correspond to the formation of an abnormal transverse tubule system (Schmid et al., 1984; Kawamoto and Baskin, 1986). The increase in receptors after denervation by transection is maximal at 15 days and then the number of these receptors (channels) begins to decline. Muscle atrophy and degeneration may contribute to this effect (Schmid et al., 1984). No study on increases in Ca^{++}-channels during OPIDN has appeared in the literature, but the time course of their increase and decrease in numbers corresponds to the time course of ataxia and pathological lesions induced by a single administration of neuropathy-inducing OPs (see Section 1.6:b).

Perhaps the most profound functional manifestation of denervation is that an effective depolarization of muscle

can be evoked at any point or at many points along the muscle fiber, based on muscle's cable properties (Axelsson and Thesleff, 1957; Bowman and Marshall, 1971). This implies that smaller concentrations of transmitter can act on a larger area (Bowman and Marshall, 1971). Fibrillation of denervated muscle, therefore, occurs 5 to 15 days later due to spontaneous depolarizations (Dowben, 1980). These begin at the motor endplate, particularly when nerve axotomy is more distal (i.e. nearer the end plate [Luco and Eyzaguirre, 1955]), and are propagated in both directions along the length of the muscle fibers. Unlike normal contractions, they are asynchronous, possessing a lower conduction velocity than that of innervated muscle (Axelsson and Thesleff, 1957; Dowben, 1980). Fibrillation is seen during electromyographical examination of humans and experimental animals with OPIDN (Roberts, 1977; Baker and Lowndes, 1980; Vasilescu and Florescu, 1980; Vasilescu et al., 1984; Abou-Donia et al., 1986).

b) Contribution of Ca^{++} to the Capability of Corticosteroids to Modify OPIDN

Adrenocorticoids have been used in the treatment of a number of neuromuscular disorders (Ellison, 1984). It has been speculated that the glucocorticoids may exert their effect via a specific receptor (Riker et al., 1982). Indeed, such receptors have been identified in the spinal cord using ^{3H}dexamethasone, where they enhance serotonergic transmis-

sion and synaptic reflexes (Clark et al.,1981). Glucocorticoid receptors also occur in the cytosol of muscle, and their number demonstrated to increase during atrophy (DuBois and Almon, 1980; Konagaya et al., 1986). Increases in these receptor sites may be followed by modulation of levels of proteolytic enzymes (Florini, 1987). As stated above, one proteolytic enzyme is CANP, which contributes to nerve and muscle degeneration (Schlaepfer, 1987; Baker and Margolis, 1987). There appears to be a variability in the beneficial use of glucocorticoids in treatment of neuromuscular diseases, and the variability may depend on muscle fiber type affected, as glucocorticoids stimulate protein degradation or inhibit protein synthesis in fast muscle, but are less likely to do so in slow muscle (Kelly et al., 1986; Florini, 1987).

A recent study reports that the actions of a potent glucocorticoid antagonist (RU38486) could both prevent the binding of glucocorticoid to its receptor and inhibit dexamethasone-induced muscle atrophy (Konagaya, 1986). The adverse effects of corticosteroids on skeletal muscle, where they increase protein degradation and decrease protein synthesis may occur without any alteration in muscle activity, even as these drugs exert their beneficial effect on nerve (Goldberg, 1969). Detrimental effects of corticosteroids on muscle appear to be augmented by muscle denervation (Rouleau et al., 1987).

Numbers of glucocorticoid receptors in muscle, similar to ACh-receptors of denervated muscle, increase 1-3 days following denervation (DuBois and Almon, 1981). This increase increases the probability of subsequent muscle atrophy as more glucocorticoids are bound and, subsequently, protein synthesis is inhibited while degradation is stimulated. This phenomenon may explain the adverse effects of some corticoids in exacerbating clinical signs during OPIDN (Ehrich et al., 1985; 1986a; 1988). Paradoxically, some steroids (e.g. prednisolone) are capable of inducing desensitization of ACh-receptors, thus providing protection from enhanced stimulation and subsequent increases in muscle activity (Ribera and Nastuk, 1988). This last observation, as well as capability of corticoids to exert benificial effects on nerves (Goldberg, 1969), may explain the ability of some corticosteroids in particular doses to alleviate clinical signs during OPIDN (Baker et al., 1982; Baker and Stanec, 1985; Ehrich et al., 1985; 1986a; 1988).

The action of corticosteroids and Ca^{++} in muscle damage may also be linked because hydrocortisone and methylprednisolone have both been demonstrated to significantly attenuate Ca^{++} influx during necrosis induced by exposure of cardiac muscle to epinephrine. This was accompanied by reduction in CPK depletion (Kraikitpanitch et al., 1976).

PART III

OBJECTIVES, EXPERIMENTAL DESIGN AND METHODS

CHAPTER 3

OBJECTIVE OF THE RESEARCH PROPOSED

The objective of the dissertation research was to delineate the possible involvement of increased Ca^{++} mobilization on events within nerve and muscle that may contribute to some of the detrimental changes occuring in OPIDN. Ca^{++} may be implicated because it contributes to neurotransmitter release, degradation of axonal cytoskeletal structures, myelin integrity, excitation-contraction couplin, and degradation of muscle. All of these parameters are altered as an animal develops OPIDN.

Experimental evidence indicates that use of Ca^{++}-channel blockers and/or reduction of free intracellular Ca^{++} may modify the course of degenerative changes in both nerve and muscle. Therefore, the primary objective of this proposal is to study effects of the Ca^{++}-channel blockers verapamil and nifedipine on Ca^{++}-mediated events occuring in nerve and muscle during OPIDN.

TESTING OF THE HYPOTHESIS:

The specific aims of this research were:

1. To use Ca^{++}-channel blockers to attenuate clinical, biochemical, and electrophysiological changes occuring during OPIDN. These studies used the animal model of OPIDN (the chicken) (Bickford, 1984) to evaluate the effects of verapamil and nifedipine on ataxia, activities of enzymes usually altered during OPIDN (NTE [Johnson, 1987], muscle AChE, plasma CPK [Cisson and Wilson, 1982]), function of nerve and muscle

(strength-duration relationships) (Robertson et al., 1987), denervation hypersensitivity of muscle to ACh, and histopathological changes in nerve (Bouldin and Cavanagh, 1979) by light and electron microscopy.

2. To determine if OPIDN, with and without Ca^{++}-channel blockers, altered the activities of a proteolytic enzyme, CANP, in neural and muscle tissues (Schlaepfer, 1987).

3. To determine at what point in time Ca^{++}-channel blockers, and thus Ca^{++}, affected the development of OPIDN. This included evaluation of biochemical, electrophysiological and morphological parameters at intervals prior to and during appearance of clinical deficits. These parameters were also evaluated in chickens that appeared to recover from ataxia, so nerve regeneration was also assessed.

CHAPTER 4

EXPERIMENTAL PROTOCOL AND METHODS

4.1. Experimental Protocol

In order to test the hypothesis, which was to determine if Ca^{++} is involved in OPIDN, Ca^{++}-channel blockers were used to modify the course of this chemically-induced disease, in the following studies:

STUDY #1. This study established the isolated adult hen biventer cervicis neuromuscular preparation as a suitable model for detection of detrimental effects on nerve and muscle during the course of OPIDN, using electrophysiological, pharmacological and pathological evaluation.

STUDY #2. This study determined the earliest detectable signs of neuromuscular damage following administration of neuropathy-inducing OP, the protoxicant TOTP, and its active congener phenyl saligenin phosphate (PSP). The parameters described below were evaluated days 4, 7, 10, 15, 21, and 37 after p.o. administration of TOTP (360 mg/kg), and i.m. administration of PSP (2.5 mg/kg) (Ehrich and Gross, 1983; Jortner and Ehrich, 1987).

Methods used for evaluation of OPIDN were as follows:

1. **Clinical evaluation, using a scoring system of ataxia ranging from 0 (unaffected) to 5 (paralysis of both hindlimbs and wings) (Sprague et al., 1980).**

2. Biochemical evaluation

(a) brain neuropathy esterase (NTE)

(b) plasma CPK

(c) nerve and muscle CANP

(d) muscle AChE

3. Physiology and Pharmacology using nerve-muscle preparations

(a) in vitro: biventer cervicis nerve-muscle.

(1) establishing strength-duration curves (SDC) for the preparation as an assessment of neuromuscular damage.

(2) determination of sensitivity and contractile tension development in response to ACh.

(b) in vivo: sciatic/tibial-gastrocnemius muscle and close-arterial injection.

(1) establishing SDC for sciatic and tibial nerves, and gastrocnemius muscle.

(2) responses of muscle to close-arterial ACh injection.

4. Histopathological evaluation

(a) tibial nerve

(b) biventer cervicis nerve

STUDY #3. This study examined modification of nerve and muscle damage using Ca^{++}-channel blockers during the time course for development of OPIDN induced by PSP. Suitable dosages and routes of administration of Ca^{++}-channel blockers (verapamil and nifedipine) relied

on published information (Hudecki et al., 1984; Dretchen et al., 1986), results obtained after acute administration, and evaluation of clinical observations and mortality.

4.2. Methods

4.2.1. Methods for Meeting Requirements of Study #1, which was to establish the biventer cervicis nerve-muscle as a suitable model for the detection of electrophysiological, pharmacological, and pathological changes following onset of OPIDN were as follows:

a) Animals: Adult (>6 months old, 1.2-1.8 kg) White Leghorn hens were used for these experiments. This specie and age group is the accepted animal model of OPIDN (Bickford, 1984). Hens were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. They were from a long maintained flock of Cornell random-bred birds, raised and kept under veterinary supervision. They were vaccinated against Merek's disease, and came from a flock with no infectious disease problems. They were suitably housed in group cages, 5 hens per cage, with access to food and water ad libitum.

b) Dosing: Organophosphorus compounds, including the protoxicant TOTP (Eastman Chemical Co., Rochester, N.Y.) and an active congener PSP (synthesized as described by Jortner and Ehrich, 1987) were used to induce delayed neuropathy. Chickens received TOTP by oral administration (360 mg/kg), and PSP intramuscularly (2.5 mg/kg). These are doses previously used in the Toxicology Laboratory to induce OPIDN (Ehrich and Gross; 1983; Jortner and Ehrich, 1987). OPs

were dissolved such that the mg/kg dose was in 1 ml corn oil (for oral TOTP), or 0.25 ml dimethyl sulfoxide (for im PSP).

c) Clinical Evaluation: Clinical signs of delayed neuropathy were assessed according to the designation of Sprague et al., (1980). Scores of 0= normal; 1= altered gait; 2= difficulty standing and walking; 3= severe ataxia; 4= hindlimb paralysis; 5= paralysis of both hindlimb and wings were used.

d) Physiology and Pharmacology of OPIDN using The Biventer Cervicis Nerve-Muscle Preparation: This *in vitro* technique designed for the chick by Ginsborg and Warriner (1960) was modified for accomodation of adult hens (Figure 1).

(1) Strength-Duration Curves (SDC) determine the inverse relationship between the stimulus intensity producing a minimal perceptible depolarization, as indicated by muscle twitch response, for various stimulus durations (20-500 μsec) of single electrical pulses. Rheobase (minimal intensity of stimulus of prolonged duration necessary to excite the tissue) and chronaxie (minimum time required to excite the tissue for a stimulus twice the strength of rheobase) are derived from the SDC (Harris, 1971; Wynn Parry, 1971; Heckman, 1972; Friedli and Meyer, 1984). Graphical representation of the SDC is shown in Figure 2.

(2) Sensitivity and Contractile Tension of Muscle are determined by establishing a concentration-response curve in

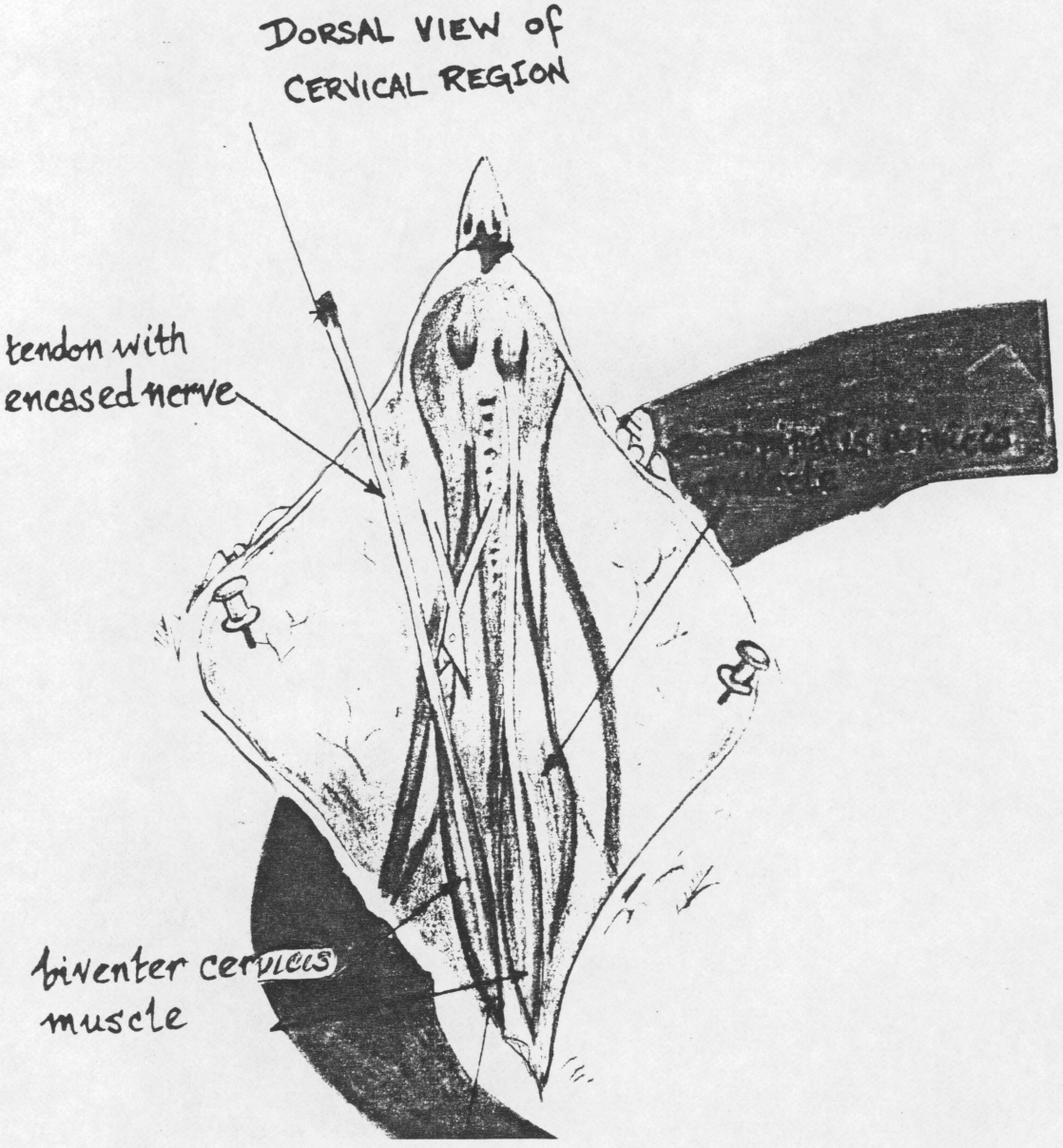

DORSAL VIEW of
CERVICAL REGION
tendon with
encased nerve
biventer cervicis
muscle

Figure 1. Schematic representation of the hen biventer cervicis nerve-muscle preparation (after Ginsborg and Warriner, 1960).

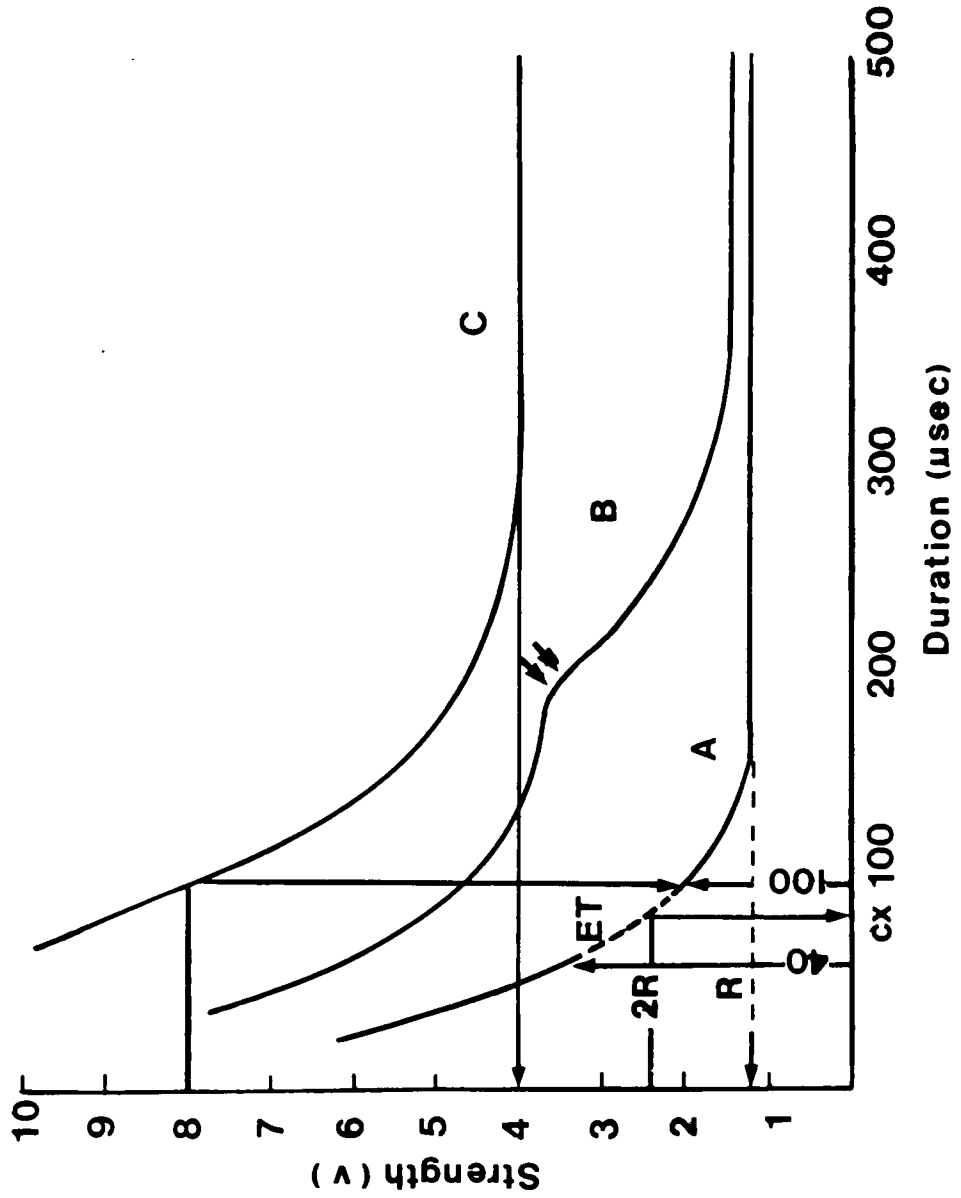

Strength (v)
10
9
8
7
6
5
4
3
2
1
C
B
A
2R
R
ET
100
0
cx 100
200
300
400
500
Duration (µsec)

Figure 2. Graphic representation of the strength-duration curve (SDC) and the data derived from it. A = normal nerve showing the inflexion region (40-100 μsec) representing the excitability threshold (ET), R = rheobase; C_X = chronaxie (duration at 2R). B = partial innervation, showing "kinks" or discontinuities in the curve to indicate this (arrows). C = complete denervation shifts the curve to the right and raises the rheobase (after Wynn Parry, 1971).

response to ACh application in an isolated organ bath.

e) Data Analysis: Quantitative values obtained from clinical evaluation, biochemical assays, physiological assessment and some pathological evaluation were subjected to statistical analysis. Mean, standard deviation, and standard error were calculated. Differences between groups were compared using either the Student's t-test or analysis of variance, where appropriate, and the Newman-Keuls method of multiple differences.

2.2.2. Methods for Meeting the Requirements of Study #2, which was to determine the time of earliest detectable changes following administration of an OP inducing delayed neuropathy were as follows:

As delineated above for Study #1, adult White Leghorn hens, TOTP and PSP were used in these studies of OPIDN. Clinical assessment and physiology and pharmacology of the biventer cervicis nerve-muscle preparation were used to evaluate OPIDN as described for Study #1. In addition, OPIDN was evaluated using biochemical markers, additional electrophysiological measurements, and morphology.

a) Biochemical Evaluation:

(1) NTE: Spectrophotometric determination of NTE measures the hydrolysis of phenyl valerate. Advantage is taken of the enzyme's sensitivity to inhibition by mipafox after

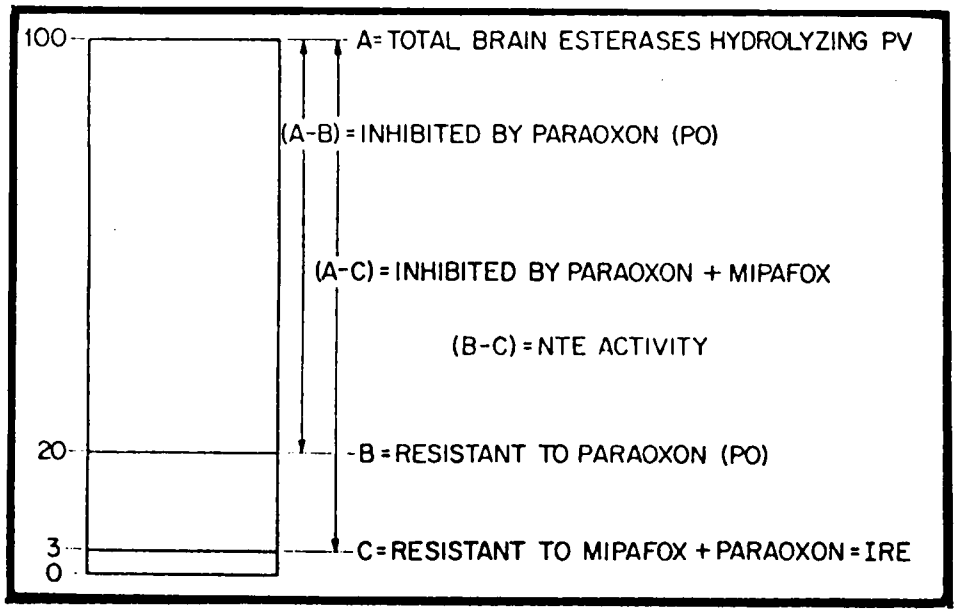

100
20
3
0
A= TOTAL BRAIN ESTERASES HYDROLYZING PV
(A-B) = INHIBITED BY PARAOXON (PO)
(A-C) = INHIBITED BY PARAOXON + MIPAFOX
(B-C) = NTE ACTIVITY
B = RESISTANT TO PARAOXON (PO)
C = RESISTANT TO MIPAFOX + PARAOXON = IRE

Figure 3. Schematic showing the selective inhibition of phenyl valerate (PV) hydrolysis by brain homogenate. NTE is defined as the difference between B (PV esterase activity resistant to paraoxon inhibition), and C (Pv esterase activity resistant to mipafox + paraoxon inhibition) (from Davis and Richardson, 1980).

inhibition of irrelevant carboxylesterases by paraoxon. A schematic depiction of this is given in Figure 3. Samples are read at absorbance 510 nm (Sprague et al., 1981).

(2) Plasma CPK: CPK will be determined spectrophotometrically as described by Oliver (1955) and Hess et al., (1968). This method relies on the coupling of the CPK reaction with two other enzyme reactions. The activity is assessed as the rate of NADPH reduction at 340 mu as follows:

$$\text{ADP} + \text{PC} \xrightarrow{\text{CPK}} \text{ATP} + \text{Creatine}$$

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

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G6DPH}} \text{6-phosphogluconate} + \text{NADPH}$$

where PC= phosphocreatine, HK= hexokinase, G-6-P= glucose 6-phosphate, G6DPH= glucose-6-phosphate dehydrogenase.

One unit of CPK activity is defined as that amount of activity which leads to the formation of 1 μM NADPH/ minute. This assay was done by the Clinical Pathology Laboratory, using a Baker Instruments (Allentown, PA) automated system and reagent kit.

(3) Nerve and Muscle CANP: This is a spectrophotometric determination that relies on the digestion of the protein chromogenic derivative, azocasein, by CANP extracted from tissue homogenates (Waxman, 1981; Sarath, de la Motte, and Wagner [Protease assay methods], unpublished). Following incubation of enzyme and substrate for 30 minutes at 30^{o}c, the reaction was stopped with 10% trichloroacetic acid, and the sample read at 440 nm absorbance. Protease activity was presented as the change in absorbance/ 30 min/mg protein.

This value was that of total protease activity. A duplicate sample treated with EDTA determined Ca^{++}-independent protease activity. CANP activity is determined as follows:

CANP activity= Protease $Activity_{total}$ - Ca^{++}-independent activity

(4) Muscle AChE: The spectrophotometric method of Ellman et al., (1961), relies on the hydrolysis of acetylthiocholine iodide substrate by homogenized tissue AChE, and the subsequent interaction between thiocholine and the chromogenic dithiobisnitrobenzoate (DNB) to produce a yellow color:

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

$$\text{thiocholine} + \text{DNB} \longrightarrow \text{yellow color (410 nm)}$$

In addition a final concentration of 10^{-6} M of iso-OMPA was added to inhibit nonspecific esterase (Ehrich, personal communication).

b) <u>Additional Methods For Physiological and Pharmacological Evaluation</u>

(1) The sciatic/tibial-gastrocnemius muscle preparation with close arterial injection is an adaptation of the preparation used by Brown and Harvey (1938a) used for electrophysiological assessment of hens with OPIDN by Robertson et al., (1987). Close arterial injection was added as suggested by Brown and Harvey (1938b), to allow specific targeting of injected ACh to the muscle.

Following pentobarbital anesthesia, the hen was tied to a surgical board and maintained under a surgical lamp to prevent hypothermia. The thigh and gastrocnemius muscle were exposed by removal of all feathers and incising the skin. The sciatic nerve was exposed by blunt dissection and retraction of the muscle. Care was taken to separate the nerve from all surrounding fat, and to expose the sciatic artery for later cannulation. The tibial branch innervating the gastrocnemius was also exposed, and covered with cotton wool soaked in mineral oil. The tendon anchoring the gastrocnemius muscle was threaded with a length of suture prior to cutting at the tibiotarsal joint. The suture was attached to an isometric force displacement transducer. An appropriate resting load was placed on the muscle. The limb was fixed into position by clamping drill bits inserted through the knee and ankle joints as described by Eyre and Goff (1968). The incised skin was loosely sutured and the limb wrapped in warm gauze soaked in physiological saline to prevent drying and heat loss. The tibial arterial branch was cannulated proximal to the muscle with a needle cannula having an injection port. All branches of the artery perfusing other muscles were ligated (Figure 4).

(a) Establishing SDC for the sciatic and tibial nerves used standard bipolar stimulating and recording electrodes. The SDC was described above under Study #1.

(b) Responses of muscle to ACh injection were determined prior to, during, and following cumulative injection of ACh,

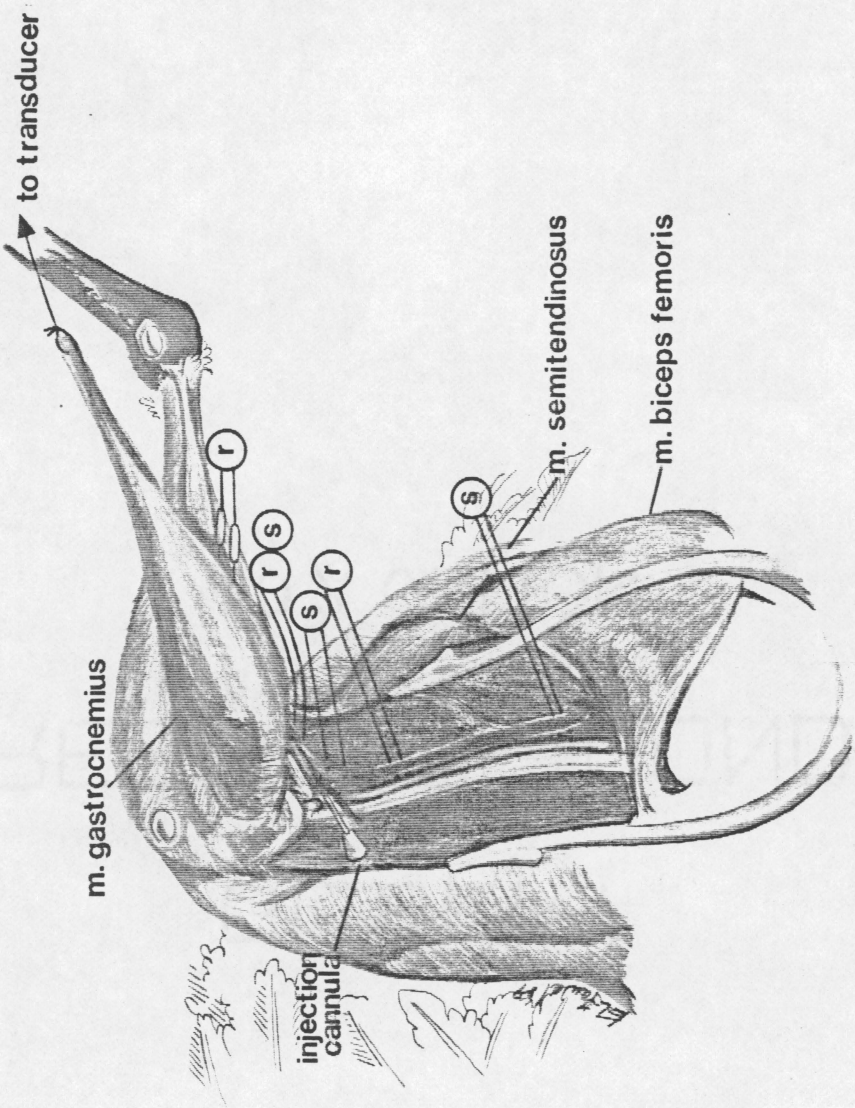

to transducer
m. gastrocnemius
injection cannula
r
s
r
s
r
s
m. semitendinosus
m. biceps femoris

Figure 4. Schematic representation of the isolation of the sciatic and tibial nerves and gastrocnemius muscle for *in vivo* measurements. Placement of stimulating (s) and recording (r) electrodes are identified.

as a trace of muscle contraction during continuous stimuli (12 pulses/min) was recorded and a dose-response curve plotted.

c) Histopathological Evaluation: The distal (over the muscle belly) region of the biventer cervicis nerve, and the tibial branch to the head of the gastrocnemius muscle were dissected out from all freshly killed hens used in the above experiments. Samples were positioned straight on cards and placed in a fixative of the following composition: 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4. After 24-48 hours of fixation at 4^{o} C segments of nerve were rinsed in buffer, post fixed in 2% osmium tetroxide and embedded in Epon^{R} epoxy resin. Cross sections of 1.0 um were cut and stained in toluidine blue and safranin for light microscopy examination. Electron microscopy was performed on selected samples.

4.2.3. Methods for Meeting the Requirements of Study #3, which is to determine if Ca^{++} channel blockers can modify OPIDN, are as follows:

Regimens for treatment with Ca^{++} channel blockers were determined as described in the Experimental Protocol. Evaluation of their capability to modify OPIDN used methods described for studies #1 and #2. Both verapamil and nifedipine were used because they have different molecular mechanisms (Miller, 1987) and both have been used to modify acute

OP intoxication (Dretchen et al., 1986) and other neuromuscular disorders (Hudecki et al., 1984).

PART IV

RESULTS

CHAPTER 5

THE BIVENTER CERVICIS NERVE-MUSCLE PREPARATION OF ADULT HENS: EFFECTS OF PHENYL SALIGENIN PHOSPHATE ADMINISTRATION

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ABSTRACT

A biventer cervicis nerve-muscle preparation was used to assess in vitro neuromuscular function in adult white leghorn hens with clinical signs of delayed neuropathy induced by phenyl saligenin phosphate (PSP). Denervation of fast-twitch muscle fibers 13-15 days after PSP was indicated by higher excitability thresholds and by discontinuities of the strength-duration curves. Nerve degeneration was also indicated by significantly elevated rheobase values for all three experimental groups (2, 6 and 10 mg/kg PSP, im) and by shorter chronaxie for preparations from hens receiving 6 and 10 mg/kg. Chronaxie values for preparations from hens given 2 mg/kg PSP were longer than controls, indicating only partial denervation. Biventer cervicis muscle from all PSP-treated hens was 100-1000x more sensitive to acetylcholine (ACh) than muscle from untreated hens, a response typical of denervated slow-tonic muscle. Tension development in response to ACh was 20-45x greater than control in muscle of PSP-treated hens. The greatest sensitivity and tension development in response to ACh was encountered in muscles from hens given 10 mg/kg PSP. Denervation was also indicated histologically by the extensive degeneration and loss of larger myelinated nerve fibers. This study indicates that alteration in neuromuscular function and morphology occurs in the neck region of chickens during OPIDN and that deficits in nerves

innervating both fast-twitch and slow-tonic muscles can be differentiated by nerve stimulation and by denervation hypersensitivity to ACh.

INTRODUCTION

Among the organophosphorus compounds (OPs) used today, some have a potential to induce delayed neuropathy in man and other susceptible animal species following a single exposure (Abou-Donia, 1981; Murphy, 1986). Organophosphorus-induced delayed neuropathy (OPIDN) is characterized by a latent period of 6-14 days after exposure before appearance of axonal degeneration of long fibers and a secondary degeneration of myelin. Clinically this is presented as incoordination, ataxia and flaccid paralysis (Abou-Donia, 1981; Metcalf, 1984). Among species susceptible to OPIDN, the adult chicken is generally recognized as providing the most reliable animal model (Davis and Richardson, 1980; Abou-Donia, 1981; Bickford, 1984).

Methods of OPIDN evaluation have included clinical (Sprague et al., 1980) or histopathological scoring (Cavanagh, 1954; Prentice and Roberts, 1984; Jortner and Ehrich, 1987) and a rapidly evolving inhibition of neurotoxic esterase activity (Johnson, 1982). Recently, electrophysiological studies have been used to demonstrate a loss of sensory and motor function (Lapadula et al., 1982; Drakontides and Baker, 1983; Durham and Ecobichon, 1984; Robertson et al., 1987).

Another component of OPIDN appears to be muscle degeneration and atrophy, characteristic changes that occur after denervation (Cisson and Wilson, 1982). Denervation of skeletal muscle

is also accompanied by the development of increased sensitivity to acetylcholine (Axelsson and Thesloff, 1957; Kandel, 1985). The present study was undertaken to determine if biventer cervicis nerve-muscle preparations from adult chickens could be used as an *in vitro* model for assessing degenerative and denervation changes in neuromuscular function during OPIDN.

Previous preparations used to evaluate neuromuscular function during OPIDN have included the cat soleus muscle, chicken plantaris muscle and the hen gastrocnemius muscle. These are composed predominantly of slow-twitch, slow-tonic, and fast-twitch fibers, respectively (Lowndes *et al.*, 1974; Durham and Ecobichon, 1984; Abou-Donia *et al.*, 1986; Robertson *et al*, 1987). Unlike these preparations, the biventer cervicis muscle contains both fast-twitch and slow-tonic muscle fiber types (Ginsborg, 1960). Furthermore, the effects of both physical or chemical denervation, such as occurs in OPIDN, can be differentiated for both types of fibers using a single preparations. This is done by measuring responses to nerve stimulation in fast-twitch muscle fibers and by recording sustained contractures produced by exogenous acetylcholine in slow-tonic muscle fibers (Gordon *et al.*, 1976; Harvey and Marshall, 1986). Avian slow-tonic muscles are believed to be equivalent to mammalian slow-twitch muscle (Barnard *et al.*, 1982) and, indeed, responses of both to denervation by development of hypersensitivity to neurotransmitter are

similar (Gordon et al., 1976).

MATERIALS AND METHODS

Animals and housing. White leghorn hens (> 7 months old, 1.2-1.8 kg) used in this study were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. They were suitably housed in group cages, and had access to food and water ad libitum. Hens were divided into 4 groups, with 5-7 hens in each. Three of the four groups were treated with different doses of phenyl saligenin phosphate (PSP), which causes OPIDN (Jortner and Ehrich, 1987). Controls consisted of untreated hens. An additional group of 5 untreated one-week-old white leghorn chicks was included in the study to allow for comparison with biventer-cervicis preparations described in the literature (Ginsborg and Warriner, 1960; Marshall, 1971).

Treatment. Phenyl saligenin phosphate (PSP, 2-phenoxy-4H-1-1,3,2-benzodioxaphosphorin-2-oxide), a prototype of the cyclic saligenin phosphates, representing the neurotoxic metabolite of tri-ortho-tolyl phosphate (TOTP; Eto et al., 1962), was synthesized as previously described (Jortner and Ehrich, 1987). PSP was dissolved in dimethyl sulfoxide (DMSO) so that the 2, 6, and 10 mg/kg dosages were available in a volume of 0.25 ml/kg. This compound was administered by divided injection into the breast muscle, half on each side. Dosages for PSP were based on previous experiences in our laboratory (Jortner and Ehrich, 1987).

Clinical signs. Following dosing, chickens were observed daily for clinical signs. Independent blind assessment by at least two individuals was recorded and averaged. Scores were designated as 0 for normal, 1 for altered gait, 2 for difficulty in walking and standing, 3 for severe ataxia, 4 for leg paralysis and 5 for paralysis with both leg and wing involvement (Sprague et al., 1980).

Electrophysiological and pharmacological studies. Electrophysiological and pharmacological methods for evaluating the effects of OPIDN were conducted on biventer cervicis nerve-muscle preparations obtained after hens had been euthanized by iv injection of T-61 solution (Hoescht Corp., Somerville, N.J.). This preparation used a modification of the procedure developed by Ginsborg and Warriner (1960) that allowed for accommodation of adult hens. To remove the biventer cervicis nerve and muscle, the skin at the back of the neck was incised along the midline from the skull to below the base of the neck, exposing the biventer cervicis muscle on either side of the midline. A suture was tied to the proximal end of the tendon prior to detachment from the skull. By retracting on the suture, the tendon with the ensheathed nerve was exposed and separated from the underlying semispinalis cervicis muscle. Tendon and muscle were carefully removed. The muscle was anchored at the base of a 150 ml organ bath (custom-made) and the tendon passed through a custom-made bipolar electrode. The tendon was attached by the suture to

a Model FT03 force displacement isometric transducer, leading to a coupler-amplifier-recorder system (Grass Medical Instruments, Quincy, MA). Two grams of resting tension was placed on each muscle, as this was determined by pilot studies to be optimal. The organ bath contained Krebs-Henseleit solution [composition in g/l: NaCl, 6.9; $MgSO_4 \cdot 7H_2O$, 0.29; HK_2PO_4, 0.15; glucose, 2.0; $NaHCO_3$, 2.1; KCl, 0.35; $CaCl_2$, 0.28] maintained at 37°C and aerated with a 95% O_2-5% CO_2 mixture.

The terminals of the biventer cervicis electrode were connected to a rectangular pulse stimulator (Model S44 Stimulator, Grass Medical Instruments, Quincy, MA). The voltage required to produce a minimally perceptible response was determined for various durations ranging from 20-500 μsec. For each of the pulse durations detection thresholds were obtained using the method of descending limits and viewed on an oscilloscope (Model 5115 storage oscilloscope, Tektronix, Inc., Beaverton, OR).

Strength-duration curves (SDC) for the biventer cervicis nerve muscle preparations were obtained by plotting strength (volts) producing a least perceptible depolarization, as indicated by muscle twitch response, against stimulus duration (μsec). Rheobase (minimal intensity of stimulus of prolonged duration necessary to excite the tissue) and chronaxie (minimum time required to excite the tissue for a stimulus twice the strength of rheobase) are derived from the curve. The inflexion region of the curve (50-100 μsec) was converted

to linear form by using a double logarithmic plot of abscissa and ordinate as performed by Robertson et al. (1987), to allow comparison of threshold excitability between groups.

Log concentration-response curves in response to acetylcholine chloride (ACh, Sigma Chemical Co., St. Louis, MO) were constructed by plotting cumulative increments in concentration of ACh against gram-tension developed by the muscle. All stock solutions of ACh (10^{-1} M) were prepared on the day of the experiment in distilled water. Subsequent serial molar concentrations (final bath concentrations: 10^{-8} M-$2x10^{-3}$ M) were prepared by dilution in Krebs-Henseleit solution.

Histology. For histologic study on days 13 (6 and 10 mg groups) and day 15 (2 mg group) the distal region (lying over the belly of the muscle) of nerve supplying the twin biventer cervicis muscle was dissected away from the freshly removed specimen and placed in a fixative of the following composition: 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4. After 24-48 hours of fixation at 4°C segments of the nerve were rinsed in buffer, post fixed in 2% osmium tetroxide and embedded in Epon® epoxy resin. Cross sections were cut at one μm thickness, stained with toluidine blue and safranin and examined by light microscopy.

Statistics. All data were analyzed using an analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences between control and experimental groups with $p < 0.05$ considered significant.

All data are expressed as mean ± standard error (S.E.), except where otherwise indicated.

RESULTS

Clinical signs of OPIDN were evident in all PSP-treated chickens 7-8 days after administration of the organophosphorus compound. OPIDN progressed in severity over the next several days (Fig. 1) Hens given 6 and 10 mg/kg PSP had the highest clinical scores until day 13, when they were sacrificed for electrophysiological and pharmacological studies.

Strength-duration curves generated for the biventer cervicis nerve-muscle revealed an increase in threshold excitability in preparations from hens given PSP (Fig. 2). The shape of the curves for all three experimental groups, particularly those given 2 mg/kg PSP, show "kinks" or discontinuities (Fig. 3). Rheobase values for nerves of PSP-treated hens were significantly higher than for controls (Table 1). Chronaxie values were significantly shorter for nerves from hens given 6 and 10 mg/kg, although not different from each other. Chronaxie values for nerves from hens given 2 mg/kg PSP were longer than controls (Table 1).

Log concentration-response-curves showing tension development of the biventer cervicis muscle preparation after exposure to exogenous ACh are shown in Fig. 4. Preparations treated with PSP were 1000x, 100x and 100x more sensitive to ACh, for 10, 6, and 2 mg/kg, respectively, than control preparations. Contractile-tension development was increased significantly, to 20-45 fold over control in all preparations from hens

treated with PSP at the 10^{-4}M concentration of ACh. Responses of muscle from 1-week old chicks (which were included to verify the method for adult birds) were similar to those of muscles from hens treated with 10 mg/kg PSP both in sensitivity and contractility.

Microscopic examination of the distal portion of nerve to biventer cervicis muscle 15 days following treatment with PSP revealed a spectrum of lesions in larger myelinated fibers that were not found in controls (Fig. 5a, b). Axonal swelling and pallor of staining frequently associated with intra-axonal debris and thinning or fragmentation of the myelin sheath were noted. This apparently led to phagocytosis and complete degeneration of involved fiber profiles. More advanced changes were evidenced by Schwann cell tubes devoid of intact axons, but sometimes containing residual debris. The overall result of the process was a diminution of intact myelinated fibers.

While variation in intensity and stages of evolution of lesions in the biventer cervicis nerve were seen within each dosage group at 13 and 15 days, the following generalization can be made: There was very extensive, often advanced, involvement of larger myelinated fibers in the 6 and 10 mg/kg PSP groups with few such neurites unaffected (Fig. 5c). It was not possible to distinguish pathologically between these two dosage groups. More myelinated fibers in the 2 mg/kg PSP group appeared histologically intact, although

even here lesions were widespread. No lesions were seen in the biventer cervicis nerves from controls or nerves from hens given an organophosphate that does not induce OPIDN (malathion).

DISCUSSION

In this study we demonstrated that effects of the delayed neurotoxicity produced by phenyl saligenin phosphate, on nerve and muscle function could be examined using the *in vitro* chicken biventer cervicis nerve-muscle preparation of Ginsborg and Warriner (1960), modified to accommodate adult hens. The biventer cervicis muscle contains both slow-tonic and fast-twitch muscle fibers (Ginsborg, 1960). The former are multi-innervated whereas the latter possess a single neuromuscular junction in each muscle fiber (i.e. focally-innervated) (Ginsborg, 1960; Harvey and Van Halden, 1981). The effects of OPIDN on the function of nerves innervating both muscle types can be conveniently examined *in vitro* in one preparation, since twitch responses of fast muscle are elicited by nerve stimulation, while contraction of slow-tonic fibers is produced by compounds mimicking ACh (Ginsborg, 1960; Hess, 1961, Gordon et al., 1976; Harvey and Marshall, 1986). Although mammalian skeletal muscles, with the exception of extraocular and striated esophageal muscle fibers, are all focally innervated, avian slow tonic muscles are believed to be equivalent to mammalian slow-twitch muscles (Barnard *et al.*, 1982), particularly in their response to denervation (Gordon, *et al*, 1976).

Preparations previously used to assess neuromuscular function during OPIDN involved only one muscle type. The

cat soleus muscle used by Lowndes et al. (1974) and Abou-Donia et al. (1986) is a slow-twitch muscle, while the chicken plantaris muscle used by Durham and Ecobichon (1984) is a slow-tonic muscle. The gastrocnemius muscle, whose innervation has recently been evaluated (Robertson et al., 1987), is predominantly a fast-twitch muscle. Results presented here indicate that the effects of OPs-inducing delayed neuropathy occur in nerves that go to both types of muscle fibers.

Strength-duration curves (SDC), which reflect the function of nerves innervating fast-twitch muscle fibers, were plotted for nerve-muscle preparations after development of OPIDN. The shapes of the SDC for the three experimental groups show "kinks" or discontinuities, most prominently in preparations from hens given 2 mg/kg. Presence of "kinks" in SDC has generally been interpreted as indicating partial denervation (Wynn Parry, 1971; Heckman, 1972). The shift of SDC for preparations from 6 and 10 mg/kg PSP-treated hens suggests that denervation is further advanced than in those receiving 2 mg/kg PSP (Wynn Parry, 1971). Rheobase values derived from SDC were significantly higher in PSP-treated hens, particularly those receiving 6 and 10 mg/kg PSP, than in controls. Chronaxie values for nerve-muscle preparations of hens given the two higher doses of PSP were significantly shorter. These observations are consistent with peripheral neuropathies due to other toxicants (Harris, 1971). On the

other hand, chronaxie values for preparations from hens receiving 2 mg/kg PSP were longer, an observation suggesting only partial denervation (Harris, 1971). Both rheobase and chronaxie are used to indicate degeneration; high rheobase accompanied by a short chronaxie suggests that degeneration has occurred (Wynn Parry, 1971), particularly when the shape of the curve indicates denervation.

Desensitization of skeletal muscle to exogenous ACh has previously been reported to occur in muscle preparations from newborn animals, as complete innervation is gradually established (Child and Zaimis, 1960; Gordon et al, 1976). This was confirmed in the biventer cervicis preparation when we used chicks from 1-5 weeks of age, as they showed an age-dependent decrease in sensitivity to exogenous ACh (unpublished data). Untreated adult controls in the present study also showed low sensitivity to exogenous ACh when compared to 1-week chicks. However, preparations from adult hens treated with PSP all exhibited an increased sensitivity to ACh. Furthermore, preparations from hens with OPIDN were comparable to 1-week old chicks both in sensitivity and in tension development following response to ACh. Effects of PSP were not duplicated by administration of malathion, an organophosphorus compound incapable of causing OPIDN, either within hours or later (15 days) after administration of doses that caused cholinergic signs (unpublished data). Denervation of slow-tonic postural muscle and the ensuing hypersensitivity

to ACh is believed to account for muscle fibrillation (Guyton, 1986), a not uncommon observation during OPIDN. Shortening of muscle, particularly postural muscle in later stages, would also explain contorted positions common in denervation (Guyton, 1986).

The sensitivity to ACh of muscle in preparations from PSP-treated hens is consistent with reports of skeletal muscle (slow-twitch or tonic) hypersensitivity to ACh following denervation (Axelsson and Thesleff, 1957; Miledi, 1960; Marshall, 1971; Gordon et al., 1976; Kandel, 1985). Mechanisms for hypersensitivity to ACh after denervation have not been precisely defined. It has been suggested, however, to be due to de novo synthesis of ACh receptors (Grampp et al., 1972; Hartzell and Fambrough, 1972; Chang and Tung, 1974; Kandel, 1985). Such an increase in ACh receptors is believed to occur 3 days to several weeks following denervation (Almon and Appel, 1976; Lindsley and Holmes, 1984). These periods are consistent with the time course of OPIDN. This extrajunctional proliferation following denervation is believed to be due to the absence of trophic neuronal regulation (Lomo and Rosenthal, 1972; McArdlle, 1983). The function of these de novo synthesized receptors under pathological conditions is unknown, although they are believed to be a prerequisite for reinnervation (Gordon et al., 1976).

The denervation of biventer cervicis muscle in PSP-treated hens, as indicated by SDC and responses to ACh, is

supported by the histopathological observations for all experimental groups. Peripheral nerve lesions were qualitatively similar to those reported previously in studies of this compound (Jortner and Ehrich, 1987) Damage was more extensive in nerves from hens given 6 and 10 mg/kg PSP, an observation consistent with the clinical scores and responses to nerve stimulation for these two groups.

The present study, therefore, describes a nerve which is subject to OPIDN that can be used as an in vitro nerve-muscle preparation allowing for the differentiation and evaluation of neuromuscular function in both slow and fast muscle fibers. Its usefulness in evaluation of nerve and muscle function during OPIDN was demonstrated by dose-related responses that were noted in preparations from hens given PSP.

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REFERENCES

ABOU-DONIA, M.B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-548.

ABOU-DONIA, M.B., TROFFATER, L.P., GRAHAM, D.G. AND LAPDULA, D.M. (1986). Electromyographic, neuropathologic, and functional correlates in the cat as the result of tri-o-cresyl phosphate delayed neurotoxicity. Toxicol. Appl. Pharmacol. 83:126-141.

ALMON, R.R. AND APPEL, S.H. (1976). Cholinergic sites in skeletal muscle. I. Denervation effects. Biochem. J. 15: 3662-3667.

AXELSSON, J. AND THESLEFF, S. (1957). A study of super-sensitivity in denervated mammalian skeletal muscle. J. Physiol. 149:. 178-193.

BARNARD, E.A., LYLES, J.M. AND PIZZEY, J.A. (1982). Fiber types in chicken skeletal muscles and their changes in muscular dystrophy. J. Physiol. 331: 333-354.

BICKFORD, A.A. (1984). The hen as a test animal for delayed neurotoxicity studies: Introductory comments. In: Delayed Neurotoxicity (J.M. Cranmer, E.J Hixson, Eds.) pp 24-25, Intox Press, Little Rock.

CAVANAGH, J.B. (1954). The toxic effects of tri-ortho-cresyl-phosphate on the nervous system. An experimental study in hens. J. Neurol. Neurosurg. Psychiat. 17: 163-172.

CHANG, C.C. AND TUNG, L.H. (1974). Inhibition by actinomycin D of the generation of acetylcholine receptors induced by denervation in skeletal muscle. Eur. J. Pharmacol. 26: 386,388.

CHILD, K.J. AND ZAIMIS, E. (1960). A new biological method for the assay of depolarizing substances using the isolated semispinalis muscle of the chick. Brit. J. Pharmacol. 15: 412-416.

CISSON, C.M. AND WILSON, B.W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity. Altered acetylcholinesterase molecular forms and increased plasma creatine kinase. Toxicol. Appl. Pharmacol. 64: 289-305.

DAVIS, C.S. AND RICHARDSON, R.J. (1980). Organophosphorus compounds. In: Experimental and Clinical Neurotoxicity (P.S. Spencer, H.H. Schaumburg, Eds.) pp 527-544, William and Wilkins, Baltimore.

DRAKONTIDES, A.B. AND BAKER, T. (1983). An electrophysiologic and ultrastructural study of the phenylmethanesulfonyl fluoride protection against a delayed organophosphorus neuropathy. Toxicol. Appl. Pharmacol. 70: 411-422.

DURHAM, H.D. AND ECOBICHON, D.J. (1984). The function of motor nerves innervating slow tonic skeletal muscle in hens with delayed neuropathy induced by tri-o-tolyl phosphate. Can. J. Physiol. Pharmacol. 62: 1268-1273.

ETO, M., CASIDA, J.E. AND ETO, T. (1962). Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11: 337-351.

GINSBORG, B.L. (1960). Some properties of avian skeletal muscle fibres with multiple neuromuscular junctions. J. Physiol. 154: 581-598.

GINSBORG, B.L. AND WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. Brit. J. Pharmacol. 15: 410-411.

GORDON, T., JONES, R. AND VRBOVA, G. (1976). Changes in chemosensitivity of skeletal muscles as related to endplate formation. Prog. Neurobiol. 6:103-136.

GRAMPP, W., HARRIS, J.B. AND THESLEFF, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221: 743-754.

GUYTON, A.C. (1986). Contraction of skeletal muscle. In: Textbook of Medical Physiology. pp 120-135 W.B. Saunders Co., Philadelphia.

HARRIS, R. (1971). Chronaxy. In: Electrodiagnosis and Electromyography, (S Licht Ed.) pp 218-239, Elizabeth Licht. New Haven.

HARTZELL, H.C. AND FAMBROUGH, D.M. (1972). Acetylcholine receptors; distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J. Gen. Physiol. 50: 248-262.

HARVEY, A.L. AND MARSHALL, I.G. (1986). Muscle. In: Avian Physiology, (P.D. Sturkie Ed.) pp 74-86, Springer-Verlag, New York.

HARVEY, A.L. AND VAN HELDEN, D. (1981). Acetylcholine receptors in singly and multiply innervated skeletal muscle fibers in the chicken during development. J. Physiol. 317: 397-411.

HECKMAN, J.R. (1972). Excitability curve: a new technique for assessing human peripheral nerve excitability in vivo. Neurology 22:224-230.

HESS, A. (1961). Structural differences of fast and slow extrafusal muscle fibers and their nerve endings in chickens. J. Physiol. 157: 221-231.

JOHNSON, M.K. (1982). The target for initiation of delayed neurotoxicity by organophosphorus esters. Biochemical studies and toxicological applications. In: Reviews in Biochemical Toxicology, Vol 4, (E. Hodgson, J.R. Bend, R.M. Philpot Eds.) pp 141-212, Elsevier Biomedical Publishers, New York.

JORTNER, B.S AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

KANDEL, E.R. (1985). Synapse formation, trophic interactions between neurons, and the development of behavior. In: Principles of Neural Science. (E.R. Kandel and J.H. Schwartz Eds.) pp 743-756, Elsevier Publishing Co. New York.

LAPADULA, D.M., KINNES, C.G., SOMJEN, G.G. AND ABOU-DONIA, M.B. (1982). Monosynaptic reflex depression in cats with organophosphorus neuropathy: effects of tri-o-cresyl phosphate. NeuroToxicology 3: 51-62.

LINDSLEY, D.F. AND HOLMES, J.E. (1984). Basic Human Neurophysiology. pp 124-144, Elsevier Publishing. New York.

LOMO, T., ROSENTHAL, J. (1972). Control of ACh sensitivity by muscle activity in the rat. J. Physiol. 221: 493-513.

LOWNDES, H.E., BAKER, T., RIKER, F. (1974). Motor nerve dysfunction in delayed DFP neuropathy. Eur. J. Pharmacol. 29:66-73.

MARSHALL, I.G. (1971). Actions of acetylcholine and carbachol on the chick biventer cervicis muscle. Brit. J. Pharmacol. 42: 462-472.

MCARDLE, J.J. (1983). Molecular aspects of the trophic influence of nerve on muscle. Prog. Neurobiol. 21: 135-198.

METCALF, R.L. (1984). Historical perspective of organophosphorus ester-induced delayed neurotoxicity. In: Delayed Neurotoxicity. (J.M. Cranmer, E.J. Hixson Eds.) pp 7-2, Intox Press, Little Rock.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. J Physiol 151: 1-23.

MURPHY, S.D. (1986). Pesticides. In: Casarett and Doull's Toxicology, The Basic Science of Poisons, 3rd ed. (J.J. Doull, C.D. Klaasen, M.O. Andur Eds.) pp 519-581, Macmillan Publishing Co., New York.

PRENTICE, D.E. AND ROBERTS, N.L. (1984). Acute delayed neurotoxicity in hens dosed with tri-ortho-cresyl phosphate (TOCP): correlation between clinical ataxia and neuropathological findings. In: Delayed Neurotoxicity. (J.M. Cranmer, E.J. Hixson Eds.) pp 139-144, Intox Press, Little Rock.

ROBERTSON, D.G., SCHWAB, B.W., SILLS, R.D., RICHARDSON, R.J. AND ANDERSON, R.J. (1987). Electrophysiologic changes following treatment with organophosphorus-induced neuropathy-producing agents in adult hens. Toxicol. Appl. Pharmacol. 87: 420-429.

SPRAGUE, G.L., SANDVICK, L.L., BICKFORD, A.A. AND CASTLES, T.R. (1980). Evaluation of a sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. Life. Sci. 27: 2523-2528.

WYNN, PARRY CB. (1971). Strength-duration curves. In: Electrodiagnosis and Electromyography. (S. Licht Ed.) pp 241-271, Elizabeth Licht. New Haven.

TABLE 1. ELECTROPHYSIOLOGICAL PARAMETERS DERIVED FROM STRENGTH-DURATION CURVES FOLLOWING PSP ADMINISTRATION

Group	Chronaxie[a] (msec)	Rheobase[b] (volts)
Controls	0.14 ± .011	1.36 ± .07
PSP 2 mg/kg	0.15 ± .059	3.52 ± .40[c]
PSP 6 mg/kg	0.06 ± .002[c]	4.56 ± .22[c]
PSP 10 mg/kg	0.06 ± .002[c]	4.35 ± .29[c]

[a]Chronaxie derived from individual strength-duration curves, $\bar{x}$ ± S.E., n = 4 - 5.

[b]Rheobase derived from Figure 3, $\bar{x}$ ± S.E., n = 4 - 5

[c]Significantly different from control values, $p < 0.05$, Newman-Keuls test for multiple comparisons.

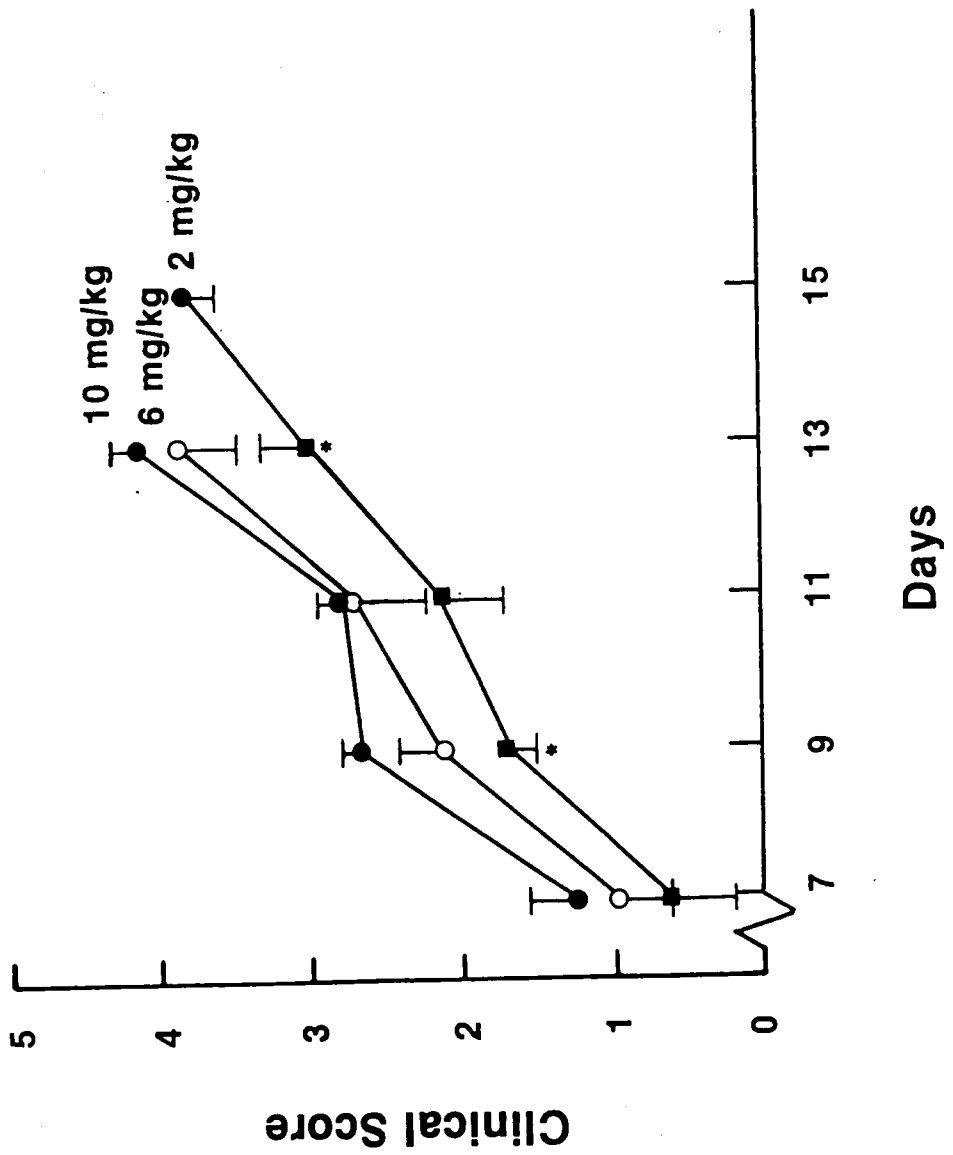

10 mg/kg
6 mg/kg
2 mg/kg
Clinical Score
Days
0
1
2
3
4
5
7
9
11
13
15
*
*

Figure 1. Development of clinical signs in hens after administration of phenyl saligenin phosphate (PSP). Results are presented as mean ± S.D., n = 4 - 5. Significant differences between 2 mg/kg and the other two groups of hens from PSP are indicated by asterisks. Increasing clinical scores reflect progression in deficits as detailed in methods.

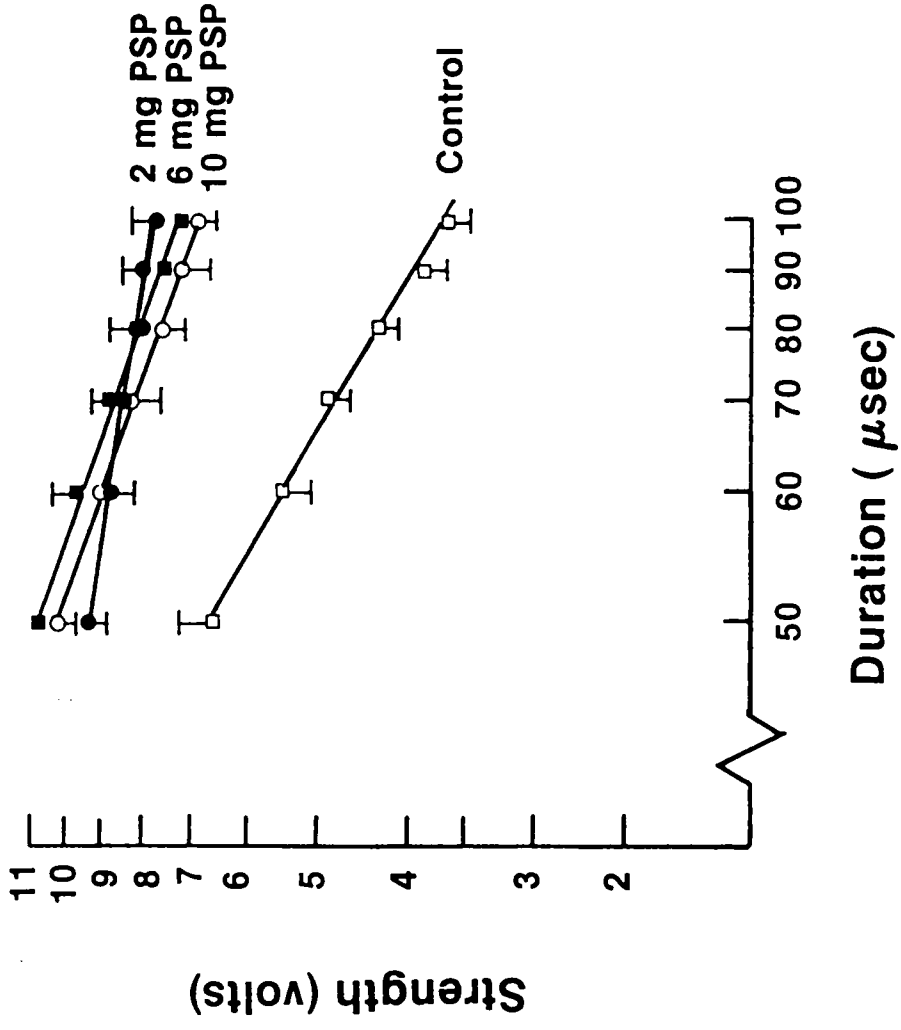

2 mg PSP
6 mg PSP
10 mg PSP
Control
Strength (volts)
11
10
9
8
7
6
5
4
3
2
50
60
70
80
90
100
Duration (μsec)

Figure 2. Log-log plot of the inflexion region (50-100 μsec) of strength-duration curves from biventer cervicis nerve-muscle preparation following PSP treatment. Preparations from PSP-treated hens required a higher threshold for stimulation. All points represent the mean ± S.E., ($n = 4 - 5$). Results were examined for statistical differences, indicated by an analysis of variance and Newman-Keuls test for multiple comparison ($p < 0.05$). All PSP-treated groups were different from control, but not different from each other.

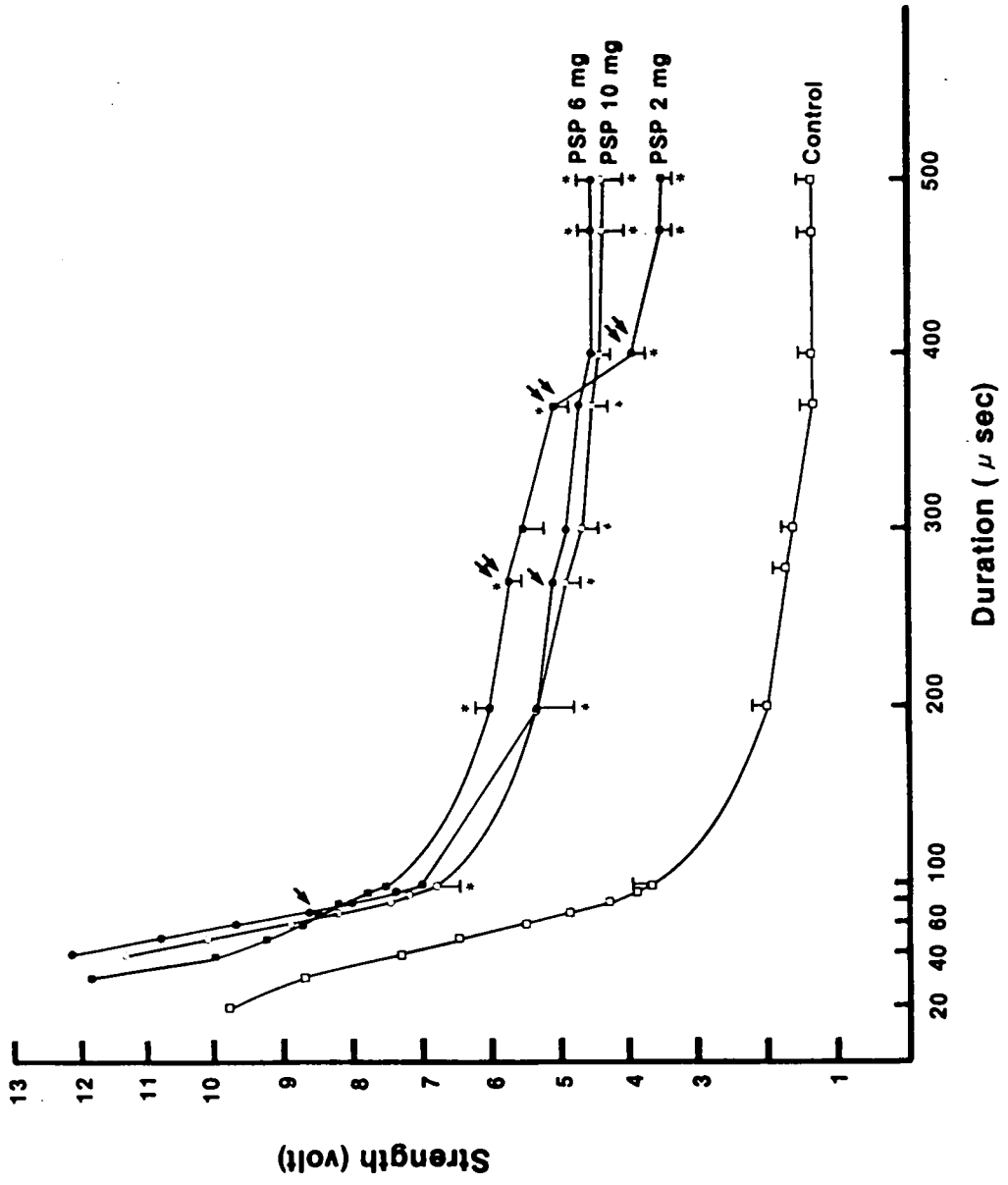

PSP 6 mg
PSP 10 mg
PSP 2 mg
Control
Strength (volt)
Duration (μ sec)
13
12
11
10
9
8
7
6
5
4
3
1
20
40
60
100
200
300
400
500

Figure 3. Strength-duration curves for rectangular pulses on the biventer cervicis nerve-muscle preparations from controls and PSP-treated hens. "Kinks" (discontinuities) indicative of partial denervation are indicated by single arrows (6 and 10 mg/kg) and by double arrows (2 mg/kg). All points represent the mean ± S.E., (n = 4-5). Results were examined for statistical differences by an analysis of variance. Points at which PSP-treated groups were significantly different from control are noted by asterisks (Newman-Keuls test for multiple comparisons, $p < 0.05$).

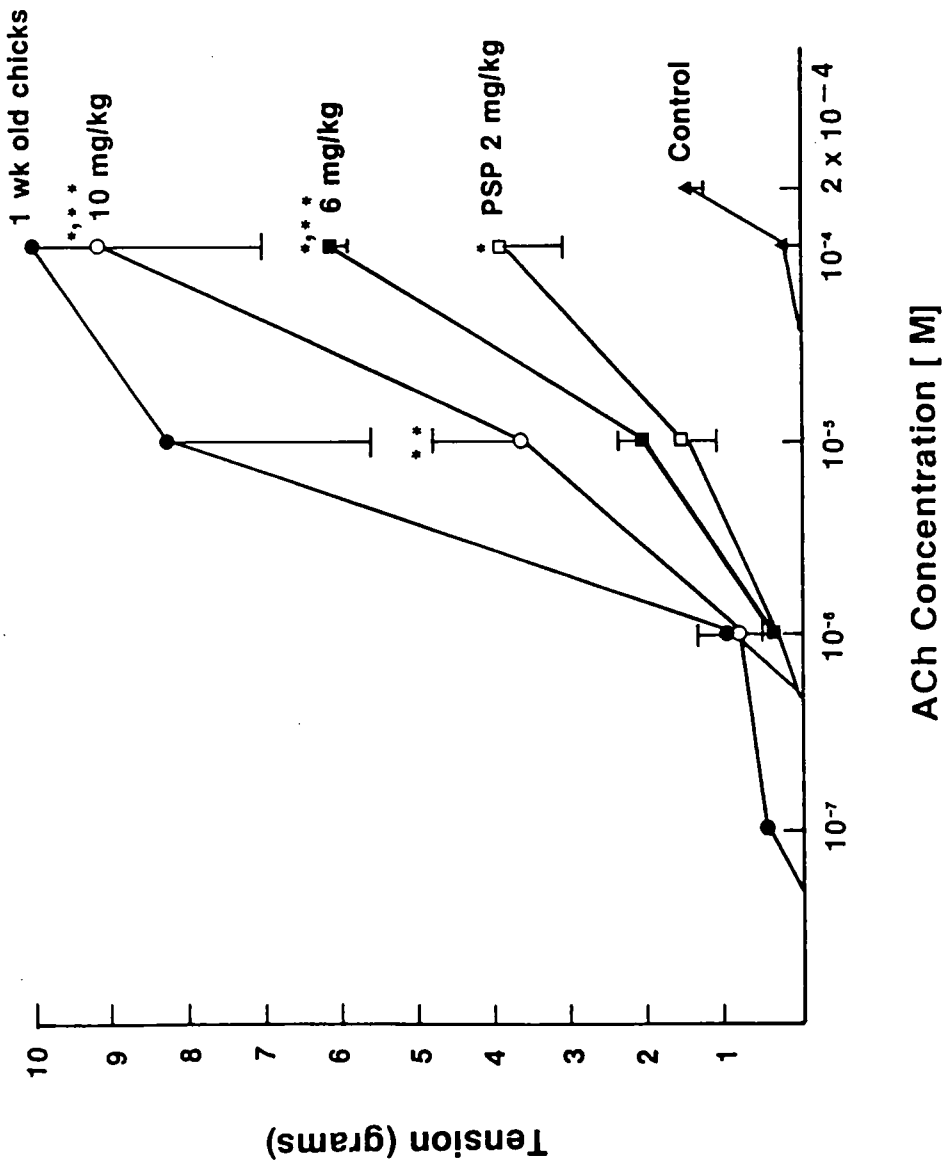
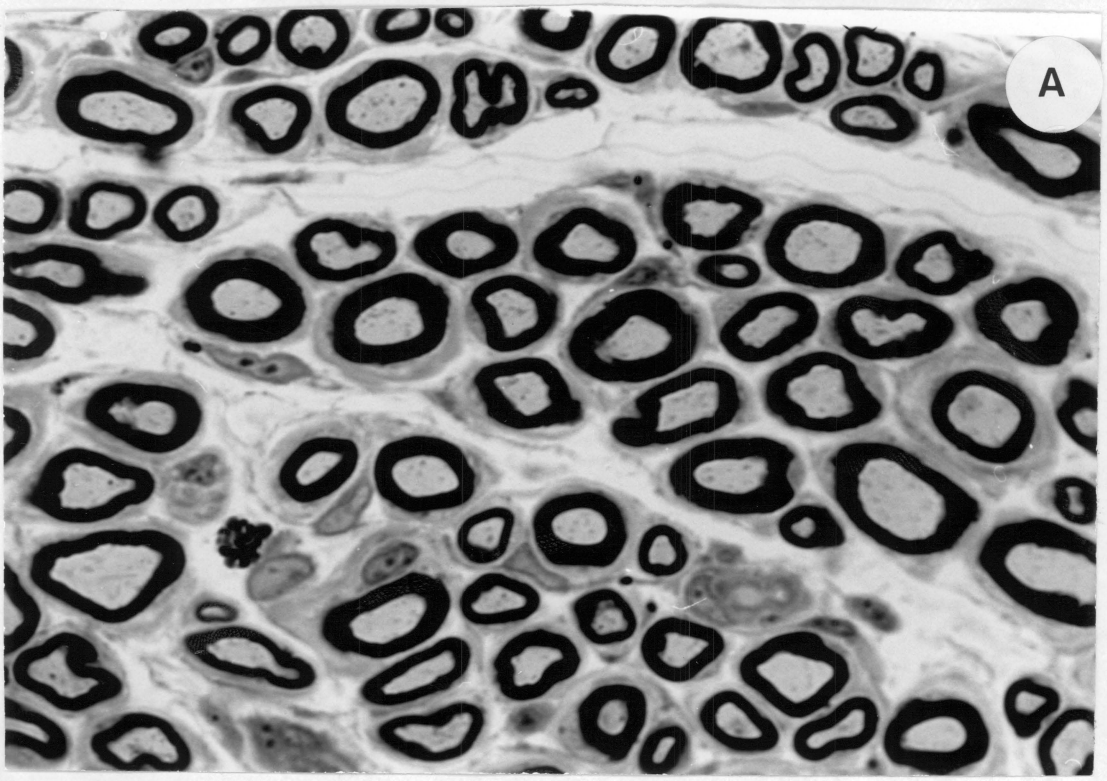
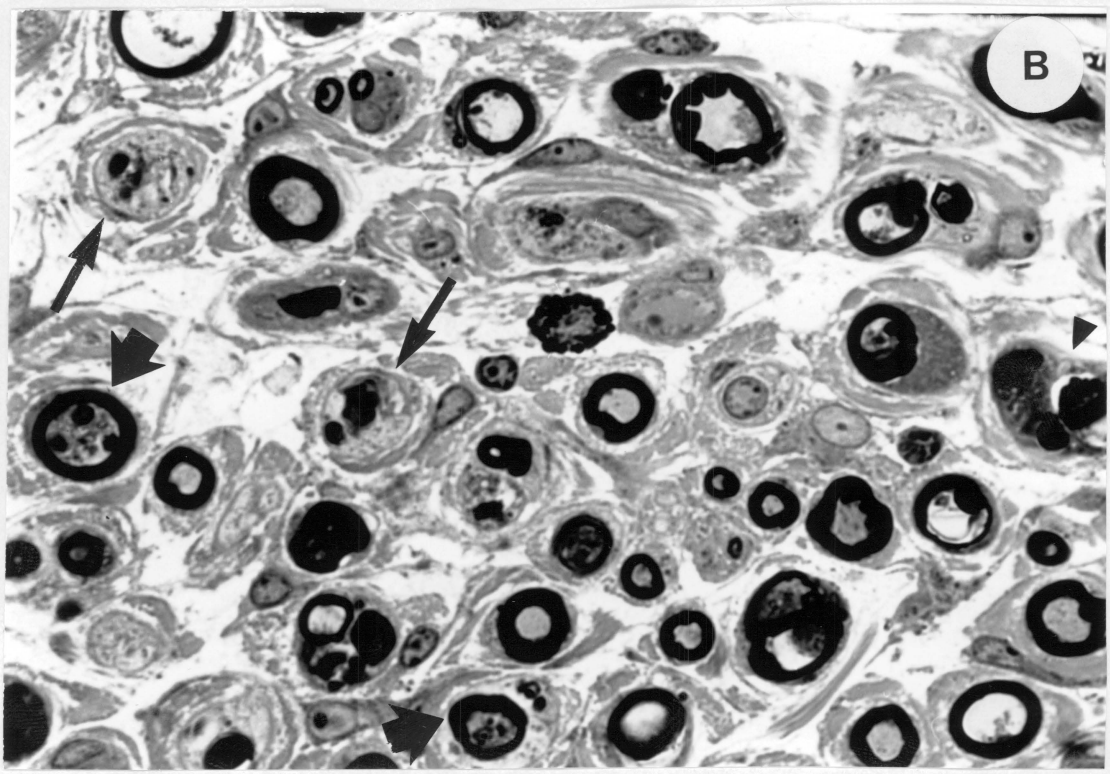

1 wk old chicks
, * 10 mg/kg
, * 6 mg/kg
* PSP 2 mg/kg
Control
**
Tension (grams)
ACh Concentration [M]
10
9
8
7
6
5
4
3
2
1
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
2 x 10⁻⁴

Figure 4. Tension generation in the biventer cervicis nerve-muscle preparation in response to ACh from 1-week-old chicks, control and PSP-treated hens. All points represent the mean ± S.E., (n = 4 - 7). Points significantly different from controls are identified by asterisks, as determined by an analysis of variance and Newman-Keuls test for multiple comparisons ($p < 0.05$). Double asterisks indicate significant differences between preparations given 2 mg/kg PSP and those given 6 or 10 mg/kg PSP.


A


B

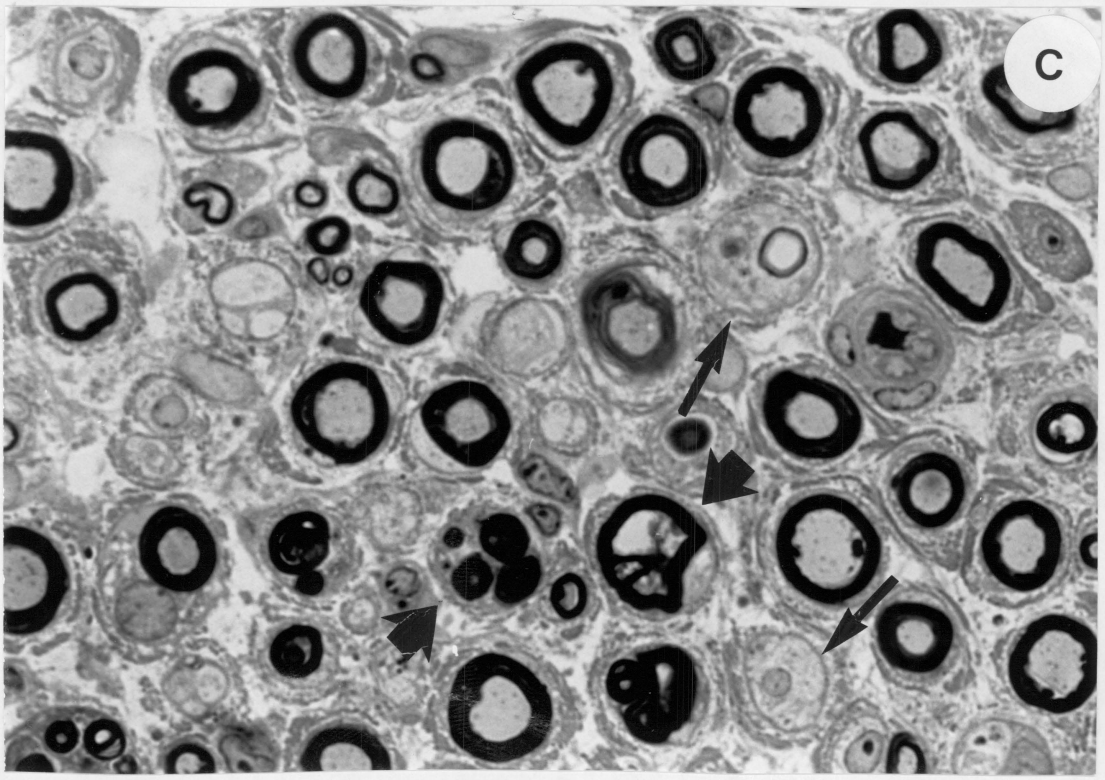

C

Figure 5. Cross-sections of distal region of nerve to biventer cervicis muscle (x 1120). Figure 5a = untreated control showing myelinated nerve fibers; Figure 5b = from hen given 2 mg/kg PSP 15 days earlier. Note the large myelinated fibers in various stages of degeneration, including the presence of intra-axonal densities (large arrows) and complete fiber breakdown (arrowheads). Schwann cell tubes devoid of intact axons, but residual debris are also seen (small arrows); Figure 5c = from hen given 10 mg/kg PSP 13 days earlier. In contrast to Figure 5b, fewer actively degenerating fibers are present (arrows), and Schwann cell tubes are more prominent (small arrows). Toluidine blue safranin stain.

pbh

4ME.1

CHAPTER 6

USE OF THE BIVENTER CERVICIS NERVE-MUSCLE PREPARATION TO DETECT EARLY CHANGES FOLLOWING EXPOSURE TO ORGANOPHOSPHATES INDUCING DELAYED NEUROPATHY.

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ABSTRACT

Indicies of organophosphorus-induced delayed neuropathy (OPIDN) in the hen model have traditionally been restricted to the early inhibition of neuropathy target esterase (NTE), which soon returns to control levels, and ataxia with associated pathological changes in hind limb peripheral nerve or spinal cord which occur more than 7 days after OP exposure. The biventer cervicis nerve-muscle preparation was used to evaluate OPIDN in adult hens that had been treated with either the protoxicant tri-o-tolyl phosphate (TOTP), 360 mg/kg po, or the active congener phenyl saligenin phosphate (PSP), 2.5 mg/kg im. Hens were sacrificed 2, 4, 7, 10, 15, 21, and 37 days later. NTE activity was 20 and 45% of control for TOTP and PSP, respectively, 4 days after administration. Clinical signs were notable by 10 days and progressed in severity to paralysis by 21 days. Partial clinical recovery was evident at 37 days. Denervation hypersensitivity of biventer cervicis muscle to acetylcholine (ACh) was evident as early as 4 days following TOTP or PSP treatment. The sensitivity to ACh was greatest 21 days after OP administration, with partial recovery at 37 days. Strength-duration curves (SDC) showed an increase in excitability thresholds and elevated rheobase with shorter chronaxie than controls as early as 4 days following treatment with either compound. SDC at 37 days indicated partial reinnervation. The pattern of nerve degeneration and regeneration was fur-

ther apparent on histopathological examination. This study suggests that the biventer cervicis nerve-muscle preparation may prove useful for detection of functional and morphological changes that occur during the interval between NTE inhibition and appearance of clinical deficits.

INTRODUCTION

Organophosphorus compounds (OPs) are widely used in agriculture as pesticides and in industry as petroleum additives and modifiers of plastics. Some of these compounds have the potential to induce a delayed neuropathy in humans and other susceptible animal species following a single exposure (Abou-Donia, 1981; Murphy, 1986). The adult chicken is generally recognized as providing the most reliable animal model among species susceptible to organophosphorus-induced delayed neuropathy (OPIDN) (Davis and Richardson, 1980; Abou-Donia, 1981; Bickford, 1984). Traditional descriptions of early events in OPIDN have relied on initial inhibition of a neural esterase (neuropathy target esterase or neurotoxic esterase, NTE). This enzyme has been proposed to be the initial molecular site of action of neurotoxic OPs because reductions of 70-80% in NTE activity are predictive of OPIDN (Richardson, 1984). However, although NTE activity is initially inhibited, enzyme activity recovers and may be comparable to those in controls 7 or more days prior to onset of ataxia (Abou-Donia, 1981; Davis et al., 1985; Jortner and Ehrich, 1987). In susceptible species, the clinical signs appearing following the delay period include incoordination and ataxia, which progress to a flaccid paralysis. Initially the hind limbs are affected, but neuropathy may occur in the forelimbs in severe cases (Johnson, 1975; Davis and Richardson, 1980; Abou-

Donia, 1981). Histological examination up to 10 days following single exposure to neuropathy-inducing organophosphorus esters indicates axonal degeneration of long fibers and a secondary degeneration of myelin and muscle atrophy (Cavanagh, 1964; Prentice and Roberts, 1984; Cisson and Wilson, 1982; Jortner and Ehrich, 1987).

Until recently, few studies have addressed events which may occur between the initial event, NTE inhibition, and the development of the clinical and morphological deficits 7 to 14 days later. For example, most electrophysiological evaluations have been performed at periods when neuropathy was well developed (Lowndes et al., 1974; Baker et al., 1977; Lapadula et al., 1982; Durham and Ecobichon, 1984; Robertson et al., 1987). In previous studies we found the biventer cervicis nerve-muscle preparation could be used to detect electrophysiological and pharmacological changes in adult hens with OPIDN (El-Fawal et al., 1988). The present study uses this preparation to determine if it has value in detecting changes that occur prior to and during the course of clinical and pathological deficits of OPIDN.

MATERIALS AND METHODS

Animals and housing. White leghorn hens (> 6 months old, 1.2-1.8 kg) used in this study were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. Chickens were housed in groups of 4-5 with access to food and water ad libitum. Hens were divided according to treatment [control, tri-ortho tolyl phosphate (TOTP) and phenyl saligenin phosphate (PSP)], with 35 hens in each treatment group.

Dosing. Protoxicant tri-ortho-tolyl phosphate (TOTP, Eastman Chemical Co., Rochester, N.Y.) was dissolved in corn oil such that the dosage of 360 mg/kg could be administered at 1 ml/kg oral gavage. PSP, an active prototype of the cyclic saligenin phosphates, toxic metabolites of TOTP (Eto et al., 1962), was synthesized as previously described (Jortner and Ehrich, 1987) and dissolved in dimethyl sulfoxide (DMSO) so that the 2.5 mg/kg dosage was available in 0.25 ml/kg and administered into the breast muscle. Dosages for TOTP and PSP were based on previous experience in our laboratory (Ehrich and Gross, 1982; Jortner and Ehrich, 1987).

Clinical signs. Hens were observed daily for clinical signs. Independent blind assessment by at least three individuals were recorded and averaged. Scores were designated as 0 for normal, 1 for altered gait, 2 for difficulty in walking and standing, 3 for severe ataxia, 4 for leg paraly-

sis, and 5 for paralysis with both leg and wing involvement (Sprague et al., 1980).

Electrophysiological and pharmacological studies. These methods for evaluating the effects of OPIDN using the biventer cervicis nerve have been previously described (El-Fawal et al., 1989). Evaluations were performed on days 2, 4, 7, 10, 15, 21 and 37 following exposure to either OP. The biventer cervicis nerve-muscle was removed after hens were killed by iv injection of T-61 solution (Hoescht Corp., Somerville, N.J.). Incision along the midline from the skull to below the base of the midline revealed the twin biventer cervicis nerve-muscle. A suture was tied to the proximal end of the nerve tendon prior to detachment from the skull. Retracting the suture, the preparation was separated from the underlying semispinalis muscle. The muscle was anchored at the bottom of a 150 ml organ bath (custom made) and the tendon in which the nerve is ensheathed passed through a custom made version of the biventer cervices electrode. The tendon was attached by the suture at the proximal end to a Model FTO3 force displacement isometric transducer, leading to a coupler-amplifier-recorder system (Grass Medical Instruments, Quincy, MA). Two grams of resting tension was placed on the muscle, as this was deremined by pilot studies to be optimal to ensure uniformity of isometric contraction. The organ bath contained Krebs-Henseleit solution maintained at 37^{o} C and aerated with a 95% O_2-5% CO_2 mixture.

The terminals of the biventer cervicis bipolar electrode

were connected to a square pulse stimulator (Grass Medical Instruments). Strength-duration curves (SDC) were constructed by measuring the voltage required to elicit a minimally perceptible response at a durations of 20 to 500 μsec. Rheobase and chronaxie were derived from these curves. In addition, the region of inflexion (40-100 μsec) was linearized by a log-log plot of both the ordinate and absicissa as described by Robertson et al. (1987).

Log concentration-response curves in response to acetylcholine chloride (ACh, Sigma Chemical Co., St.Louis, MO) were established by plotting cumulative concentrations of ACh against gram-tension developed by the muscle. Stock solutions of ACh (10^{-1}M) were prepared in distilled water on the day of the experiment. Subsequent serial molar concentrations (final bath concentrations of 10^{-11} to 10^{-3}M) were prepared by dilution in Krebs-Henseleit solution.

Histology. The distal region (lying over the belly of the muscle) of nerve supplying the twin biventer cervices was dissected away from the freshly removed specimen and placed in a fixative of the following composition: 2.5% glutaraldehyde in 0.05M sodium coacodylate at pH 7.4. Following a period of 24-48 hr of fixation at 4^{o} C, segments of the nerve were rinsed in buffer, post-fixed in 2% osmium tetroxide and embedded in Epon epoxy resin. Cross sections were cut at one um thickness, stained with toluidine blue and safranin, and examined by light microscopy.

Statistics. Except in the case of clinical scores, where the Student t-test was used, all other data was analyzed by analysis of variance with the Newman-Keuls test of multiple comparisons for determination of statistical differences between control and experimental groups. Differences were considered significant when $p < 0.05$. All data are expressed as means $\pm$ standard error.

RESULTS

Clinical signs of OPIDN were evident in all OP-treated chickens 10 days after administeration of either TOTP or PSP. OPIDN progressed in severity over the next several days (Fig. 1). By day 15 post exposure clinical signs began to stabilize for both treatment groups. Hens began to recover from clinical signs of TOTP treatment after 18 days and from PSP treatment after 20 days. By 30 days following treatment clinical signs in both groups began to stabilize and remained essentially unchanged until the experiment was terminated on day 37.

Strength-duration curves for the biventer cervicis nerve-muscle preparations indicated an elevation of threshold excitability as early as 4 days following administration of either TOTP or PSP (Fig. 2a and b). Elevation of thresholds continued until days 15 and 21 for TOTP- and PSP-treated hens, respectively. The shape of the curves for preparations from hens treated with either TOTP at days 4, 7, 10, and 37 or PSP show kinks or discontinuities (Fig. 3a and b).

Rheobase values for preparations from hens treated with either TOTP or PSP were significantly higher than controls. Chronaxie values were significantly shorter than controls for nerves from hens treated with either compound (Table 1).

Log concentration-response-curves showing tension development of the biventer cervicis muscle preparations on addition of exogenous ACh are presented in Fig. 4a and b.

Thresholds and maximum tension developed are summarized in Table 1. Responses seen at day 2 post-exposure were not different from control. Four days after OP treatment preparations from chickens treated with TOTP were 5X more sensitive to ACh as controls, whereas those from PSP-treated hens were 10X more sensitive. The increase in sensitivity progressed in both experimental groups until 21 days post exposure, becoming 10^6X more sensitive than control preparations. By 37 days post-exposure sensitivity to ACh in experimental groups was again 5X more sensitive than controls in biventer muscles from OP-treated hens. Contractile-tension development was significantly higher than in control muscle at all days tested, reaching a maximum at day 4 and 7 for the TOTP-and PSP-treated groups, respectively.

Light microscopic examination of the distal portion of the nerve to the biventer cervicis muscle 4 days following treatment with TOTP showed no obvious lesions. However, nerves from the PSP-treated group showed a few fibers undergoing degeneration 4 days after administration (Fig. 5a and b). By 7 days after administration, nerves from hens given both TOTP and PSP showed lesions, but they were more pronounced in hens given PSP (Fig. 5c, d, and e). By day 10 nerves from hens treated with either compound showed significant progressive fiber degeneration and by day 15 few intact fibers were noted, especially in the group treated with PSP (Fig. 5f and g). Although a paucity of intact fibers and many empty Schwann cell tubes were noted by day 21,

regenerating myelinated fibers were evident in sections viewed at higher power (Fig. 5h and i). Thirty-seven days following exposure to either TOTP or PSP many thinly myelinated and nonmyelinated regenerating axons were seen (Fig. 5j and k).

The intensity and stage of lesion development in the biventer cervicis nerve varied between the time intervals at which nerves were taken but all stages were well represented. These ranged from axonal swelling at the onset to pallor of staining with associated intra-axonal debris in many cases as degeneration progressed, as well as the thinning and fragmentation of the myelin sheath. More advanced changes were evidenced by "empty" Schwann cells, representing axons fully degenerated at the levels of examination. In later stages (21 days) occasional regenerating, but not yet myelinated or thinly myelinated, axons were recognized in such Schwann cells. Myelination of these regenerating fibers was apparently more advanced at 37 days, although fiber diameter was qualitatively less than before.

DISCUSSION

This study demonstrated that administration of two OPs inducing delayed neuropathy significantly changed the electrophysiological and pharmacological parameters evaluated using the biventer cervicis nerve-muscle preparation before the appearance of clinical signs. The protoxicant, TOTP, and the active congener, PSP, had pronounced effects on strength-duration curves (SDC), a parameter which indicates the function of nerves innervating fast-twitch muscle fibers (El-Fawal et al., 1988). The shapes of the SDC for the two treatment groups showed discontinuities, most prominently in preparations from the early time-points (days 4, 7, and 10). The presence of these "kinks" or discontinuities is generally interpreted as signifying a state of partial innervation (i.e. occurrence of denervation or re-innervation)(Wynn Parry, 1971; Heckman, 1972). Consistent with this is the reappearance of these discontinuities at day 37 as reinnervation occurs. The greater shift in the SDC at later time intervals (days 15 and 21) suggests that denervation is further advanced than at the early intervals. Rheobase values were significantly higher in experimental groups than in control preparations, regardless of the compound used or the time interval examined, while chronaxie values were significantly shorter. The elevation of rheobase and shortening of chronaxie is consistent with nerve degeneration (Harris, 1971; Wynn Parry, 1971) and denervation associated with

OPIDN seen in an earlier report from our laboratory (El-Fawal et al., 1988). Recovery from OPIDN, evident at day 37, caused a drop in the rheobase both when TOTP and PSP were given. The inflexion region of SDC, which represents the excitability threshold, further confirmed the occurrence of functional deficits early after TOTP and PSP treatments. Our studies confirm previous studies suggesting SDC as an early indicator of OPIDN (Robertson et al., 1987; Anderson et al., 1988). Results with TOTP and PSP contrast to those with di-n-butyl-2,2-dichlorovinyl phosphate (DBCV), as DBCV studies showed elevated thresholds 24 hr after exposure followed by partial recovery by days 7 and 14 (Robertson et al., 1988). Further differences between OPIDN caused by TOTP and PSP and OPIDN caused by DBCV existed because DBCV significantly altered rheobase and chronaxie values 21 days after administration (Robertson et al., 1988). The discrepancy between the two studies may reflect differences between the compounds or in the sensitivities of the <u>in vitro</u> preparation used in the present study and that done by Robertson et al. (1988) using the sciatic and tibial nerves.

Denervation hypersensitivity to ACh has been previously described for the biventer cervicis nerve-muscle preparation during OPIDN (El-Fawal et al., 1988; 1989). In the present study we demonstrated that sensitivity to ACh, which reflects the deficits occurring in the innervation of slow-tonic postural muscle, can be detected as early as 4 days following exposure to neurotoxic OPs. The progressive

increase in denervation hypersensitivity to the neurotransmitter was consistent with the progressive severity of clinical signs begining day 10 and the partial recovery noted by day 37. The development of denervation hypersensitivity to ACh is believed to account for muscle fibrillation, while shortening of these postural muscle would explain contorted positions common in denervation (Guyton, 1986).

The course of electrophysiological and pharmacological changes reported here were closely mirrored in the histopathological changes seen early after TOTP and PSP treatments. Peripheral nerve lesions were qualitatively similar to those reported previously (Jortner and Ehrich, 1987), but previous studies did not examine nerve damages as early as in the present experiments. Moreover, previous studies did not examine early histopathological damage to the biventer cervicis nerve (Cavanagh, 1964; Jortner, 1984; El-Fawal et al., 1988).

The electrophysiological changes occuring as early as 4 days following exposure to neurotoxic OPs are consistent with Wynn-Parry's (1971) statement that the SDC may indicate changes within 4 to 5 days of trauma. The development of denervation hypersensitivity and its detection at day 4 following administration of PSP or TOTP could correlate with increased de novo synthesis of ACh receptors within 3 days of denervation (Almon and Appel, 1976). The present study, therefore, suggests that functional denervation may begin before morphological denervation. Loss of nerve influence

on muscle could explain development of denervation hypersensitivity (Lomo and Rosenthal, 1972; McArdle, 1983). This appeared especially evident in the biventer cervicis nerve. The biventer cervicis also appears to provide a sensitive nerve for detection of early histopathological changes. All early functional and morphological changes seen in the biventer cervicis nerve occurred prior to the appearance of clinical deficits and later than the most significant inhibition of NTE (Davis and Richardson, 1980). This study demonstrates that SDC and sensitivity to ACh in the biventer cervicis nerve-muscle preparation may be used as sensitive and earlier indicators of OPIDN.

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REFERENCES

ABOU-DONIA, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-148.

ALMON, R. R. AND APPEL, S. H. (1976). Cholinergic sites in skeletal muscle. I. Denervation effects. Biochem. 13: 3662-3667.

ANDERSON, R. J., ROBERTSON, D. G., HENDERSON, J. D. AND WILSON, B. W. (1988). DFP-induced elevation of strength-duration threshold in hen peripheral nerve. NeuroToxicology. 9: 47-52.

AXELSSON, J. AND THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. 147: 178-193.

BAKER, T., GLAZER, E. AND LOWNDES, H. E. (1977). Subacute neuropathy effects of diisopropylfluorophosphate at the cat soleus neuromuscular junction. Neuropath. Appl. Neurobiol. 3: 377-390.

BICKFORD, A. A. (1984). The hen as a test model for delayed neurotoxicity studies: introductory comments. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 24-25 Intox Press, Little Rock.

CAVANAGH, J. B. (1964). Peripheral nerve changes in orthocresyl phosphate poisoning in the cat. J. Pathol. Bact. 87: 365-383.

CAVANAGH, J. B. (1985). Peripheral nervous system toxicity: a morphological approach. In Neurotoxicology (K. Blum and L. Manzo, Eds.), pp. 1-44 Marcel Dekker, Inc., New York.

CISSON, C. M. AND WILSON, B. W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity: altered acetylcholinesterase molecular forms and increased plasma creatine phosphokinase activity. Toxicol. Appl. Pharmacol. 64: 289-305.

DAVIS, C. S. AND RICHARDSON, R. J. (1980). Organophosohorus compounds. In Experimental and Clinical Neurotoxicology (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527-544 Williams and Wilkins, Baltimore.

DAVIS, C. S., JOHNSON, M. K. AND RICHARDSON, R. J. (1985). Organophosphorus compounds. In Neurotoxicity of Industrial and Commercial Chemicals (J. L. O'Donoghue, Ed.),

pp. 1-23 CRC Press, Fl.

DURHAM, H. D. AND ECOBICHON, D. J. (1984). The function of motor nerves innervating slow tonic skeletal muscle in hens with delayed neuropathy induced by tri-o-tolyl phosphate. Can. J. Physiol. Pharmacol. 62: 1268-1273.

EHRICH, M. AND GROSS, W. B. (1982). Effect of corticosterone on toxicity of malathion and TOTP in hens. Neurobehav. Toxicol. Teratol. 4: 789-792.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1988). The biventer cervicis nerve-muscle preparation of adult hens: Effects of phenyl saligenin phosphate administration. NeuroToxicology 9: 625-636.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989). Effect of verapamil on organophosphate-induced delayed neuropathy (OPIDN) in hens. Toxicol. Appl. Pharmacol. 97: 500-511.

ETO, M., CASIDA, J. E. AND ETO, T. (1962). Hydroxylation and cyclization reactions in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11: 337-352.

GUYTON, A. C. (1986). Contraction of skeletal muscle. In Textbook of Medical Physiology, pp. 120-135. W. B. Saunders, Philadelphia.

HARRIS, R. (1971). Chronaxy. In Electrodiadnosis and Electromyography (S. Licht, Ed.), pp. 218-239. Elizabeth Licht, New Haven.

HECKMAN, J. R. (1972). Excitability curve: a new technique for assessing human peripheral nerve excitability in vivo. Neurology 22: 224-230.

JOHNSON, M. K. (1975). Organophosphorus esters causing delayed neurotoxic effects. Arch. Toxicol. 34: 259-288.

JORTNER, B. S. (1984). Pathology of organophosphorus-induced delayed neurotoxicity. In Proceedings of the Fourteenth Conference on Environmental Toxicology (J. D. MacEwan and E. H. Vernot, Eds.), pp. 106-117. National Information Service, Springfield, Va.

JORTNER, B. S. AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

LAPADULA, D. M., KINNES, C. G., SOMJEN, G. G. AND ABOU-DONIA, M. B. (1982). Monosynaptic reflex depression in cats with organophosphorus neuropathy: effects of tri-

o-cresyl phosphate. NeuroToxicology. 3: 51-62.

LOMO, T. AND ROSENTHAL, J. (1972). Control of ACh sensitivity by muscle activity in the rat. J. Physiol. 221: 493-513.

LOWNDES, H. E., BAKER, T. AND RIKER, W. F. (1974). Motor nerve dysfunction in delayed DFP neuropathy. Eur. J. Pharmacol. 29: 66-73.

McARDLE, J. J. (1983). Molecular aspects of the trophic influence of nerve on muscle. Prog. Neurobiol. 21: 135-198.

MURPHY, S. D. (1986). Pesticides. In Casarett and Doull's Toxicology, The Basic Science of Poisons, (J. Doull, C. D. Klassen and M. O. Amdur, Eds.), 3rd ed., pp. 519-581. Macmillan Publishing Co., New York.

PRENTICE, D. E. AND ROBERTS, N. L. (1984). Acute delayed neurotoxicity in hens dosed with tri-ortho-cresyl phosphate (TOCP): Correlation between clinical ataxia and neuropathological findings. In Delayed Neurotoxicity, (J. M. Cranmer and E. J. Hixson, Eds.), pp. 139-144. Intox Press, Little Rock.

RICHARDSON, R. J. (1984). Neurotoxic esterase: normal and pathogenic roles. In Cellular and Molecular Neurotoxicology (T. Narahashi, Ed.), pp. 285-295 Raven Press, New York.

ROBERTSON, D. G., SCHWAB, B. W., SILLS, R. D., RICHARDSON, R, J. AND ANDERSON, R. J. (1987). Electrophysiologic changes following treatment with organophosphorus-induced delayed neuropathy-producing agents in the adult hen. Toxicol. Appl. Pharmacol. 87: 420-429.

ROBERTSON, D. G., MATTSON, A. M., BESTERVELT, L. L., RICHARDSON, R. J. AND ANDERSON, R. J. (1988). Time course of electrophysiologic effects induced by di-n-butyl-2,2-dichlorovinyl phosphate (DBVC) in the adult hen. J. Toxicol. Enviorn. Hlth. 23: 283-294.

SPRAGUE, G. L., SANDVIK, L. L., BICKFORD, A. A. AND CASTLES, T. R. (1980). Evaluation of a sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. Life Sci. 27: 2523-2528.

WYNN PARRY, C. B. (1971). Strength-duration curves. In Electrodiagnosis and Electromyography (S. Licht, Ed.), pp. 241-271. Elizabeth Licht, New Haven.

TABLE 1. ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL PARAMETERS DERIVED FROM STRENGTH-DURATION AND CONCENTRATION-RESPONSE CURVES FOLLOWING TREATMENT WITH EITHER TOTP OR PSP.

Treatment	Rheobase (volts)[a]	Chronaxie (msec)[b]	Threshold (M)[c]	Tension (g) at 2 x 10^{-3}
Control	2.6 ± .26	.09 ± .009	10^{-4}	2.07 ± 0.59
TOTP (360 mg/kg)				
Day 4	4.3 ± 0.19[c]	0.06 ± 0.010[c]	$5x10^{-5}$ (5x)[d]	9.96 ± 0.46[c]
Day 7	4.8 ± 0.24[c]	0.06 ± 0.005[c]	$5x10^{-5}$ (5x)	8.87 ± 1.72[c]
Day 10	5.1 ± 0.16[c]	0.06 ± 0.004[c]	10^{-6} (10^{2}x)	3.96 ± 0.84[c]
Day 15	5.7 ± 0.59[c]	0.04 ± 0.002[c]	$5x10^{-10}$(10^{5}x)	4.98 ± 0.42[c]
Day 21	8.1 ± 3.00[c]	0.07 ± 0.02[c]	$5x10^{-11}$(10^{6}x)	6.45 ± 0.59[c]
Day 37	4.8 ± 0.31[c]	0.06 ± 0.005[c]	$5x10^{-5}$ (5x)	3.37 ± 0.45[c]
PSP (2.5 mg/kg)				
Day 4	4.8 ± 0.55[c]	0.05 ± 0.002[c]	$5x10^{-5}$ (5x)	7.38 ± 0.75[c]
Day 7	5.32 ± 0.42[c]	0.05 ± 0.005[c]	10^{-5} (10x)	8.75 ± 1.48[c]
Day 10	4.8 ± 0.40[c]	0.05 ± 0.004[c]	$5x10^{-9}$ (10^{4}x)	5.71 ± 0.54[c]
Day 15	10.0 ± 3.33[c]	0.03 ± 0.010[c]	$5x10^{-10}$(10^{5}x)	6.87 ± 0.64[c]
Day 21	6.6 ± 0.42[c]	0.05 ± 0.00	$5x10^{-11}$(10^{6}x)	6.90 ± 1.51[c]
Day 37	5.7 ± 0.34[c]	0.05 ± 0.002[c]	$5x10^{-5}$ (5x)	6.01 ± 1.01[c]

a. Rheobase derived from Figure 3a and b, X ± S.E., n = 5.
b. Chronaxie derived from individual SDC, X ± S.E., n = 5.
c. Significantly different from control values, $p < 0.05$. Newman-Keuls test for multiple comparisons.
d. Degree of sensitivity to ACh compared to control in parentheses.

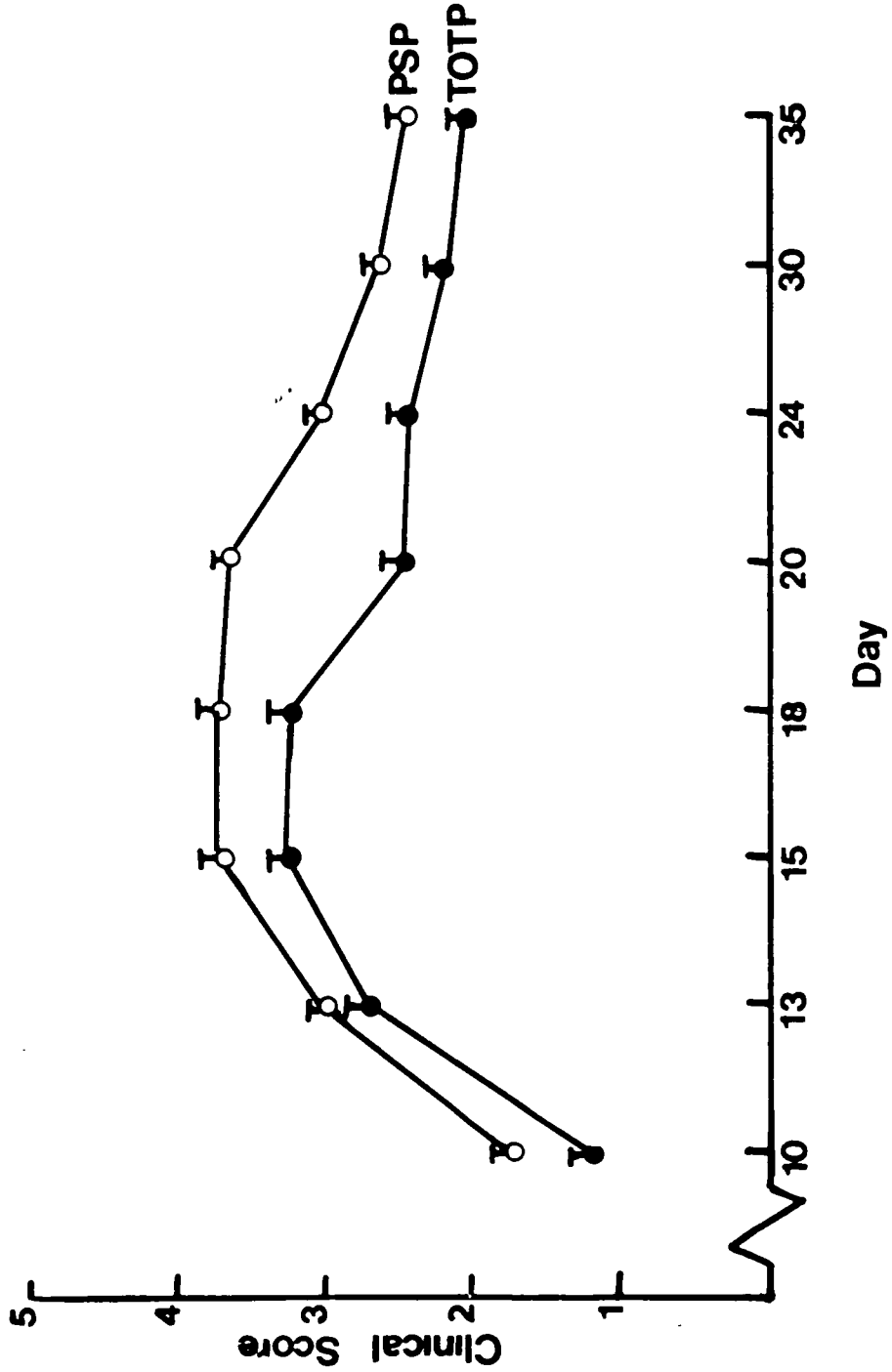
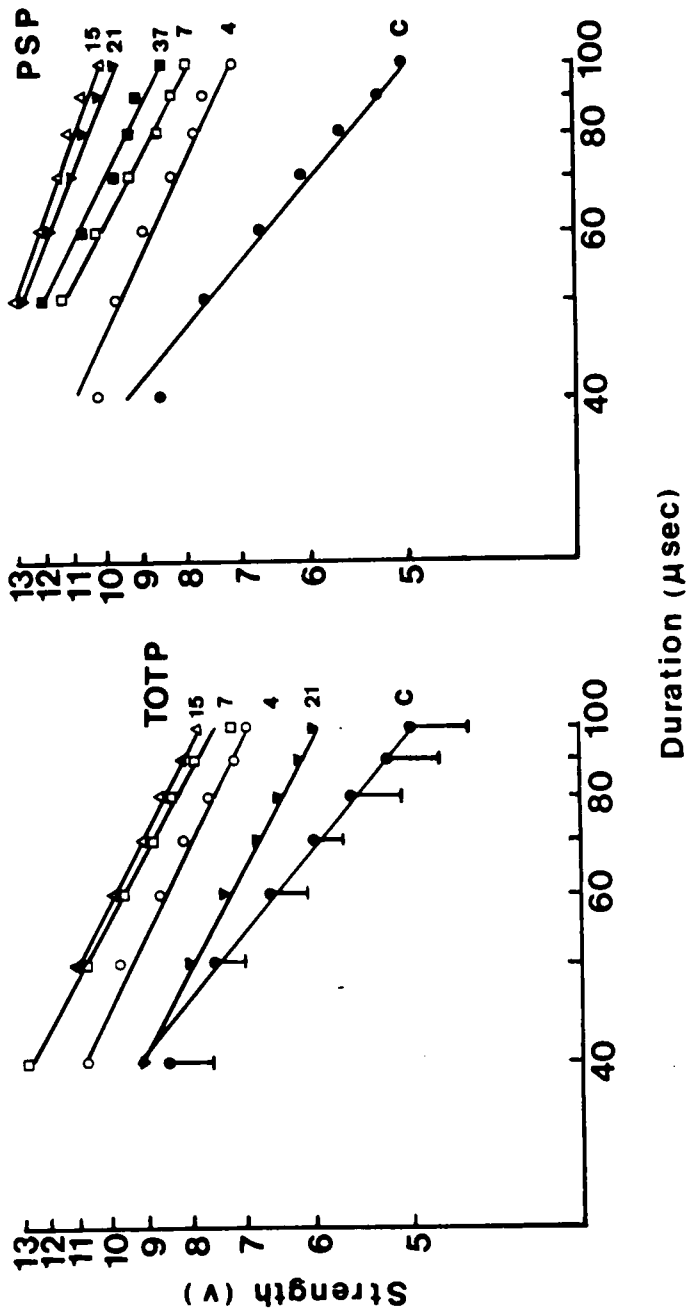
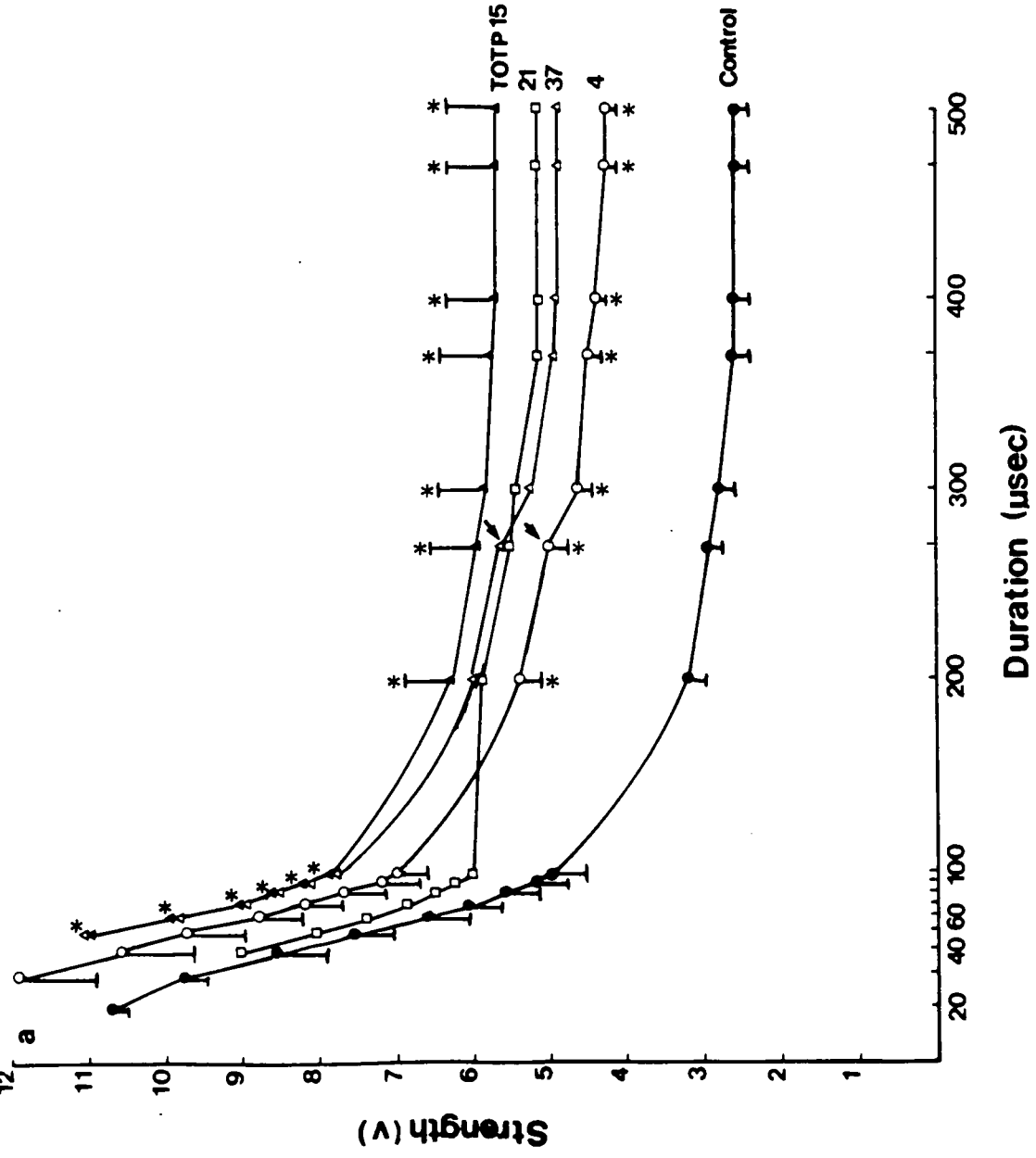
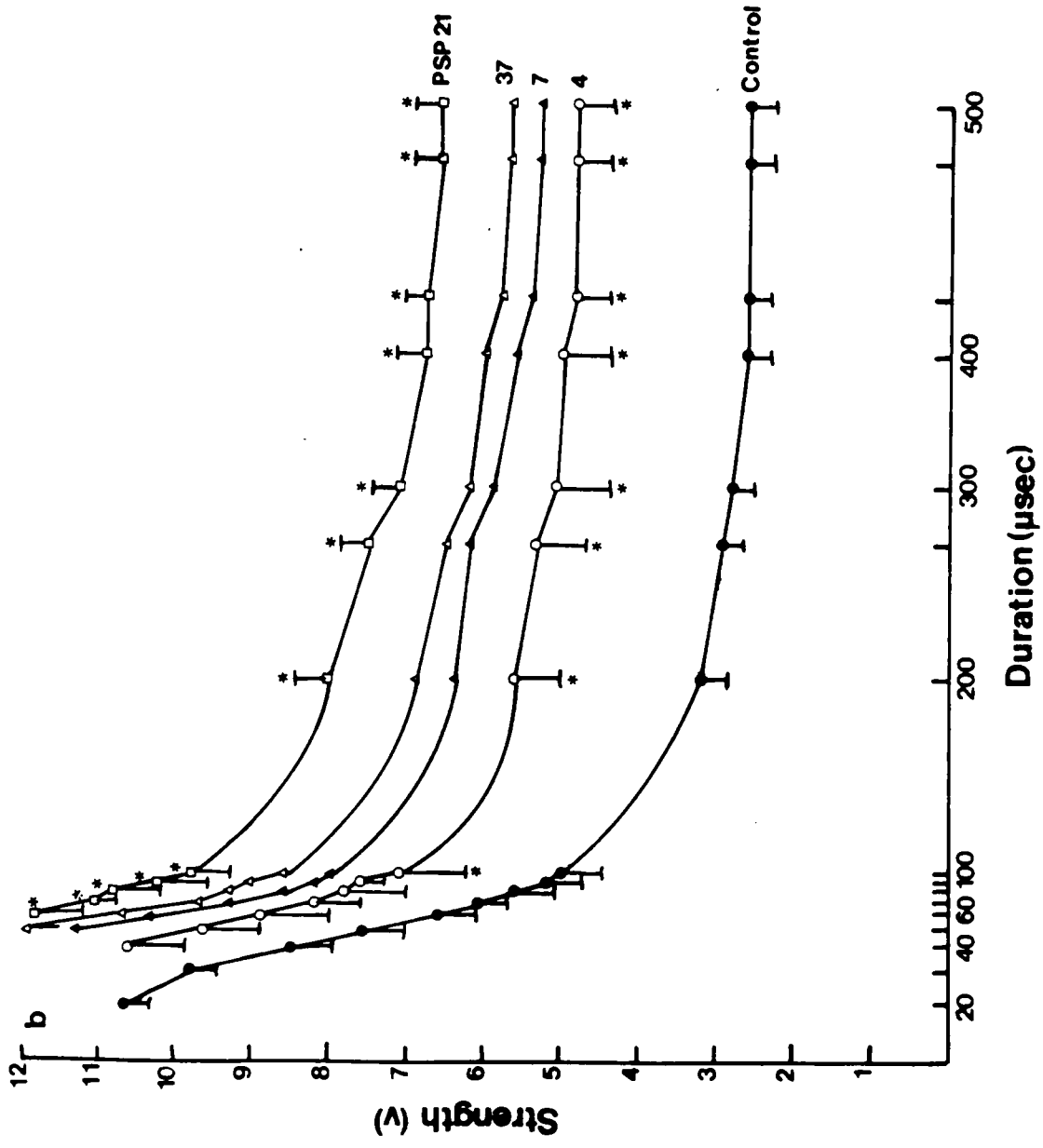

PSP
TOTP
Clinical Score
Day
1
2
3
4
5
10
13
15
18
20
24
30
35

Figure 1. Development of clinical signs in hens after TOTP or PSP administration. Results are presented as mean ± S.E., n = 9-10. 1 = altered gait; 2 = difficulty in walking and standing; 3 = severe ataxia; 4 = leg paralysis; 5 = paralysis with both leg and wing involvement.

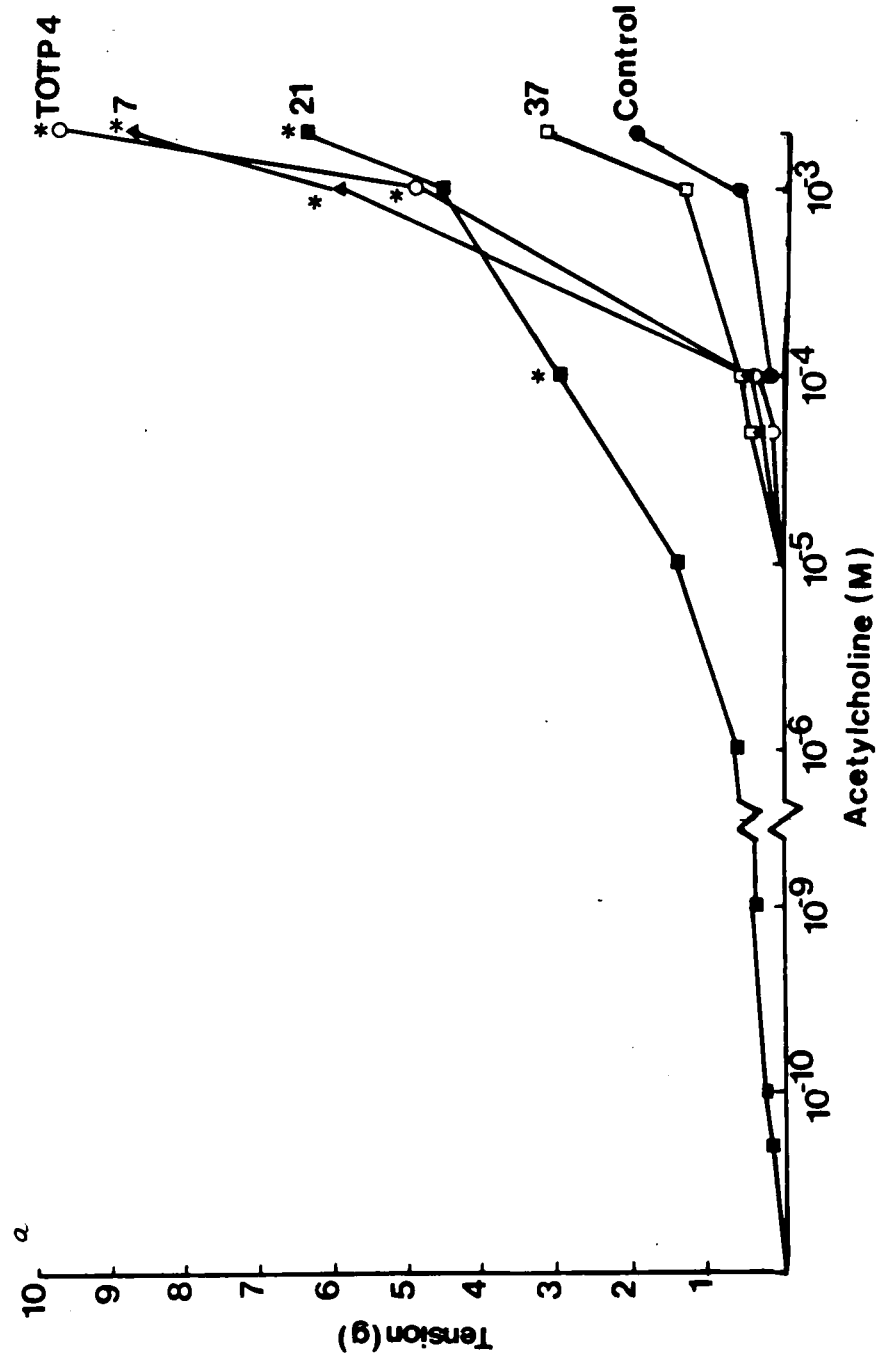

PSP
15
21
37
7
4
C
TOTP
15
7
4
21
C
13
12
11
10
9
8
7
6
5
40
60
80
100
Strength (V)
Duration (μsec)

Figures 2a and b. Log-log plots of the inflexion region (40-100 μsec) of strength-duration curves from biventer cervicis nerve-muscle preparations days 4, 7, 10, 15, 21, and 37 following TOTP (Fig. 2a) or PSP (Fig. 2b) administration. Preparations treated with either compound required a higher threshold for stimulation. All points represent the mean $\pm$ S.E., (n = 5). Results were examined for statistical differences by an ANOVA and Newman-Keuls test for multiple comparisons ($p < 0.05$). Inflexion regions were significant for TOTP days 4-15 and for PSP days 4-21.


a
Strength (V)
Duration (µsec)
TOTP 15
21
37
4
Control
20
40
60
100
200
300
400
500
1
2
3
4
5
6
7
8
9
10
11
12


b
PSP 21
37
7
4
Control
Strength (V)
Duration (μsec)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
100
200
300
400
500

Figures 3a and b. Strength-duration curves for square pulses on biventer cervicis nerve-muscle preparations from controls and TOTP (Fig. 3a) or PSP (Fig. 3b)-treated hens. "Kinks" indicative of partial denervation are noted by arrows. All points represent the mean ± S. E., (n = 5). Results were examined for statistical differences by an ANOVA. Points at which OP-treated groups were significantly different from control are noted by asterisks (Newman-Keuls test for multiple comparisons, $p < 0.05$).


*TOTP 4
*7
*21
37
Control
Tension (g)
Acetylcholine (M)
10
9
8
7
6
5
4
3
2
1
10^{-3}
10^{-4}
10^{-5}
10^{-6}
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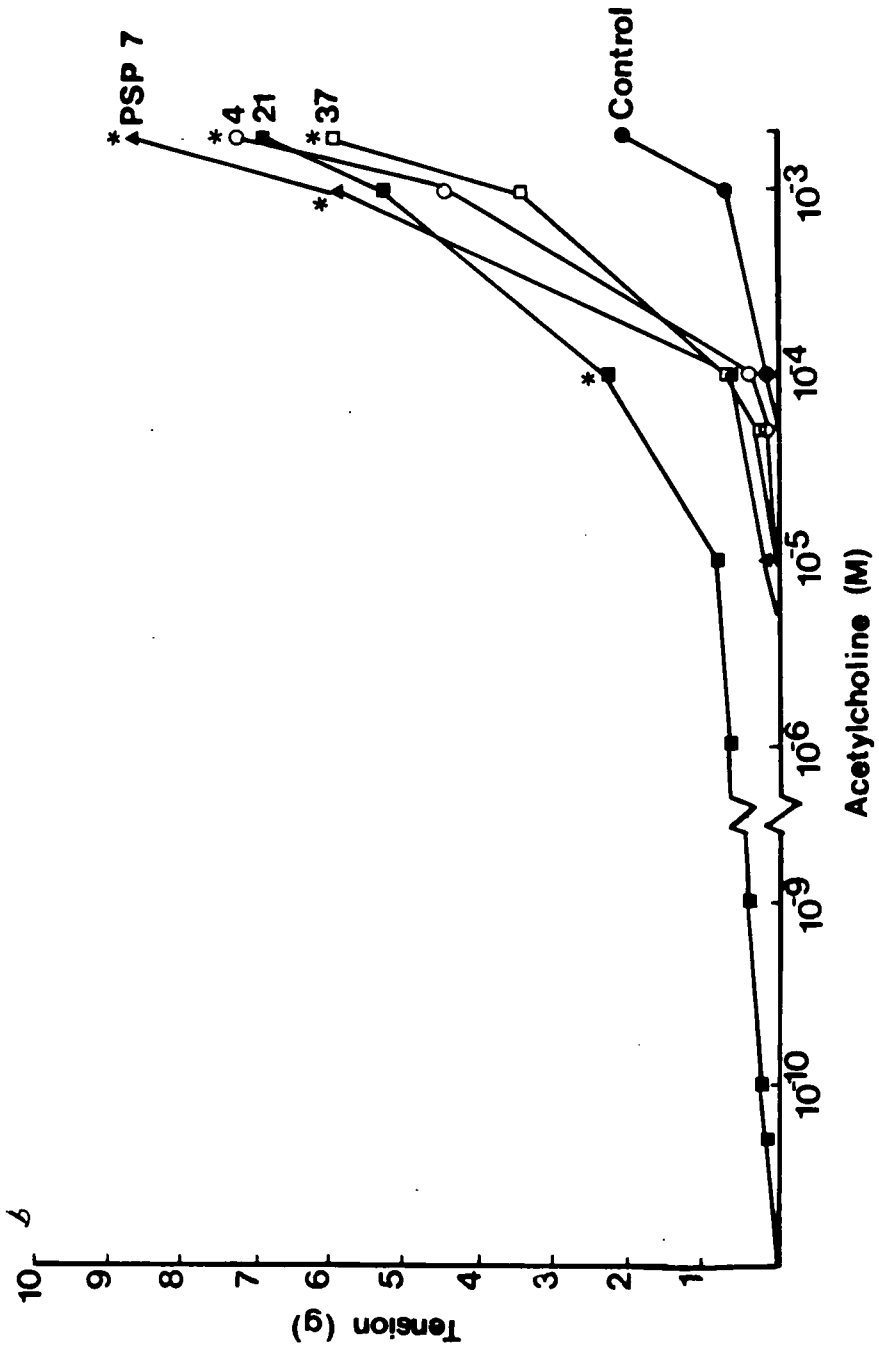
PSP 7
4
21
37
Control
Tension (g)
Acetylcholine (M)
10
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8
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10^{-10}
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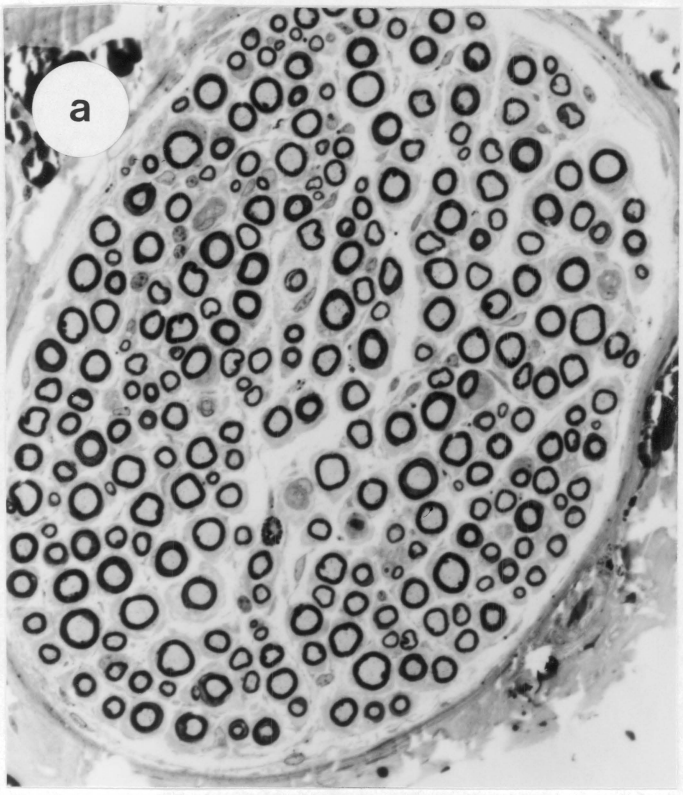
Figure 4a and b. Tension development in the biventer cervicis nerve-muscle preparation in response to ACh following treatment with TOTP (Fig. 4a) or PSP (Fig. 4b). All points represent the mean ± S.E., (n=5). Points significantly different from controls are identified by asterisk, as determined by an analysis of variance and Newman-Keuls test for multiple comparisons ($p < 0.05$). Thresholds and maximum tension for days not shown are included in Table 1.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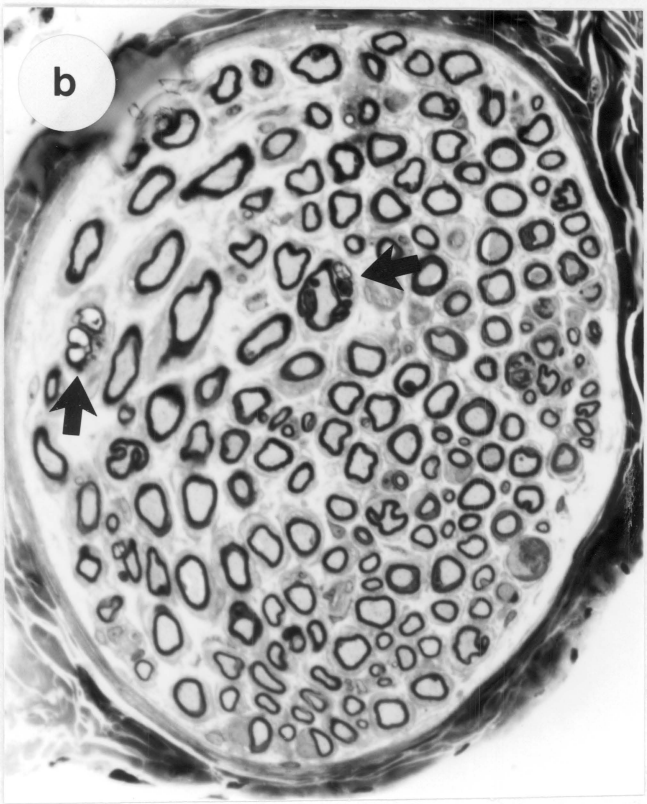
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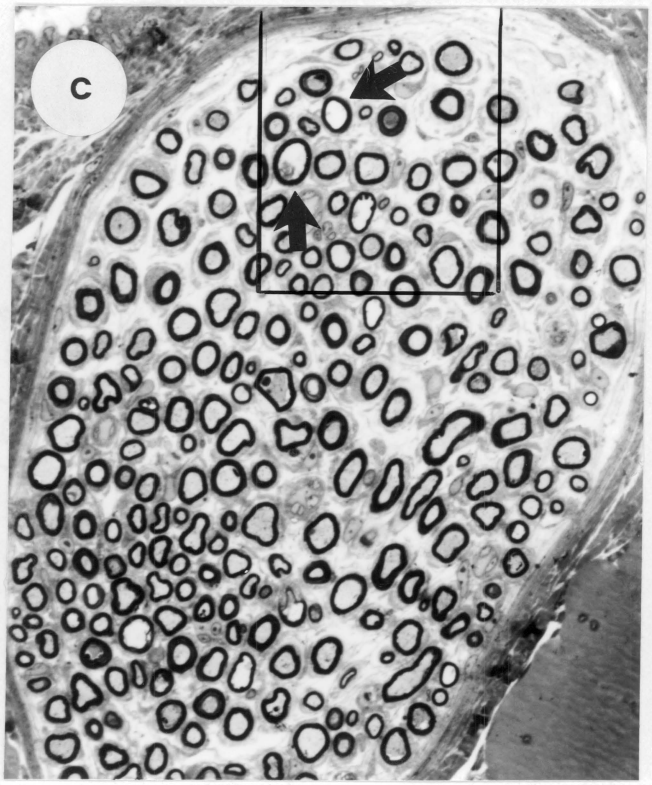
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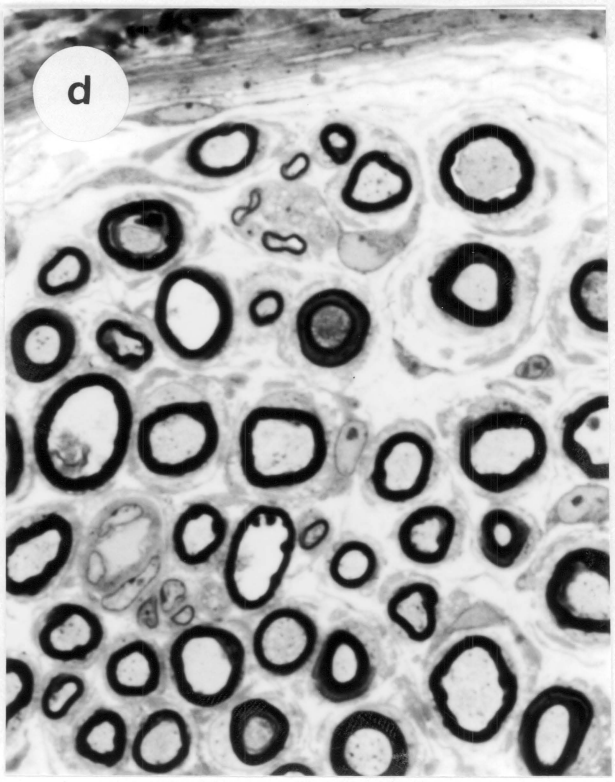
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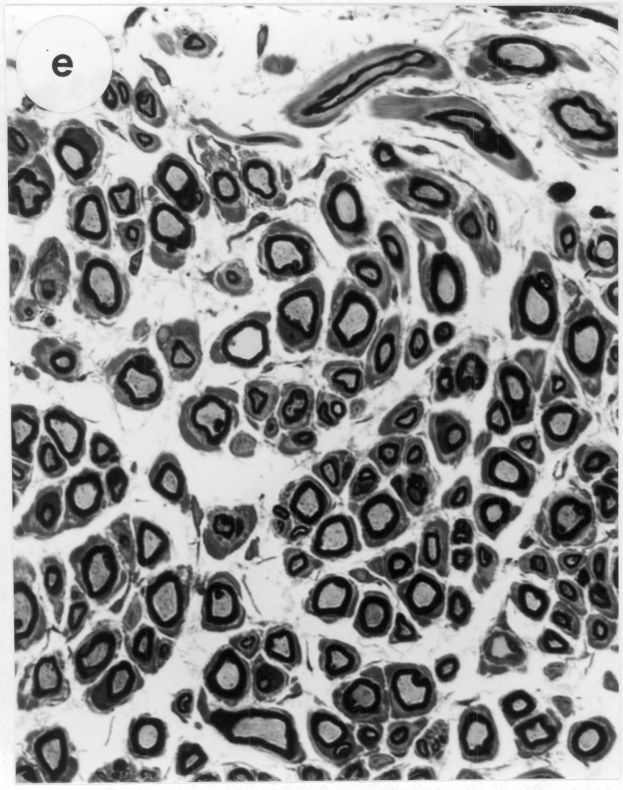
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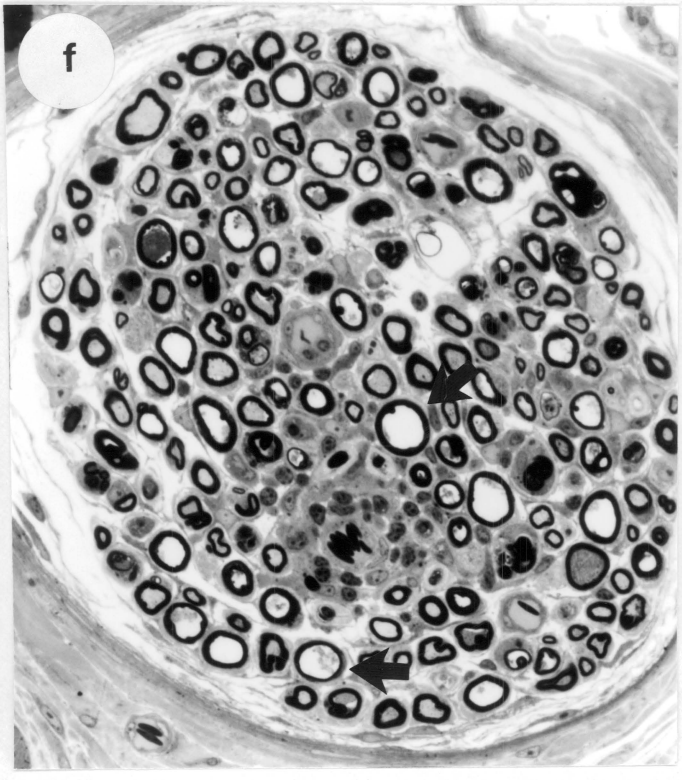
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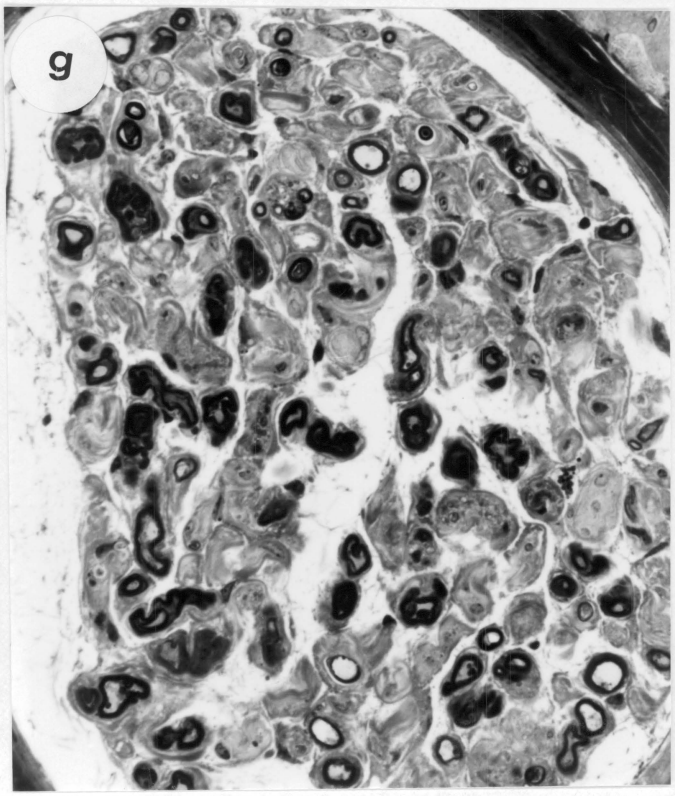
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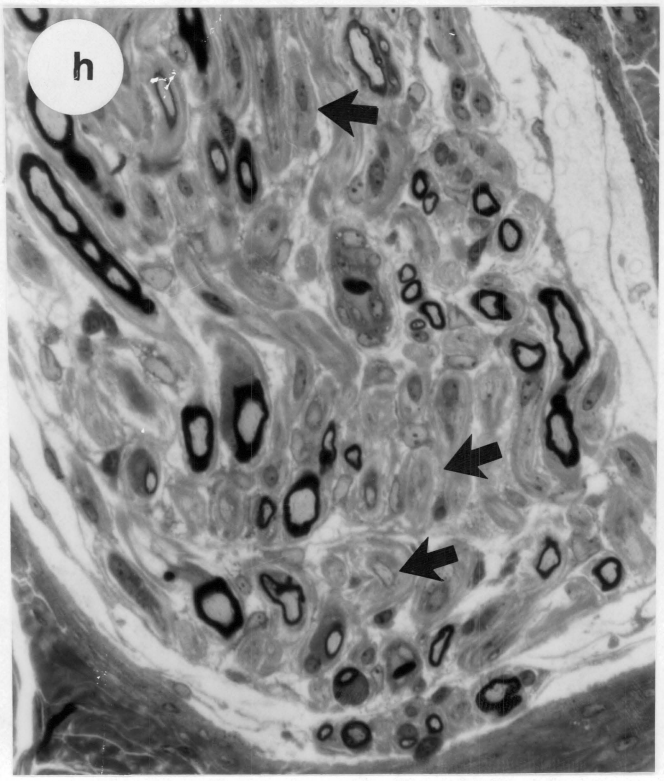
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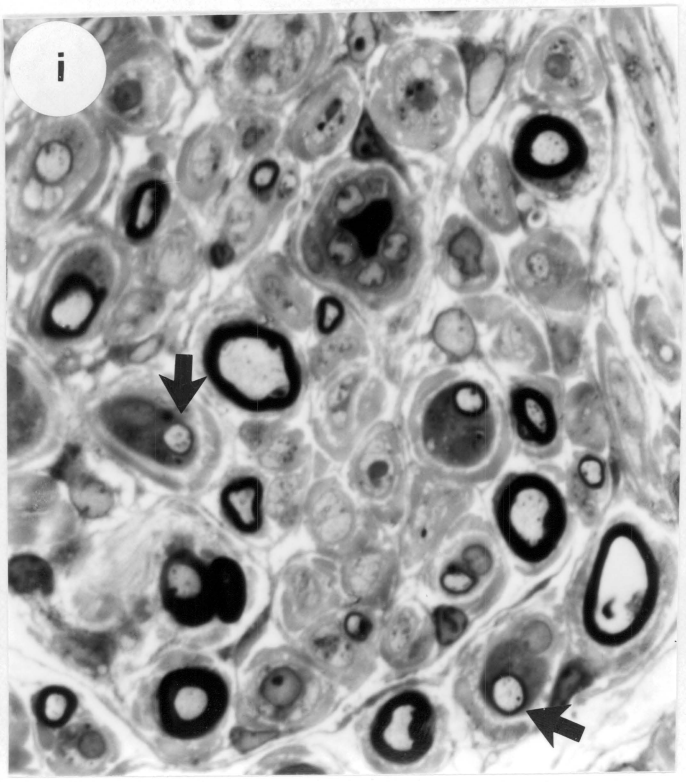
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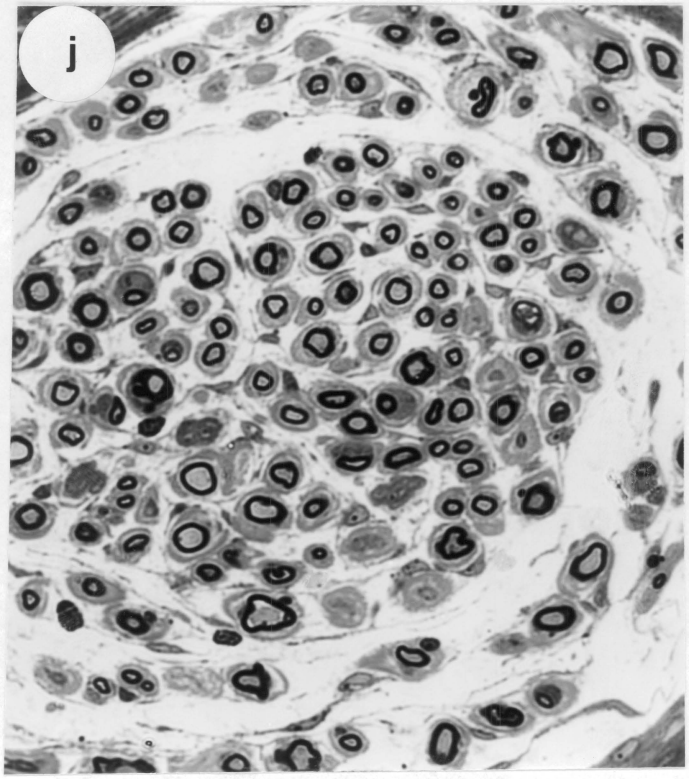
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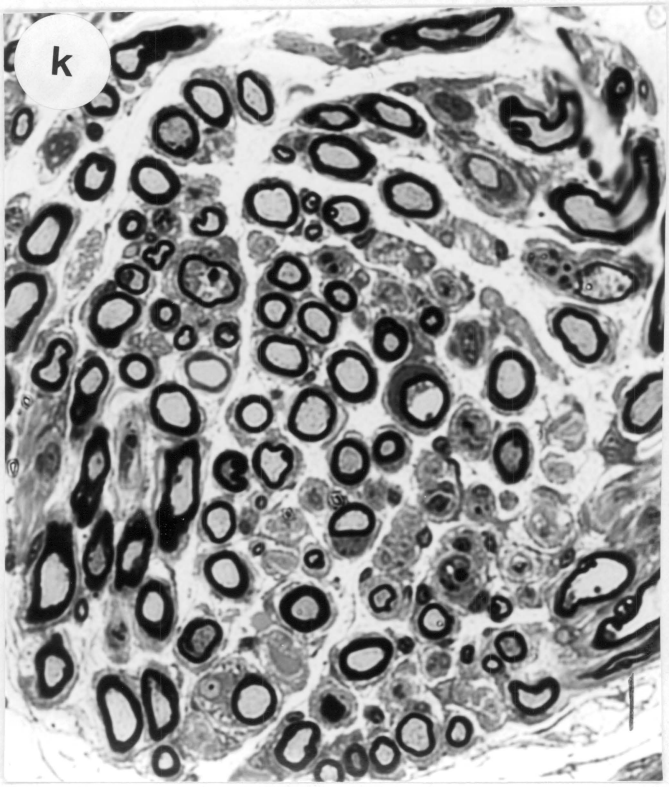
k

Figures 5 a-k. Progressive degeneration in cross sections of the distal region of nerve to biventer cervicis muscle. Fig. 5a is from a control hen and Fig. 5b is from a hen given PSP 4 days earlier. Note the presence of few fibers undergoing degeneration (arrows). Fig. 5c is from a hen given PSP 7 days earlier. Note the increased number of degenerating myelinated fibers. Fig. 5d is high power examination of the area outlined in Fig. 5c. Fig. 5e is from a hen receiving TOTP 7 days earlier, less degeneration is noted than in that receiving PSP at the same time (Fig. 5c). Fig. 5f at 10 days following PSP administration. Advanced fiber degeneration is noted (arrows). At 15 days following PSP treatment (Fig. 5g) few intact fibers remain. Fig. 5h and 5i at 21 days after PSP, many empty Schwann cell tubes are noted (arrows) at low power, and at high power (Fig. 5i) regenerating thinly myelinated fibers are present (arrows). Fig. 5j and 5k are of nerves taken 37 days after PSP and TOTP, respectively. Note the presence of small apparently newly myelinated fibers (Figures 5a, 5b, 5c, 5d, 5e, 5f, 5g, 5h, 5j, and 5k at X260. Figures 5d, and 5i at X650).

CHAPTER 7

EFFECT OF VERAPAMIL ON ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY (OPIDN) IN HENS

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ABSTRACT

Verapamil, a calcium channel blocker, was administered to adult white leghorn hens to determine if inhibition of calcium entry could alter delayed neuropathy induced by administration of phenyl saligenin phosphate (PSP). Verapamil was given im in doses of 7 mg/kg/day for four days beginning 24 hr before administration of PSP (2.5 mg/kg im). Ataxia was less pronounced in hens given PSP plus verapamil than in hens given PSP alone during observations made 8-28 days after PSP administration. Myelinated fiber lesions were less extensive and regeneration more notable in the biventer cervicis nerve in chickens given PSP plus verapamil, with samples obtained both 17 and 28 days after PSP. In the absence of verapamil, rheobase and chronaxie values of strength duration curves were higher and shorter, respectively, and sensitivity to acetylcholine was increased in biventer cervicis nerve-muscle preparations from hens given PSP. Verapamil did not alter PSP-induced inhibition of neurotoxic esterase, indicating that the mechanism involved in amelioration of these indices of delayed neuropathy was not associated with initial enzyme inhibition caused by this organophosphorus ester.

INTRODUCTION

Organophosphorus compounds (OPs) are widely used today in agriculture and industry. Some of these compounds have potential to induce a delayed neuropathy in man and other susceptible animal species following a single exposure (Abou-Donia, 1981). Organophosphorus-induced delayed neuropathy (OPIDN) is characterized by initial inhibition of a neural esterase (neuropathy target esterase or neurotoxic esterase, NTE), a latent period of one to two weeks between exposure and appearance of axonopathy, and degeneration of long myelinated fibers (Davis and Richardson, 1980; Johnson, 1987). Clinically OPIDN is manifest by incoordination, ataxia and flaccid paralysis (Abou-Donia, 1981; Metcalf, 1984). The adult chicken, among species susceptible to OPIDN, is generally recognized as providing the most reliable model for human OPIDN (Davis and Richardson, 1980; Bickford, 1984).

Precise mechanisms responsible for OPIDN have not been defined, but breakdown of axonal cytoskeletal elements associated with increased levels of free calcium (Ca^{++}) might be a factor. This association has been made for other neuropathies (Schlaepfer and Bunge, 1973; Schlaepfer, 1987). Ca^{++} appears to contribute to such degenerative changes both in nerve and muscle by facilitating the activities of Ca^{++}-associated proteases and kinases (Schlaepfer and Hasler, 1979; Kamakuru et al., 1983; Banik et al., 1984; 1985;

Llados, 1985; Berlet, 1987). Changes in these enzymes have been noted following administration of OPs that induce delayed neuropathy (Abou-Donia et al., 1984; Patton et al., 1985; Suwita et al., 1986). Based on the studies cited above and the known role of Ca^{++} in cell injury (Wrogemann and Pena, 1976; Schanne et al., 1979; Farber, 1981), and previous investigations on the use of verapamil to alter neuromuscular disorders (Dretchen et al., 1987; Hudecki et al., 1984; Entrikin et al., 1981), the present investigation was performed to determine if treatment with this Ca^{++}-channel blocker would alter the outcome of OPIDN. These agents inhibit both Ca^{++} entry into the cell and its mobilization from intracellular storage sites (Needleman et al., 1985). The effects of the Ca^{++}-channel blocker verapamil on OPIDN were assessed by clinical observations, biochemistry, neuropathological study and electrophysiological and pharmacological evaluation of an *in vitro* nerve-muscle preparation.

MATERIALS AND METHODS

Animals and housing. White leghorn hens (> 6 months old, 1.2-1.6 kg) used in this study were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. They were housed in groups of 4-5, and had access to food and water ad libitum. Hens were divided according to treatment (control, verapamil, verapamil + phenyl saligenin phosphate (PSP), and PSP alone), with 10-15 hens in each group.

Dosing. PSP (2-phenoxy-4H-1-1, 3, 2-benzodioxaphosphorin-2-oxide), a prototype of the cyclic saligenin phosphates, toxic metabolites of tri-o-tolyl phosphate (Eto et al., 1962), was synthesized as previously described (Jortner and Ehrich, 1987). PSP was dissolved in dimethyl sulfoxide (DMSO) so that the 2.5 mg/kg dosage was available in 0.25 ml/kg and administered into the breast muscle, with half the injection given on each side. This regimen was used because previous studies had shown denervation hypersensitivity to acetylcholine to occur as early as 4 days after PSP or TOCP administration (El-Fawal, Jortner and Ehrich, unpublished data). Verapamil (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water and 7.0 mg/kg administered im for 4 days. The first of the four doses of verapamil was given one day prior to PSP administration to ensure adequate bioavailability (Anonymous, 1981).

Clinical signs. Chickens were observed daily for

clinical signs. Independent blind assessment by at least two individuals was recorded and averaged. Scores were designated as 0 for normal, 1 for altered gait, 2 for difficulty in walking and standing, 3 for severe ataxia, 4 for leg paralysis and 5 for paralysis with both leg and wing involvement (Sprague et al., 1980).

Enzyme assays. NTE activity was measured spectrophotometrically in quick-frozen brains of 3-4 hens sacrificed by cervical dislocation 72 hours after PSP administration (24 hours after the last dose of verapamil), using the method of Sprague et al. (1981). Protein concentrations were determined using the assay of Bio-Rad Laboratories, Richmond, CA. Spectrophometric measurement of acetylcholinesterase (AChE) activities was performed using the method of Ellman et al. (1961), with 0.8 mg brain on the same tissue used for NTE activity measurements. Plasma creatine phosphokinase (CPK) activity was assayed 15 days after PSP administration using an automated analyzer and kit (Baker Instruments, Allentown, PA).

Electrophysiological and pharmacological studies. These methods for evaluating the effects of OPIDN were conducted on biventer cervicis nerve-muscle preparations obtained after euthanasia of hens performed by iv injection of T-61 solution (Hoescht Corp., Somerville, NJ). This preparation used a modification of the procedure developed by Ginsborg and Warriner (1960) that allowed for accommodation

of adult hens. The muscle contains both slow-tonic and fast-twitch fibers (Ginsborg and Warriner, 1960; El-Fawal et al., 1988). To remove the biventer cervicis nerve and muscle, the skin at the back of the neck was incised along the midline from the skull to below the base of the midline. A suture was tied to the proximal end of the tendon prior to detachment from the skull. By retracting on the suture, the tendon with the ensheathed nerve was exposed and separated from the underlying semispinalis cervicis muscle. Tendon and muscle were carefully removed. The muscle was anchored at the bottom of a 150 ml organ bath (custom-made) and the tendon passed through a custom-made version of the biventer cervicis muscle electrode. The tendon was attached by the suture to a Model FT03 force displacement isometric transducer, leading to a coupler-amplifier-recorder system (Grass Medical Instruments, Quincy, MA). Two grams of resting tension was placed on each muscle, as this was determined by pilot studies to be optimal to assure uniformity of isometric contraction. The organ bath contained Krebs-Henseleit solution [composition in g/l: NaCl, 6.9; $MgSO_4.7H_2O$, 0.29; HK_2PO_4, 0.15; glucose, 2.0; $NaHCO_3$, 2.1; KCl, 0.35; $CaCl_2$, 0.28] maintained at 37°C and aerated with a 95% O_2-5% CO_2 mixture.

The terminals of the biventer cervicis electrode were connected to a rectangular pulse stimulator (Grass Medical Instruments, Quincy, MA). The voltage required to produce a

minimally perceptible response was determined for various durations ranging from 20-500 μsec. For each of the pulse durations detection thresholds were obtained using the method of descending limits and viewed on an oscilloscope (Model 5115 storage oscilloscope, Tektronix, Inc., Beaverton, OR).

Strength-duration curves for the biventer cervicis nerve-muscle preparations were obtained by plotting strength (volts) producing a least perceptible depolarization, as indicated by muscle twitch response, against stimulus duration (μsec). Rheobase (minimal intensity of stimulus of prolonged duration necessary to excite the tissue) and chronaxie (minimum time required to excite the tissue for a stimulus twice the strength of rheobase) were derived from the curve.

Log concentration-response curves in response to acetylcholine chloride (ACh, Sigma Chemical Co., St. Louis, MO) were constructed by plotting cumulative increments in concentration of ACh against gram-tension developed by the muscle. All stock solution of drug (10^{-1} M) were prepared on the day of the experiment in distilled water. Subsequent serial molar concentrations (final bath concentrations: 10^{-8} M-$2x10^{-3}$ M) were prepared by dilution in Krebs-Henseleit solution.

Histology. The distal region (lying over the belly of the muscle) of nerve supplying the twin biventer cervicis muscle was dissected away from the freshly removed specimen and placed in a fixative of the following composition: 2.5%

glutaraldehyde in 0.05 M sodium coacodylate at pH 7.4. After 24-48 hours of fixation at 4°C segments of the nerve were rinsed in buffer, post-fixed in 2% osmium tetroxide and embedded in Epon epoxy resin. Cross sections were cut at one μm thickness, stained with toluidine blue and safranin and examined by light microscopy.

Statistics. Except in the case of clinical scores, where the Student-t test was used, all other data was analyzed using an analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences between control and experimental groups with $p < 0.05$ considered significant. All data are expressed as means ± standard error (SE), except where otherwise indicated.

RESULTS

Clinical signs of OPIDN were evident in hens treated with PSP alone 8 days following administration. No signs of ataxia were evident in hens treated with both verapamil and PSP on this day. OPIDN progressed in severity over the next several days (Fig. 1). By day 13 all chickens given PSP, with or without verapamil, showed clinical evidence of OPIDN, but hens given PSP alone were more severely affected. Scores for verapamil and PSP-treated chickens were consistently lower during the entire duration of the 28 day study. Hens given verapamil only did not develop ataxia.

Activity of NTE was significantly depressed in hens treated with either PSP alone or verapamil and PSP 72 hours after PSP administration (Table 1). Verapamil treatment alone had no effect on NTE. PSP with and without verapamil also significantly depressed brain AChE activity, but in neither case was inhibition sufficient to cause cholinergic signs. Plasma CPK levels at 15 days were greatly increased in hens given PSP alone, although this did not prove statistically significant (Table 1).

Strength-duration curves generated for the biventer cervicis nerve 17 and 28 days after PSP showed that PSP alone caused an increase in threshold excitability of the nerve (Fig. 2a, b). This was more pronounced at 28 days (Fig. 2b) than at 17 days (Fig. 2a). The shape of the curves for preparations from chickens given PSP with and without verapamil

at 17 days shows "kinks" or discontinuities. Rheobase values for nerves of PSP-treated hens were significantly higher than for controls, verapamil alone or the verapamil plus PSP-treated groups both at 17 and 28 days (Table 2). Chronaxie values were significantly shorter in nerves from hens given PSP only compared to the other groups (Table 2).

Concentration-response curves showing contraction of the biventer cervicis muscle preparation after exposure to exogenous ACh are shown in Fig. 3. Preparations from chickens treated with PSP 28 days earlier were 1000-fold more sensitive to ACh than were controls; preparations from chickens treated with verapamil plus PSP were 10-fold more sensitive. Results with verapamil alone were similar to controls. Contractile-tension development increased significantly as concentration of ACh increased in preparations from chickens given PSP alone. Although less pronounced at 17 days than at 28 days, sensitivity to ACh in biventer cervicis preparations was increased 100-fold and 5-fold over preparations from control hens in preparations from PSP-alone and from verapamil plus PSP groups, respectively (thresholds: 10^{-4} M for control or verapamil alone, 10^{-6} M for PSP alone, and $5x10^{-5}$ M for verapamil and PSP). Contractile-tension development in muscle preparations exposed to ACh was similar in all groups of chickens at 17 days.

Microscopic examination of the distal portion of nerve to biventer cervicis muscle 17 days following treatment with PSP revealed few intact myelinated fibers and numerous "empty" (without recognizable axons) Schwann cells, repre-

senting axons fully degenerated at the level of examination (Fig. 4). Nerves from hens treated with both verapamil and PSP revealed a large number of intact myelinated fibers, with a small number of fibers undergoing Wallerian-like degeneration. In addition only a small number of "empty" Schwann cells were seen (Fig. 5). Occasional regenerating, but not yet myelinated, or thinly (newly) myelinated axons were recognized in such Schwann cells by light and electron microscopy (Figs. 5, 6). At 28 days axonal regeneration was more prominent in nerves from hens given both verapamil and PSP than in nerves from hens given PSP alone (Figs. 7, 8, respectively). This was particularly evident with regard to the presence of newly myelinated regenerating fibers. The lesions described above were not seen in nerves from control hens or those from hens given verapamil alone.

DISCUSSION

In this study, we demonstrated that the calcium-channel blocker verapamil could modify the neurotoxic effects of PSP on physiological, pharmacological and pathological parameters used to assess nerve and muscle function. Strength-duration curves, for example, for the biventer cervicis nerve-muscle preparations from hens treated 17 days earlier with PSP alone or with verapamil and PSP showed "kinks" or discontinuities. This is accepted as indicative of partial denervation (Wynn Parry, 1971; Heckman, 1972). Higher rheobase values and short chronaxie values for nerve-muscle preparations of hens treated with PSP 17 and 28 days earlier were consistent with our previous study (El Fawal et al., 1988) and with reports of peripheral neuropathies due to other toxicants (Harris, 1981). High rheobase and short chronaxie values are used to indicate degeneration while low or normal rheobase and long chronaxie values are used to indicate regeneration of nerves (Wynn Parry, 1971). Increase in rheobase values is consistent with the trend observed by Robertson et al. (1987) following administration of tri-ortho-tolyl phosphate. We found that treatment with verapamil lengthened chronaxie values in preparations from PSP-treated hens.

The more advanced denervation of biventer cervicis muscle in PSP-treated hens, as indicated by the strength-duration curves, was supported by histopathological observations. More degenerating myelinated fibers were noted in distal level of the biventer cervicis nerves from hens give PSP

alone than in nerves from hens given verapamil and PSP. Large numbers of intact myelinated fibers remained and more active axonal regeneration was evident in biventer cervicis nerves of hens treated with both verapamil and PSP. At 28 days, consistent with the pattern indicated by the strength-duration curves, axonal regeneration was more pronounced in nerves from hens given verapamil and PSP than in hens given PSP alone. This may account for the improved electrophysiological and pharmacological responses of the verapamil-treated hens. Pathological damage was more extensive in the biventer cervicis nerve of hens given PSP than in branches of the tibial and dorsal metatarsal nerves seen in an earlier study (Jortner and Ehrich, 1987).

Denervation was further indicated by the hypersensitivity of muscle to exogenous ACh (Axelsson and Thesleff, 1957; Kandel, 1985). This was observed in the present study as increased responsiveness of the biventer cervicis muscle to the neurotransmitter 17 and 28 days after PSP. Muscle from verapamil and PSP-treated hens was, however, less sensitive than muscle from hens given PSP alone. Denervation hypersensitivity to ACh is believed to occur as a result of the synthesis and incorporation of new ACh-receptors into the muscle membrane in the absence of intact nerve. This synthesis has been reported to occur within 3 days to weeks following denervation (Gramp et al., 1972; Chang and Tung, 1974; Kandel, 1985). An observation of denervation hypersensitivity to ACh is consistent with the time course of OPIDN (El-Fawal

et al., 1988).

In our studies, a drug capable of altering intracellular Ca^{++} levels modified OPIDN. Ca^{++} plays a pivotal role as a messenger linking external stimuli to the intracellular environment in nerve and muscle cells (Cavero and Spedding, 1983; Miller, 1987). However Ca^{++}, when unregulated, is implicated in degeneration and death of cells, including nerve and muscle (Wrogemann and Pena, 1976; Schanne et al., 1979; Farber, 1981). Increases in intraneuronal Ca^{++} levels and induction of Wallerian degeneration of nerve fibers is thought to be facilitated by the activation of a Ca^{++}-activated neutral protease (CANP) in the axoplasm (Schlaepfer and Hasler, 1979; Kamakuru et al., 1983; Schlaepfer and Zimmerman, 1984; Mata et al., 1986) and in myelin (Banik et al., 1984, 1985; Berlet, 1987). A related enzyme is found in muscle and is activated by sarcoplasmic increases in Ca^{++}. This biochemical change produces muscle degeneration and atrophy (Reddy et al., 1975; Llados, 1985). Removal of Ca^{++} attenuated or abolished degenerative changes in the studies cited above.

Increases in intracellular Ca^{++} have also been shown to increase ACh-receptor synthesis that occurs in denervated muscle. Such synthesis may be suppressed by decreasing intracellular Ca^{++} or by using a Ca^{++}-channel blocker (Metafora et al., 1980; McManaman et al., 1981; Kallo and Steinhardt, 1983). In the present study, denervation, as indicated by muscle sensitivity to ACh and morphological examination, was attenuated by verapamil. Suppression of receptor synthesis

or receptor incorporation into muscle membrane may provide an explanation for these observations. Increases in plasma CPK, characteristic of muscle denervation and degeneration occurring during OPIDN (Cisson and Wilson, 1982), were prevented by verapamil. This has also been noted in other studies employing Ca^{++}-channel blockers to study muscle degeneration (Anand and Emery, 1982; Bhattacharya et al., 1982).

It cannot be said that verapamil alters the development of OPIDN by protecting the target enzyme NTE, or by preventing the inhibition of brain AChE, as neither were changed when PSP was given with the Ca^{++}-channel blocker.

The present study provides impetus for further investigations into the role of Ca^{++} during OPIDN. It is possible that Ca^{++} itself may not be the initial cause of OPIDN, but intracellular increases occurring as a consequence of a triggering event, possibly NTE inhibition and aging, may predispose peripheral nerves to a cascade of detrimental events.

ACKNOWLEDGEMENTS

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REFERENCES

ABOU-DONIA, M.B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21, 511-548.

ABOU-DONIA, M.B., PATTON, S.E., AND LAPADULA, D.M. (1984). Possible role of endogenous protein phosphorylation in organophosphorus compound-induced delayed neurotoxicity. In Cellular and Molecular Pharmacology (T. Narahashi, Ed.), pp. 265-283. Raven Press, New York, N.Y.

ANAND, R., AND EMERY, A.E.H. (1982). Verapamil and calcium stimulated enzyme efflux from skeletal muscle. Clin. Chem. 28, 1482-1484.

ANONYMOUS (1981). Verapamil for arrhythmias. The Medical Letter 23, 29.

AXELSSON, R.R., AND THESLEFF, S. (1957). A study of super sensitivity in denervated mammalian skeletal muscle. J. Physiol. 149, 178-193.

BANIK, N.L., HAGAN, E.L., AND MCALHANEY, W.W. (1984). A role for calcium and activation of neutral proteinase in myelinosis. In Experimental Allergic Encephalomyelitis: a Useful Model for Multiple Sclerosis, pp. 145-152. Alan R. Liss, Inc., New York, N.Y.

BERLET, H.H. (1987). Calcium-dependent neutral protease activity of myelin from bovine spinal cord: evidence for soluble cleavage products of myelin proteins. Neurosci. Lett. 73, 266-270.

BHATTACHARYA, S.L., PALMIERI, G.M., BERTORINI, T.E., AND NUTTING, D.F. (1982). The effects of diltiazem in dystrophic hamsters. Muscle & Nerve 5, 73-78.

BICKFORD, A.A. (1984). The hen as a test animal for delayed neurotoxicity studies: Introductory comments. In Delayed Neurotoxicity (J.M. Cranmer and E.J. Hixson, Eds.), pp. 24-25. Intox Press, Little Rock, AR.

CAVERO, I., AND SPEDDING, M. (1983). "Calcium Antagonists": a class of drugs with a bright future. Part I. Cellular calcium homeostasis and calcium as a coupling messenger. Life Sci. 33, 2571-2581.

CHANG, C.C., AND TUNG, L.H. (1974). Inhibition by actinomycin D of the generation of acetylcholine receptors induced by denervation in skeletal muscle. Eur. J. Pharmacol. 26, 386-388.

CISSON, C.M., AND WILSON, B.W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity: Altered acetylcholinesterase molecular forms and increased plasma creatine phosphokinase activity. Toxicol. Appl. Pharmacol. 64, 289-305.

DAVIS, C.S., AND RICHARDSON, R.J. (1980). Organophosphorus compounds. In Experimental and Clinical Neurotoxicology. (P.S. Spencer and H.H. Schaumburg, Eds.), pp. 527-544. Williams and Wilkins, Baltimore, MD.

DRETCHEN, K.L., BOWLES, A.M., AND RAINES, A. (1986). Protection by phenytoin and calcium channel blocking agents against the toxicity of diisopropyl fluoridate. Toxicol. Appl. Pharmacol 83:584-589.

EL-FAWAL, H.A.N., JORTNER, B.S., EYRE, P., AND EHRICH, M. (1988). The biventer cervicis nerve-muscle preparation of adult hens: Effects of phenyl saligenin phosphate administration. Neuro Toxicol. 9: in press.

ELLMAN, G.L., COURTNEY, K.D., ANDRES, V., AND FEATHERSTONE, R.M. (1961). A new and rapid calorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

ENTRIKIN, R.K., PATTERSON, G.I., AND WILSON, B.W. (1981). Bactofen, procainamide, verapamil, and prenylamine in hereditary muscle dystrophy of the chicken. Expt. Neurol. 74:86-92.

ETO, M., CASIDA, J.E., AND ETO, T. (1962). Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11, 337-351.

FARBER, J.L. (1981). The role of calcium in cell death. Life Sci. 28, 1289-1295.

GINSBORG, B.L., AND WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. Brit. J. Pharmacol. 15, 410-411.

GRAMP W., HARRIS, J.B., AND THESLEFF, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221, 743-754.

HARRIS, R. (1971). Chronaxy. In Electrodiagnosis and Electromyography. (S. Licht, Ed.), Elizabeth Licht, New Haven, CT.

HECKMAN, J.R. (1972). Excitability curve: a new technique for assessing human peripheral nerve excitability in vivo. Neurology 22, 224-230.

HUDECKI, M.S., POLLINA, C.M., AND HEFFNER, R.R. (1984). In vivo effects of three calcium blockers on chickens with inherited muscular dystrophy. Expt. Neurol. 84:512-523.

JOHNSON, M.K. (1987). Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. Trends Pharmacol. Sci. 8, 174-179.

JORTNER, B.S., AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. Neuro-Toxicology 8, 97-108.

KALLO, J.R., AND STEINHARDT, R.A. (1983). The regulation of extrajunctional acetylcholine receptors in the denervated rat diaphragm. J. Physiol. 344, 433-452.

KAMAKURU, K., ISHIURA, S., SUGITA, H., AND TOYOKURA, Y, (1983). Identification of Ca^{2+}-activated neutral protease in the peripheral nerve and its effect on neurofilament degeneration. J. Neurochem. 40, 908-913.

KANDEL, E.R. (1985). Synapse formation, trophic interactions between neurons, and the development of behavior. In Principles of Neural Science (E.R. Kandel and J.H. Schwartz, Eds.), pp. 743-756. Elsevier Publishing Co., New York, N.Y.

LLADOS, F.T. (1985). Neurogenic origin of an experimental myopathy induced by the ionophore A23187. J. Neurol. Sci. 69, 171-182.

MATA, M., STAPLE, J., AND FINK, D.J. (1986). Changes in intra-axonal calcium distribution following nerve crush. J. Neurobiol. 17, 449-467.

MCMANAMAN, J.L., BLOSSER, J.C., AND APPEL, S.H. (1981). The effects of calcium on acetylcholine receptor synthesis. J. Neurosci. 1, 771-776.

METAFORA, S., FELSANI, A., COTRUFO, R., TAJANA, G.F., DIIORIO, G.D., DEL RIO, A., DE PRISCO, P.P. AND ESPOSITO, V. (1980). Neural control of gene expression in the skeletal muscle fiber: the nature of the lesion in the muscular protein-synthesizing machinery following denervation. Proc. Res. Soc. Lond. B. 209, 239-255.

METCALF, R.L. (1984). Historical perspective of organophosphorus ester-induced delayed neurotoxicity. In Delayed Neurotoxicity. (J.M. Cranmer and E.J. Hixson, Eds.), pp. 7-22. Intox Press, Little Rock, AR.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. Science 235, 46-52.

NEEDLEMAN, P., CORR, P.B., AND JOHNSON, E.M. JR. (1985). Drugs used for treatment of angine: Organic nitrates, calcium channel blockers, and ß-adrenergic antagonists. In Goodman and Gilman's the Pharmacological Basis of Therapeutics, 7th ed. (A.G. Gilman, L.S. Goodman, T.W. Rall and F. Murad, Eds.), pp. 806-826. Macmillan Publishing Co., New York.

PATTON, S.E., LAPADULA, D.M., O'CALLAGHAN, J.P., MILLER, D.B., AND ABOU-DONIA, M.B. (1985). Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-o-cresyl phosphate to hens. J. Neurochem. 45, 1567-1577.

REDDY, M.K., ETLINGER, J.D., RABINOWITZ, M., FISCHMAN, D.A., AND ZAK, R. (1975). Removal of Z-lines and -actin from isolated myofibrils by a calcium-activated neutral protease. J. Biol. Chem. 250, 4278-4284.

ROBERTSON, B.G., SCHWAB, B.W., SILLS, R.D., RICHARDSON, R.J., AND ANDERSON, R.G. (1987). Electrophysiological changes following treatment with organophosphorus-induced neuropathy-producing agents in adult hens. Toxicol. Appl. Pharmacol. 87:420-429.

SCHANNE, F.A.X., KANE, A.B., YOUNG, E.E., AND FARBER, J.L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science 206, 700-702.

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

SCHLAEPFER, W.W., AND BUNGE, R.P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J. Cell. Biol. 59, 456-470.

SCHLAEPFER, W.W., AND HASLER, M.B. (1979). Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. Brain Res. 168, 299-3019.

SCHLAEPFER, W.W., AND ZIMMERMAN, U.P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. In Axonal Transport in Neuronal Growth and Regeneration. (J.S. Elam and P. Cancalon, Ed.), pp. 261-273. Plenum Press, N.Y., N.Y.

SPRAGUE, G.L., SANDVIK, L.L., BICKFORD, A.A., AND CASTLES, T.R. (1980). Evaluation of a sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. Life Sci. 27, 2523-2528.

SPRAGUE, G.L., SANDVIK, L.L., BROOKINS-HENDRICKS, M.J., AND BICKFORD, A.A. (1981). Neurotoxicity of two organophosphorus ester flame retardents in hens. J. Toxicol. Environ. Hlth. 8, 507-518.

SUWITA, E., LAPADULA, D.M., AND ABOU-DONIA, M.B. (1986). Calcium and calmodulin enhanced in vitro phosphorylation of hen brain cold stable microtubules and spinal cord neurofilament triplet proteins after a single oral dose of tri-o-cresyl phosphate. Proc. Natl. Acad. Sci. 83, 6174-6178.

WROGEMAN, K., AND PENA, S.D.J. (1976). Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle disease. The Lancet 1, 672-673.

WYNN PARRY, C.B. (1971). Strength-duration curves. In Electrodiagnosis and Electromyography (S. Licht, Ed.), pp. 241-271. Elizabeth Licht, New Haven, CT.

TABLE 1: BIOCHEMICAL PARAMETERS FOLLOWING ADMINISTRATION OF PHENYL SALIGENIN PHOSPHATE (PSP) OR VERAPAMIL AND PSP

Treatment	NTE[a] nmol/min/mg protein (% of control)	AChE Brain[a] mol/min/g tissue	Plasma CPK[b] (U/l)
Control	23.8±0.7	13±1	649±137
Verapamil	22.0±0.5 (92%)	15±1 (119%)	531±24 (82%)
Verapamil+ PSP	5.9±0.1[c] (25%)	9±1[c] (71%)	635±134 (98%)
PSP	6.3±0.4[c] (27%)	10±1[c] (76%)	1066±238 (164%)

[a]72 hours post-PSP administration, mean ± SE, n=3-4.

[b]Creatinine phosphokinase (CPK) activity 15 days post-PSP administration, mean ± SE, n=3-5.

[c]Significantly different from control values, $p < 0.05$, Newman-Keuls test for multiple comparisons

TABLE 2: ELECTROPHYSIOLOGICAL PARAMETERS DERIVED FROM STRENGTH-DURATION CURVE AT 17 AND 28 DAYS AFTER ORGANOPHOSPHATE ADMINISTRATION

Treatment		Chronaxie (msec)[a]	Rheobase (volts)[b]
Control		0.143±0.008	1.31±0.06
Verapamil	day 17+28:	0.114±0.012	1.11±0.04
Verapamil+PSP	day 17:	0.174±0.031	1.44±0.07
	day 28:	0.163±0.006	1.45±0.19
PSP	day 17:	0.104±0.008[c,d]	1.92±0.18[c,d]
	day 28:	0.104±0.007[c,d]	2.76±0.35[c,d]

[a]Chronaxie derived from individual strength-duration curves, mean ± S.E., n=4-7.

[b]Rheobase derived from Fig. 2, mean ± S.E., n=4-7.

[c]Significantly different from control values, $p < 0.05$, Newman-Keuls test for multiple comparisons.

[d]Significantly different from verapamil + PSP groups, $p < 0.05$, Newman-Keuls test for multiple comparisons.

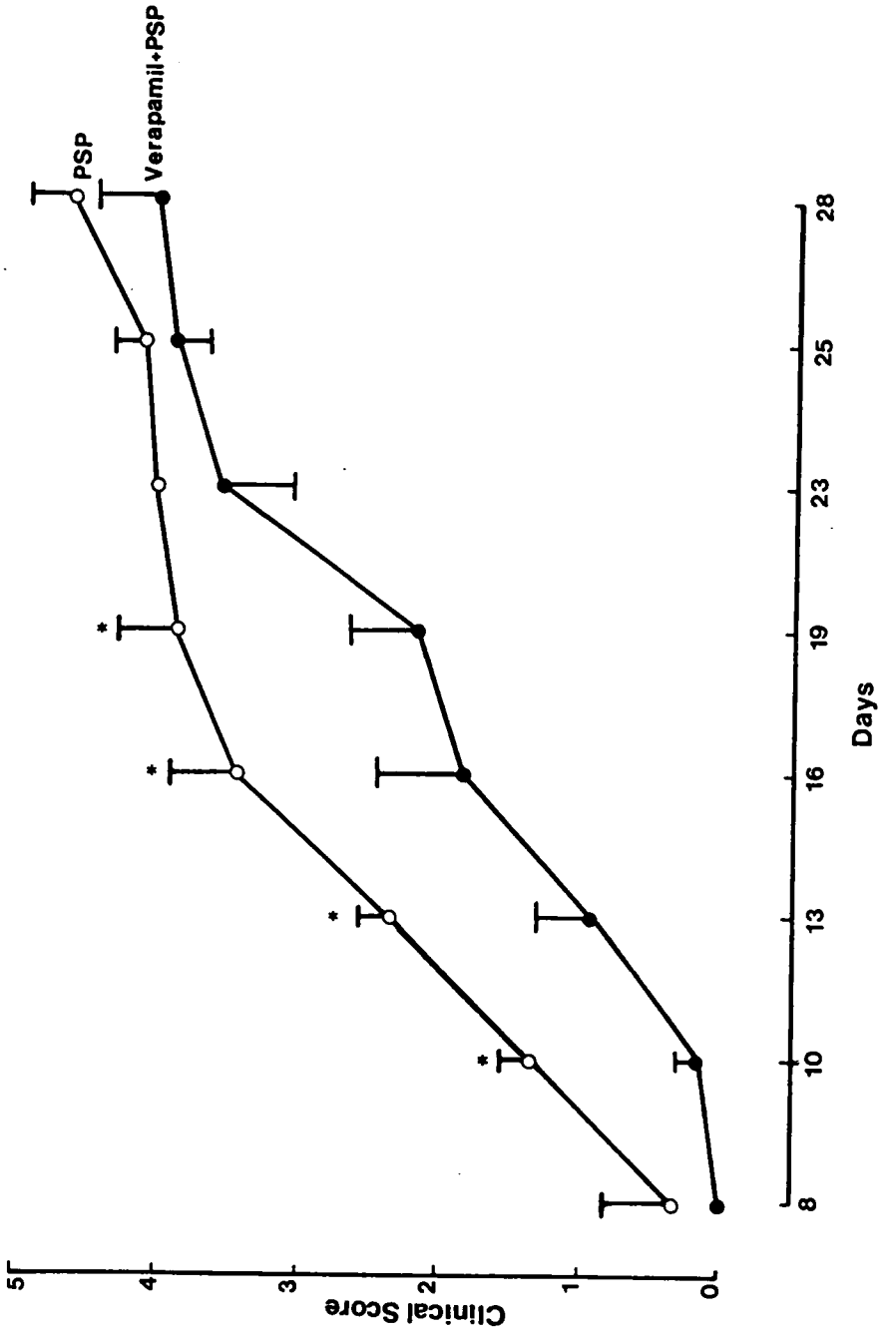
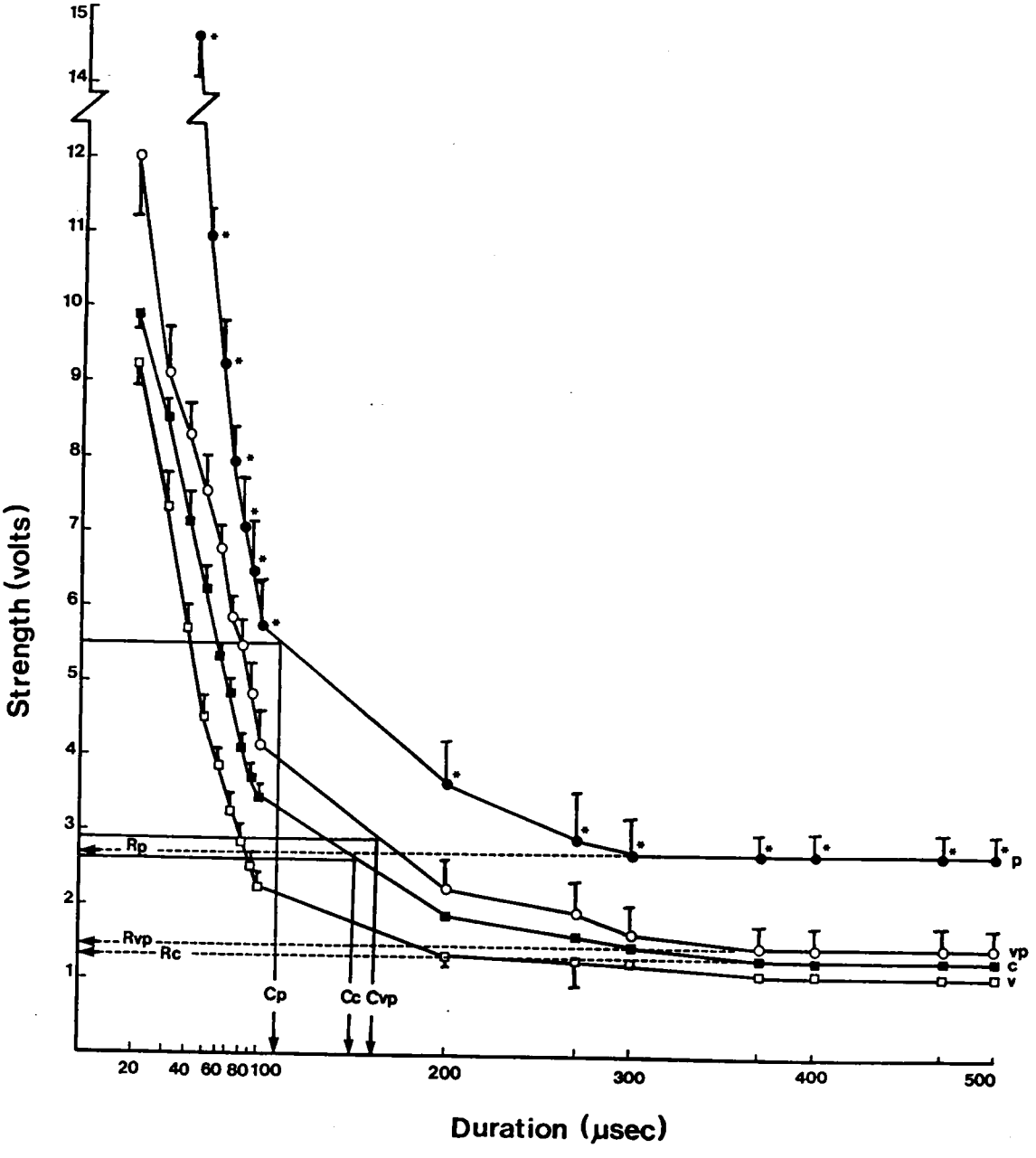

PSP
Verapamil+PSP
Clinical Score
0
1
2
3
4
5
8
10
13
16
19
23
25
28
Days
*
*
*
*
*

Figure 1. Development of clinical signs in hens after administration of phenyl saligenin phosphate (PSP) and verapamil + PSP. Results are presented as mean ± SD, n=4-6. The Student's t-test was used to compare the two groups; significant differences are indicated by asteriks. Increasing clinical scores reflect progression in deficits as detailed in Methods.


Strength (volts)
15
14
12
11
10
9
8
7
6
5
4
3
2
1
Rp
Rvp
Rc
Cp
Cc
Cvp
20
40
60
80
100
200
300
400
500
Duration (μsec)
p
vp
c
v

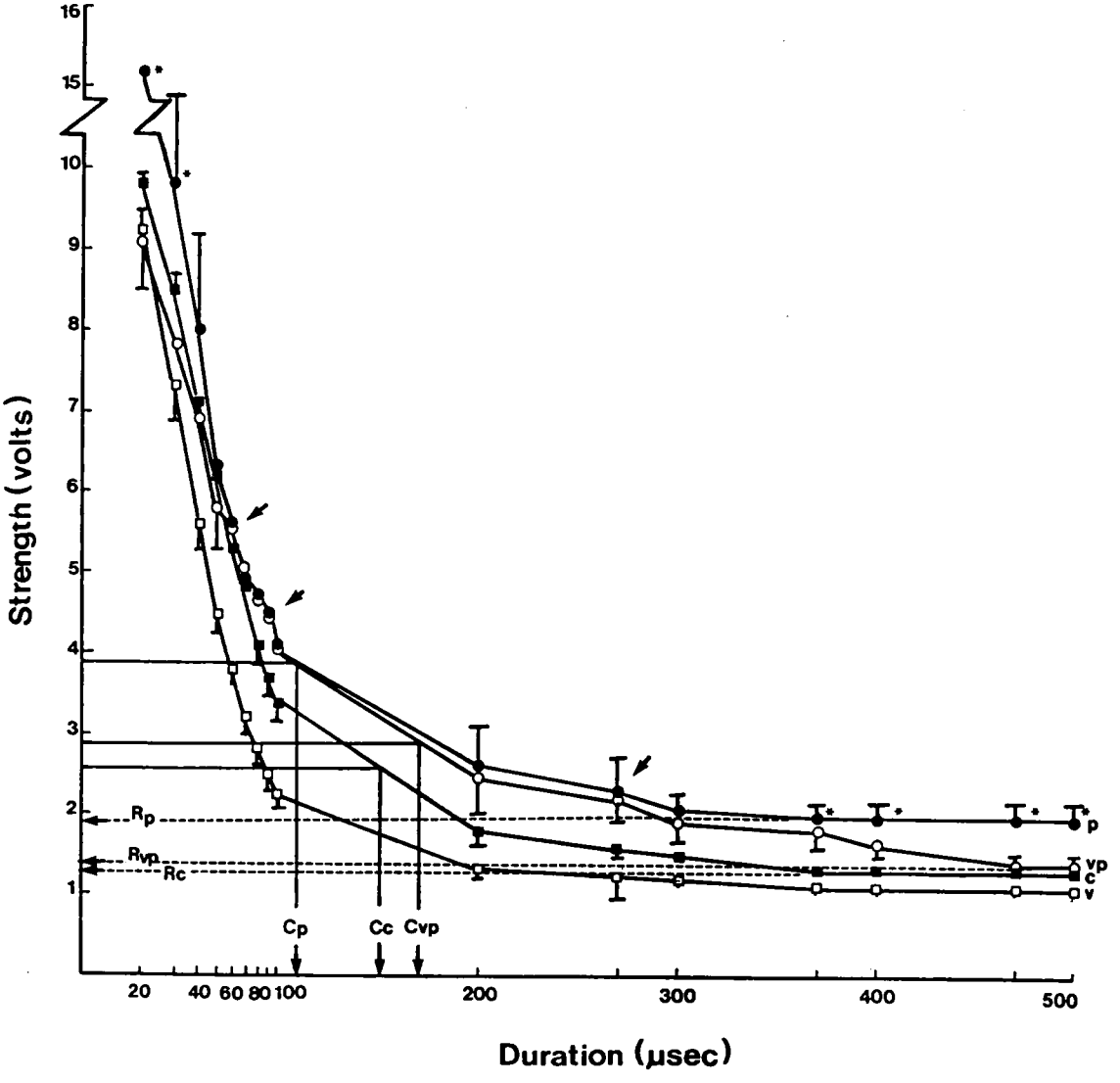

Strength (volts)
16
15
10
9
8
7
6
5
4
3
2
1
Rp
Rvp
Rc
Cp
Cc
Cvp
20
40
60
80
100
200
300
400
500
Duration (μsec)
p
vp
c
v

Figure 2. Strength-duration curves for rectangular pulses on the biventer cervicis nerve-muscle preparations from controls (c), verapamil (v), verapamil and PSP (vp), and PSP- (p) treated hens at 17 days (Fig. 2a) and 28 days (Fig. 2b). Note the "kinks" (discontinuities: arrows) in Fig. 2a, indicative of partial denervation. R = rheobase; C = chronaxie for control (c), verapamil and PSP (vp) and PSP alone (p) lines, which are denoted by subscript. All points represent the mean ± SE, n = 4-7. Results were examined for statistical differences by an analysis of variance, and points significantly different from control noted by asteriks (Newman-Keuls test for multiple comparisons, $p < 0.05$).

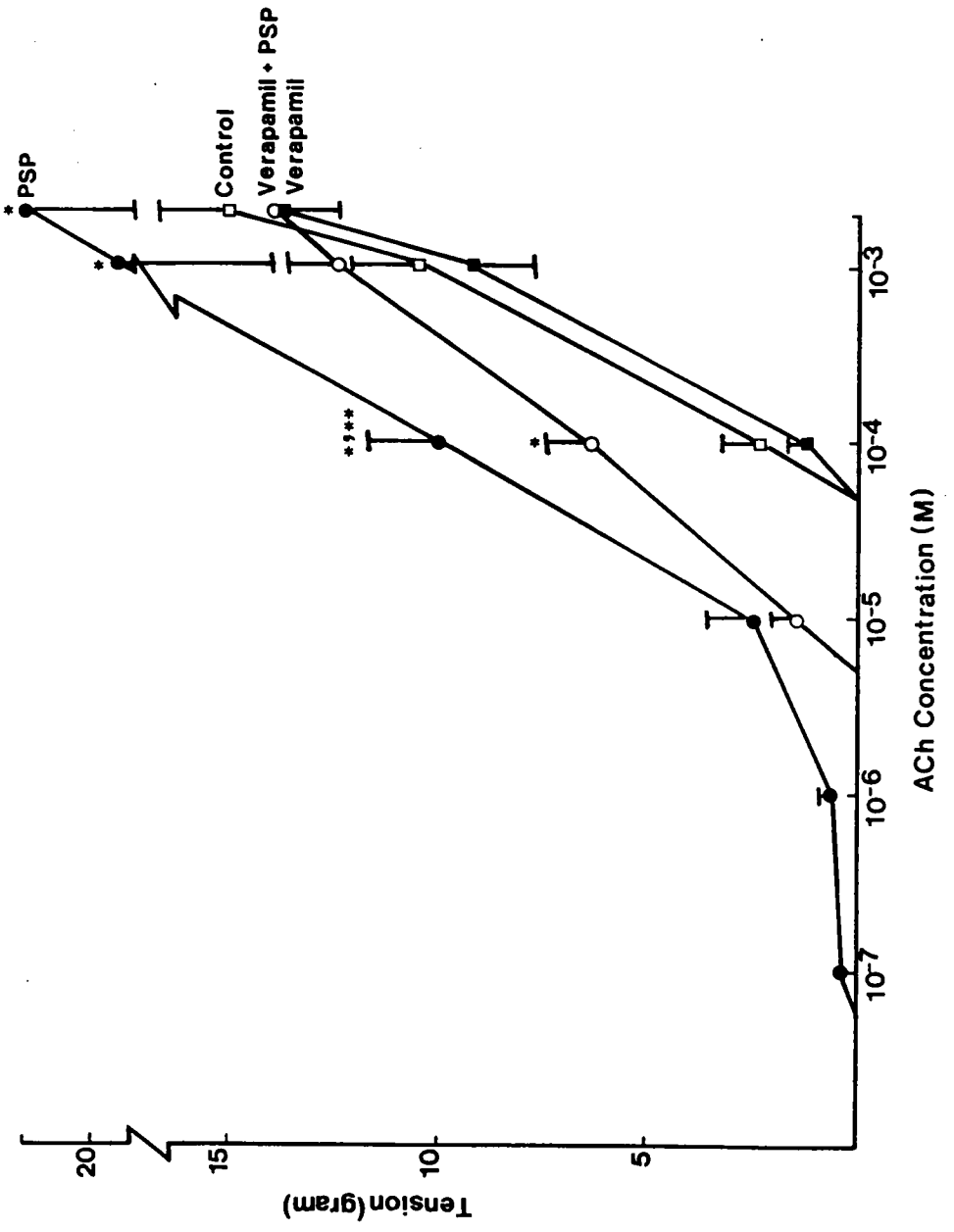
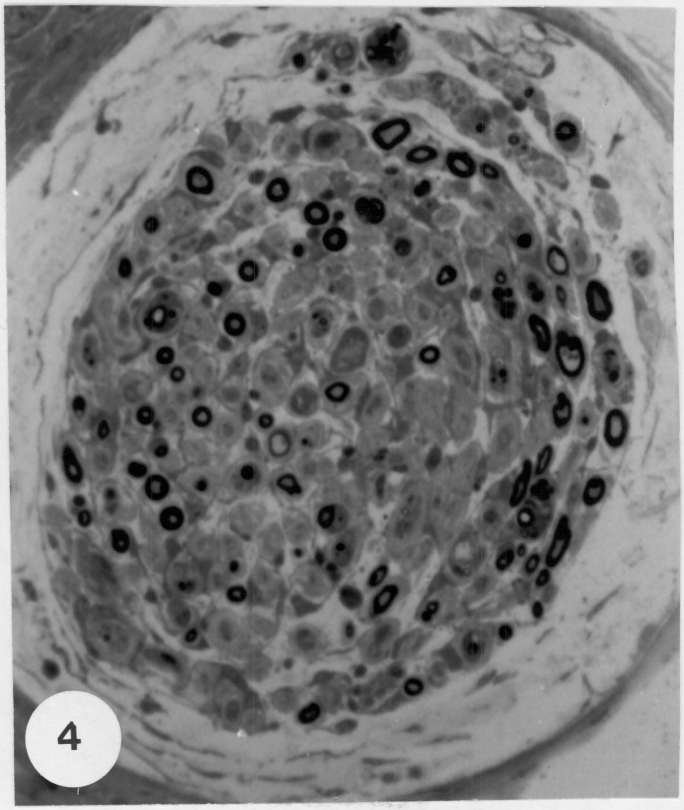

PSP
Control
Verapamil + PSP
Verapamil
Tension (gram)
20
15
10
5
ACh Concentration (M)
10-7
10-6
10-5
10-4
10-3

Figure 3. Tension generation in the biventer cervicis nerve-muscle preparation in response to ACh in controls, verapamil, verapamil and PSP, and PSP-treated hens 28 days after PSP administration. All points represent the mean ± SE, n = 4-7. Points significantly different from controls or verapamil are identified by (*), those different from verapamil + PSP are identified by (**) as determined by an analysis of variance and Newman-Keuls test for multiple comparisons, ($p < 0.05$).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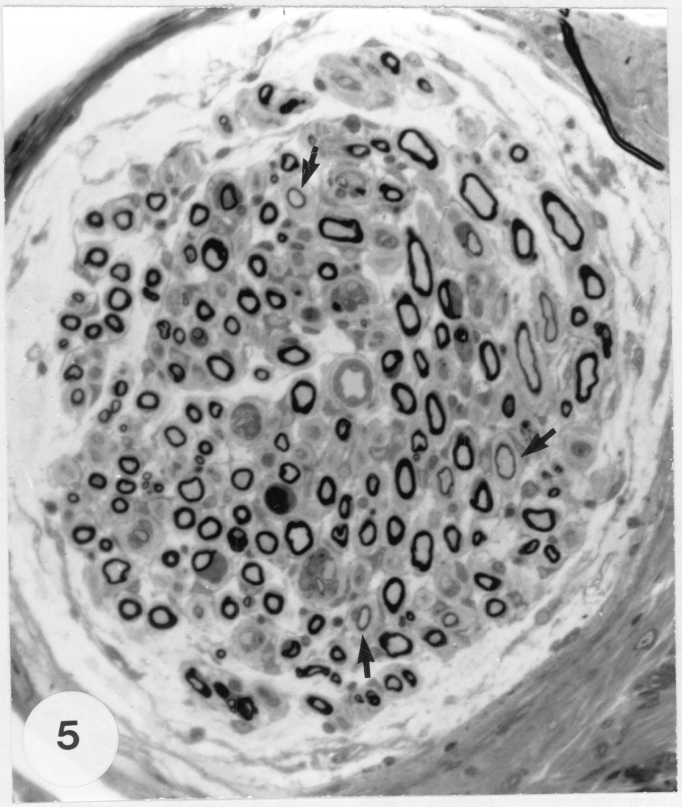
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Figure 4 and 5. Cross sections of the distal region of nerve to biventer cervicis muscle. Figure 4: from hen given PSP 17 days earlier. Figure 5: from hen given verapamil and PSP 17 days earlier. Note the presence of a greater number of apparently intact myelinated fibers in Figure 5 as compared to 4. Also, thinly myelinated (arrows) and non-myelinated regenerating axons are prominent in Fig. 5. Toluidine blue-safranin strain, x183.

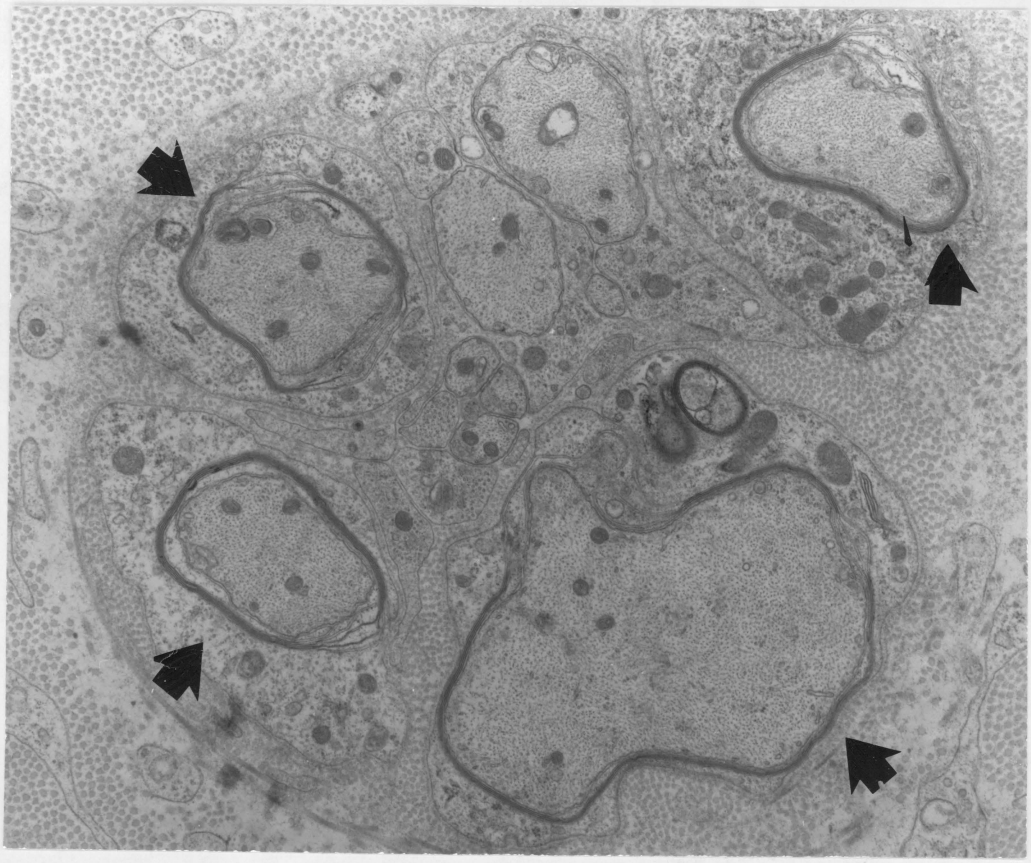
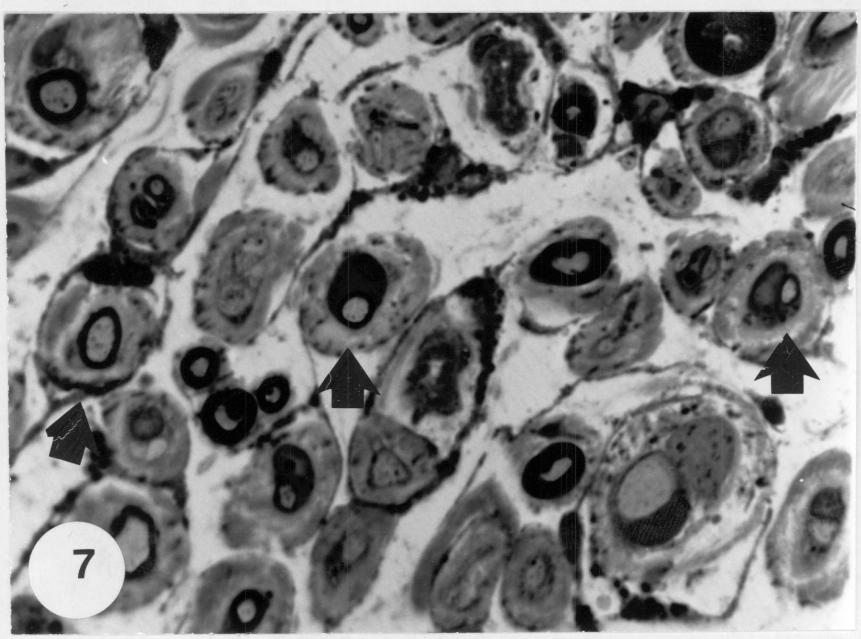


Figure 6. Cluster of thinly myelinated and premyelinated regenerating axons (arrows) and associated Schwann cells. Biventer cervicis nerve from verapamil treated chicken 17 days after PSP administration, x12857.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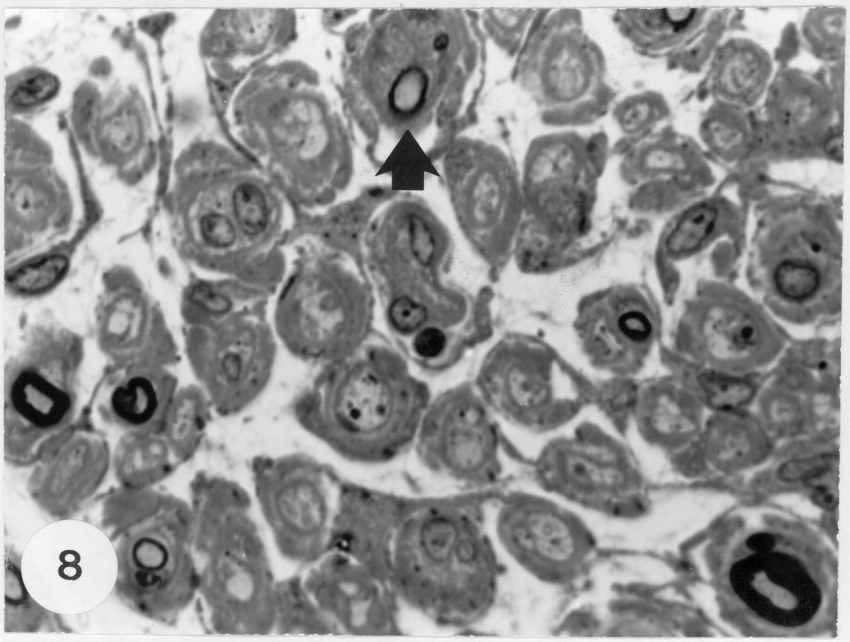
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Figure 7 and 8. Distal region of nerve to biventer cervicis muscle. Figure 7: hen given verapamil and PSP (28 days earlier). Note the numerous regenerating, newly myelinated axons (arrows). Figure 8: hen given only PSP 28 days earlier. Note the extensive fiber degeneration. Only a few regenerating axons are seen (arrows). Toluidine blue - safranin stain, x1344.

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CHAPTER 8

MODIFICATION OF ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY (OPIDN) WITH CALCIUM CHANNEL BLOCKERS: I. IN VIVO USING THE SCIATIC-GASTROCNEMIUS PREPARATION

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ABSTRACT

Calcium channel blockers, nifedipine and verapamil, were administered to adult white leghorn hens to determine if inhibition of calcium entry or intracellular mobilization could modify delayed neuropathy induced by administration of phenyl saligenin phosphate (PSP, 2.5 mg/kg im). Hens were given either nifedipine (1.0 mg/kg im for 5 days), or verapamil (7.0 mg/kg im for 4 days) begining 24 hr before administration of PSP. Calcium channel blockers alone did not affect parameters indicative of OPIDN. Ataxia was less pronounced in hens given PSP plus either calcium channel blocker than in hens given PSP alone during observations made 7-30 days after PSP administration. Strength-duration curves for sciatic and tibial nerves and for gastrocnemius muscle were established in anaesthetized hens at 4-5, 7-8, 15-16, and 21-22 days after PSP administration. These revealed significant increases in excitability threshold for preparations from hens receiving PSP only compared to preparations from control hens, or to preparations from hens treated with PSP and calcium channel blockers. High rheobase and long chronaxie values indicative of nerve degeneration and reduced conductivity were encountered earlier in hens treated with only PSP than in those receiving either nifedipine or verapamil in conjunction with PSP. Changes similar to those caused by PSP alone did not appear until 15-16 days for these latter groups. Qualitatively, histology of the branch of tibial nerve to the lateral head of the

gastrocnemius muscle showed similar lesions of Wallerian degeneration in all groups. Close-arterial injection of acetylcholine to the gastrocnemius muscle indicated early development of denervation hypersensitivity that was more pronounced in hens treated with PSP only. This study indicates that agents modulating calcium entry or intracellular movement could attenuate functional deficits associated with OPIDN.

INTRODUCTION

Some organophosphorus compounds (OPs) have the potential to induce a delayed neuropathy in man and other susceptible species following a single exposure (Abou-Donia, 1981). Organophosphorus-ester-induced delayed neuropathy (OPIDN) is characterized by the inhibition of a neural esterase, neuropathy target esterase (or neurotic esterase, NTE) and a delay period of 7-14 days after exposure before the appearance of clinical deficits. These deficits appear as incoordination, ataxia and flaccid paralysis (Abou-Donia, 1981). About the time clinical deficits appear, morphological indications of axonopathy, "dying-back" Wallerian degeneration, in large myelinated axons appear (Davis and Richardson, 1980; Johnson, 1987).

The mechanisms involved in the pathogenesis of OPIDN have not been previously defined. The breakdown of axonal cytoskeletal elements related to increases in free calcium (Ca^{++}) levels has been suggested, however, as contributing to neuropathies of various origin (Schlaepfer and Bunge, 1973; Komulainen and Bondy, 1988). Ca^{++} apparently contributes to such degenerative changes in both nerve and muscle by the enhanced activation of Ca^{++}-dependent proteases and kinases (Schlaepfer and Hasler, 1979; Kamakura et al., 1983; Banik et al., 1985; Llados, 1985; Berlet, 1987). Changes in some of these enzymes have been noted following administration of OPs inducing delayed neuropathy (Abou-Donia et al.,

1984; Patton et al., 1985; Suwita et al., 1986).

Because Ca^{++} can contribute to nerve and muscle degeneration and general cell injury and death (Wrogemann and Pena, 1976; Schanne et al., 1979; Farber, 1981), and a previous investigation showed verapamil could modify OPIDN (El-Fawal et al., 1989a), the present study was conducted to determine when the effects of verapamil occurred, whether effects of verapamil could be duplicated by another Ca^{++} channel blocker, nifedipine, and if the effects noted in an *in vitro* system (El-Fawal et al., 1989a) also occurred in the *in vivo* preparation described by others as a sensitive indicator of OPIDN (Robertson et al., 1987; 1988).

MATERIALS AND METHODS

Animals and Housing. White leghorn hens (>6 month-old, 1.2-1.8 kg) used in this study were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. They were housed in groups of five in cages (57 x 84 x 61 cm) and had access to food and water ad libitum. Hens were divided according to treatment [control, nifedipine, verapamil, nifedipine + phenyl saligenin phosphate (PSP), verapamil + PSP, and PSP alone], with 10-35 hens in each group.

Treatments. PSP, a prototype of the cyclic saligenin phosphates which are toxic metabolites of tri-o-tolyl phosphate, (TOTP) was dissolved in dimethyl sulfoxide (DMSO) so that the 2.5 mg/kg dosage was available in 0.25 ml/kg for administration into the breast muscle. Nifedipine (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and 1.0 mg/kg administered im for 5 days. Verapamil was dissolved in distilled water and 7.0 mg/kg administered im for 4 days. The first of the doses of either Ca^{++} channel blocker was given one day prior to PSP administration to ensure adequate bioavailability (Anonymous, 1981). This regimen of administration for 5 (nifedipine) or 4 (verapamil) days was used because previous studies had shown denervation changes could occur as early as 4 days after PSP or TOTP administration (El-Fawal et al., 1989b).

Clinical signs. Hens were observed daily for clinical signs. Independent blind evaluations by at least two individuals were recorded and averaged. Scores were designated as 0 for normal, 1 for altered gait, 2 for difficulty in walking and standing, 3 for severe ataxia, 4 for leg paralysis, and 5 for both leg and wing involvement (Sprague et al., 1980).

Electrophysiological and pharmacological studies. Hens were examined for electrophysiological changes 4-5, 7-8, 15-16, and 21-22 days after dosing. Hens were anesthetized with sodium pentobarbital (30 mg/kg) (Green, 1982) administered to effect via a cannula placed in the brachial vein which allowed additional injection when needed. A tracheotomy was performed and hens were ventillated at a frequency of 15-35 breaths/min, tidal volume of 35 ml and minute volume of 0.7 l/min. Hens were kept under a heat lamp which maintained rectal body temperature at 37.6°C. To expose the sciatic nerve, the biceps femoris muscle was separated from the underlying semitendinosus muscle along the entire ventral-caudal extent. The biceps was then cut at the knee joint. The semitendinosus was retracted to reveal the sciatic trunk, which was isolated along its entire length from the ilium to the tibial nerve bifurcation and the caput laterale of the gastrocnemius muscle. Prior to isolation of the gastrocnemius muscle the sciatic and tibial nerve were immersed in mineral oil warmed to 37° C and covered with warm, moist cotton gauze. New oil was period-

ically added. The gastrocnemius muscle was prepared as described by Brown and Harvey (1938). The skin incised along the posterior aspect of the leg from the tarsus to the upper end of the femur. The gastrocnemius was detached by its tendon just proximal to the tibio-tarsal joint. A length of suture was passed through the tendon with a needle and tied. The external head of the muscle was separated from the middle head and from the muscles covering the tibia as far as the knee joint. The suture tied to the muscle was attached to an FT03 isometric transducer (Grass Medical Instruments, Quincy, MA), and placed under 30g resting tension prior to recording. The muscle was also covered with warm oil and wrapped in moist, warm cotton gauze to prevent heat and moisture losses. The limb was fixed by clamping drill bits inserted through the knee and ankel joints (Durham and Ecobichon, 1984). For establishing strength-duration curves (SDC), nerves were positioned on pairs of bipolar stimulating and recording electrodes. In the case of the gastrocnemius muscle SDC, stimulation was via the tibial branch to the lateral head of the muscle. A pair of Grass needle electrodes placed in the body of the muscle acted as the recording electrodes. The distance between stimulating and recording electrodes ranged between 30 and 35 mm for all preparations, nerve and muscle. The voltage required to produce a minimally perceptible threshold depolarization of nerve or muscle at stimulus durations from 20 to 500 μsec were viewed on a Tektronix 5115 oscilloscope

(Tektronix Inc., Beaverton, OR). Rheobase and chronaxie were derived from these curves as previously described (El-Fawal et al., 1988) and the inflexion region (40-100 μsec) linearized as performed by Robertson et al., (1987), to allow comparison of threshold excitability between groups.

After completion of electrophysiological measurements, the anterior tibial vein was ligated and cut close to its junction with the sciatic vein and retracted by the suture at the cut end. This exposed the anterior tibial artery into which a cannula (filed 29 gauge insulin needle) was eventually placed. All branches of the posterior tibial artery were ligated and cut with the exception of that supplying the lateral head of the gastrocnemius muscle. The anterior tibial artery could now be clamped and the cannula inserted into the distal end. When injections were done, a measured volume of fluid, usually 0.5 ml (heparinized saline or acetylcholine), was rapidly injected into the cannula, the circulation was stopped by clamping the main sciatic artery proximal to its bifurcation.

Acetylcholine chloride (ACh; Sigma Chemical Co., St. Louis, MO) was prepared in 9% saline such that each concentration (0.0025-100 μg) was contained within a 0.5 ml volume. Prior to measurement of responses to ACh, a bolus of saline was injected to establish that responses seen were not due to change in muscle volume.

On completion of electrophysiological and pharmacological measurements, hens were euthanized with an overdose of

anesthetic. Both sciatic nerves and the contralateral gastrocnemius nerve were removed and snap frozen in liquid nitrogen for later biochemical analysis. The biventer cervicis nerve-muscle preparation was also dissected out for further physiological measurements (see El-Fawal et al., 1989c).

Histology. The branch of the tibial nerve innervating the lateral head of the gastrocnemius muscle was dissected from the contralateral leg and placed in a fixative of the following composition: 2.5% glutaraldehyde in 0.05M sodium coacodylate at pH 7.4. Following a period of 24-48 hr of fixation at 4° C the nerve was rinsed in buffer, post-fixed in 2% osmium tetroxide and embedded in Epon epoxy resin. Cross sections were cut at 1 um thickness, stained with toluidine blue and safranin, and examined by light microscopy.

Statistics. All data was analyzed by analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences between control and experimental groups with $p<0.05$ considered significant. All data are expressed as mean $\pm$ standard error.

RESULTS

Clinical signs of ODIPN were evident in hens treated with PSP alone 7 days following administration. No signs of ataxia were evident in hens treated with either nifedipine and PSP or verapamil and PSP on this day. OPIDN progressed in severity over the next several days (Fig. 1). By day 13 all chicken given PSP, with or without Ca^{++} channel blocker, showed clinical evidence of OPIDN, but hens given PSP alone were more severly affected. Scores for hens given PSP or either Ca^{++} channel blocker were consistently and significantly lower during the entire duration of the experiment. Clinical scores for verapamil and PSP-treated chickens were lower than those for nifedipine and PSP treated birds during the 15 days they were on the experiment.

Strength-duration curves generated for the sciatic, tibial, and gastrocnemius muscle revealed an elevation in threshold excitability in hens given PSP alone as early as 4-5 days following PSP administration (Fig. 2a). The difference in excitability thresholds between preparations from PSP-treated hens and controls continued to increase to days 7-8 (Fig. 2b) and remained elevated through days 15-16 (Fig. 2c). Excitability thresholds for the verapamil and PSP-treated group began to increase on day 7 (Fig. 2b) and remained elevated in the sciatic nerve on day 15, but these thresholds were lower than thresholds for the PSP group (Fig. 2b). Thresholds for the nifedipine and PSP group

remained comparable to controls from day 4 through day 15 (Fig. 2a,b,c). Excitability thresholds at 21-22 days continued to be elevated for hens treated with PSP only (Fig. 2d). It was not until this time that excitability thresholds in sciatic and tibial nerves and gastrocnemius muscles of hens treated with nifedipine and PSP were significantly different from controls.

Rheobase and chronaxie values derived from SDC are shown in Table 1. Rheobase values for sciatic and tibial nerves were significantly higher for hens treated with PSP alone at 4-5 days than for controls or hens treated with nifedipine and PSP. PSP did not significantly increase rheobase in gastrocnemius muscle until 7-8 days after administration. Chronaxie for sciatic and gastrocnemius muscle was affected as early as 4-5 days after PSP administration, but not in any of the preparations given nifedipine + PSP. Rheobase and chronaxie values continued to be higher and longer, respectively, in preparations from PSP-treated chickens than in chickens given nifedipine until 15-16 after PSP administration (Table 1). Effects of nifedipine on rheobase and chronaxie values were greater than effects of verapamil during this time. By 15 days after PSP, nifedipine could no longer completely attenuate the deleterious effect of PSP on rheobase and chronaxie.

Typical original tracings of responses of gastrocnemius muscle to close-arterial injection of ACh are shown in Fig. 3. Responses of the gastrocnemius muscle of hens to close-

arterial injection of ACh at various times after PSP treatment are shown in Fig. 4. The gastrocnemius muscle was 25, 50, 125, and 13 X times more sensitive at 4-5, 7-8, 15-16, and 21-22 days after PSP, respectively, when compared to control muscle. Those of hens treated with Ca^{++} channel blockers and PSP remained comparable to control throughout most of the experiment.

Light microscopic examination of the epoxy embedded branch of the tibial nerve supplying the lateral head of the gastrocnemius muscle at 15-16 days revealed a spectrum of alterations characteristic of Wallerian degeneration. Among the axonal changes seen were regions with pallor of axoplasmic staining and thinning of the myelin sheath (Fig. 5). There was accumulation of stained intra-axonal debris. Qualitatively, nerves from hens given PSP or hens treated with PSP and with Ca^{++} channel blockers did not appear to be different, although lesions appeared to be less extensive, especially in nerves from the group treated with verapamil and PSP (Fig. 5c).

DISCUSSION

In this study it was demonstrated that the Ca^{++} channel blockers nifedipine or verapamil could modify the neurotoxic effects of PSP on electrophysiological and pharmacological parameters used to assess nerve and muscle function. Excitability thresholds, for example, for sciatic, and tibial nerves, and for the gastrocnemius muscle were less affected in preparations from hens given calcium channel blockers and PSP. The elevation of excitability thresholds in PSP-treated hens is consistent with other reports using this compound (El-Fawal et al., 1988) and other OPs (Robertson et al., 1987, 1988; Anderson et al., 1988) and has been suggested to represent a compromise of nerve conductivity.

The elevation of rheobase and lengthening of chronaxie are in agreement with patterns of nerve degeneration reported for other neuropathies (Wynn Parry, 1971), and in delayed neuropathy due to di-n-butyl-2,2-dichlorovinyl phosphate (DBCV) (Robertson et al., 1988). The increase in chronaxie seen in hens treated with PSP and in the group treated with nifedipine and PSP (15 days) has been suggested to represent an increased thresholds for nerve conduction (Robertson et al., 1987) or hypoexcitability (Harris, 1971; Wynn Parry, 1971).

Responses to close-arterial injection of ACh following OP-induced denervation were not qualitatively different from normal muscle, although the muscle was much more sensitive

to this neurotransmitter. This is consistent with reports on effects seen following denervation due to axotomy of mammalian, amphibian, or avian muscle (Brown, 1937; Brown and Harvey, 1938). The development of denervation hypersensitivity to ACh following administration of neurotoxic OPs seen in the present study is consistent with earlier reports from our laboratory (El-Fawal et al., 1988, 1989a).

It was difficult to assess the histological differences between groups recieving PSP only or PSP and Ca^{++}-channel blockers without further quantitative studies. This may be due in part to the fact that only 10-20% of all fibers are affected in the tibial branch supplying the lateral head of the gastrocnemius muscle (Jortner and Ehrich, 1987). Unlike the tibial branch used in the present study, lesions in the biventer cervicis nerve are much more definitive (El-Fawal et al., 1988, 1989a).

The attenuation of clinical, electrophysiological and pharmacological changes due to OPIDN by the Ca^{++}-channel blockers, verapamil and nifedipine in this study is consistent with our earlier report on the effect of verapamil on OPIDN using the biventer cervicis nerve-muscle preparation (El-Fawal et al., 1989a). Here, however, we also used a dihydropyridine blocker which, unlike verapamil, blocks both surface voltage-sensitive calcium channels and acts intracellularly to inhibit release of Ca^{++} from its stores (mitochondria, sarcoplasmic reticulum). Verapamil blocks only membrane channels (Miller, 1987). Results reported here are

further substantiated by studies using an in vitro preparation following PSP administration, with and without both calcium channel blockers (El-Fawal et al., 1989c).

Calcium plays a key role in signal transduction, linking external stimuli to the intracellular environment in nerve, muscle and other cell types (Cavero and Spedding, 1983; Miller, 1987). Unregulated intracellular Ca^{++} levels, however, are implicated in death of nerve and muscle cells (Wrogemam and Pena, 1976; Schanne et al., 1979; Faber, 1981). The induction of "dying-back", Wallerian degeneration of nerve fibers is believed to be facilitated by the activation of a Ca^{++}-activated neutral protease (CANP or calpain) in the axoplasm (Schlaepfer and Hasler, 1979; Kamakuru et al., 1983; Schlaeepfer and Zimmerman, 1984; Mata et al., 1986) and in myelin (Banik et al., 1985; Berlet, 1987). A related enzyme is found in muscle, its activation contributes to muscle degeneration and atrophy (Reddy et al., 1975; Llados, 1985). Removal of Ca^{++} attenuates or abolishes some of the degenerative changes cited above. The increased activation of CANP in brain, nerve, and muscle has been demonstrated in OPIDN, as has its attenuation by calcium channel blockers (El-Fawal et al., 1989d). Furthermore, activation of L (long) type voltage sensitive calcium channels, which are blocked by nifedipine, are said to be associated with the activation of CANP. Their blockade may subsequently contribute to the ability of nifedipine to modify OPIDN.

Increases in intracellular Ca^{++} have also been shown to enhance the ACh receptor synthesis that occurs in denervated muscle. Denervation (following axotomy) also increases the number of functional voltage sensitive calcium channels. These channels also function as receptors for dihydropyridines such as nifedipine (Schmid et al., 1984; Miller, 1987). The time course of increases and decreases in ACh receptors and dihydropyridine receptors in axotomy corresponds to that of OPIDN, with notable effects at 3 days and maximum effects at 15 days. Furthermore, the de novo synthesis of the ACh receptors is suppressed by decreased intracellular Ca^{++} using Ca^{++} channel blockers such as verapamil (Metafora et al., 1980; McManaman et al., 1981; Kallo and Steinherdt, 1983). This may explain why hens treated with nifedipine or verapamil did not develop as notable hypersensitivity to ACh following OP administration.

In the present study drugs capable of altering Ca^{++} movement in cells could modify OPIDN. The effectiveness of Ca^{++} channel blockers as modifiers of OPIDN may be due to attenuation of some detrimental consequences of increased intracellular free Ca^{++}, such as activation of degradative enzymes, in both nerve and muscle.

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REFERENCES

ABOU-DONIA, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-148.

ABOU-DONIA, M. B., PATTON, S. E. AND LAPADULA, D. M. (1984). Possible endogenous protein phosphorylation in organophosphorus compound-induced delayed neurotoxicity. In Cellular and Molecular Neurobiology (T. Narahashi, Ed.), pp. 265-283 Raven Press, New York.

ALMON, R.R. AND APPEL, S.H. (1976). Cholinergic sites in skeletal muscle. I. Denervation effects. Biochem. 13: 3662-3667.

ANDERSON, R. J., ROBERTSON, D. G., HENDERSON, J. D. AND WILSON, B. W. (1988). DFP-induced elevation of strength-duration threshold in hen peripheral nerve. NeuroToxicology 9: 47-52.

BANIK, N. L., McALHANEY, W. W. AND HOGAN, E. L. (1985). Calcium-stimulated proteolysis in myelin: Evidence for a Ca^{++}-activated neutral proteinase associated with purified myelin of rat CNS. J. Neurochem. 45: 581-588.

BERLET, H. H. (1987). Calcium-dependent neutral protease activity of myelin from bovine spinal cord: Evidence for soluble cleavage products of myelin proteins. Neurosci. Lett. 73: 266-270.

BICKFORD, A. A. (1984). The hen as a test model for delayed neurotoxicity studies: Introductory comments. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 24-25 Intox Press, Little Rock.

BROWN, G. L. (1937). The actions of acetylcholine on mammalian and frog skeletal muscle. J. Physiol. 89: 438-461.

BROWN, G. L. AND HARVEY, A. M. (1938). Reactions of avian muscle to acetylcholine and eserine. J. Physiol. 94: 101-117.

CAVERO, I., AND SPEDDING, M. (1983). Calcium antagonists: a class of drugs with a bright future. Life Sci. 33: 2571-2581.

DAVIS, C. S. AND RICHARDSON, R. J. (1980). Organophosohorus compounds. In Experimental and Clinical Neurotoxicology (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527-544 Williams and Wilkins, Baltimore.

DURHAM, H. D. AND ECOBICHON, D. J. (1984). The function of motor nerves innervating slow tonic skeletal muscle in hens with delayed neuropathy induced by tri-o-tolyl phosphate. Can. J. Physiol. Pharmacol. 62: 1268-1273.

EL-FAWAL, H. A. N., JORTNER, B. S., EYRE, P. AND EHRICH, M. (1988). The biventer cervicis nerve-muscle preparation of adult hens: Effects of phenyl saligenin phosphate administration. NeuroToxicology 9: 625-636.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989a). Effects of verapamil on organophosphate-induced delayed neuropathy (OPIDN). Toxicol. Appl. Pharmacol. 97: 500-511.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989b). The use of the biventer cervicis nerve-muscle preparation to detect early changes following exposure to organophosphates inducing delayed neuropathy. Ph.D. Dissertation, VPI & SU.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989c). Modification of organophosphate-induced delayed neuropathy (OPIDN) with calcium channel blockers: II. in vitro using the biventer cervicis nerve-muscle preparation. Ph.D. Dissertation, VPI & SU.

EL-FAWAL, H. A. N., CORRELL, L., GAY, L. AND EHRICH, M. (1989d). Modification of organophosphate-induced delayed neuropathy with calcium channel blockers: III. altered activities of esterases and calcium-activated neutral protease (CANP). Ph.D. Dissertation, VPI & SU.

ETO, M., CASIDA, J. E. AND ETO, T. (1962). Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11: 337-352.

FARBER, J. L. (1981). The role of calcium in cell death. Life Sci. 29: 1289-1295.

HARRIS, R. (1971). Chronaxy. In Electrodiadnosis and Electromyography (S. Licht, Ed.), pp. 218-239. Elizabeth Licht, New Haven.

JOHNSON, M. K. (1987). Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. Trends Pharmacol. Sci. 8: 174-179.

JORTNER, B. S. AND EHRICH, M (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

KAMAKURA, K., ISHIURA, S., SUGITA, H. AND TOYOKURA, Y. (1983). Identification of Ca^{++}-activated neutral protease in peripheral nerve and its effects on neurofilament degeneration. J. Neurochem. 40: 908-913.

KOMULAINEN, H. AND BONDY, S. C. (1988). Increased free intracellular Ca^{2+} by toxic agents: an index of potential neurotoxicity? Trends Pharmacol. Sci. 9: 154-156.

LLADOS, F. T. (1985). Neurogenic origin of an experimental myopathy induced by the ionophore A23187. J. Neurol. Sci. 69: 171-182.

MATA, M., STAPLE, J. AND FINK, D. J. (1986). Changes in intra-axonal calcium distribution following nerve crush. J. Neurobiol. 17: 449-467.

McMANAMAN, J. L., BLOSSER, J. C. AND APPEL, S. (1981). The effects of calcium on acetylcholine receptor synthesis. J. Neurosci. 1: 771-776.

METAFORA, S., FELSANI, A., COTRUFO, R., TAJANA, G. F., DI IORIO, D., DEL RIO, A., DE PRISCO, P. P. AND ESPOSITO, V. (1980). Neural control of gene expression in the skeletal muscle fiber: the nature of the lesion in the muscular protein-synthesizing machinery following denervation. Proc. R. Soc. Lond. B. 209: 239-255.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. Science. 235: 46-235.

PATTON, S. E., LAPADULA, D. M., O'CALLAGHAN, J. P., MILLER, D. B. AND ABOU-DONIA, M. B. (1985). Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-o-cresyl phosphate to hens. J. Neurochem. 45: 1567-1577.

REDDY, M. K., ETLINGER, J. D., RABINOWITZ, M., FISCHMAN, D. A. AND ZAK, R. (1975). Removal of Z-lines and a-actinin from isolated myofibrils by a calcium-activated neutral protease. J. Biol. Chem. 250: 4278-4284.

ROBERTSON, D. G., SCHWAB, B. W., SILLS, R. D., RICHARDSON, R, J. AND ANDERSON, R. J. (1987). Electrophysiologic changes following treatment with organophosphorus-induced delayed neuropathy-producing agents in the adult hen. Toxicol. Appl. Pharmacol. 87: 420-429.

ROBERTSON, D. G., MATTSON, A. M., BESTERVELT, L. L., RICHARDSON, R. J. AND ANDERSON, R. J. (1988). Time course of electrophysiologic effects induced by di-n-butyl-2,2-dichlorovinyl phosphate (DBVC) in the adult hen. J. Toxicol. Enviorn. Hlth. 23: 283-294.

SCHANNE, F. A. X., KANE, A. B., YOUNG, E. E. AND FARBER, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science. 206: 700-702.

SCHLAEPFER, W. W. AND BUNGE, R. P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J. Cell. Biol. 59: 456-470.

SCHLAEPFER, W. W. AND HASLER, M. B. (1979). Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. Brain Res. 168: 299-309.

SCHLAEPFER, W. W. AND ZIMMERMAN, U.P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. In Axonal Transport in Neuronal Growth and Regeneration (J. S. Elam and P. Cancalon, Eds.), pp. 261-273 Plenum Press, New York.

SCHMID, A., KAZAZOGLOU, T., RENAUD, J. F. AND LAZDUNSKI, M. (1984). Comparative changes of levels of nitrendipine Ca^{++} channels, of tetrodotoxin-sensitive Na^{+} channels and oubain-sensitive $(Na^{+}+K^{+})$-ATPase following denervation of rat and chick skeletal muscle. FEBS 172: 114-118.

SPRAGUE, G. L., SANDVIK, L. L., BICKFORD, A. A. AND CASTLES, T. R. (1980). Evaluation of a sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. Life Sci. 27: 2523-2528.

SUWITA, E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986b). Calcium and calmodulin-enhanced in vitro phosphorylation of hen brain cold-stable microtubules and spinal cord neurofilament triplet proteins after a single oral dose of tri-o-cresyl phosphate. Proc. Natl. Acad. Sci. 83: 6174-6178.

WROGEMAN, K. AND PENA, S. D. J. (1976). Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle disease. Lancet. 7961: 672-673.

WYNN PARRY, C. B. (1971). Strength-duration curves. In Electrodiagnosis and Electromyography (S. Licht, Ed.), pp. 241-271. Elizabeth Licht, New Haven.

TABLE 1. ELECTROPHYSIOLOGICAL PARAMETERS DERIVED FROM STRENGTH-DURATION CURVE FOLLOWING ADMINISTRATION OF PSP, NIFEDIPINE AND PSP, OR VERAPMIL AND PSP.

Treatment	Tissue	Rheobase (volts)[a]	Chronaxie (msec)[a]
ontrol	sciatic	0.97 ± 0.02	0.037 ± 0.002
	tibial	1.15 ± 0.05	0.040 ± 0.001
	gastrocnemius	1.10 ± 0.06	0.042 ± 0.002
ifedipine	sciatic	1.02 ± 0.17	0.037 ± 0.002
	tibial	1.12 ± 0.15	0.042 ± 0.002
	gastrocnemius	1.20 ± 0.08	0.042 ± 0.002
erapamil	sciatic	1.05 ± 0.05	0.040 ± 0.00
	tibial	1.25 ± 0.09	0.052 ± 0.002
	gastrocnemius	1.35 ± 0.12	0.042 ± 0.002
ay 4-5			
PSP	sciatic	1.60 ± 0.21[b]	0.060 ± 0.009[b]
	tibial	1.72 ± 0.21[b]	0.040 ± 0.008
	gastrocnemius	1.30 ± 0.28	0.070 ± 0.006[b]
Nifedipine + PSP	sciatic	0.93 ± 0.03	0.036 ± 0.006
	tibial	0.86 ± 0.08	0.033 ± 0.008
	gastrocnemius	1.16 ± 0.15	0.043 ± 0.008
Verapamil + PSP	sciatic	1.40 ± 0.115[b]	0.053 ± 0.003[b]
	tibial	1.35 ± 0.125[b]	0.082 ± 0.14[b]
	gastrocnemius	1.25 ± 0.095[b]	0.075 ± 0.006[b]
ay 7-8			
PSP	sciatic	1.62 ± 0.25[b]	0.120 ± 0.014[b]
	tibial	1.75 ± 0.33[b]	0.090 ± 0.005[b]
	gastrocnemius	2.40 ± 0.50[b]	0.070 ± 0.008[b]
Nifedipine + PSP	sciatic	1.10 ± 0.15	0.036 ± 0.012
	tibial	1.40 ± 0.58	0.036 ± 0.003
	gastrocnemius	1.06 ± 0.18	0.036 ± 0.003
Verapamil + PSP	sciatic	1.33 ± 0.17	0.063 ± 0.003
	tibial	1.60 ± 0.11	0.056 ± 0.003
	gastrocnemius	1.46 ± 0.17	0.050 ± 0.00

ay 15-16			
PSP	sciatic	1.85 ± 0.18[b]	0.090 ± 0.009[b]
	tibial	2.20 ± 0.57[b]	0.100 ± 0.202[b]
	gastrocnemius	2.53 ± 0.63[b]	0.100 ± 0.029[b]
Nifedipine + PSP	sciatic	1.20 ± 0.02[b]	0.080 ± 0.014
	tibial	0.81 ± 0.01	0.080 ± 0.030[b]
	gastrocnemius	1.70 ± 0.06[b]	0.060 ± 0.004[b]
Verapamil + PSP	sciatic	1.26 ± 0.06	0.053 ± 0.003
	tibial	1.50 ± 0.09[b]	0.056 ± 0.008
	gastrocnemius	1.60 ± 0.11[b]	0.046 ± 0.006
ay 21-22			
PSP	sciatic	1.93 ± 0.06[b]	0.050 ± 0.005[b]
	tibial	2.53 ± 0.35[b]	0.060 ± 0.008[b]
	gastrocnemius	3.40 ± 0.23[b]	0.070 ± 0.003[b]
Nifedipine + PSP	sciatic	4.20 ± 0.11[b]	0.050 ± 0.00
	tibial	1.20 ± 0.11	0.056 ± 0.003
	gastrocnemius	0.73 ± 0.35[b]	0.050 ± 0.011

Rheobase and chronaxie derived from the strength duration curves. Results expressed as mean ± S.E. n=3-6. Values for preparations from control hens and from hens given either nifepidine or verapamil did not differ at any time period at which they were measured and results presented, therefore, are pooled values over 4-22 days.

Significantly different from control. Analysis of variance and Newman-Kuels test for multiple comparisons.

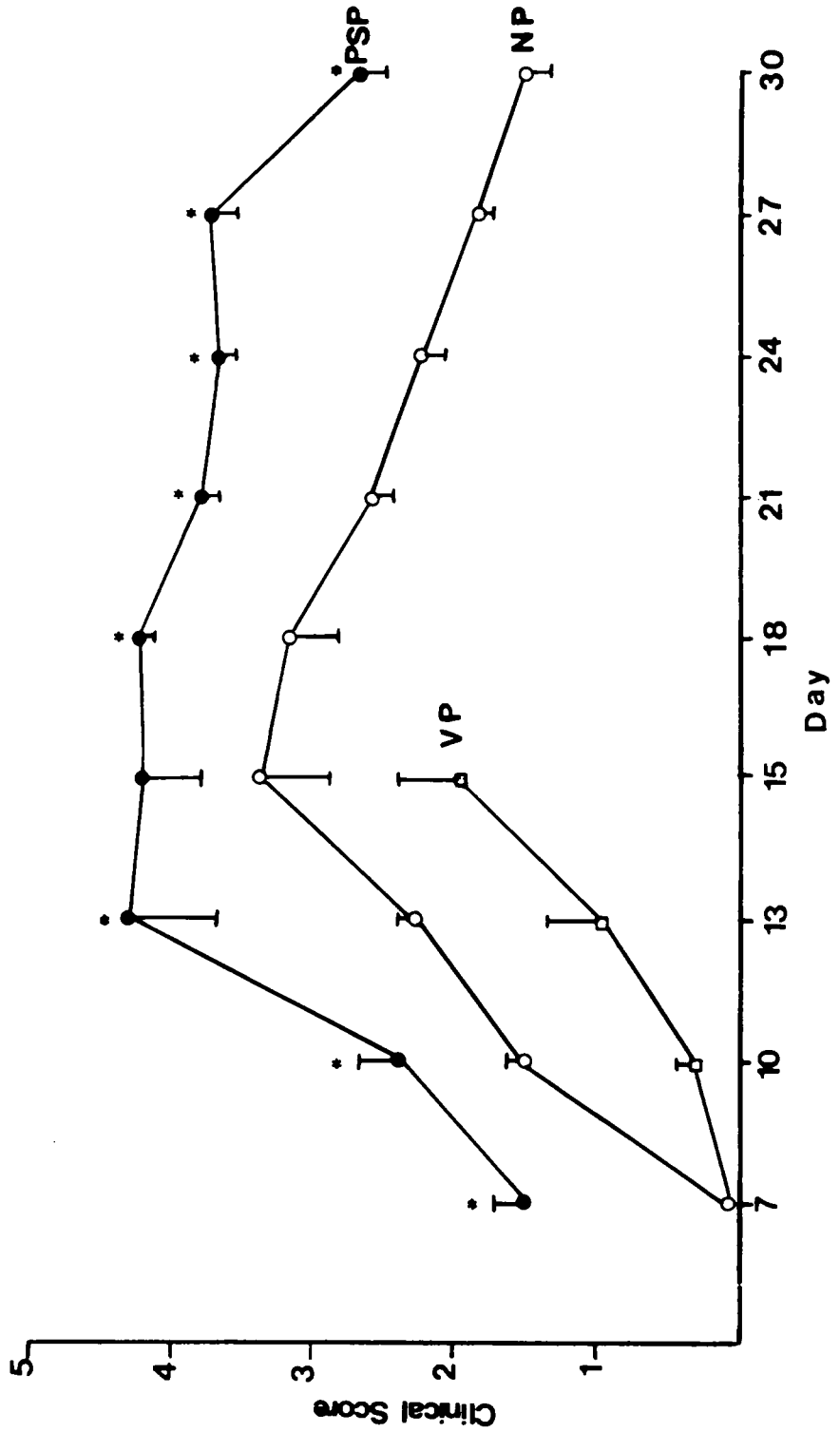
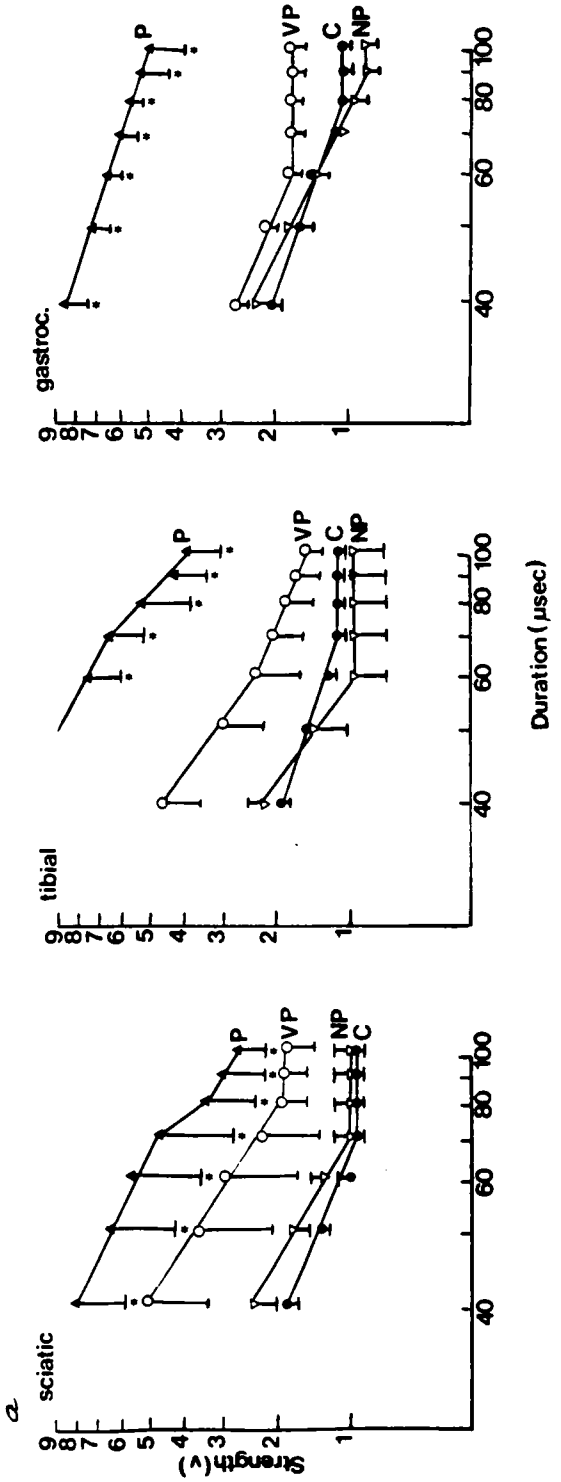
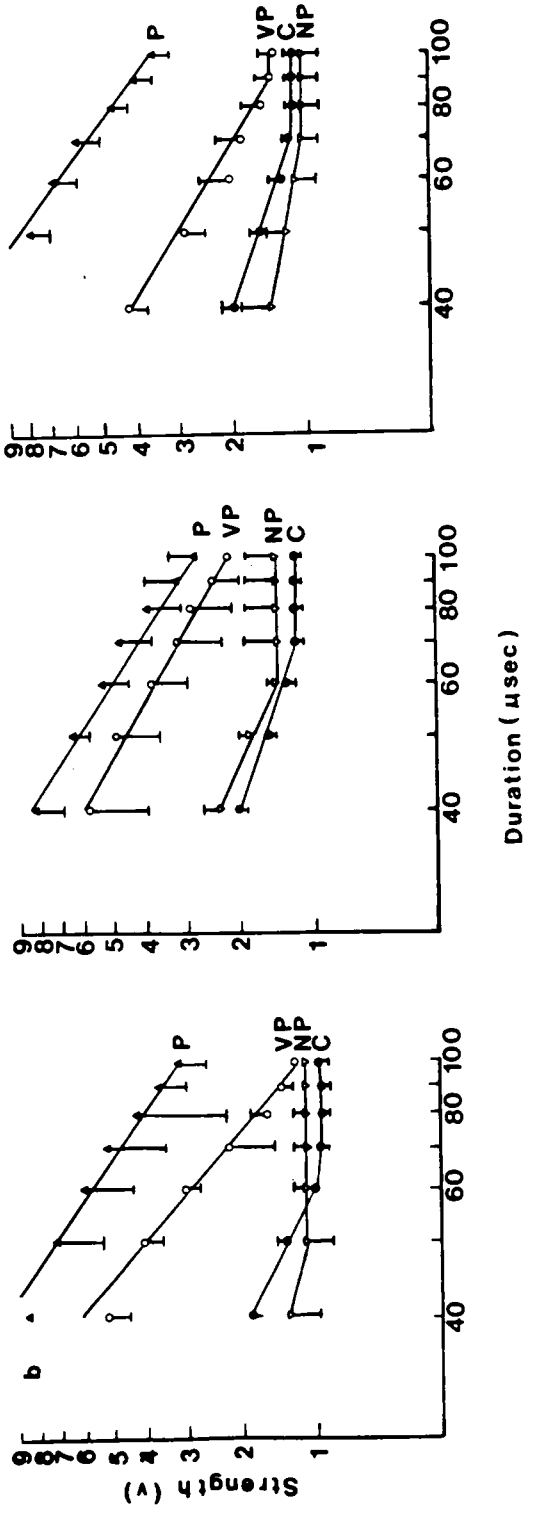

PSP
NP
VP
Clinical Score
Day
1
2
3
4
5
7
10
13
15
18
21
24
27
30

Figure 1. Development of clinical deficits and partial recovery after administration of phenyl saligenin phosphate (PSP), nifedipine plus PSP (NP) or verapamil plus PSP (VP). Results are presented as mean ± S.E., (n=5-10). Differences between PSP-treated groups from NP or VP are denoted by asterisks. ANOVA with Newman-Keuls test for multiple comparisons ($p < 0.05$). 1 = altered gait; 2 = difficulty in walking and standing; 3 = severe ataxia; 4 = leg paralysis; 5 = paralysis with both leg and wing involvement.


a
sciatic
tibial
gastroc.
Strength(v)
Duration (μsec)
P
VP
C
NP
9
8
7
6
5
4
3
2
1
40
60
80
100


P
VP
NP
C
b
Strength (V)
Duration (μsec)
1
2
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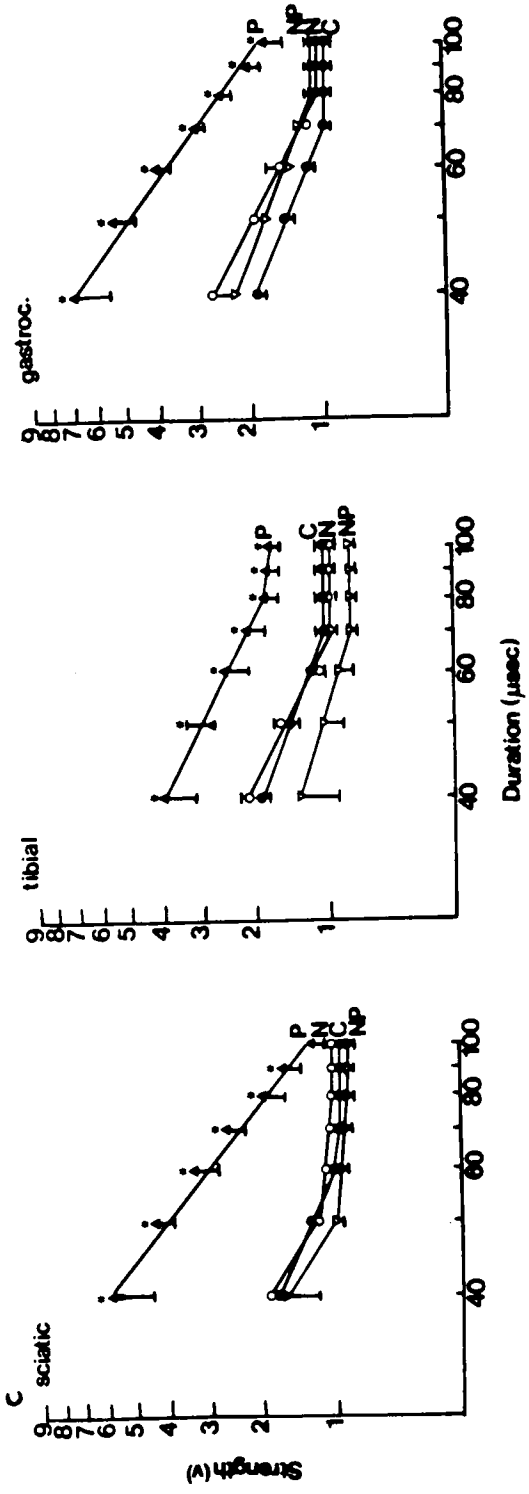
6
7
8
9
40
60
80
100


gastroc.
tibial
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C
Strength (V)
Duration (μsec)
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C
NP

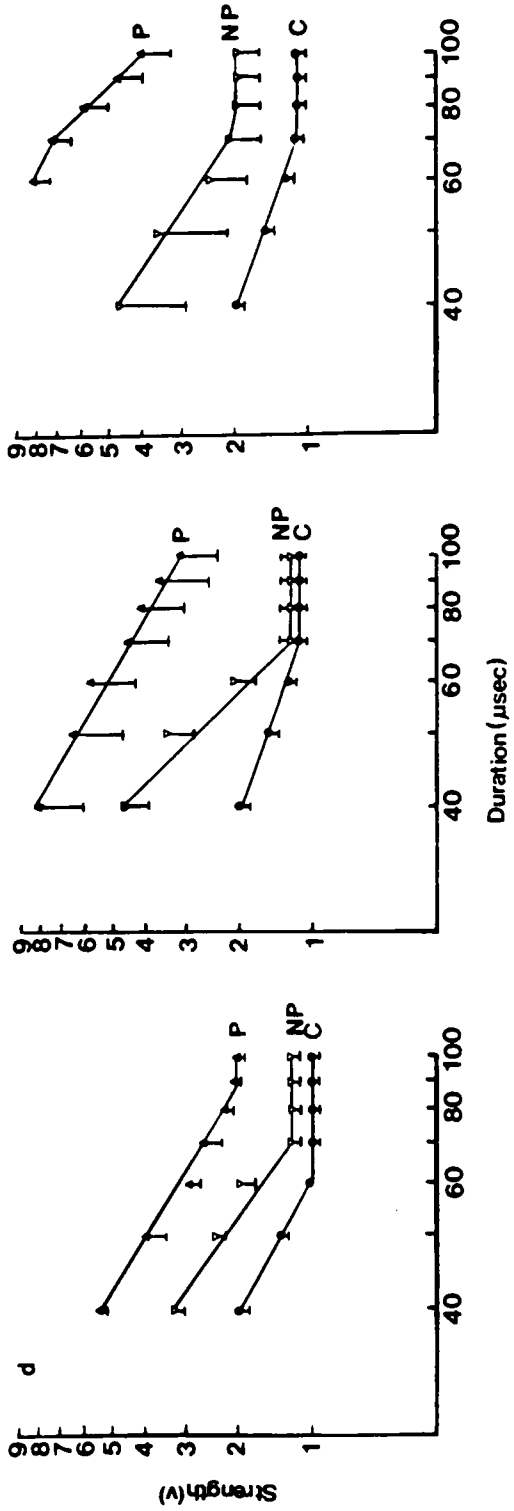

Strength(V)
Duration (μsec)
P
NP
C
d
1
2
3
4
5
6
7
8
9
40
60
80
100

Figure 2a, b, c, and d. Log-log plots of inflexion region (40-100 μsec) of strength-duration curves for sciatic and tibial nerves and gastrocnemius muscle at 4-5 (Fig. 2a), 7-8 (Fig. 2b), 15-16 (Fig. 2c), and 21-22 (Fig. 2d) days after PSP (P), nifedipine plus PSP (NP) or verapamil plus PSP (VP) treatments. C = control, N = nifedipine only. Preparations from groups receiving only PSP required a significant higher threshold for stimulation on all days tested. Those from the NP group were more comparable to control as were preparations from the VP group. All points represent the mean $\pm$ S. E. (ANOVA, and Newman-Keuls test for multiple comparison; $p < 0.05$; $n = 3-5$).

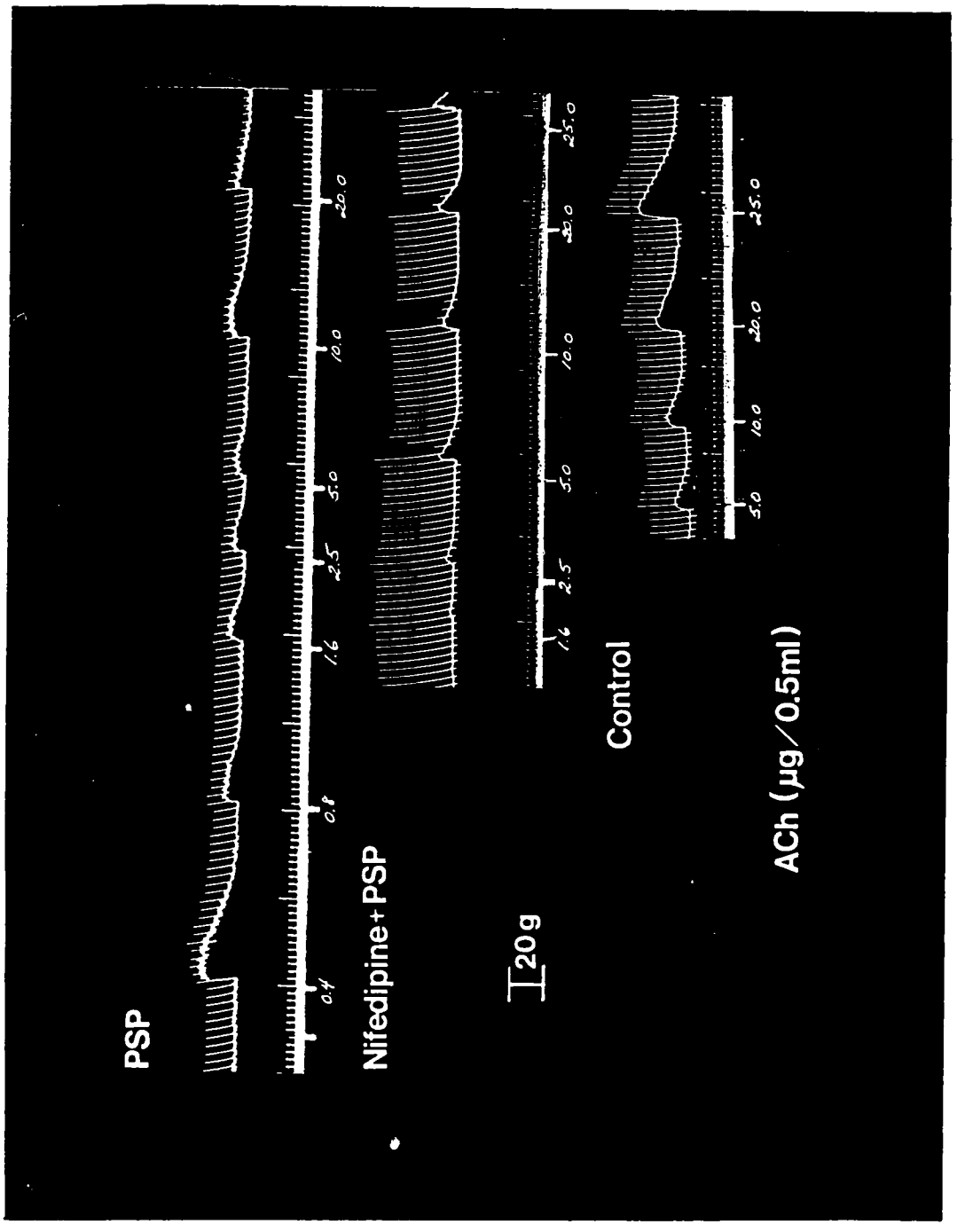

PSP
Nifedipine+PSP
20g
Control
ACh (μg/0.5ml)
0.4
0.8
1.6
2.5
5.0
10.0
20.0
25.0

Figure 3. Typical original trace of responses of the gastrocnemius muscle to close-arterial injection of ACh on day 21 following administration of PSP. Stimulation frequency = 12 ppm, event marker shows time and concentration of ACh injection.

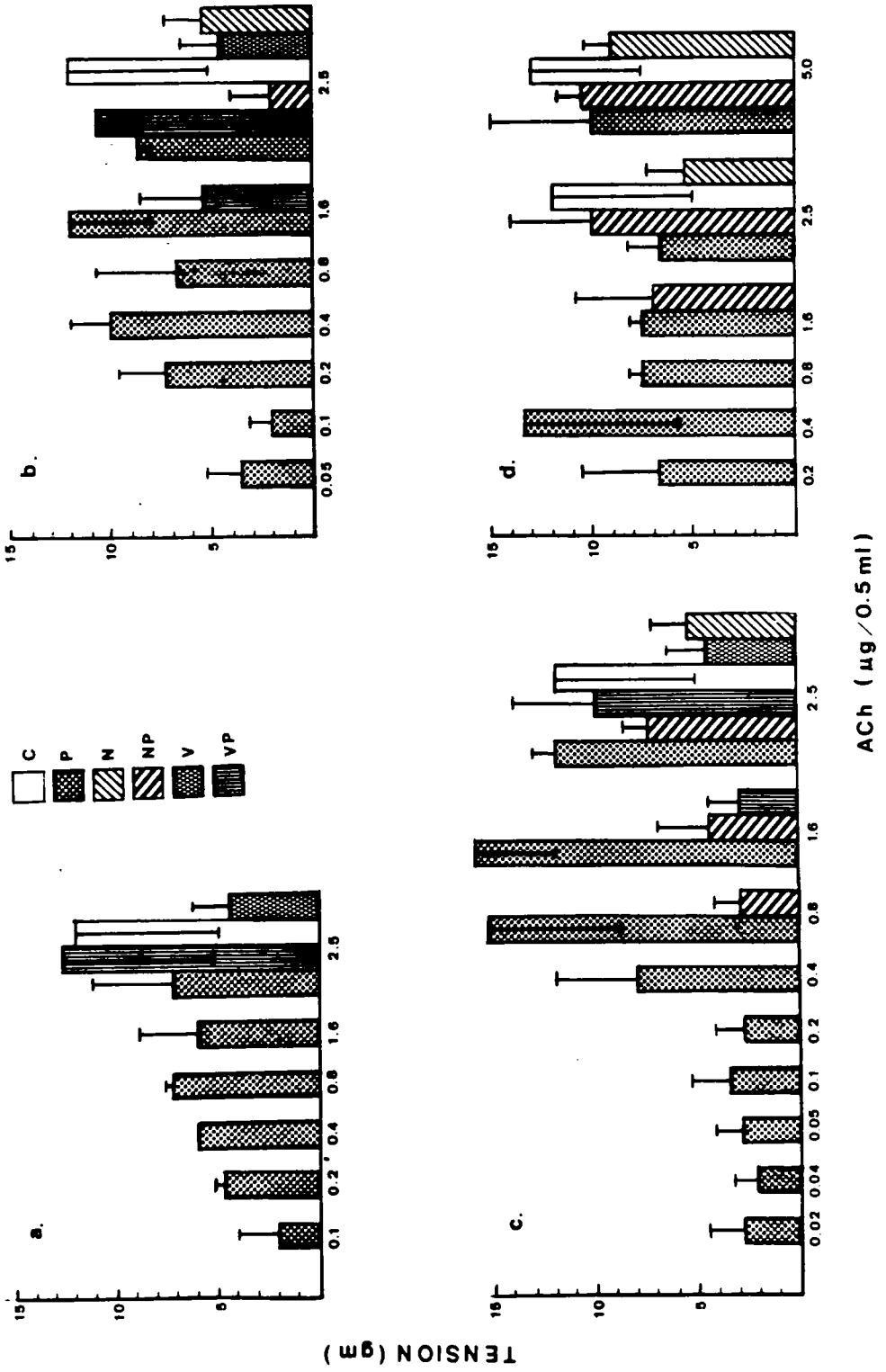
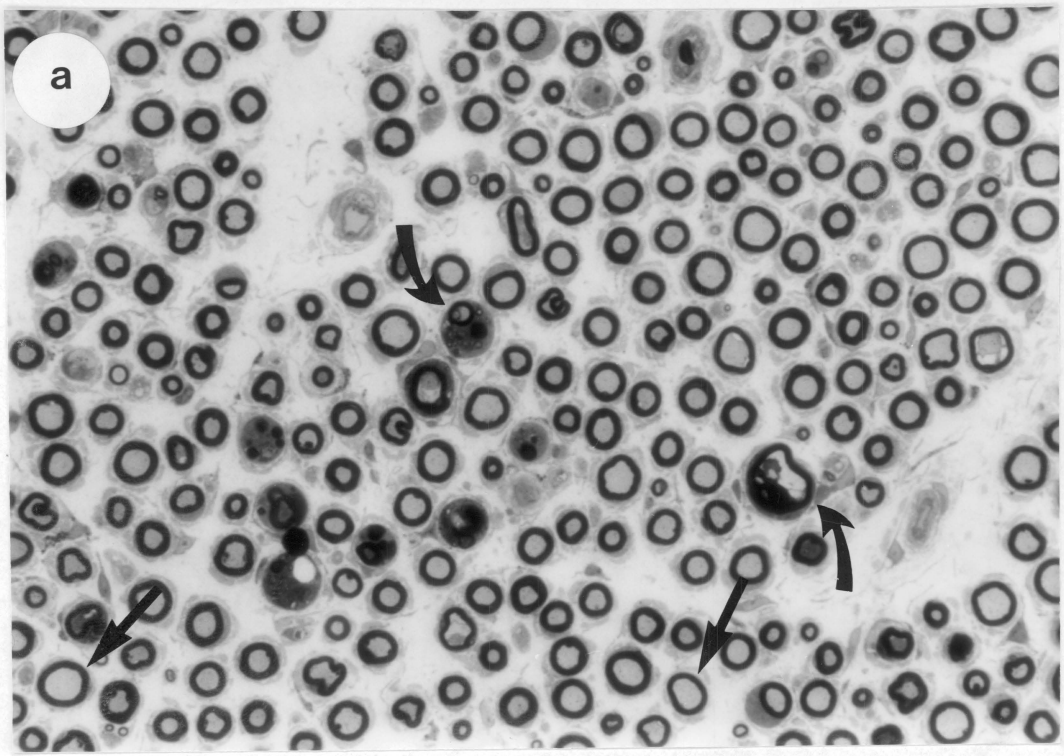
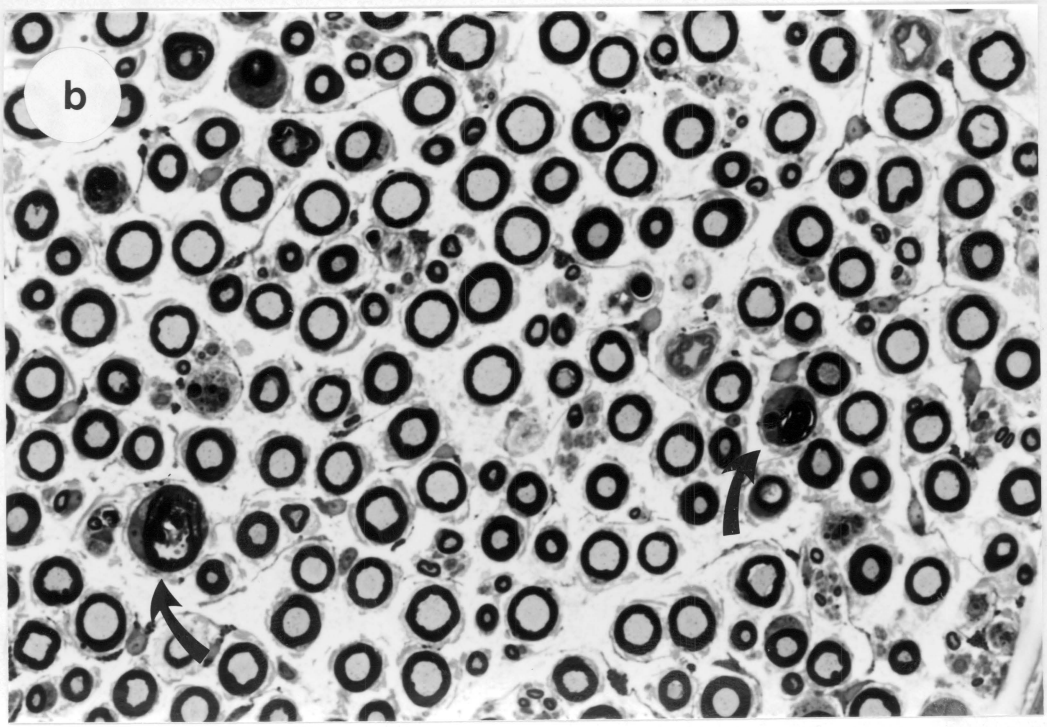

C
P
N
NP
V
VP
a.
b.
c.
d.
TENSION (gm)
ACh (µg/0.5ml)

Figure 4 a, b, c, and d. Tension development in gastrocnemius muscle in response to close arterial injection of ACh on days 4-5 (Fig. 4a), 7-8 (Fig. 4b), 15-16 (Fig. 4c), and 21-22 (Fig. 4d) following PSP administration. C = control, N = nifedipine-treated, V = verapamil-treated, NP = nifedipine plus PSP-treated, and VP = verapamil plus PSP-treated (n = 3-5). No response was seen in any but the PSP-treated group at the doses of ACh shown until more than one bar is presented. Control groups and group treated only with Ca^{++} channel blockers did not respond until 2.5 mg/0.5 ml ACh was administered.


a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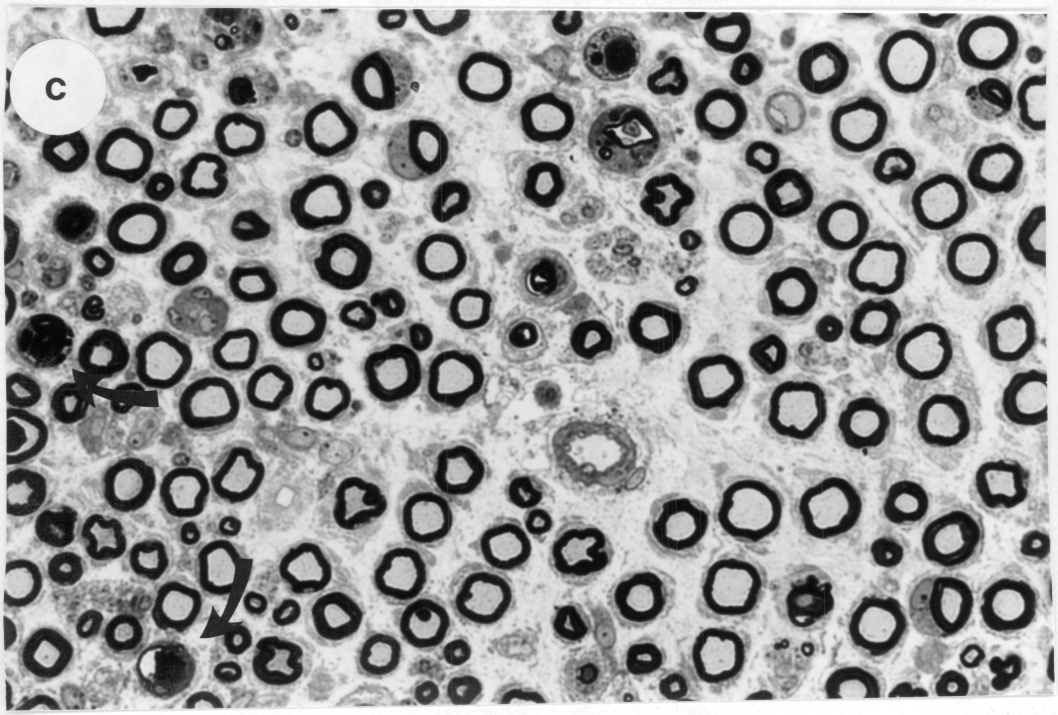
C

Figures 5 a, b, and c. Cross sections of tibial nerve branch to the lateral head of the gastrocnemius muscle, 15 days after administration of PSP (Fig. 5a), nifedipine plus PSP (Fig. 5b), or verapamil plus PSP (Fig. 5c). Note the fibers undergoing Wallerian-like degeneration (curved arrows) in nerves of hens from all three groups, and the more advanced thinning of myelin in those receiving PSP only (straight arrows), 260 X.

CHAPTER 9

MODIFICATION OF ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY (OPIDN) WITH CALCIUM CHANNEL BLOCKERS: II. IN VITRO USING THE BIVENTER CERVICIS NERVE-MUSCLE PREPARATION

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Abstract

The modification of OPIDN was attempted using calcium channel blockers, nifedipine (1.0 mg/kg im for 5 days) and verapamil (7.0 mg/kg im for 4 days) in white leghorn hens treated with phenyl saligenin phosphate (PSP, 2.5 mg/kg im) 24 hr following the first dose of Ca^{++} channel blocker. An equal number of hens received the same dose of PSP without either agent. Nerve and muscle functions were assessed 4-5, 7-8, 15-16, 21-22, 37, and 64 days after PSP administration in the biventer cervicis nerve-muscle preparation, in vitro. Strength-duration curves (SDC) revealed earlier elevation of excitability thresholds and rheobase in preparations from hens receiving PSP only when compared to controls or hens receiving calcium channel blockers and PSP. The shape of SDC at 21-22 days showed discontinuities indicative of regeneration in preparations from hens receiving both nifedipine and PSP. Denervation hypersensitivity to acetylcholine appeared earlier in preparations from hens administered PSP only. Myelinated fiber lesions were less extensive in nerves of hens receiving calcium channel blockers. This study served to verify the validity of the biventer cervicis nerve-muscle preparation in the detection of OPIDN, including its greater sensitivity in detecting functional and morphological deficits during OPIDN, compared to in vivo preparations. It also confirmed the ability of calcium channel blockers to modify OPIDN, and implicated Ca^{++} in development

of neuropathy.

INTRODUCTION

Organophosphorus-induced delayed neuropathy (OPIDN) in hens is indicated by ataxia and morphological damage to peripheral nerves that occurs weeks after single dose exposure in susceptible species including chickens, cats, sheep and man (Abou-Donia, 1981). Although first described in the 1930's, the mechanisms involved in the development of the delayed neuropathy induced by some organophosphorus compounds (OPs) have not yet been precisely defined (Abou-Donia, 1981). It has been noted, however, that an essential initiating event occurs in OPIDN, the inhibition of a neural esterase (neuropathy target esterase, NTE) (Davis and Richardson, 1980; Johnson, 1987). NTE inhibition has not been considered the sole event responsible for OPIDN because the activity of this enzyme returns to normal levels during the latent period of 1 to 2 weeks between exposure and onset of clinical deficits. The clinical signs observed in affected animals include incoordination, ataxia and flaccid paralysis (Abou-Donia, 1981; Metcalf, 1984). Clinical deficits are usually mirrored by the development of histological deficits characterized as a "dying-back", Wallarian degeneration involving the breakdown of the axon and a secondary degeneration of myelin and muscle atrophy (Davis and Richardson, 1980; Cisson and Wilson, 1982; Jortner and Ehrich, 1987).

Although not demonstrated in OPIDN until recently (LoPachin et al., 1988), excesses of intracellular calcium (Ca^{++}) have been suggested to lead to the development of Wallerian degeneration in other neuropathies including axotomy (Schlaepfer and Bunge, 1973; Schlaepfer, 1987; Komulainen and Bondy, 1988). Wallerian degeneration, breakdown and accumulation of cytoskeletal elements, myelin degeneration and muscle wasting associated with axotomy, for example, may be induced by the enhanced activity of a Ca^{++}-activated neutral protease (CANP or calpain) (Schlaepfer and Hasler,1977; Kamakuru et al., 1983; Banik et al., 1984; Llados, 1985; Berlet 1987) or changes in Ca^{++}-dependent kinases. Changes in activities of some Ca^{++}-dependent enzymes have been shown to occur following administration of neurotoxic OPs (Abou-Donia et al., 1984; Patton et al., 1985; Suwita et al., 1986). In addition, Ca^{++} has a known role in cell injury and death (Wrogeman and Pena, 1976; Schanne et al., 1979; Farber, 1981).

In a previous study we implicated Ca^{++} in the pathogenesis of OPIDN because the Ca^{++} channel blocker verapamil ameloriated functional and morphological changes associated with this neuropathy (El-Fawal et al., 1989a). In the present study, the effects of Ca^{++} channel blockers on the course of OPIDN were extended to include the use of a dihydropyridine Ca^{++} channel blocker, nifedipine. These studies include the time course of modification of electrophysiological, pharmacological and neuropathological parameters in

the biventer cervicis of the hen, an in vitro nerve-muscle preparation that we previously noted to be useful for assessment of changes that occur earlier than clinical deficits after exposure to neuropathy-inducing OPs (El-Fawal et al., 1988).

MATERIALS AND METHODS

Animals and housing. White leghorn hens (> 6 months old, 1.2-1.8 kg) used in this study were obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. Chickens were housed in groups of 4-5 with access to food and water ad libitum. Hens were divided according to treatment [control, nifedipine, verapamil, nifedipine + phenyl saligenin phosphate (PSP), verapamil + PSP, and PSP alone], with 10-35 hens in each group.

Treatments. PSP, an active neurotoxic OP compound, is a prototype of the cyclic saligenin phosphates, toxic metabolites of tri-o-tolyl phosphate (TOTP)(Eto et al.,1961). It was dissolved in dimethyl sulfoxide (DMSO) so that the 2.5 mg/kg dosage was contained within 0.25 ml/kg and administered into the breast muscle. Nifedipine (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and 1.0 mg/kg administered im for 5 days. Verapamil was distilled water and 7.0 mg/kg administered im for 4 days. The first of the doses of either nifedipine or verapamil was given one day prior to PSP administration to ensure adequate bioavailability (Anonymous, 1981). The regimen of administration for several days was used because previous studies had shown denervation changes in this nerve-muscle preparation could be detected as early as 4 days after PSP or TOTP administration (El-Fawal et al., 1989b).

Electrophysiological and pharmacological studies. Hens were examined for electrophysiological and pharmacological changes 4-5, 7-8, 15-16, 21-22, 37 and 64 days after dosing with PSP. The use of the biventer cervices nerve-muscle preparation has been described elsewhere (El-Fawal et al., 1988). Briefly, hens were euthanized with an iv overdose of sodium pentobarbital following in vivo electrophysiological assessment (El-Fawal et al., 1989c). The biventer cervices nerve-muscle preparation was isolated from the surrounding musculature of the neck and the proximal end of the nerve tendon tied with a length of suture prior to detachment from the skull. The muscle was anchored at the bottom of a 150 ml organ bath and the tendon with the ensheathed nerve passed through a custom made version of the biventer cervices electrode. The tendon was attached by the suture at the proximal end to a Model FTO3 force displacement isometric transducer, leading to a coupler-amplifier-recorder system (Grass Medical Instruments, Quincy, MA). Two grams of resting tension were placed on the muscle. The organ bath contained Krebs-Henseleit solution maintained at 37°C and aerated with a 95% O_2-5% CO_2 mixture.

For establishing strength-duration curves (SDC), the voltage required to produce a minimally perceptible threshold depolarization, as indicated by muscle twitch, at stimulus durations of 20 to 500 μsec were viewed an a Tektronix 5115 oscilloscope (Tektronix Inc., Beaverton, OR). Rheobase and chronaxie were derived from these curves as previously

described (El-Fawal et al., 1988) and the inflexion region (40-100 μsec) linearized as performed by Robertson et al. (1987) to allow comparison of excitability thresholds between groups.

Log concentration-response curves in response to acetylcholine chloride (ACh, Sigma Chemical Co., St.Louis, MO) were established by plotting cumulative concentrations of ACh against gram-tension developed by the muscle. Stock solution of ACh (10^{-1}M) were prepared in distilled water on the day of the experiment. Subsequent serial molar concentrations (final bath concentrations of 10^{-11} to 10^{-3}M) were prepared by dilution in Krebs-Henseleit solution.

Histology. The distal region (lying over the belly of the muscle) of nerve supplying the twin biventer cervices was dissected away from the freshly removed specimen and placed in a fixative of the following composition: 2.5% glutaraldehyde in 0.05M sodium coacodylate at pH 7.4. Following a period of 24-48 hrs. of fixation at 4°C segments of the nerve were rinsed in buffer, post-fixed in 2% osmium tetroxide and embedded in Epon epoxy resin. Cross sections were cut at oneum thickness, stained with toluidine blue and safranin, and examined by light microscopy.

Statistics. All data was analyzed by analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences between control and experimental groups with $p<0.05$ considered significant. All data are expressed as mean ± standard error.

RESULTS

Excitability thresholds for biventer cervices nerve-muscle preparations are provided in Figure 1. Hens treated with only PSP had thresholds that were significantly higher than those of control as early as 4 days following exposure. Thresholds remained significantly elevated when compared to controls at all days tested (days 4-5 7-8, 15-16, 21-22, 37 and 64). On day 4, thresholds for prepatations from hens treated only with nifedipine or verapamil, with nifedipine and PSP, or with verapamil and PSP were lower than those for preparations from controls, with differences noted between 60 to 100 μsec (Fig. 1a). At 7 days following exposure to PSP, excitability thresholds of preparations from hens treated with verapamil and PSP were still significantly lower than control (Fig. 1b). Thresholds for preparations from hens treated with nifedipine and PSP did not differ from controls after day 4 (Fig. 1b, c, d, e, and f).

Full SDC plots for preparations from hens given PSP, nifedipine and PSP, 21-22, 37, and 64 days earlier and untreated controls are shown in Figure 2. The plot at 21 days (Fig. 2a) shows the subtle development of "kinks" or discontinuities which continued to be evident for the nifedipine and PSP group at days 37 and 64 (Fig. 2b, c), These kinks did not appear in SDC for preparations from hens treated with PSP alone until day 37 (Fig. 2b,c).

Rheobase values extracted from SDC for nerves of PSP-treated hens were significantly higher than for all other groups on days 4 and 7 following exposure to OP and continued to be significantly elevated over control for the duration of the experiment. After day 15 rheobase was also elevated in preparations from hens treated with nifedipine and PSP, but the elevation was not as pronounced as the group treated with PSP only (Table 1). Rheobase did not rise in the group treated with verapamil and PSP (Table 1). Chronaxie values were significantly shorter in nerves from hens given only PSP at all days tested up to day 64. Chronaxie was also shortened in preparations from hens given PSP and the Ca^{++} channel blockers, nifedipine and verapamil.

Pharmacological assessment of the biventer cervices muscle was done by measuring tension generation after exposure to cumulative concentration of ACh for days 4-5, 15-16 and 64 are shown in Fig. 3. Thresholds, sensitivities and maximum tension at the concentration of $2x10^{-3}$ are included in Table 1. Begining day 4, preparations from chickens treated with only PSP were 60 to 10^5x more sensitive than control. The greatest sensitivity was noted on days 15-16, 21-22 and 37. Preparations from hens treated with nifedipine and PSP were 5 to 10^3x more sensitive than controls begining day 7, with peak sensitivity on day 21, while those from the group treated with PSP plus verapamil were 5 to 10x more sensitive than control muscle begining on day 7.

Microscopic examination of cross sections taken at the distal level of biventer cervicis nerve from hens given PSP only 7 days earlier revealed severe neuropathy (Fig. 4a). This contrasts to the relatively mild lesions in nerve from hens receiving nifedipine and PSP (Fig. 4b), or verapamil and PSP (Fig. 4c). At 15 days, nerves from hens administered PSP alone revealed advanced degenerative lesions, loss of myelinated fibers and associated presence of empty Schwann cell tubes (Fig. 5a). The degenerative process was at an earlier stage when nifedipine or verapamil were administered in conjunction with PSP (Fig. 5b, c) Regeneration was more prominant at 37 days in both, although myelination appeared more advanced in nerves form chickens which had received both nifedipine and PSP.

A spectrum of lesions were represented throughout the time course of the study following treatment with PSP. Axonal swelling, often associated with thinning or fragmintation of the myelin sheath and intra-axonal debris was noted. This apparently progressed to include the phagocytosis and complete degeneration of involved fibers, accounting for their absence at later stages. Advanced changes were manifested by Schwann cell tubes with the absence of intact axons. The end result being a diminution of intact myelinated fibers. In later stages (37 days) occasional regenerating, thinly (newly) myelinated, or not yet myelinated axons were recognized in such Schwann cells by light microscopy.

DISCUSSION

In this study, two drugs capable of regulating Ca^{++} movement, nifedipine and verapamil, modified the neurotoxic effects of PSP on electrophysiological, pharmacological and pathological parameters used to assess nerve and muscle function in an in vitro preparation. For example, excitability thresholds derived from the strength-duration curve were significantly elevated most prominently for nerves of hens receiving PSP alone. The excitability threshold is reported to be one of the few electrophysiological parameters which apparently relates changes in axonal function to OPIDN (Robertson et al., 1987; Anderson et al., 1988). High rheobase and short chromaxie values predominating in nerve-muscle preparations of hens treated with PSP alone were consistent with earlier reports on this preparation (El-Fawal et al., 1988; 1989a) and with reports of peripheral neuropathies due to other toxicants (Harris, 1971). Rheobase and chronaxie values taken together are used to indicate nerve degeneration (Wynn Parry, 1971). According to our data, nerve degeneration was most notable in hens given PSP only. The shorter chronaxie appearing early (day 4-5) following treatment with nifedipine alone, or following combinations of nifedipine and verapamil with PSP treatment may indicate hyperexcitability. Caution, however, is recommended when interpreting the significance of changes in chronaxie alone (Harris, 1971).

The appearance of "kinks" or discontinuities in the SDC in later intervals of the study signify incomplete innervation of the muscle. In this case, incomplete innervation was due to the establishment of reinnervation (Wynn-Parry, 1971). These discontinuities appeared earlier in the group treated with nifedipine and PSP than in SDC from hens given only PSP.

The more advanced degenerative changes as indicated by electrophysiological parameters measured here may reflect the involvement of Ca^{++} in the degenerative process. Unregulated increases in intra-axonal Ca^{++} and the development of Wallerian degeneration, the primary lesion during OPIDN, have been suggested in neuropathies developing following axotomy or trauma (Schlaepfer and Hasler, 1979; Kamakuru et al., 1983; Schlaepfer and Zimmerman, 1984; Mata et al., 1986). The degenerative changes typical of Wallerian degeneration are facilitated by the enhanced activation of a Ca^{++}-activated neutral protease (CANP) found in both the axoplasm, myelin, and muscle. In the studies cited above reduction of Ca^{++} attenuated or abolished degenerative changes. Furthermore, the L-type voltage sensitive Ca^{++} channel, which is modulated by dihydropyridine antagonists such as nifedipine, may be linked to the activity of CANP (Miller, 1987). We have investigated the involvement of CANP in a time course study parallel to this electrophysiological study and found that activity of this enzyme was indeed increased in brain, sciatic nerve, and gastrocmemius

muscle. Treatment with these same Ca^{++} channel blockers ameliorated this change in enzyme activity (El-Fawal et al., 1989d).

The nerve degeneration suggested by the SDC measurements reported here and predominating in preparations of those treated with PSP alone was supported by our histopathological observations. Degeneration of myelinated fibers was more advanced in distal level of the biventer nerves from hens given PSP alone than in those receiving either nifedipine and PSP or verapamil and PSP. More intact myelinated fibers remained in these two latter groups, although eventually these too disappeared. These observations are consistent with those previously reported when nerves of hens treated with verapamil and PSP were examined 17 and 28 days after administration of the OP (El-Fawal et al., 1989a).

The more advanced denervation of biventer cervicis muscle in hens given only PSP was further indicated by the hypersensitivity of muscle to exogenous ACh (Axelsson and Thesleff, 1957; Kandel, 1985). Enhanced responsiveness of muscle to the neurotransmitter as early as 4-5 days following PSP-treatment was evident for preparations from hens receiving the neurotoxicant only, and continued to be more pronounced in this group throughout the duration of the study. This is consistent with previous results measuring sensitivity to ACh in muscle from hens with OPIDN using both *in vitro* and *in vivo* preparations (El-Fawal et al., 1989a, b, and c). Denervation hypersensitivity to ACh is thought

to occur as a result of newly synthesized ACh receptors in the absence of intact innervation (Grampp et al., 1972; Chang and Tung, 1974). Increased receptor synthesis occuring following denervation has been shown to be enhanced by increasing intracellular Ca^{++} and may be suppressed by use of Ca^{++} channel blockers (Metafora et al., 1980; McManaman et al., 1981; Kallo and Steihardt, 1983). The synthesis of new ACh receptors and their detection has been reported to occur as early as 3 days following denervation (Almon and Appel, 1976), a pattern consistent with the development of OPIDN and detection denervation hypersinsitivity (El-Fawal et al., 1989b). The use of Ca^{++} channel blockers in the present study prevented the early appearance as well as attenuating muscle sensitivity throughout the course of this study.

One feature of this study is that it allowed us to verify that the type of responses seen in the biventer cervicis nerve-muscle compared to those seen in the sciatic-gastrocnemius preparation cited in the literature on OPIDN (Robertson et al., 1987; 1988; Anderson et al., 1988). Responses in the two preparations were similar, but the biventer cervicis preparation proved to be more sensitive to early and late functional alterations during OPIDN. Unlike the gastrocnemius muscle, it is possible to detect the subtlties of partial denervation or reinnervation, as indicated by discontinuities in the SDC, which did not appear in the <u>in vivo</u> preparations (El-Fawal et al., 1989b). The biventer

preparation also provides the advantage of a quick assay of these alterations, as parameters on each individual preparation could be obtained in less than 30 minutes once the technique was mastered. the in vitro preparation also avoids the complications associated with procedures of invasive surgery.

An observation of keen interest relative to the biventer cervicis nerve-muscle is that, although the popular view claims that OPIDN is an axonopathy of the largest and longest distal nerve fibers (Cavanagh, 1964; Abou-Donia, 1981; Jortner, 1984), the axonal fibers of this preparation are qualitatively smaller compared to the branch of the tibial innervating the gastrocnemius muscle yet lesions are more extensive. This branch of the tibial is used frequently in the evaluation of OPIDN. In addition to being smaller than the tibial, the biventer cervicis nerve is shorter, its innervation arising immediately from the cervical region of the spinal cord. The greater susceptibility of the biventer nerve to injury may explain this preparation's sensitivity to functional alterations. Whether other relatively small and short peripheral nerves are equally susceptible to OPIDN would require further investigations.

A possible role for Ca^{++} in the pathogenesis of OPIDN has been suggested in the present study. Two Ca^{++} channel blockers, verapamil and nifedipine, which inhibit Ca^{++} entry into the cell and/or prevent its mobilization from intracellular storage sites, prevented or attenuated the development

of OPIDN as judged by electrophysiological, pharmacological and histological parameters in a sensitive neuromuscular preparation. This attenuation may reflect the underlying detrimental biochemical mechanisms involving Ca^{++}.

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REFERENCES

ABOU-DONIA, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-148.

ABOU-DONIA, M. B., PATTON, S. E. AND LAPADULA, D. M. (1984). Possible endogenous protein phosphorylation in organophosphorus compound-induced delayed neurotoxicity. In Cellular and Molecular Neurobiology (T. Narahashi, Ed.), pp. 265-283 Raven Press, New York.

ALMON, R. R. AND APPEL, S. H. (1976). Cholinergic sites in skeletal muscle. I. Denervation effects. Biochem. 13: 3662-3667.

ANDERSON, R. J., ROBERTSON, D. G., HENDERSON, J. D. AND WILSON, B. W. (1988). DFP-induced elevation of strength-duration threshold in hen peripheral nerve. NeuroToxicology. 9: 47-52.

AXELSSON, J. AND THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. 147: 178-193.

BANIK, N. L., HOGAN, E. L. AND McALHANEY, W. W. (1984). A role for calcium and activation of neutral proteinase in myelinolysis? In Experimental Allergic Encephalomyelitis. pp. 145-152 Alan R. Liss, Inc., New York.

BERLET, H. H. (1987). Calcium-dependent neutral protease activity of myelin from bovine spinal cord: evidence for soluble cleavage products of myelin proteins. Neurosci. Lett. 73: 266-270.

CAVANAGH, J. B. (1964). Peripheral nerve changes in orthocresyl phosphate poisoning in the cat. J. Pathol. Bact. 87: 365-383.

CISSON, C. M. AND WILSON, B. W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity: altered acetylcholinesterase molecular forms and increased plasma creatine phosphokinase activity. Toxicol. Appl. Pharmacol. 64: 289-305.

DAVIS, C. S. AND RICHARDSON, R. J. (1980). Organophosphorus compounds. In Experimental and Clinical Neurotoxicology (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527-544 Williams and Wilkins, Baltimore.

EL-FAWAL, H. A. N., JORTNER, B. S., EYRE, P. AND EHRICH, M. (1988). The biventer cervicis nerve-muscle preparation

of adult hens: effect of phenyl saligenin phosphate administration. NeuroToxicology 9: 625-636.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989a). Effect of verapamil on organophosphate-induced delayed neuropathy. Toxicol. Appl. Pharmacol. 97: 500-511.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989b). Use of the biventer cervicis nerve-muscle preparation to detect early changes following exposure to organophosphates-inducing delayed neuropathy. Ph.D. Dissertation. VPI & SU.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989c). Modification of organophosphate-induced delayed neuropathy (OPIDN) with calcium channel blockers: I. in vivo using the sciatic-gastrocnemius nerve-muscle preparation. Ph.D. Dissertation. VPI & SU.

EL-FAWAL, H. A. N., CORRELL, L., GAY, L. AND EHRICH, M. (1989d). Modification of organophosphate-induced delayed neuropathy (OPIDN) with calcium channel blockers: III. Altered esterases and calcium-activated neutral protease (CANP). Ph.D. Dissertation, VPI & SU.

ETO, M., CASIDA, J. E. AND ETO, T. (1962). Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11: 337-352.

FARBER, J. L. (1981). The role of calcium in cell death. Life Sci. 29: 1289-1295.

GRAMPP, W., HARRIS, J. B. AND THESLEFF, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221: 743-754.

HARRIS, R. (1971). Chronaxy. In Electrodiadnosis and Electromyography (S. Licht, Ed.), pp. 218-239. Elizabeth Licht, New Haven.

JOHNSON, M. K. (1987). Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. Trends Pharmacol. Sci. 8: 174-179.

JORTNER, B. S. (1984). Pathology of organophosphorus-induced delayed neurotoxicity. In Proceedings of the Fourteenth Conference on Environmental Toxicology (J. D. McEwan and E. H. Vernot, Eds.), pp. 106-117. National Information Service, Va.

JORTNER, B. S. AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

KALLO, J. R. AND STEINHARDT, R. A. (1983). The regulation of extrajunctional acetylcholine receptors in the denervated rat diaphragm muscle in culture. J. Physiol. 344: 433-452.

KAMAKURA, K., ISHIURA, S., SUGITA, H. AND TOYOKURA, Y. (1983). Identification of Ca^{++}-activated neutral protease in peripheral nerve and its effects on neurofilament degeneration. J. Neurochem. 40: 908-913.

KOMULAINEN, H. AND BONDY, S. C. (1988). Increased free intracellular Ca^{2+} by toxic agents: an index of potential neurotoxicity? Trends Pharmacol. Sci. 9: 154-156.

LLADOS, F. T. (1985). Neurogenic origin of an experimental myopathy induced by the ionophore A23187. J. Neurol. Sci. 69: 171-182.

MATA, M., STAPLE, J. AND FINK, D. J. (1986). Changes in intra-axonal calcium distribution following nerve crush. J. Neurobiol. 17: 449-467.

McMANAMAN, J. L., BLOSSER, J. C. AND APPEL, S. (1981). The effects of calcium on acetylcholine receptor synthesis. J. Neurosci. 1: 771-776.

METAFORA, S., FELSANI, A., COTRUFO, R., TAJANA, G. F., DI IORIO, D., DEL RIO, A., DE PRISCO, P. P. AND ESPOSITO, V. (1980). Neural control of gene expression in the skeletal muscle fiber: the nature of the lesion in the muscular protein-synthesizing machinery following denervation. Proc. R. Soc. Lond. B. 209: 239-255.

METCALF, R. L. (1984). Historical perspective of organophosphorus ester-induced delayed neurotoxicity. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 7-22 Intox Press, Little Rock.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. Science 235: 46-235.

PATTON, S. E., LAPADULA, D. M., O'CALLAGHAN, J. P., MILLER, D. B. AND ABOU-DONIA, M. B. (1985). Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-o-cresyl phosphate to hens. J. Neurochem. 45: 1567-1577.

REDDY, M. K., ETLINGER, J. D., RABINOWITZ, M., FISCHMAN, D. A. AND ZAK, R. (1975). Removal of Z-lines and a-actinin from isolated myofibrils by a calcium-activated neutral

protease. J. Biol. Chem. 250: 4278-4284.

ROBERTSON, D. G., SCHWAB, B. W., SILLS, R. D., RICHARDSON, R, J. AND ANDERSON, R. J. (1987). Electrophysiologic changes following treatment with organophosphorus-induced delayed neuropathy-producing agents in the adult hen. Toxicol. Appl. Pharmacol. 87: 420-429.

ROBERTSON, D. G., MATTSON, A. M., BESTERVELT, L. L., RICHARDSON, R. J. AND ANDERSON, R. J. (1988). Time course of electrophysiologic effects induced by di-n-butyl-2,2-dichlorovinyl phosphate (DBVC) in the adult hen. J. Toxicol. Environ. Hlth. 23: 283-294.

SCHANNE, F. A. X., KANE, A. B., YOUNG, E. E. AND FARBER, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science. 206: 700-702.

SCHLAEPFER, W. W. (1987). Neurofilaments: structure, metabolism and implication in disease. J. Neuropath. Exp. Neurol. 46: 117-129.

SCHLAEPFER, W. W. AND BUNGE, R. P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J. Cell. Biol. 59: 456-470.

SCHLAEPFER, W. W. AND HASLER, M. B. (1979). Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. Brain Res. 168: 299-309.

SCHLAEPFER, W. W. AND ZIMMERMAN, U.P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. In Axonal Transport in Neuronal Growth and Regeneration (J. S. Elam and P. Cancalon, Eds.), pp. 261-273 Plenum Press, New York.

SUWITA, E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986). Calcium and calmodulin stimulated in vitro phosphorylation of rooster brain tubulin and MAP-2 following a single oral dose of tri-o-cresyl phosphate. Brain Res. 374: 199-203>

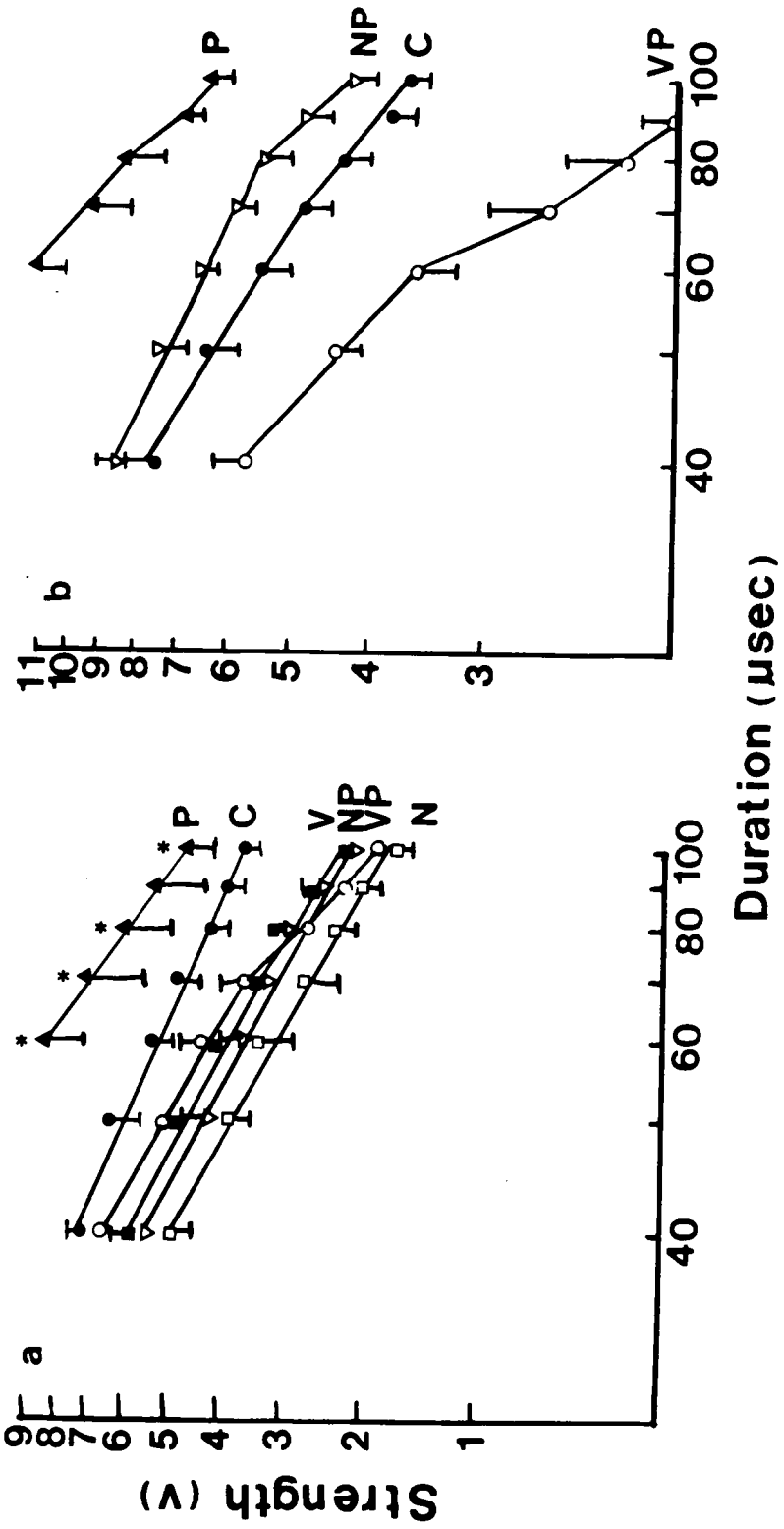
WROGEMAN, K. AND PENA, S. D. J. (1976). Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle disease. Lancet 7961: 672-673.

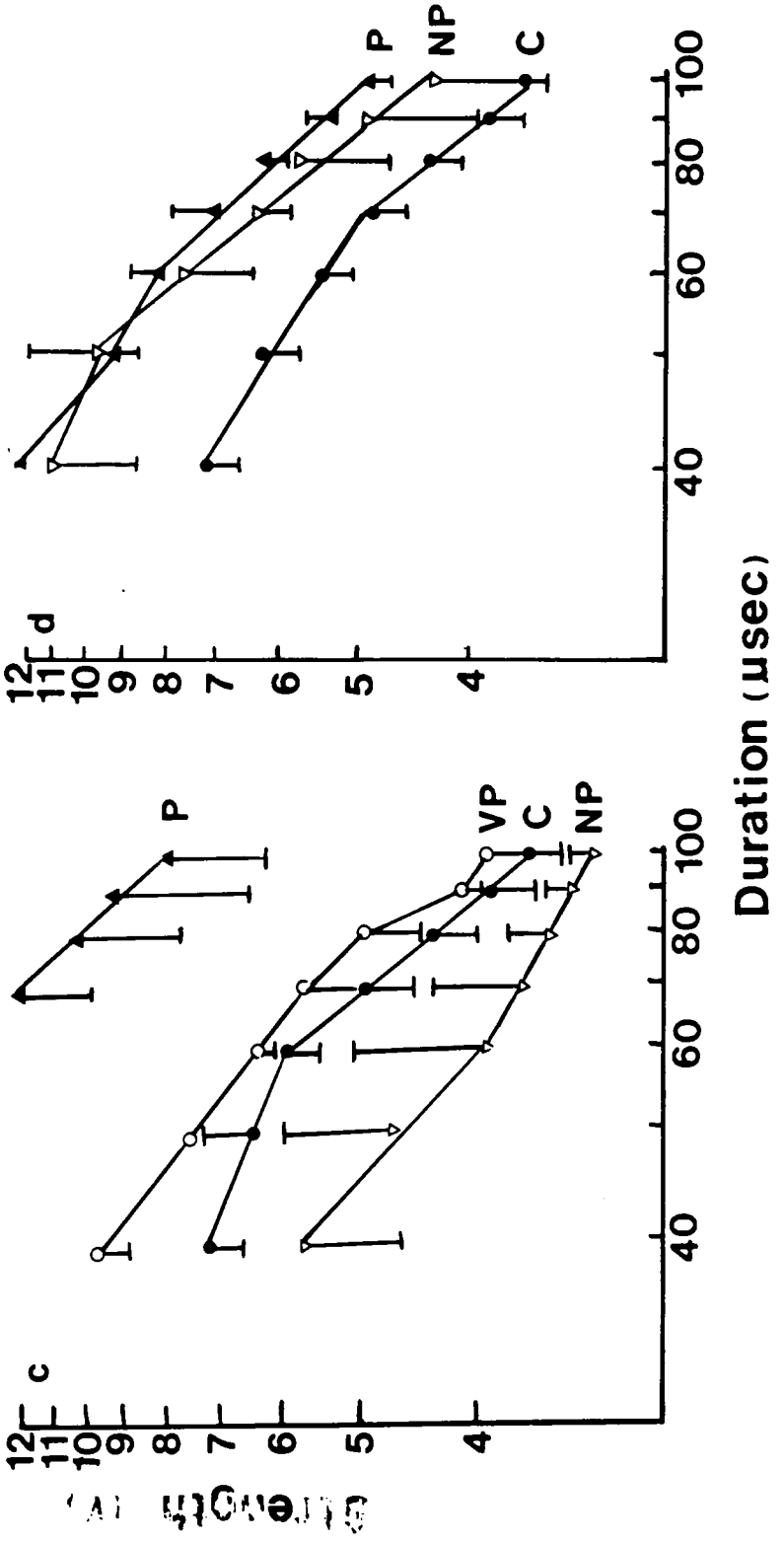
WYNN PARRY, C. B. (1971). Strength-duration curves. In Electrodiagnosis and Electromyography (S. Licht, Ed.), pp. 241-271. Elizabeth Licht, New Haven.

TABLE 1. ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL PARAMETERS DERIVED FROM STRENGTH-DURATION AND CONCENTRATION-RESPONSE CURVES FOLLOWING ADMINISTRATION OF PSP, NIFEDIPINE AND PSP OR VERAPAMIL AND PSP.

Treatment	Rheobase (volts)[b]	Chronaxie (msec)[a]	Threshold (M)[c]	Tension (g) at 2 x 10^{-3}
Control	1.31 ± 0.05	0.14 ± 0.008	10^{-4}	5.03 ± 0.25
Nifedipine	1.25 ± 0.09	0.07 ± 0.006[c]	10^{-4}	5.74 ± 1.10
Verapamil	1.11 ± 0.04	0.11 ± 0.012	10^{-4}	4.53 ± 0.40
Day 4				
PSP	2.95 ± 0.45[c,d,e]	0.13 ± 0.020	5 x 10^{-6} (60x)[f]	9.66 ± 0.73[c]
Nifedipine + PSP	1.26 ± 0.06	0.08 ± 0.003[c]	10^{-4}	5.66 ± 0.22
Verapamil + PSP	1.40 ± 0.11	0.08 ± 0.003[c]	10^{-4}	4.88 ± 0.69
Day 7				
PSP	3.60 ± 0.41[c,d,e]	0.08 ± 0.005[c,e]	10^{-7} (10^{3}x)	7.20 ± 1.40[c]
Nifedipine + PSP	1.93 ± 0.29	0.11 ± 0.020	5 x 10^{-5} (5x)	3.51 ± 0.36
Verapamil + PSP	1.26 ± 0.17	0.07 ± 0.003[c,d]	5 x 10^{-5} (5x)	6.33 ± 0.46
Day 15				
PSP	4.55 ± 0.49[c,d,e]	0.09 ± 0.010[c,e]	10^{-9} (10^{5}x)	7.31 ± 1.93[c]
Nifedipine + PSP	2.44 ± 0.14[c]	0.10 ± 0.010[c]	10^{-6} (10^{2}x)	7.87 ± 1.17[c]
Verapamil + PSP	1.45 ± 0.18	0.16 ± 0.006[c]	10^{-5} (10x)	6.12 ± 1.31
Day 21				
PSP	2.93 ± 0.43[c,d]	0.09 ± 0.00[c,d]	10^{-9} (10^{5}x)	7.25 ± 1.56[c]
Nifedipine + PSP	2.06 ± 0.17[c]	0.11 ± 0.017	10^{-7} (10^{3}x)	5.41 ± 0.60
Day 37				
PSP	2.53 ± 0.35[c,d]	0.09 ± 0.005[c]	10^{-9} (10^{5}x)	3.96 ± 0.89
Nifedipine + PSP	1.53 ± 0.24	0.11 ± 0.014	10^{-6} (10^{2})	5.62 ± 0.83
Day 64				
PSP	2.20 ± 0.11[c]	0.12 ± 0.11	10^{-6} (10x)	8.65 ± 0.31[c]
Nifedipine + PSP	2.00 ± 0.11[c]	0.10 ± 0.008[c]	5 x 10^{-5} (5x)	5.66 ± 0.83

a. Derived from individual strength-duration curves. mean ± SE, n = 3-7.
b. Derived from rheobase curves. mean ± SE, n = 3-7.
c. Significantly different from control. $p < 0.05$.
d. Significantly different from nifedipine + PSP group $p < 0.05$.
e. Significantly different from verapamil + PSP group, $p < 0.05$.
f. Degree of difference from control in parentheses.


a
b
Strength (V)
Duration (μsec)
P
C
V
NP
VP
N
*
9
8
7
6
5
4
3
2
1
11
10
40
60
80
100


d
c
P
NP
C
VP
12
11
10
9
8
7
6
5
4
40
60
80
100
Duration (μsec)

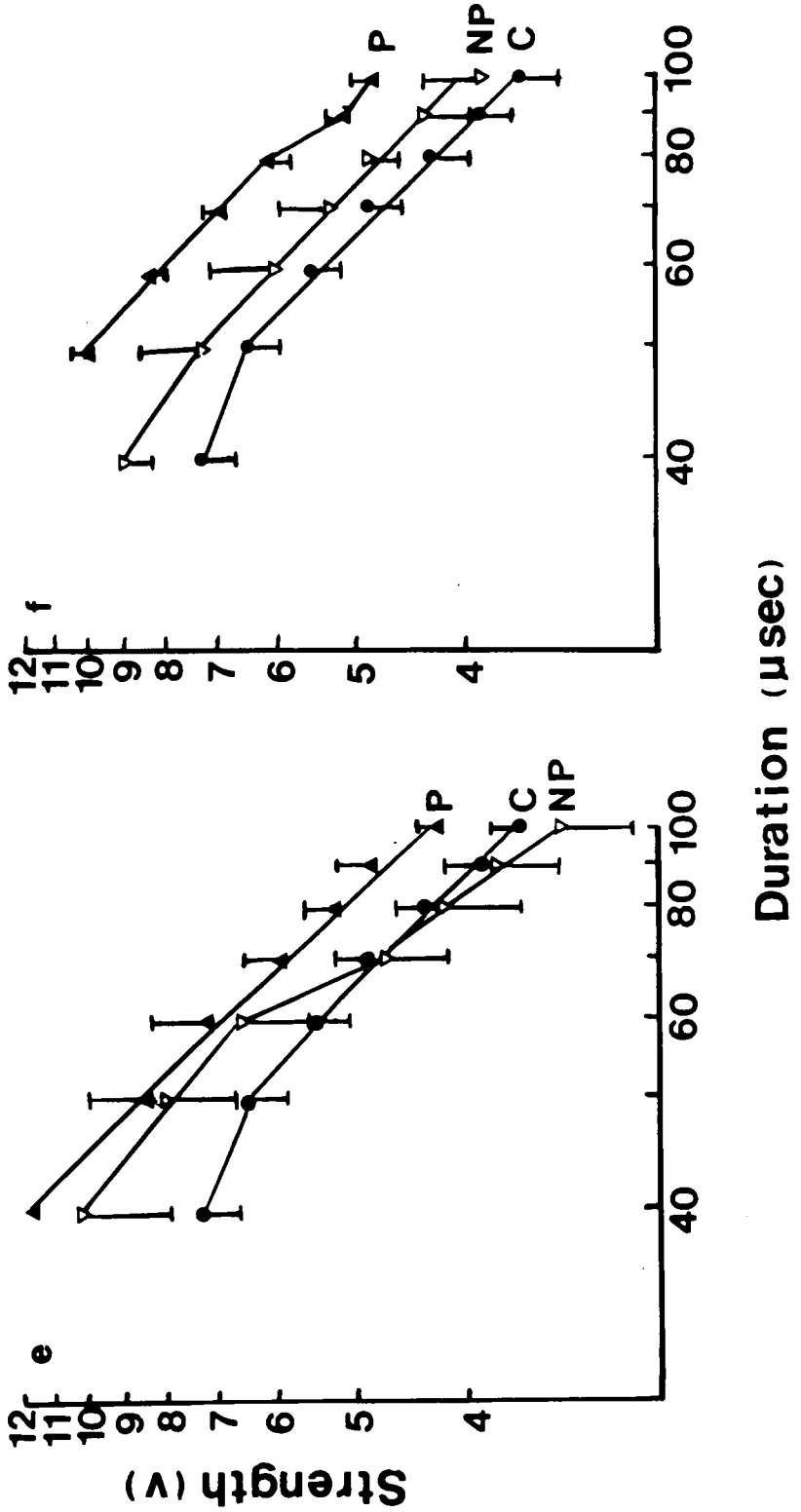
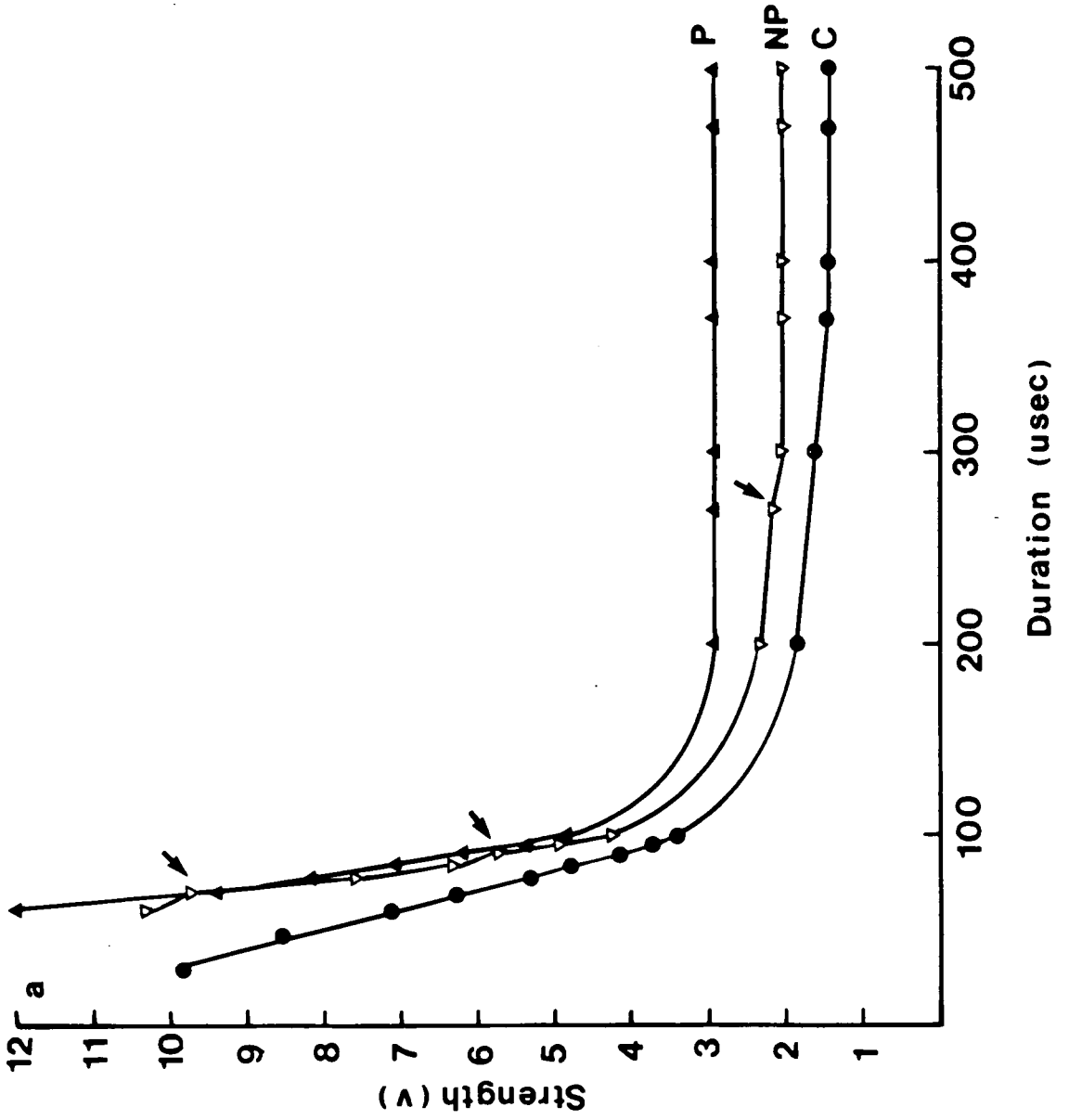
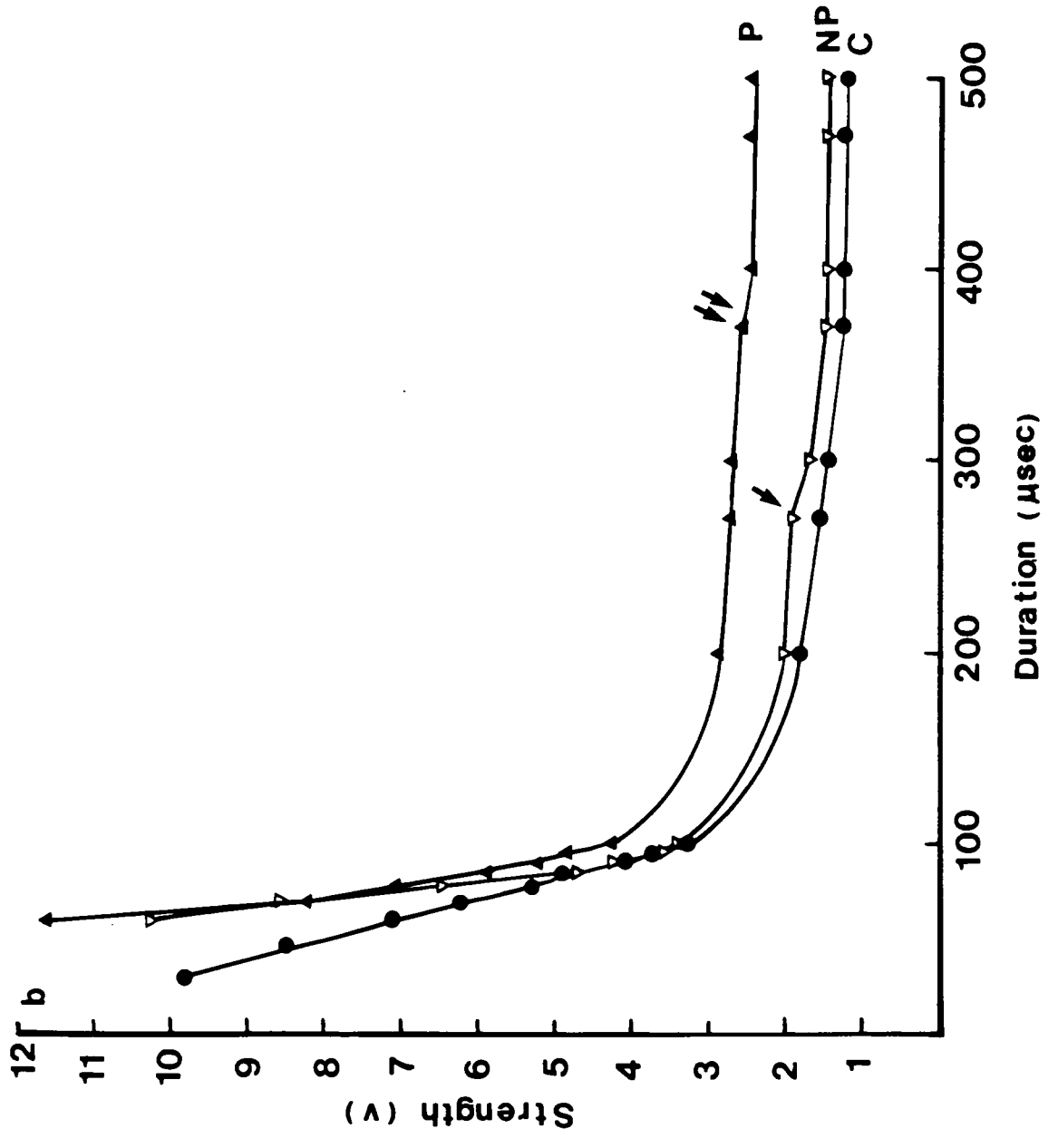

Strength (V)
Duration (μsec)
e
f
P
C
NP
12
11
10
9
8
7
6
5
4
40
60
80
100

Figure 1a, b, c, d, e, and f. Log-log plot for inflection region (40-500 μsec) of strength-duration curves from biventer cervicis nerve muscle preparation days 4-5 (Fig 1a), 7-8 (Fig. 1b), 15-16 (Fig. 1c), 21-22 (Fig. 1d), 37 (Fig. 1e), and 64 (Fig. 1f) after treatment of hens with PSP. C = control, N = nifedipine, V = verapamil, P = PSP, NP = nifedipine plus PSP, VP = verapamil plus PSP. Preparations from PSP-treated hens were always significantly different from controls and required a higher threshold of stimulation on all days tested. Those from VP and NP remained comparable to control until day 21 when regions of the NP curves were significantly higher.


a
12
11
10
9
8
7
6
5
4
3
2
1
Strength (v)
100
200
300
400
500
Duration (usec)
P
NP
C


b
12
11
10
9
8
7
6
5
4
3
2
1
Strength (v)
100
200
300
400
500
Duration (μsec)
P
NP
C

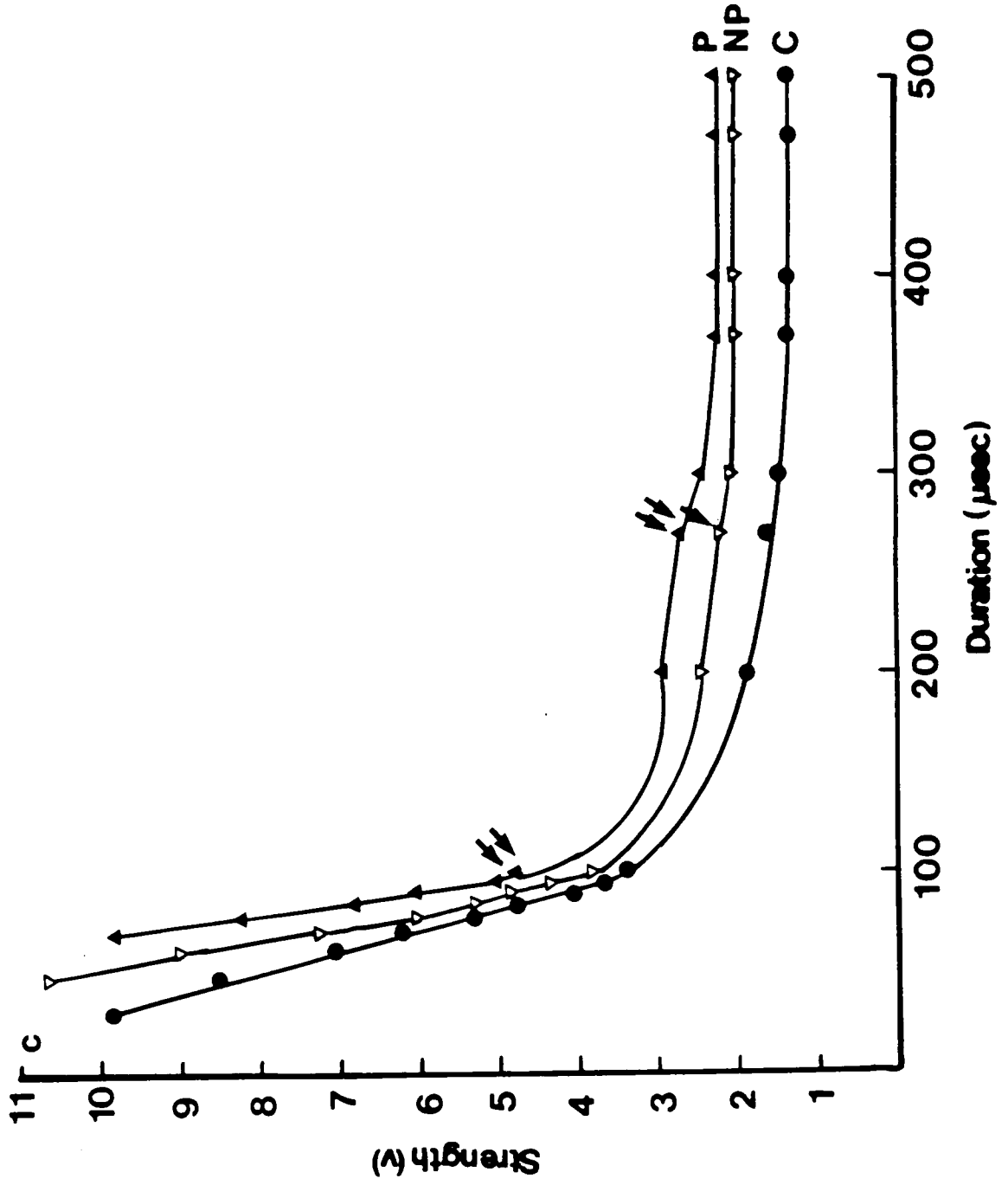
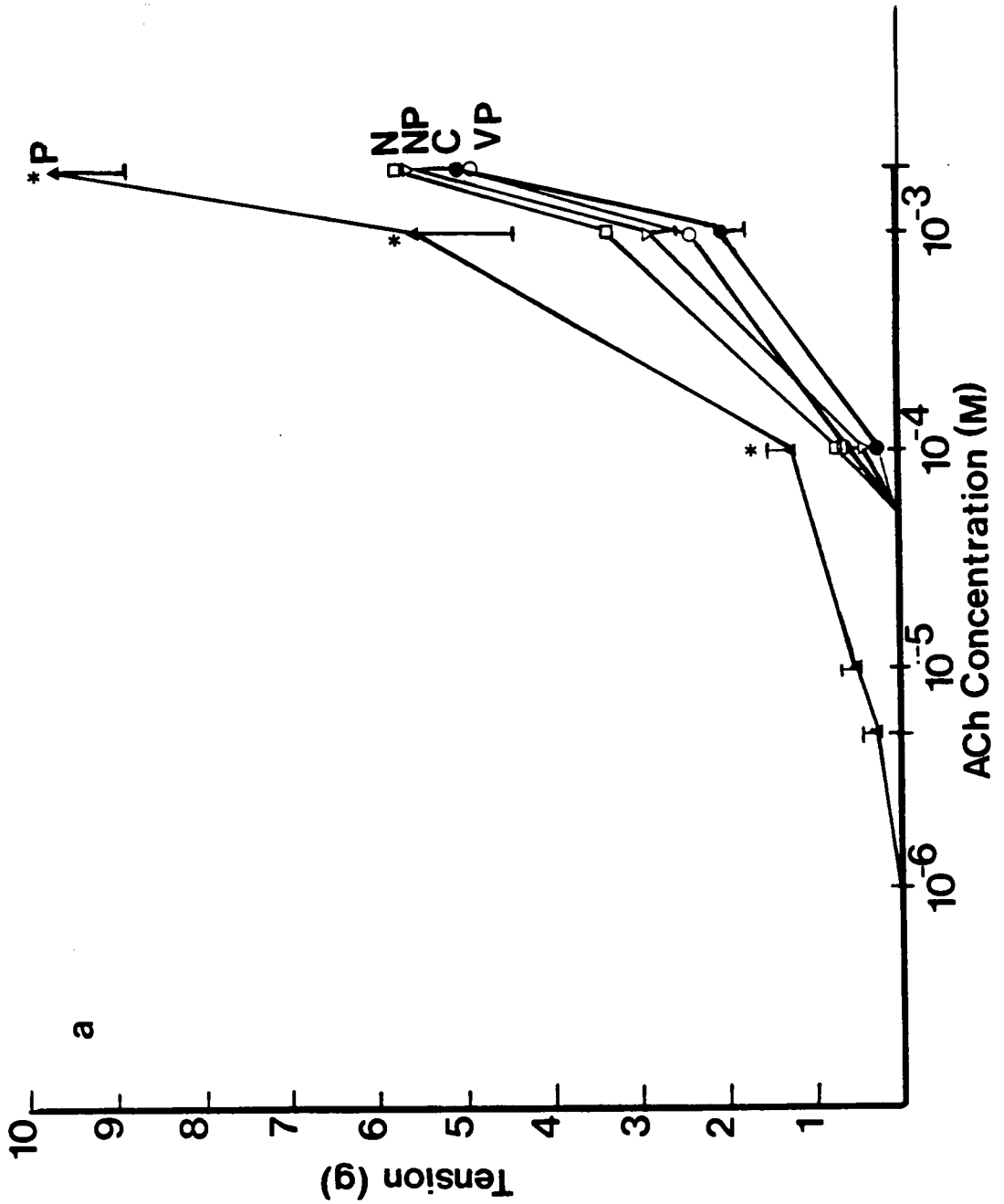
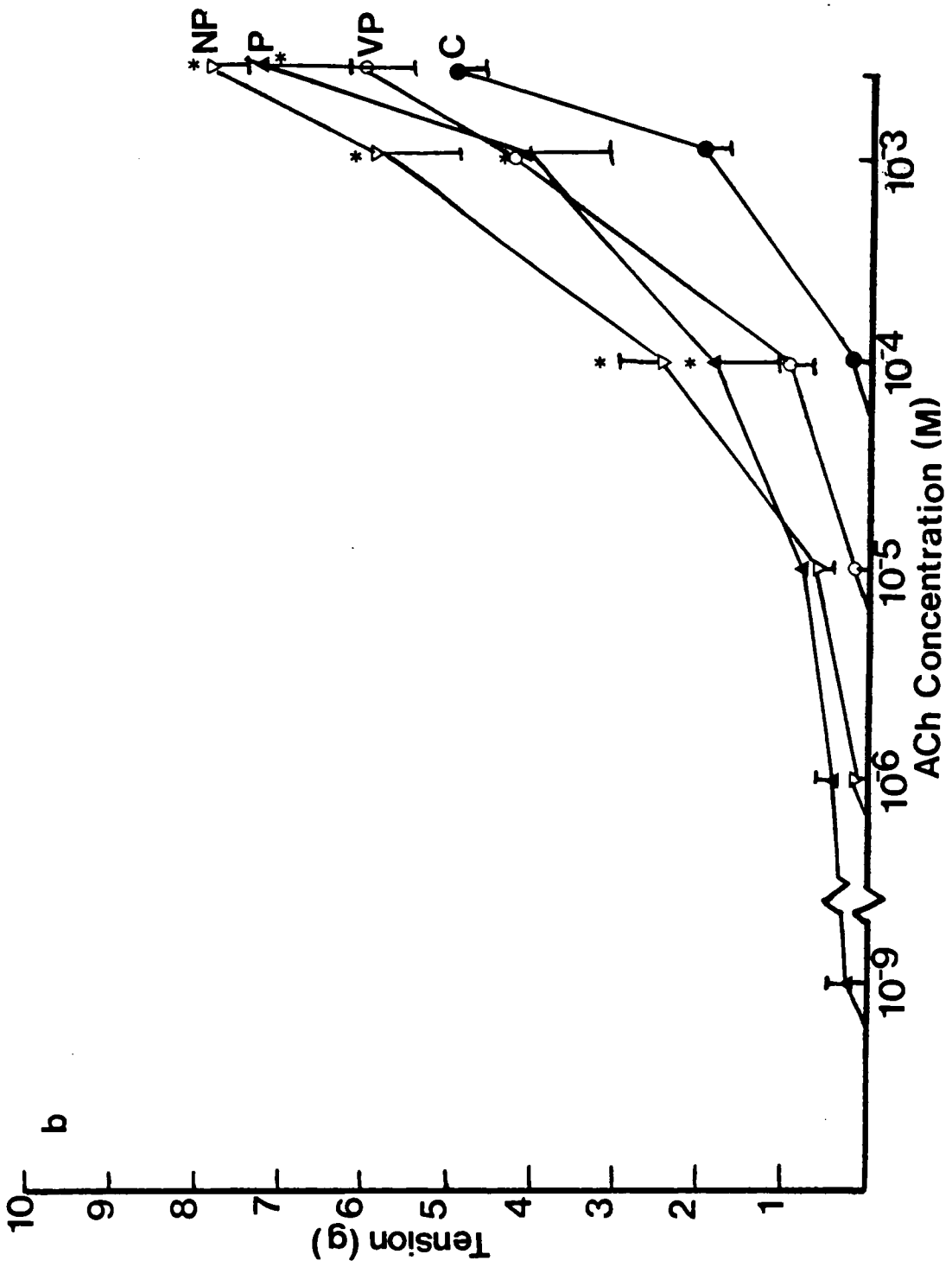

P
NP
C
Strength (V)
Duration (μsec)
11
10
9
8
7
6
5
4
3
2
1
100
200
300
400
500

Figure 2a, b, and c. Strength-duration curves of biventer cervicis nerve-muscle preparations days 21-22 (Fig. 2a), 37 (Fig. 2b) and 64 (Fig. 2c) after PSP administration. The curves show the appearance of "kinks" indicative of reinnervation in preparations from nifedipine plus PSP-treated hens (NP) [single arrows], similar kinks did not begin to appear in PSP-treated hens (P) until day 37 [double arrows].


a
Tension (g)
10
9
8
7
6
5
4
3
2
1
ACh Concentration (M)
10^{-6}
10^{-5}
10^{-4}
10^{-3}
N
NP
C
VP
*P
*
*


b
Tension (g)
10
9
8
7
6
5
4
3
2
1
*NP
*P
VP
C
10^{-9}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
ACh Concentration (M)

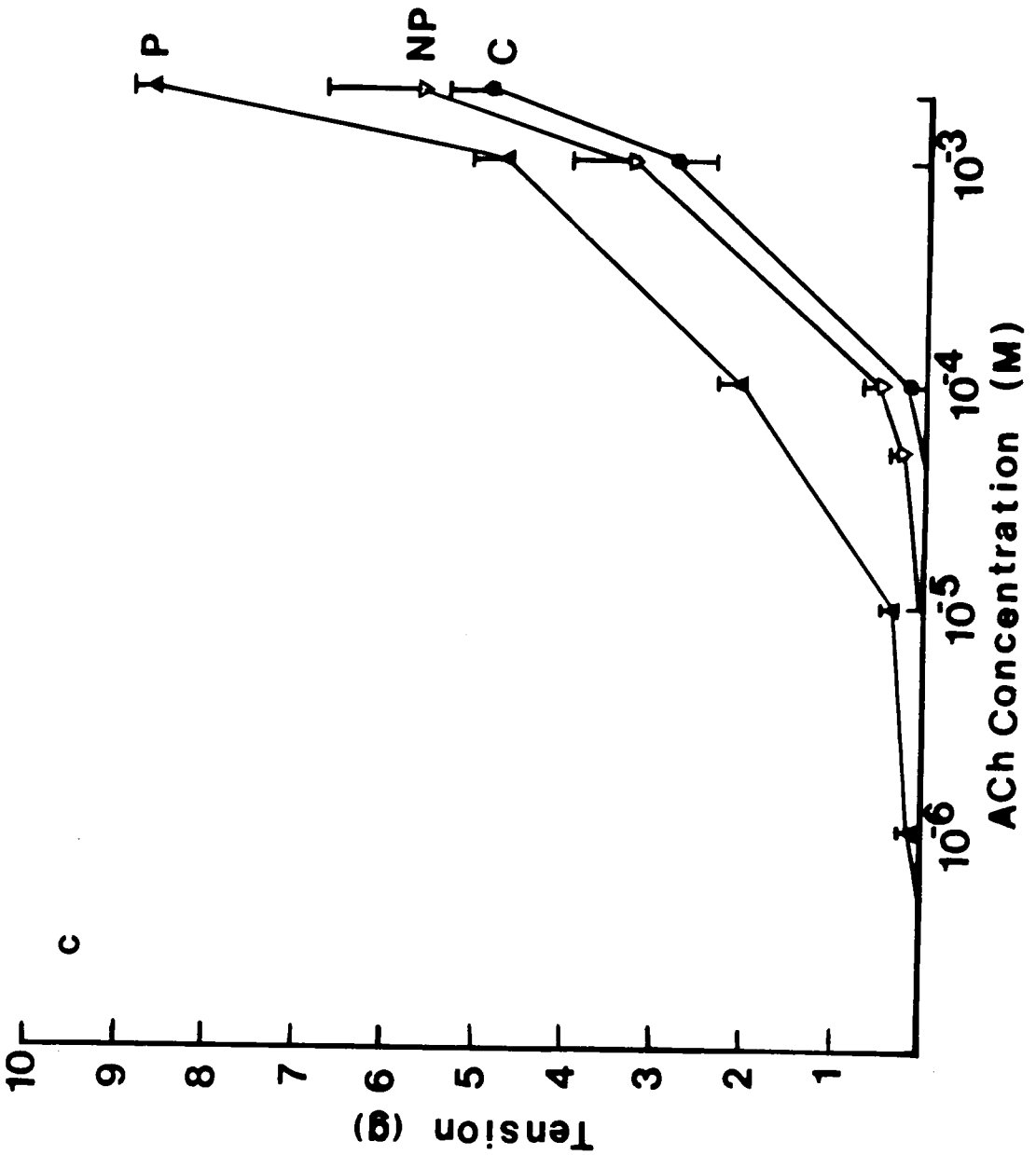
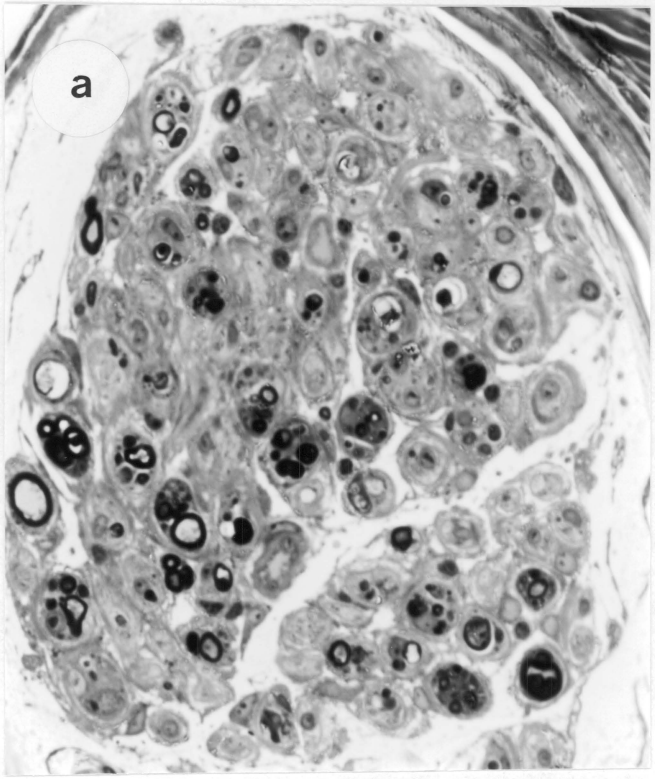
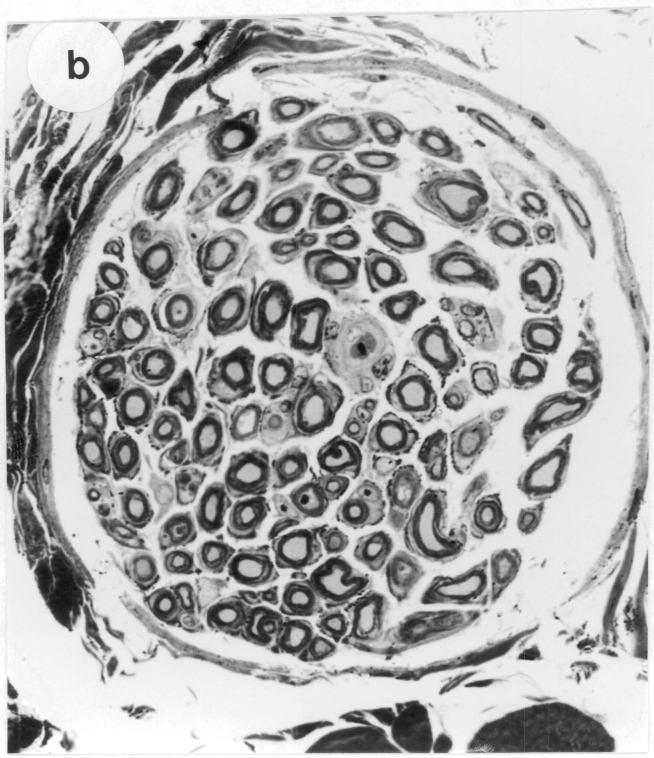
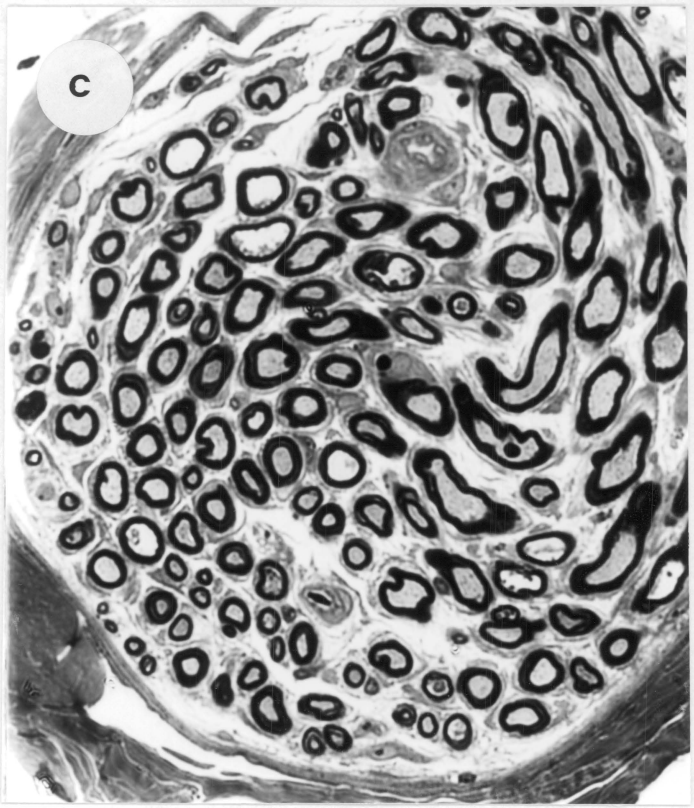

P
NP
C
c
Tension (g)
ACh Concentration (M)
10
9
8
7
6
5
4
3
2
1
10^{-6}
10^{-5}
10^{-4}
10^{-3}

Figure 3a, b, and c. Tension development in the biventer cervicis nerve-muscle preparation in response to ACh 4-5 (Fig. 3a), 15-16 (Fig. 3b), and 64 (Fig. 3c) days after PSP administration. Each point represents mean ± S. E. (n=3-7) for C (control), P (PSP-treated), N (nifedipine), NP (nifedipine plus PSP), and VP (verapamil plus PSP). Asterisks denote points significantly from controls as determined by ANOVA and Newman-Keuls test for multiple comparisons ($p < 0.05$).

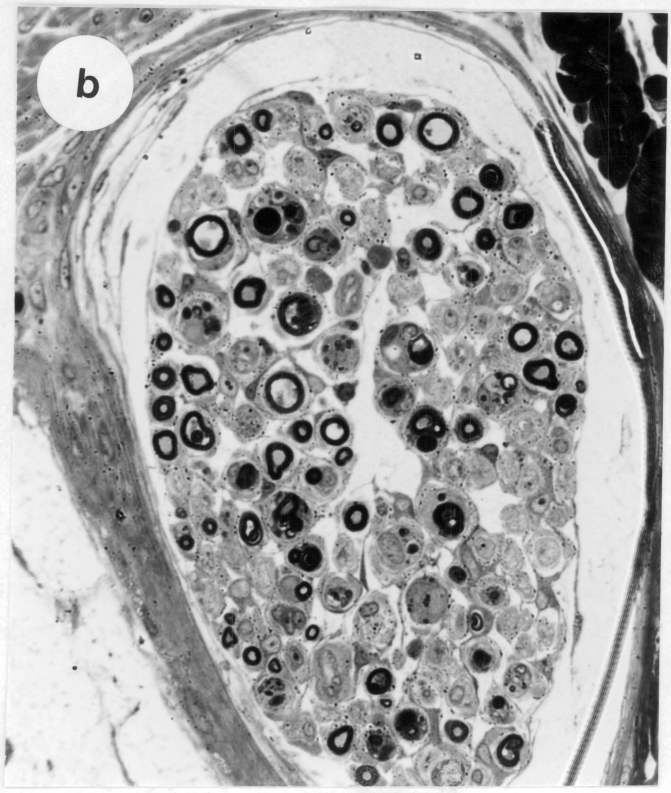

a

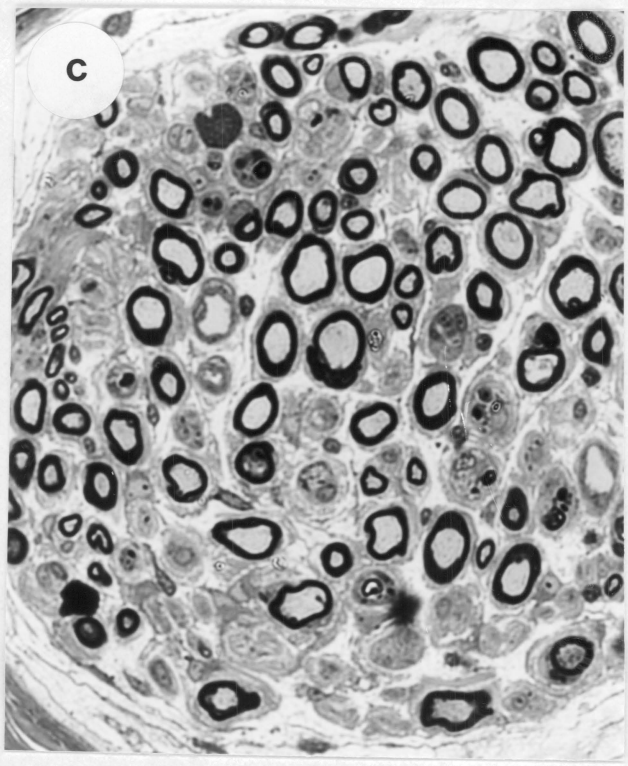

b


C

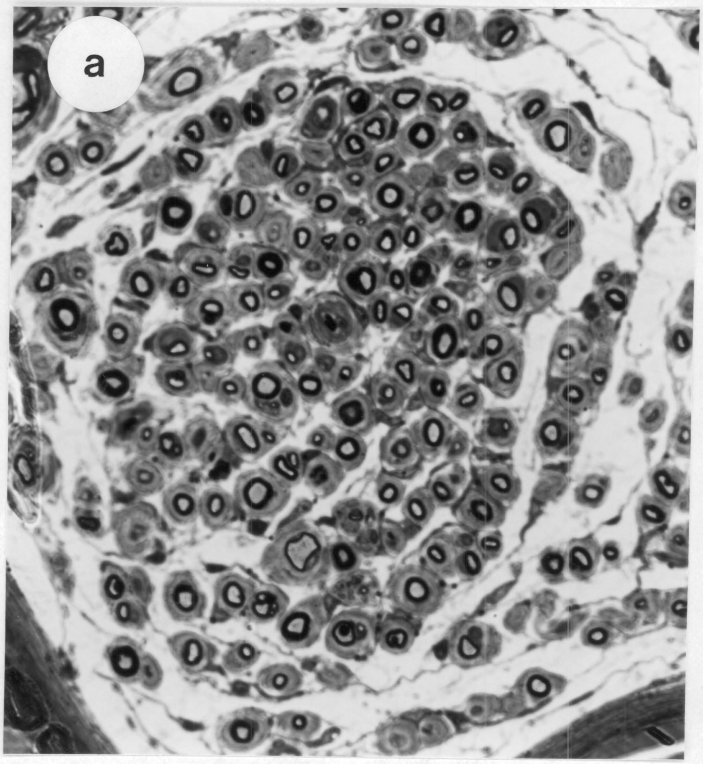
Figures 4 a, b, and c. Low power (x 260) examination of cross sections of the distal region of nerve to biventer cervicis muscle 7 days after PSP administration. Fig. 4a is from a hen given only PSP, Fig. 4b from a hen given PSP and nifedipine, and Fig. 4c from a hen given PSP and verapamil. Note the absence of but a few intact fibers in Fig. 4a as compared to Fig. 4b and Fig. 4c.

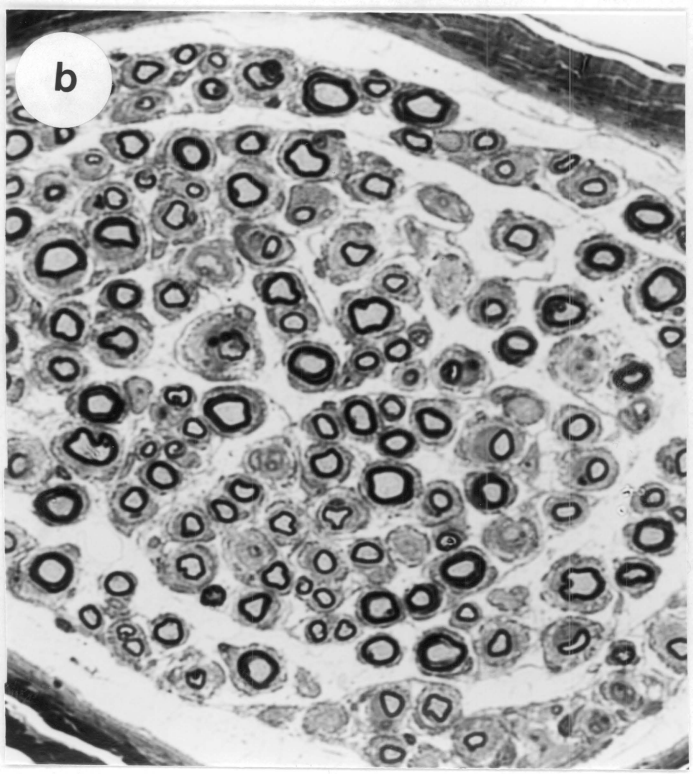

a


b


C

Figures 5 a, b, and c. Low power of cross sections of the distal region of nerve to biventer cervicis muscle from hens given PSP 15 days earlier. Fig. 5a is from a hen given only PSP, Fig. 5b from a hen given PSP and nifedipine, and Fig. 5c from a hen given PSP and verapamil. The nerve from hen given only PSP has advanced degenerative lesions with loss of myelinated fibers and associated presence of empty Schwann cell tubes. The degenerative process was at an earlier stage when nifedipine or verapamil were administered along with PSP.


a


b

Figures 6 a and b. Cross sections of biventer cervicis nerve 37 days after PSP treatment. Fig. 6a is from a hen receiving only PSP, and Fig. 6b from a hen receiving both PSP and nifedipine. Both nerves show the presence of newly myelinated regenerating fibers, although those in fig. 6b appear to be more advanced in caliber and myelination.

CHAPTER 10

MODIFICATION OF ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY (OPIDN) WITH CALCIUM BLOCKERS: III. ALTERED ACTIVITIES OF ESTERASES AND CALCIUM ACTIVATED NEUTRAL PROTEASE (CANP)

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Louisa Gay and Marion Ehrich
Virginia-Maryland Regional College
of Veterinary Medicine,
Blacksburg, Virginia

ABSTRACT

The possible involvement of Ca^{++} and Ca^{++}-activated neutral protease (CANP) in the pathogenesis of OPIDN was tested using the Ca^{++} channel blockers, nifedipine and verapamil. Nifedipine was given for 5 days (1.0 mg/kg im) and verapamil for 4 days (7.0 mg/kg im) beginning 24 hr before administration of phenyl saligenin phosphate (2.5 mg/kg im). An equal number of hens received the same dose of PSP alone. The ability of partially purified CANP to digest azocasein was assayed in quick frozen sciatic nerve and gastrocnemius muscles 4-5, 7-8, 15-16, 21-22, and 37 days following administration of PSP. Nifedipine and verapamil alone did not affect CANP activity. Combination of Ca^{++} channel blockers and PSP did, with results in sciatic nerve significantly lower than control for nifedipine-PSP combinations 4 and 7 days after PSP administration. Administration of PSP alone caused a significant increase in nerve CANP activity on days 7-8 and 21-22 following exposure. Muscle CANP activity was significantly increased 2-5 days after PSP administration The effect was transient and corresponded with cholinesterase inhibition. CANP activity of muscle in hens given PSP was significantly reduced in the presence of Ca^{++} channel blocker treatment. Increases in muscle cholinesterase due to denervation occurred only in PSP-treated hens and only as clinical signs developed. Brain CANP assayed during the course of tri-o-tolyl-phosphate (360 mg/kg) or PSP-induced

neuropathy was significantly increased throughout the 37 day study, including time periods when NTE activity was depressed. This study indicates that CANP activity increases during the course of OPIDN and can be modulated by agents modulating Ca^{++} movement. This may implicate Ca^{++}, through CANP, in the development of OPIDN.

INTRODUCTION

An irreversible, progressive neuropathy may appear in man and other suscepible species of avians and mammals after a single exposure to certain organophosporus compounds (Abou-Donia, 1981). Clinical signs of neuropathy, including incoordination, ataxia, and eventually paralysis, do not appear until 1 to 2 weeks after exposure (Davis and Richardson, 1980). Histopathological lesions characteristic of Wallerian degeneration appear at this time and include axonal degeneration, with secondary breakdown of myelin and muscle degeneration (Cavanagh, 1964; Cisson and Wilson, 1982; Prentice and Roberts, 1984; Jortner and Ehrich, 1987). Although an initial event, the inhibition of neuropathy target esterase (also known as neurotoxic esterase, NTE), has been proposed, the events occuring between exposure and clinical signs have not been precisely defined (Davis and Richardson, 1980; Abou-Donia, 1981; Johnson, 1987).

Investigators studying other neuropathies have suggested that intra-axonal elevation of free calcium (Ca^{++}) is associated with the breakdown of cytoskeletal elements and degeneration of myelin (Schlaepfer and Bunge, 1973; Schlaepfer, 1987). Ca^{++} appears to contribute to such changes by enhancement of Ca^{++}-activated neutral protease activity (CANP or calpain) (Schlaepfer and Hasler, 1979; Kamakuru et al.,1983; Banik et al., 1984; Berlet,1987), or Ca^{++}- dependent kinase. Changes in these latter enzymes have been

noted following administration of neurotoxic OPs (Abou-Donia et al., 1984; Patton et al., 1985; Suwita et al., 1986). In light of the studies cited above, the role of Ca^{++} in cell injury (Wrogeman and Pena, 1976; Schanne et al., 1979; Farber, 1981), and previous studies demonstrating the ability of Ca^{++} channel blockers to modify functional and morphological deficits of OPIDN (El-Fawal et al., 1989a,b,c), the present investigation was performed to determine if Ca^{++} and CANP activities were altered during OPIDN. The Ca^{++} channel blockers nifedipine and verapamil were also used in this study in order to determine if they could modify OP effects on Ca^{++}, CANP and esterases. Adult chickens, the generally recognized reliable model of OPIDN, were used for these studies (Davis and Richardson, 1980; Bickford, 1984).

MATERIALS AND METHODS

Animals and housing. White leghorn hens (>6 months old, 1.2 -1.8 kg) used in this study were provided by the Department of Poultry Science, Virginia Polytechnic Institute and State University. They were housed in wire bottom cages (57 x 84 x 61 cm) in groups of 4-5 and had access to food and water ad libitum. Hens were divided according to treatment [control, tri-o-tolyl phosphate (TOTP), phenyl saligenin phosphate (PSP), nifedipine, verapamil, nifedipine + PSP and verapamil + PSP] with 10 - 35 hens in each group.

Treatments. Protoxicant TOTP (Eastman Chemical Co., Rochester. N.Y.), and the active congener PSP were prepared as previously described (El-Fawal et al., 1989a), using corn oil and a dimethyl sulfoxide (DMSO) as solvents, respectively. The dosage of TOTP 360mg/kg was administered at 1 ml/kg by oral gavage, and PSP (2.5 mg/kg) by im injection in a volume of 0.25 ml/kg. Nifedipine (Sigma Chemical Co., St.Louis, MO), was dissolved in DMSO and 1.0 mg/kg administered im for 5 days beginning 1 day before PSP administration. Verapamil (Sigma Chemical Co., St.Louis. MO) was dissolved in distilled water and 7.0 mg/kg administered im for 4 days beginning one day before PSP administration. These regimens are the same as those previously used to note amelioration of OPIDN (El-Fawal et al., 1989b).

Enzyme assays. NTE activity was measured spectrophotometrically in quick-frozen brains of 4-5 hens sacrificed by

cervical dislocation at 48 hrs or by T-61 euthanasia (Hoescht Corp, Sommerville, N.J.) on days 4, 7, 10, 21 and 37, following administration of TOTP or PSP. The spectrophotometric method of Sprague et al., (1981) was used for analysis. Protein concentrations were determined using the dye-binding procedure from Bio-Rad Laboratories (Richmond, CA). Spectrophotometric measurement of brain, plasma, liver and muscle cholinesterase (Ellman et al., 1961; Bellino et al., 1978), plasma and liver carboxylesterases (Levine and Murphy, 1977) have all been described in previous publications from our laboratory (Ehrich and Larsen, 1983; Ehrich and Gross, 1983). For studies in muscle a 10^{-6}M concentration of iso-OMPA (tetramonoisoprophyl pyrophosphortetramide), a selective inhibitor of nonspecific cholinesterase, was included in the incubation procedure. Plasma creatine phosphokinase activity (CPK) was determined 7, 10, 15 and 21 days after TOTP or PSP administration using an automated analyzer and kit (Baker Instruments, Allentown, PA).

Procedures used for the partial purification and spectrophotometric determination of CANP activity were derived from the method described by Ballard et al., (1988). Briefly, brain, both sciatic nerves, or the gastrocnemius muscle were dissected immediately after sacrifice and snap-frozen in liquid nitrogen. On the day of the assay all procedures were carried out at 4^{o}C. Brain was weighed and homogenized by hand. Nerve and muscle trimmed of fat and connective tissue were minced and homogenized using a Poly-

tron honogenizer. One, 0.5 and 1.5 g wt weight of brain, nerve and muscle, respectively were homogonized with 2x volume of ice cold buffer containing 50 mM tris hydrochloride, 10 mM 2-mercaptoethanol and 5 mM-EDTA, pH 7.4. The crude homogenate was centrifuged at 100,000x g at 4°C for 30 minutes. The supernantant was passed through 1 cc tuberculin syringe columns packed with DEAE-cellulose (Whatman DE-52) which had been equilibrated with the buffer used for homogenization. The supernatant in the column was eluted with gradients of 0, 150, 250, and 300 mM NaCl prepared in the buffer that had been used to equilibriate the column. CANP activity was present in the fraction collected after equilibration at 250 mM NaCl and elution with 300 mM NaCl. Trichloroacetic acid (TCA) soluble peptides produced from azocasein by CANP proteolysis were measured in the presence of 5 mM Ca^{++} or 0.1 mM EDTA. The enzyme-substrate mixture was incubated for 30 minutes at 30°C, and the reaction stopped with 10% TCA. After precipitation of protein and centrifugation, 1N NaOH was added and absorbance read at 440 nm. CANP activity was determined as the difference between absorbances measured with 5 mM Ca^{++}, which measures total protease activity, and with 0.1 mM EDTA, which inhibits CANP. Activity was expressed as absorbance/mg protein. Protein was determined using the dye binding procedure from Bio-Rad Laboratories (Richmond, CA). Total nerve calcium was determined in nitric-perchloric acid digests of sciatic nerve analyzed by atomic absorption at a wavelength of 422.7

nm. Results were compared to a calcium carbonate standard.

Statistics. All data were analyzed using an analysis of variance with Newman-Keuls method of multiple comparisons for determination of statistical differences between control and experimental groups. Probabilities <0.05 were considered significant. All data are expressed as mean $\pm$ standard error.

RESULTS

The effects of TOTP and PSP on brain enzymes are shown in Figures 1 and 2. Activities of NTE assayed beginning day 4 and continuing through day 37 were significantly lower than for control hens following treatment with either TOTP or PSP, except on day 10 for the latter group (Fig. 1). Activity of brain CANP was elevated by 4 days after TOTP administration and by 7 days after PSP administration (Fig. 2). Activity remained elevated through 21 days after PSP administration. CANP activity was not determined in brains from hens given TOTP at every time period that it was determined in brains from hens given PSP.

Activities of plasma cholinesterases and carboxylesterases were significantly inhibited 4 days after treatment with TOTP or PSP (Table 1). Liver esterase activities were also significantly inhibited after TOTP but not after administration of PSP. Some, but not all, of these enzymes showed recovery by 7 days after administration of these organophosphates. The effects of TOTP and PSP on plasma CPK are shown in Table 2. CPK activities were highly variable and, therefore, did not differ significantly from controls.

The Ca^{++} channel blocker nifedipine did not prevent inhibition of NTE caused by PSP. NTE activity was 520 $\pm$ 28, 442 $\pm$ 17, 91 $\pm$ 6, and 87 $\pm$ 8 nmol/min/mg protein 2 days after PSP administration in brains of controls, nifedipine-treated, PSP-treated, and PSP + nifedipine-treated hens,

respectively. Treatment with nifedipine prevented the late rise in muscle cholinesterase activity seen after PSP administration (Table 3). Another calcium channel blocker, verapamil, prevented the rise in calcium accumulation in sciatic nerve seen 4 days after PSP administration (Table 3). Samples were not available for analysis at early time periods after administration of nifedipine and PSP. The effect of nifedipine and verapamil on CANP activities in sciatic nerve and gastrocnemesis muscle are shown in Figures 3 and 4. CANP activity in nerves from hens given nifedipine-PSP combinations were significantly lower than in nerves from hens given PSP only on days 7-8 and 21-22 (Fig.3). Verapamil treatment also prevented the PSP-induced increase in CANP activity at 7 days. Hens treated with nifedipine + PSP or verapamil + PSP did not have the high activity of muscle CANP seen early after administration of only PSP (Fig. 4).

DISCUSSION

Neuropathy target esterase (NTE) has been proposed as the initial biochemical site of action of neurotoxic OPs. A reduction of NTE activity to 20-30% of control is considered predictive of OPIDN (Richardson et al., 1984). NTE activity, however, may significantly recover as early as 7 days prior to the clinical appearence of ataxia after the administration of the same OPs (Abou-Donia, 1981). Few studies have addressed the time course of NTE recovery. Soliman et al., (1981) reported that recovery to greater than 50% occurred by 21 days following administration of TOTP (1000 mg/kg). On the otherhand, Carrington and Abou-Donia (1988) reported greater than 10% inhibition of NTE through 21 days following TOTP treatment (1187 mg/kg). An earlier report from this laboratory (Jortner and Ehrich,1987) indicated that the recovery of NTE occured 14 days following administration of PSP (2 and 10 mg/kg). The present study showed NTE recovery to greater than 60% of control 7 days following PSP treatment, with no significant change among activities at any subsequent time periods. NTE activity after administration of 360 mg/kg TOTP in the present study was slightly higher (28% of control) at 10 days after administration than at 7 days (11% of control) after 1000 mg/kg TOTP in Soliman's study.

Events that may occur between inhibition of NTE and the appearance of clinical deficits have not been completely

described. Moretto et al., (1987) correlated the inhibition and "aging" of NTE with a 70% reduction of retrograde axonal transport, part of the communication network between the distal axon and the nerve cell body (Price and Griffin, 1980). In the present study, CANP activity assayed in the same brain tissue used for NTE assays was noted to increase significantly at the earliest time point tested (4 days) and continued to do so throughout the 21 day time period when clinical deficits due to TOTP or PSP continued to become more notable (El-Fawal et al., 1989b). CANP is not an enzyme subject to inhibition by neurotoxic OPs. Although earlier studies by Seifert and Casida (1982) suggested that the activity of neuronal proteases could be possible targets of organophosphorus esters inducing delayed neuropathy, they did not examine activity of CANP.

The enhanced activity of CANP has been implicated in the initiating events occuring during Wallerian degeneration (e.g., granular disintegration, aggregation and accumulation of neurofilament triplet and microtubules and breakdown of myelin (Schlaepfer, 1971; Kamakura et al., 1983; Berlet et al., 1984; Banik et al., 1985), events commonly described during OPIDN (Abou-Donia, 1981; Jortner, 1984), and in the development of other neuropathies (Nixon et al., 1983; Jansco et al., 1984; Mata et al., 1986). Although brain lesions are not often found in OPIDN, Jortner (1984) reported involvemnt of the brain stem, and Tanaka and Bursian (1989) recently reported the involvement of medullary

tracts. These may explain the increase in brain CANP activity seen after administration of TOTP and PSP in the present study.

The increase in CANP activity leading to Wallerian degeneration is intimately associated with an increase in intracellular free Ca^{++} (Schlaepfer and Zimmerman, 1984; Kamakuru et al., 1983). An increase in total cell Ca^{++} was recently demonstrated by x-ray microprobe analysis following axotomy (Randall et al., 1988) and TOTP-induced delayed neuropathy (LoPachin et al., 1988). Microprobe analysis of nerves from hens given TOTP were, however, performed only at the time clinical signs were well developed (day 14). In the present study, Ca^{++}-channel blockers, agents which inhibit both Ca^{++} entry and/or its mobilization from intracellular storage sites (Needleman et al., 1985), prevented the rise in CANP activity in both distal nerve and muscle caused by administration of an active neurotoxic OP, PSP. This suggests that inhibition of Ca^{++}-influx or intracellular mobilization by these agents could contribute to reduction in CANP activity after administration of neuropathy-inducing OPs. This is consistent with reports that removal of Ca^{++} prevents Wallerian degeneration after surgically-induced axotomy (Schlaepfer, 1971; Schlaepfer and Bunge, 1973; Schlaepfer and Zimmerman, 1984). Other studies have suggested that activation of the L-type voltage-sensitive Ca^{++} channel, which is antagonized by dihydropyridine blockers such as nifedipine, is associated with activation of CANP

(Miller, 1987).

Attenuation of biochemical indicies of nerve degeneration caused by PSP in the presence of Ca^{++} channel blockers noted in the present study is consistent with amelioration of electrophysiological, pharmacological, and morphological effects reported previously (El-Fawal et al., 1989a, b, c).

The present investigation did not measure intracellular free Ca^{++}, the form responsible for the activation of CANP (Baker and Margolis, 1987). Techniques for specific measurement of intracellular free Ca^{++} in peripheral nerves of hens exposed to neuropathy-inducing OPs have not yet been developed. Atomic absorption of digested tissue measures total Ca^{++} (both free and bound). This, however, does not take in account the compartmentalization of Ca^{++} bound in cell organelles, such as mitochondria and smooth endoplasmic reticulum, and that free in the cytosol. Nevertheless, atomic absorption analysis of hen peripheral nerve in this study did indicate that levels of Ca^{++} in nerve increase following exposure to neurotoxic OPs. Ca^{++} influx may have contributed to this effect.

As in the case of nervous tissue, muscle also contains a CANP whose activity results in the breakdown of myofibril proteins and other components of the sarcomere (Reddy et al., 1975; Ishiura, 1981; Baker and Margolis, 1987). In our study we demonstrated an early transient increase in muscle CANP (day 2 and 4). This may be due to the development of a transient myopathy attributed to OPs by other investigators.

Although this myopathy is believed to be due to acute inhibition of AChE (Wecker et al., 1978a; b) and OPIDN is said to be independent of AChE inhibition (Abou-Donia, 1981), inhibition of cholinesterase, as shown in plasma and muscle at this time interval, did occur. This may have been sufficient to induce a transient myopathy that contributed to the elevation of CANP activity. The development of myopathy shortly after administration of other cholinesterase inhibitors has been demonstrated to be due to the enhanced release and accumulation of sarcoplasmic Ca^{++} with the subsequent activation of muscle CANP (Toth et al., 1981, 1983). Later increases in muscle CANP activity may coincide with the appearance of muscle degeneration reported during neuropathy following administration of TOTP (Cisson and Wilson, 1982). Muscle degeneration was not, however, indicated by increases in CPK activity in our study. This corresponds with the results of Anderson et al. (1988), who reported no change in activity of this muscle enzyme following exposure to diisopropylfluorophosphate. CPK activity was, however, elevated in plasma of hens with TOTP-induced delayed neuropathy in another study (Cisson and Wilson, 1982). It has been suggested that muscle atrophy in later stages of OPIDN is secondary to nerve damage (Anderson et al., 1988).

This study demonstrated increases in muscle cholinesterase activity as neuropathy developed following PSP administration. This is consistent with previous studies of muscle enzyme activity following surgical denervation or OP-induced

nerve degeneration in avians (Cisson and Wilson, 1982, 1983). The prevention of increases in muscle AChE caused by PSP in chickens treated with either Ca^{++} blocker may be due to attenuation of denervation-induced muscle degeneration. It could also be due to direct prevention of intra-muscular Ca^{++} accumulation, particularly since specific dihydropyridine (eg. nifidepine) receptors, which are functional Ca^{++} channels, have been identified in skeletal muscle (Schmid et al., 1986). The increase in muscle AChE may also be due to inhibition of protease activity. Alterations in muscle AChE activity following denervation have been attenuated or abolished by use of protease inhibitors (Fernandez and Duell, 1980). The mechanism by which Ca^{++} channel blockers prevent elevations of muscle cholinesterase activity in the present study however, remain to be explored.

Nifedipine administration did not alter the development of OPIDN by preventing the inhibition of NTE, since this esterase was equally inhibited in hens receiving PSP, whether or not they had been given the Ca^{++} channel blocker. This is consistent with our earlier report on the effects of verapamil on OPIDN (El-Fawal et al., 1989c).

In this study we demonstrated the use of compounds capable of modulating extracellular Ca^{++} influxes and intracellular Ca^{++} mobilization could alter the course of biochemical events occuring during OPIDN. The attenuation of biochemical events, particularly increases in activity of CANP, may be subsequent to increased Ca^{++} entry following an ini-

tiating event in nerve cells exposed to neuropathy-inducing OPs. It is possible that inhibition of protein NTE could be this initiating event. In addition to ameliorating increases in CANP activity, Ca^{++} channel blockers also preserved to a great extent the functional and structural integrity of both nervous and muscle tissue (El-Fawal et al., 1989a, b, c), further implicating this cation and CANP in degradative changes seen after administration of neuropathy-inducing OPS.

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REFERENCES

ABOU-DONIA, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-148.

ABOU-DONIA, M. B., PATTON, S. E. AND LAPADULA, D. M. (1984). Possible endogenous protein phosphorylation in organophosphorus compound-induced delayed neurotoxicity. In Cellular and Molecular Neurobiology (T. Narahashi, Ed.), pp. 265-283 Raven Press, New York.

ANDERSON, R. J., ROBERTSON, D. G., HENDERSON, J. D. AND WILSON, B. W. (1988). DFP-induced elevation of strength-duration threshold in hen peripheral nerve. NeuroToxicology. 9: 47-52.

BAKER, J. H. AND MARGOLIS, R.N. (1987). Calcium-activated protease activity in tenotomized muscle. Muscle and Nerve. 10: 34-40.

BALLARD, R., BARDSLEY, R. G., AND BUTTERLY, P. J. (1988). Changes in the activity of skeletal muscle calcium-activated neutral protease (EC 3.4.22.17) and its specific inhibitor in chickens grown at different rates in response to graded levels of dietary protein. Brit. J. Nutr. 59: 141-147.

BANIK, N. L., HOGAN, E. L. AND McALHANEY, W. W. (1984). A role for calcium and activation of neutral proteinase in myelinolysis? In Experimental Allergic Encephalomyelitis. pp. 145-152 Alan R. Liss, Inc., New York.

BELLINO M., fICARRA, M., FRONTALI, N., gHEZZO, F., GUARCINI, A. M., ORECCHIO, T., SERPIETRI, L. A. AND TRAINA, M. E. (1978). A quick simple method for the routine determination of acetyl- and butyryl- cholinesterase in blood. Brit. J. Ind. Med. 35: 161-167.

BERLET, H. H. (1987). Calcium-dependent neutral protease activity of myelin from bovine spinal cord: evidence for soluble cleavage products of myelin proteins. Neurosci. Lett. 73: 266-270.

CARRINGTON, C. D. AND ABOU-DONIA, M. B. (1988). Triphenyl phosphite neurotoxicity in the hen: inhibition of neurotoxic esterase and of prophalaxis by phenylmethylsulfonyl fluoride. Arch. Toxicol. 62: 375-380.

CAVANAGH, J. B. (1964). Peripheral nerve changes in orthocresyl phosphate poisoning in the cat. J. Pathol. Bact. 87: 365-383.

CISSON, C. M. AND WILSON, B. W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity: altered acetylcholinesterase molecular forms and increased plasma creatine phosphokinase activity. Toxicol. Appl. Pharmacol. 64: 289-305.

CISSON, C. M. AND WILSON, B. W. (1983). Percutaneous toxicity and delayed neurotoxicity of organophosphates in the scaleless hen. Toxicol. Appl. Pharmacol. 67: 310-321.

DAVIS, C. S. AND RICHARDSON, R. J. (1980). Organophosohorus compounds. In Experimental and Clinical Neurotoxicology (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527-544 Williams and Wilkins, Baltimore.

EHRICH, M. AND GROSS, W. B. (1983). Modification of triorthotolyl phosphate toxicity in chickens by stress. Toxicol. Appl. Pharmacol. 70: 249-254.

EHRICH, M. AND LARSEN, C. (1983). Drug metabolism in adult white leghorn hens -response to enzyme inducers. Comp. Biochem. Physiol. 74C: 383-386.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989a). Effect of verapamil on organophosphate-induced delayed neuropathy (OPIDN) in hens. Toxicol. Appl. Pharmacol. 97: 500-511.

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989b). Modification of organophosphate-induced delayed neuropathy (OPIDN) with calcium channel blockers: I. in vivo using the sciatic-gastrocnemius preparation. Ph.D. Dissertation, VPI & SU

EL-FAWAL, H. A. N., JORTNER, B. S. AND EHRICH, M. (1989c). Modification of organophosphate-induced delayed neuropathy (OPIDN) with calcium channel blockers: II. in vitro using the biventer cervicis nerve-muscle preparation. Ph.D. Dissertation, VPI & SU

ELLMAN, G. L., COURTNEY, K. D., ANDRES, V. AND FEATHERSTONE, R. M. (1961). A new rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1: 88-95.

FARBER, J. L. (1981). The role of calcium in cell death. Life Sci. 29: 1289-1295.

FERNANDEZ, H. L. AND DUELL, M. J. (1980). Protease inhibitors reduce effects of denervation on muscle endplate acetylcholinesterase. J. Neurochem. 35: 1166-1171.

ISHIURA, S. (1981). Calcium-dependent proteolysis in living cells. Life Sci. 29: 1079-1087.

JOHNSON, M. K. (1987). Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. Trends Pharmacol. Sci. 8: 174-179.

JORTNER, B. S. (1984). Pathology of organophosphorus-induced delayed neurotoxicity. In Proceedings of the Fourteenth Conference on Environmental Toxicology (J. D. MacEwan and E. H. Vernot, Eds.), pp. 106-117. National Information Service, Springfield, Va.

JORTNER, B. S. AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

KAMAKURA, K., ISHIURA, S., SUGITA, H. AND TOYOKURA, Y. (1983). Identification of Ca^{++}-activated neutral protease in peripheral nerve and its effects on neurofilament degeneration. J. Neurochem. 40: 908-913.

KOMULAINEN, H. AND BONDY, S. C. (1988). Increased free intracellular Ca^{2+} by toxic agents: an index of potential neurotoxicity? Trends Pharmacol. Sci. 9: 154-156.

LEVINE, B. S. AND MURPHY, S. D. (1977). Esterase inhibition and reactivation in relation to piperonyl butoxide-phosphorothionate interaction. Toxicol. Appl. Pharmacol. 40: 379-391.

LOPACHIN, R. M., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1988). Organophosphate intoxication alters distribution of elements in chicken peripheral nerve. Neuroscience Abstracts 14: 775.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. Science 235: 46-235.

MORETTO, A., LOTTI, M., SABRI, M. I. AND SPENCER, P. S. (1987). Progressive deficit of retrograde axonal transport is associated with the pathogenesis of di-n-butyl dichlorvos axonopathy. J. Neurochem. 49: 1515-1522.

NIXON, R. A., BROWN, B. A. AND MAROTTA, C. A. (1983). Limited proteolytic modification of a neurofilament protein involves a proteinase activated by endogenous levels of calcium. Brain Res. 275: 384-388.

PATTON, S. E., LAPADULA, D. M., O'CALLAGHAN, J. P., MILLER, D. B. AND ABOU-DONIA, M. B. (1985). Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-o-cresyl phosphate to hens. J. Neurochem. 45: 1567-1577.

PRICE, D. L. AND GRIFFIN, J.W. (1980). Neurons and ensheathing cells as targets of disease processes. In Experimental and Clinical Neurotoxicity (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 2-23. Williams and Wilkins, Baltimore.

PRENTICE, D. E. AND ROBERTS, N. L. (1984). Acute delayed neurotoxicity in hens dosed with tri-ortho-cresyl phosphate (TOCP): correlation between clinical ataxia and neuropathological findings. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.) pp. 139-144. Intox Press, Little Rock.

RANDALL, V. L., LOPACHIN, R. M. AND SAUBERMANN, A. J. (1988). Distribution of elements in axotomized nerves determined by microprobe analysis. The Toxicologist 8: 246.

REDDY, M. K., ETLINGER, J. D., RABINOWITZ, M., FISCHMAN, D. A. AND ZAK, R. (1975). Removal of Z-lines and a-actinin from isolated myofibrils by a calcium-activated neutral protease. J. Biol. Chem. 250: 4278-4284.

RICHARDSON, R. J. (1984). Neurotoxic esterase: normal and pathogenic roles. In Cellular and Molecular Neurotoxicology (T. Narahashi, Ed.), pp. 285-295 Raven Press, New York.

SCHANNE, F. A. X., KANE, A. B., YOUNG, E. E. AND FARBER, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science. 206: 700-702.

SCHLAEPFER, W. W. (1987). Neurofilaments: structure, metabolism and implication in disease. J. Neuropath. Exp. Neurol. 46: 117-129.

SCHLAEPFER, W. W. AND BUNGE, R. P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J. Cell. Biol. 59: 456-470.

SCHLAEPFER, W. W. AND HASLER, M. B. (1979). Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. Brain Res. 168: 299-309.

SCHLAEPFER, W. W. AND ZIMMERMAN, U.P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. In Axonal Transport in Neuronal Growth and Regeneration (J. S. Elam and P. Cancalon, Eds.), pp. 261-273 Plenum Press, New York.

SCHMID, A., KAZAZOGLOU, T., RENAUD, J. F. AND LAZDUNSKI, M. (1984). Comparitive changes of levels of nitrendipine Ca^{++} channels, of tetrodotoxin-sensitive Na^{+} channels and oubain-sensitive $(Na^{+}+K^{+})$-ATPase following denerva-

tion of rat and chick skeletal muscle. FEBS 172: 114-118.

SEIFERT, J. AND CASIDA, J. E. (1982). Possible role of microtubules and associated proteases in organophosphorus ester-induced delayed neurotoxicity. Biochem. Pharmacol. 31: 2065-2070.

SPRAGUE, G. L., BICKFORD, A. A. AND CASTLES, T. R. (1984). Neurohistological and clinical recovery in hens after administration of tri-o-tolyl phosphate. The Toxicologist 4: 26.

SUWITA, E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986). Calcium and calmodulin stimulated in vitro phosphorylation of rooster brain tubulin and MAP-2 following a single oral dose of tri-o-cresyl phosphate. Brain Res. 374: 199-203>

TANAKA, D. AND BURSIAN, S. J. (1989). Selective degeneration in the chicken central nervous system after exposure to bis (1-methyl-ethyl) phosphorofluoridate (DFP). The Toxicologist 9: 74.

TOTH, L., KARSCU, S., POBERAI, M. AND SAVAY, G. (1983). Histochemical evidence for the role of Ca^{2+} and neutral protease in the development of the subacute myopathy induced by organophosphorus compounds. Acta Histochem. 72: 71-75.

TOTH, L., KARSCU, S., POBERAI, M. AND SAVAY, G. (1981). A light and electron microscopic histochemical study on the mechanism of DFP-induced acute and subacute myopathy. J. Neuropathol. Appl. Neurobiol. 7: 399-410.

WECKER, L., KIAUTA, T. AND DETTBARN, W. D. (1978). Relationship between acetylcholinesterase inhibition and the development of a myopathy. J. Pharmacol. Exp. Therap. 206: 97-104.

WECKER, L., LASKOWSKI, M. B. AND DETTBARN, W. D. (1978). Neuromuscular dysfunction induced by acetylcholinesterase inhibition. Fed. Proc. 37: 2818-2822.

WROGEMAN, K. AND PENA, S. D. J. (1976). Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle disease. Lancet 7961: 672-673.

TABLE 1. EFFECT OF ORGANOPHOSPHORUS COMPOUNDS ON PLASMA AND LIVER ESTERASES.

Percentage of Control Enzyme Activities Present in Plasma and Liver Samples from Untreated Hens[b]

	Plasma			Liver	
Treatment[a]	AChE	BuChE	Carboxylesterase	Cholinesterase	Carboxylesterase
TOTP					
Day 4	57 ± 7[c]	35 ± 5[c]	43 ± 2[c]	66 ± 6[c]	55 ± 2[c]
Day 7	49 ± 9[c]	83 ± 12	56 ± 3[c]	68 ± 7[c]	58 ± 3[c]
PSP					
Day 4	49 ± 3[c]	40 ± 4[c]	52 ± 2[c]	86 ± 2	83 ± 1
Day 7	75 ± 7[c]	91 ± 10	61 ± 12[c]	65 ± 3[c]	80 ± 4

a. TOTP 360 mg/kg po and PSP 2.4 mg/kg im

b. X±S.E., n = 5, Values in chickens given neither TOTP or PSP: AChE = 0.261 ± 0.016; BuChE = 0.399 ± 0.032 μmol/min/ml, plasma carboxylesterase = 0.719 ± 0.038 μmol/min/ml; liver cholinesterase = 2.042 ± 0.042 μmol/min/g.

c. Significantly lower than untreated chickens, $p < 0.05$, Newman-Keuls test for multiple comparisons.

TABLE 2. EFFECT OF ORGANOPHOSPHORUS COMPOUNDS ON PLASMA CPK.

	Plasma CPK (U/L)		
	Control	TOTP[a]	PSP[a]
	500 ± 35		
Day 7		374 ± 81 (75%)	376 ± 76 (75%)
Day 10		404 ± 106 (81%)	470 ± 102 (94%)
Day 15		552 ± 158 (110%)	655 ± 471 (131%)[b]
Day 21		574 ± 170 (114%)	555 ± 109 (111%)

a. TOTP 360 mg/kg po and PSP 2.5 mg/kg im, n = 4-7.
b. Significantly higher than untreated chickens, $p < 0.05$, Newman-Keuls test for multiple comparisons.

TABLE 3. BIOCHEMICAL PARAMETERS FOLLOWING ADMINISTRATION OF PHENYL SALIGENIN PHOSPHATE (PSP), NIFEDIPINE AND PSP, OR VERAPAMIL AND PSP.

Treatment	Muscle Cholinesterase (μmol/min/g tissue)[a]	Total nerve Ca^{++} (ppm)[b]
control	1.97 ± 0.272	15.6 ± 2.03
Nifedipine	2.558 ± 0.390 (129%)	20.40 ± 1.72 (88%)
Verapamil	1.62 ± 0.483 (82%)	21.66 ± 4.52 (108%)
PSP		
Day 2	2.02 ± 0.654 (102%)	21.70 ± 3.07 (108%)
Day 4	1.82 ± 0.499 (91%)	29.13 ± 1.36 (145%)[c]
Day 7	1.73 ± 0.875 (88%)	30.26 ± 5.66 (151%)[c]
Day 15	2.50 ± 0.455 (126%)	47.27 ± 10.74 (236%)[c]
Day 21	2.84 ± 0.229 (143%)[c]	22.36 ± 2.51 (111%)
Day 37	3.59 ± 0.670 (181%)[c]	
Nifedipine + PSP		
Day 2	1.52 ± 0.419 (76%)	17.16 ± 1.24 (86%)[d]
Day 4	2.04 ± 0.612 (103%)	-
Day 7	2.40 ± 0.507 (121%)	-
Day 15	1.49 ± 0.474 (76%)	45.45 ± 10.74 (227%)[c]
Day 21	1.95 ± 0.107 (98%)	28.60 ± 3.34 (142%)[c]
Day 37	1.62 ± 0.351 (82%)	-
Verapamil + PSP		
Day 4	1.66 ± 0.532 (84%)	18.03 ± 2.87 (90%)
Day 7	1.71 ± 0.755 (86%)	29.29 ± 0.84 (146%[c])
Day 15	2.55 ± 0.302 (129%)	36.50 ± 4.67 (182%)[c]

a. 10^{-6}M iso-OMPA included for inhibition of non-specific cholinesterase (n = 3-10)
b. Flame emission atomic absorption determination following tissue digestion with concentrated HNO_3 and perchloric acid (4.1).
c. Significantly higher than control $p < 0.05$. Newman-Keuls test for multiple comparisons.
d. Significantly lower than control $p < 0.05$. Newman-Keuls test for multiple comparisons.

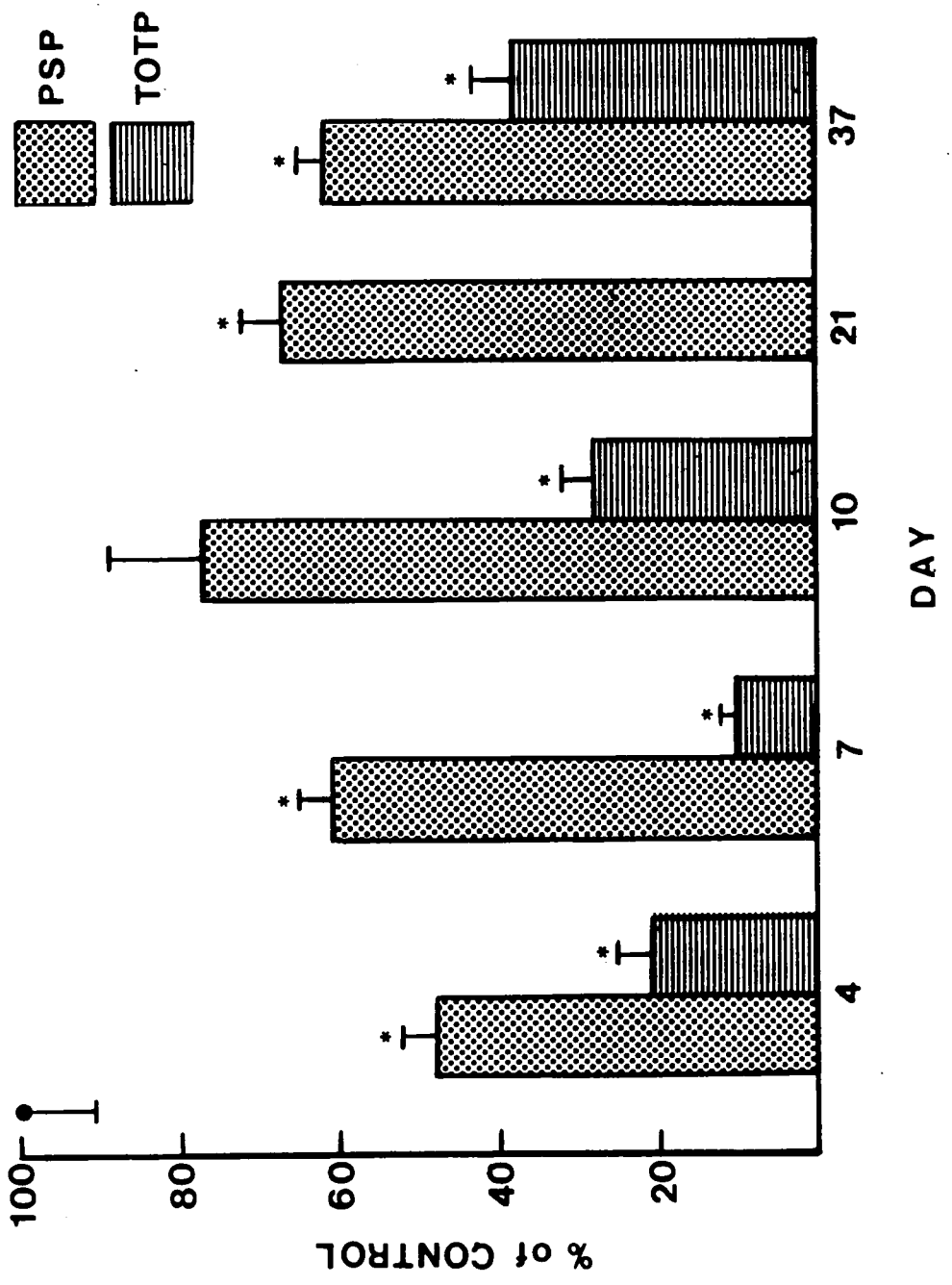
PSP
TOTP
100
80
60
40
20
% of CONTROL
4
7
10
21
37
DAY

Figure 1. Brain neuropathy target esterase (NTE) activity as percent of control (451 ± 22 nmol/15 min/mg protein) 4, 7, 10, 21, and 37 days after administration of either TOTP (360 mg/kg po) or PSP (2.5 mg/kg im). Asterisks denote significant differences when compared to controls as determined by ANOVA and Newman-Keuls test for multiple comparisons ($p < 0.05$), n=5-8. NTE activities after PSP administration were not significantly different from each other on any of the days tested after day 4.

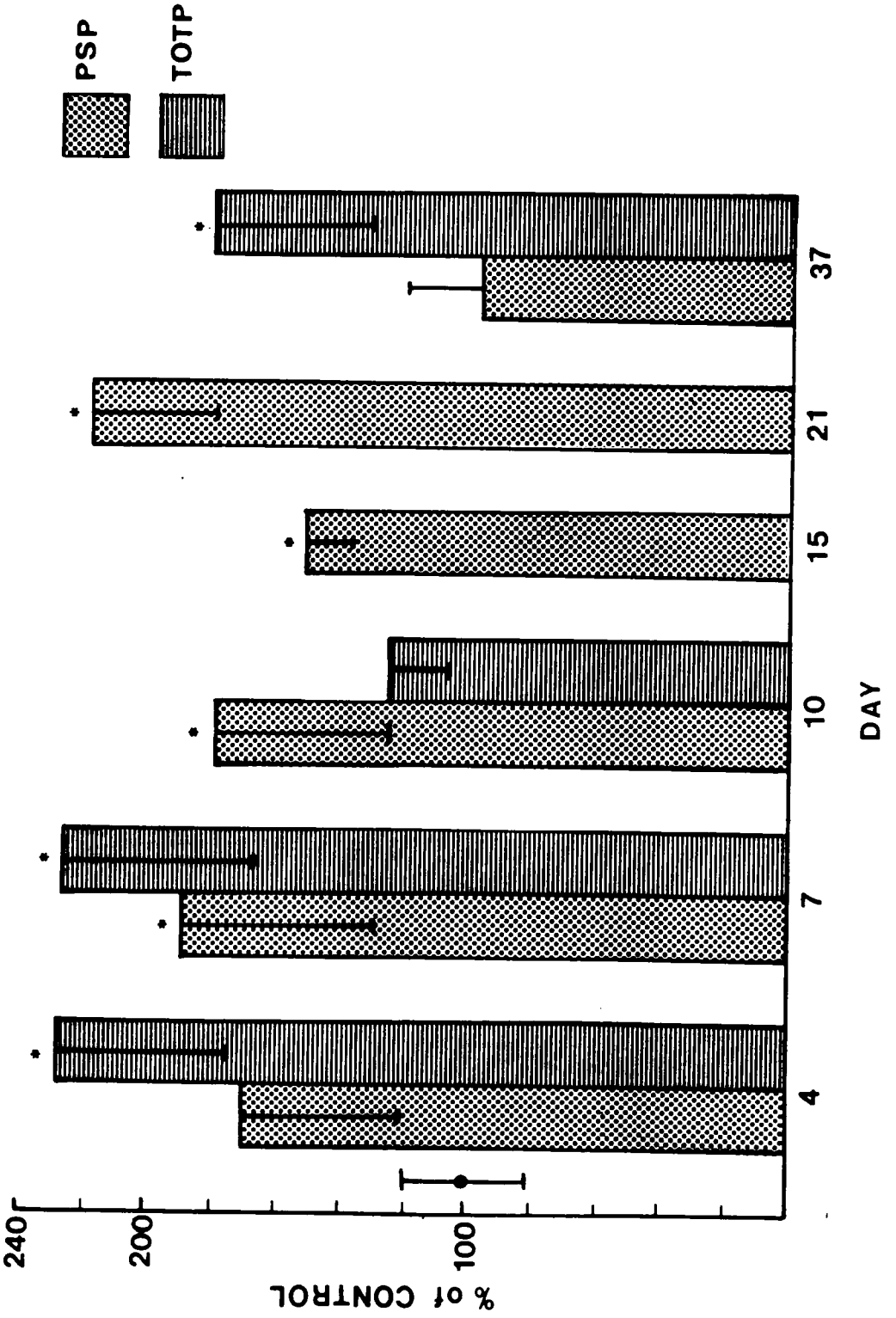
PSP
TOTP
240
200
100
% of CONTROL
4
7
10
15
21
37
DAY

Figure 2. Brain calcium-activated neutral protease (CANP) activity as percent of control (7.174 $\pm$ 1.40 absorbance units/ 30 minutes/mg protein x 10^3) 4, 7, 10, 15, 21, and 37 days after administration of either TOTP or PSP. Asterisks denote significant differences when compared to controls as determined by ANOVA and Newman-Keuls test for multiple comparisons ($p < 0.05$), n=5-8.

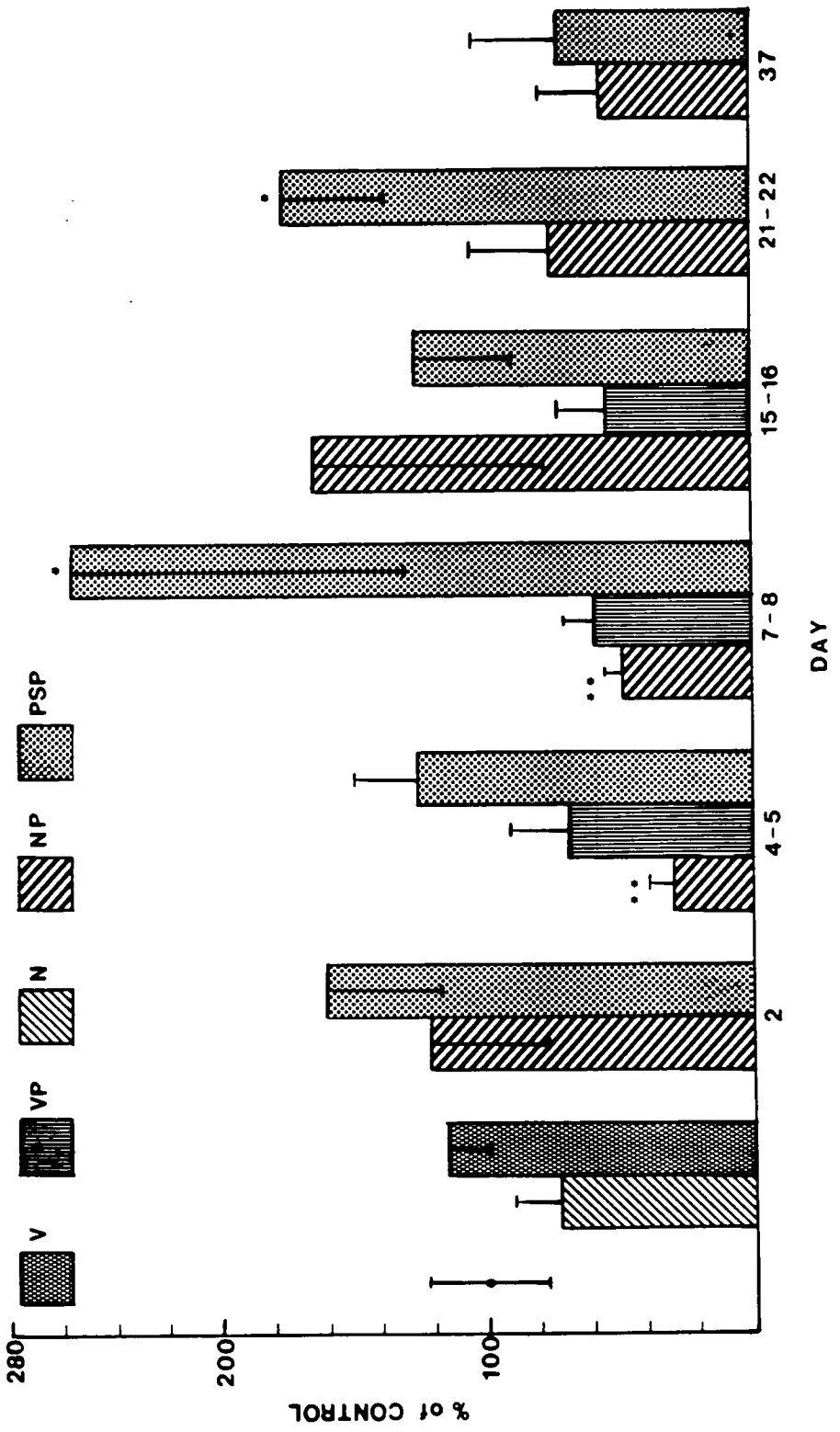
280
200
100
% of CONTROL
V
VP
N
NP
PSP
2
4-5
7-8
15-16
21-22
37
DAY

Figure 3. Sciatic nerve CANP activity as percent of control (C) (26.38 ± 6.13 absorbance units/ 30 minutes/ mg protein x 10^3) 2, 4-5, 7-8, 15-16, 21-22, and 37 days after treatment with PSP (P). NP = nifedipine plus PSP; VP = verapamil plus PSP; N = nifedipine only; V = verapamil only. NP and P groups were different from each other days 4-5, 7-8, and 21-22 after OP administration. VP and P groups were different from each other days 4-5, 7-8, and 15-16. Single asterisks denote significantly higher than controls, double asterisks denote significantly lower than controls as determined by ANOVA and Newman-keuls test for multiple comparisons ($p < 0.05$), n = 3-10.

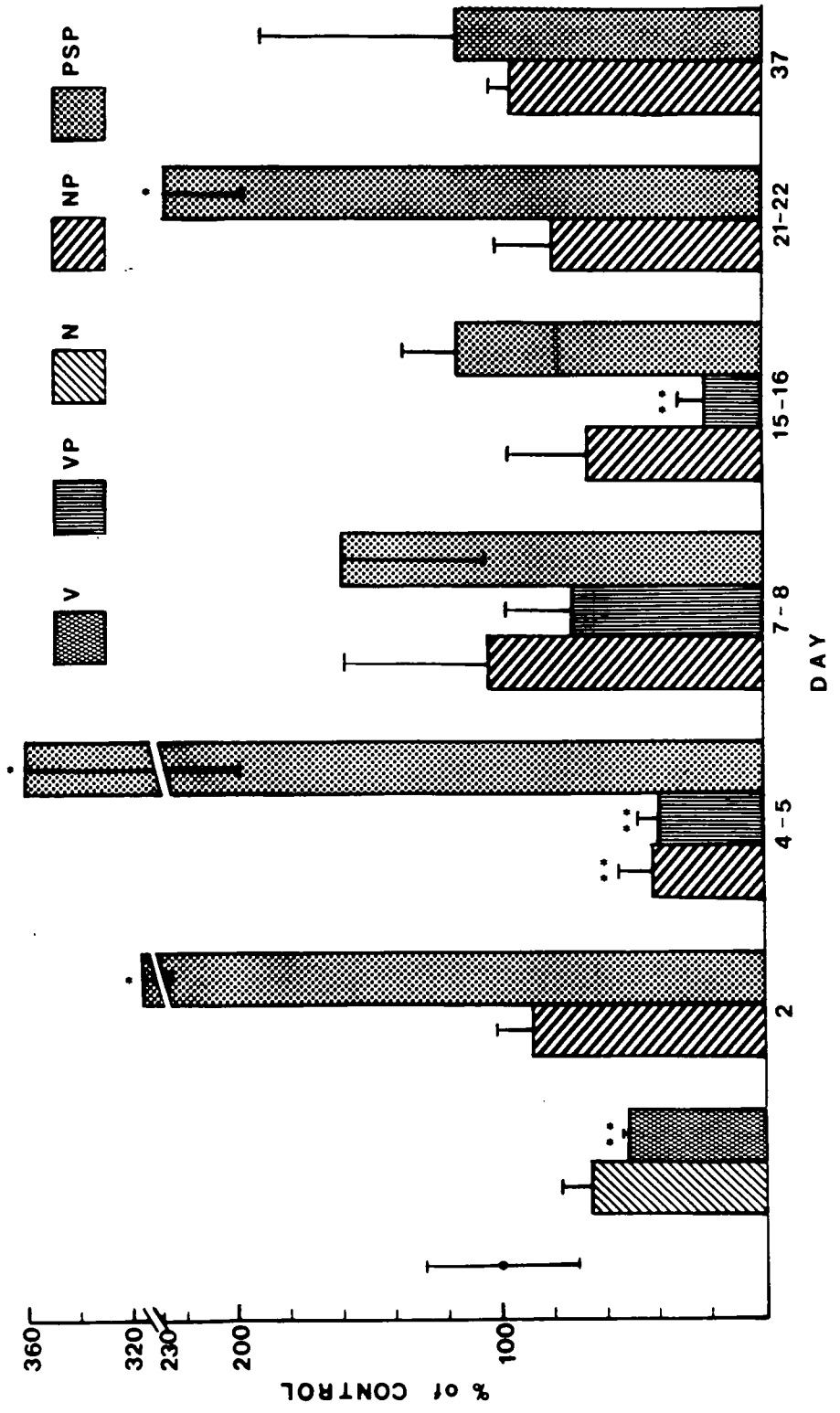
PSP
NP
N
VP
V
37
21-22
15-16
7-8
4-5
2
DAY
360
320
230
200
100
% of CONTROL

Figure 4. Gastrocnemius muscle CANP activity as percent of control (C) (109.81 ± 33 absorbance units/ 30 minutes/ mg protein x 10^3) 2, 4-5, 7-8, 15-16, 21-22, and 37 days after treatment with PSP (P). NP = nifedipine plus PSP; VP = verapamil plus PSP; N = nifedipine only; V = verapamil only. Single asterisks denote significantly higher than controls, double asterisks denote significantly lower than controls as determined by ANOVA and Newman-Keuls test for multiple comparisons ($p < 0.05$), n = 3-10.

PART V

CHAPTER 11
GENERAL DISCUSSION AND CONCLUSION

The work presented in this dissertation represents new information relative to organophosphorus-induced delayed neuropathy (OPIDN). This includes (1) the correlation of clinical, biochemical, physiological and morphological changes of developing deficits; (2) the modification of OPIDN using agents which modulate Ca^{++} movement into and within cells; and (3) the identification of some molecular events occurring between inhibition of NTE and the development of clinical signs.

The polyneuropathy due to exposure to some organophosphorus compounds was first described in 1899 when phosphocreosate, a triaryl phosphate, was used in the treatment of tuberculosis (Beck et al., 1977; Cherniak, 1986; Goldstein et al., 1988). The greatest outbreak of neuropathy ascribed to an OP occurred in the 1930s, during the Prohibition Era, as a result of the consumption of a popular remedy, "Ginger Jake" or "Jamaica Jack", which was adulterated with tri-ortho-cresol phosphate used in the extraction of the ginger. Over 4837 cases were reported and unofficial estimates were ten times higher (Beck et al., 1977; Cherniak, 1986). The resulting neurological syndrome, Ginger Paralysis or Jack-Leg (due to the hind limb paralysis), became topic for popular song of the time (Goldstein et al., 1988).

Since then interest has grown in the study of what came to be known as organophosphorus-induced delayed neuropathy (OPIDN). Studies of OPIDN have from that time described morphological alterations (Cavanagh, 1954; Jortner, 1984) and

identified metabolic pathways that contribute to its expression (Aldridge et al., 1962). Investigations into the possible mechanisms involved in production of OPIDN were essentially lacking until the late 1969's when Johnson (1969) described a phosphorylation site, an esterase, as the possible target for neuropathy-inducing organophosphorus esters. This esterase was named "neurotoxic esterase" or NTE, and was later shown to be a membrane-bound protein whose inhibition and irreversible modification (or "aging") appears to be a prerequisite for induction of OPIDN. More recently this site has been more appropriately named "neuropathy target esterase" (Johnson, 1987). Routine assay of NTE is now mandatory in the screening of potentially neurotoxic compounds (Abou-Donia, 1981; Federal Register, 1985). However, investigations on mechanisms of OPIDN have been confounded by the observation that clinical deficits do not appear until weeks to months following exposure, a time period much later than NTE inhibition, which can be noted within hours after OP exposure (Davis and Richardson, 1980).

In an attempt to identify events occurring between NTE inhibition and clinical signs, investigations in the 1970's characterized electrophysiological changes (e.g., conduction velocity, post-tetanic potentiation, etc.) that occurred after exposure to neuropathy-inducing OPs (Lowndes et al., 1974). These studies examined different animal models of OPIDN, exposure kinetics, the influence of age, and species susceptibilities (Abou-Donia, 1981). From studies conducted

from the 1930's to the 1970's the adult hen emerged as the animal model of choice, as it was capable of providing an extremely sensitive model and a reproducable syndrome that resembled that seen in humans (Bickford, 1984).

In an effort to define mechanisms responsible for OPIDN, investigators have used various pharmacological agents. These have included corticoids (Ehrich and Gross, 1982; 1983; Ehrich et al., 1985; 1986a, b; Baker et al., 1982; 1983; Drakontides et al., 1982; Baker and Stanec, 1985) and compounds that inhibit NTE without "aging" (e.g., phenyl methyl sulfonyl fluoride, PMSF) (Carrington and Abou-Donia, 1983; Caroldi et al., 1984; Veronesi and Padilla, 1985).

In the studies done for this dissertation, a new method of electrophysiological measurement was found useful in identifying events occurring between NTE inhibition and onset of clinical signs. In addition, a class of pharmacological agents, the calcium channel blockers, was found to modify OPIDN, thereby providing information on mechanisms associated with its pathogenesis.

1. The Biventer Cervicis Nerve-Muscle Preparation and Time Course of OPIDN Development.

In this study early neuropathological and morphological changes were detected using an <u>in vitro</u> neuromuscular preparation of adult hens first developed as part of the dissertation research. Past studies on neuromuscular function had used <u>in vivo</u> measurements on preparations from cats or

hens exposed to neurotoxic OPs. In vivo preparations had the disadvantage of being limited to the innervation of one muscle type. For example, the cat soleus muscle, chicken plantaris muscle and hen gastrocnemius muscle are composed primarily of slow twitch, slow tonic, and fast twitch fibers respectively (Lowndes et al., 1974; Durham and Ecobichon, 1984; Robertson et al., 1987). The biventer cervicis nerve-muscle preparation used for this research contains both fast-twitch and slow-tonic muscle fiber types (Ginsborg, 1960), and allows for the assessment of the innervation to either type. Fast-twitch muscle responds to electrical stimulation, while slow-tonic muscle reponds with contraction to the application of exogenous acetylcholine (ACh). Advantage of these properties were exploited in the present study, as innervation of muscle types affected by OPIDN could be identified.

The primary lesion in OPIDN, Wallerian degeneration, is in effect a chemically-induced axotomy or denervation of the effector organ (e.g., muscle). This loss in nerve integrity subsequently causes functional changes in muscle. Previous studies for evaluation of these changes, while relying on evaluation of nerve conduction velocities or compound action potentials, were not definitive and gave variable results. The dissertation research used strength-duration measurements, which determine the excitability properties of nerve and muscle. These measurements have long been used clinically as sensitive indicies of neuromuscular function (Har-

ris, 1971; Wynn Parry, 1971). The sensitivity of this measure was confirmed in experimental animals with OPIDN.

Another consequence of denervation is the development of denervation hypersensitivity to neurotransmitter in skeletal muscle that is primarily of the type defined as slow-tonic or slow-twitch. Sensitivity of the biventer cervicis muscle to application of ACh identified the response of this muscle type to denervation. In the research conducted for this dissertation, strength-duration curves (SDC) reflecting the function of fast-twitch muscle innervation, and supersensitivity to ACh reflecting the function of slow-tonic muscle innervation were altered during OPIDN.

In the dissertation research hens were treated with phenyl saligenin phosphate (PSP) at 3 dose levels. PSP was found to alter strength-duration relationships in a manner which indicated partial to complete denervation of fast-twitch muscle at times when neuropathy was well developed. This is consistent with observations in in vivo preparations (Robertson et al., 1987). The development of denervation hypersensitivity to neurotransmitter in slow-tonic muscle, which is primarily postural, is consistent with the production of muscle fibrillations previously described in OPIDN (Vesilescu and Florescu, 1980). The responses elicited for both muscle types of the biventer cervicis were dose-dependent.

The extensive damage to the nerve innervating the biventer cervicis muscle in hens with OPIDN was confirmed by mor-

phological methods, and was consistent with patterns of Wallerian degeneration. This extensive damage of the biventer cervicis nerve was unexpected in light of previous studies. It has generally been assumed that the distal, longest, and larger myelinated nerve fibers were preferentially affected in OPIDN (Cavanagh, 1964). The biventer cervicis nerve is relatively short, as it arises immediately from the spinal cord, and fiber diameters are smaller than those of distal nerves of the leg traditionally used to evaluate OPIDN. Furthermore, the biventer cervicis is located in the cervical region of the chicken and, with the exception od Bouldin and Cavanagh (1979), damage to nerves of the cervical region have not been reported. The lack of studies of peripheral nerve damage in the cervical region of animals with OPIDN is not unusual, particularly since clinical deficits in this area generally go unnoticed.

The biventer cervicis nerve-muscle preparation proved to be very useful in evaluation of functional and morphological changes following administration of OP-induced delayed neuropathy. Electrophysiological and pharmacological measurements require only 30 minutes (much less than for <u>in vivo</u> preparations, which involve complicated surgical procedures). The anatomy of this preparation, a twin nerve-muscle, allows the simultaneous evaluation of physiological function and collection of samples for histological study.

Advantage was taken of the conveniences afforded by this preparation to determine if functional and morphological

deficits could be detected between NTE inhibition and clinical signs of OPIDN induced by protoxicant tri-ortho-tolyl phosphate (TOTP) and active congener PSP. Results indicated changes in nerve excitability thresholds and patterns of denervation, as suggested by SDC patterns and values for rheobase and chronaxie derived from SDC, as early as 4 days following exposure to either TOTP or PSP. The shape of the SDC indicated patterns of partial to complete denervation between 4 and 15 days after OP administration, which correlated with clinical signs. The observations substantiate those of Wynn-Parry (1971), who reported changes in strength-duration relationships as early as 4-5 days following nerve trauma. The biventer cervicis muscle could also be used to detect responses indicative of partial recovery in nerve function as progression of clinical signs ceased and the hens became less ataxic after a period of time.

The time course for denervation hypersensitivity to ACh was similar to the time course of SDC during OPIDN. Progressive deficits to 21 days and a later partial recovery were demonstrated. The early detection and time course of denervation, which is believed to be due to de novo synthesis of ACh receptors, is consistent with reports that this occurs as early as 3 days following insult (Almon and Appel, 1976).

The time course and severity of developing histological lesions characteristic of OPIDN in the biventer cervicis nerve correlated with changes in SDC and development of hyp-

ersensitivity to ACh. Wallerian degeneration was noted as early as 4 days after PSP. In this regard, the biventer cervicis nerve was more sensitive to early lesions than distal nerves of the hind limbs as limb lesions usually begin about the time of clinical deficits (7 to 10 days after OP administration)(Davis and Richardson, 1980; Jortner and Ehrich, 1987). Regeneration of nerve fibers, evident as thinly myelinated to not-yet myelinated clusters in the biventer cervicis nerve, could be seen 2 to 3 weeks following exposure to PSP or TOTP.

2.Modification of OPIDN With Ca^{++} Channel Blockers.

Ca^{++} plays a key role as a messenger linking external stimuli to signal transductionin in the intracellular environment in nerve and muscle cells (Cavero and Spedding, 1983; Miller, 1987). However, when this cation is unregulated due to defects in influx or intracellular modulating mechanisms (e.g. sequestering in the mitochondria) it plays a role in degeneration and death of nerve and muscle cells (Farber, 1981).

Among the mechanisms by which Ca^{++} is thought to be involved in cell degradation is by the activation of Ca^{++}-activated neutral proteases (CANP) or Ca^{++}-dependent kinases (Schlaepfer, 1987; Abou-Donia et al., 1984). CANP is located in axoplasm and also in myelin and skeletal muscle, tissues which degenerate following axonal breakdown in OPIDN.

Another mechanism whereby increased Ca^{++} could contribute to effects seen after exposure to neuropathy-inducing OP's is its capability to stimulate synthesis of ACh receptors (Metafora et al., 1980), thereby contributing to the development of denervation hypersensitivity to ACh during OPIDN in the study discussed above.

The role of Ca^{++} in the pathogenesis of OPIDN was substantiated by this study using Ca^{++} channel blockers. The agents used in these investigations, nifedipine and verapamil, inhibit intracellular Ca^{++} mobilizations from their stores and/or inhibit Ca^{++} entry from the extracellular space (Miller, 1987). The phenylalkylamine, verapamil, has been suggested to act only at external Ca^{++} channels and to lack the specificity of nifedipine, a dihydropyridine.

Both nifedipine and verapamil beneficially modified OPIDN, as indicated by clinical signs, SDC *in vivo* and *in vitro*, denervation hypersensitivity, and histology of the biventer cervicis nerve.

Relative improvements in OP-induced effects on SDC and denervation hypersensitivity with Ca^{++} channel blockers were comparable in *in vivo* and *in vitro* preparations. However, the *in vitro* biventer cervicis nerve muscle preparation was more sensitive with regard to magnitude of changes. This part of the dissertation research served to validate the use of the simpler biventer cervicis preparation, since previous use of SDC in OPIDN was only done *in vivo* (Robertson et al., 1987; 1988).

Histological examination of the biventer cervicis nerve substantiated beneficial effects of Ca^{++} channel blockers on OPIDN induced by PSP. Nerves from hens treated with either Ca^{++} channel blocker in conjunction with PSP showed less degeneration of mylelinated fibers, and, in some instances, earlier appearance of regeneration. Ca^{++} channel blockers did not completely prevent the development of OPIDN in the present investigation, but this may reflect the dose and treatment regimen used.

The mechanism by which Ca^{++} channel blockers exerted their beneficial effect was not through direct alteration of the interaction of OP and NTE, as inhibition was similar in all hens given neuropathy-inducing PSP. The blockers may have prevented the increase in axoplasmic Ca^{++} demonstrated by X-ray microprobe analysis following axotomy (Randall et al., 1988) and more recently following TOTP-induced neuropathy (LoPachin et al., 1988). Ca^{++} channel blockers may also have contributed to the activation of Ca^{++}-dependent kinases, as they have been demonstrated to increase during OPIDN (Suwita et al., 1986a, b) causing the phosphorylation of the neurofilament triplet and tubulin peptides.

3. Biochemical Events During OPIDN

Another mechanism that could explain why Ca^{++} channel blockers ameliorated degenerative changes and the functional alterations associated with these changes may be their capability to attenuate the activiation of Ca^{++}-dependent pro-

teolytic enzyme, CANP. This study found activity of this enzyme to be less in nerves from hens given PSP and these blockers than in nerves from hens given PSP only. The general involvement of proteases had been suggested previously in OPIDN but specific activity of CANP had not been measured (Seifert and Casida, 1982). CANP is responsible for the breakdown of neurofilaments and microtubules (Schlaepfer, 1987).

In the studies done for this dissertation not only was peripheral nerve CANP elevated following PSP treatment, but CANP activity in the brain also increased, particularly during the interval between NTE inhibition and appearance of clinical signs. This may indicate that CANP activation is one of the earliest events occuring after exposure to neurotoxic OPs. Furthermore, activation of the L-type voltage-sensitive Ca^{++} channel has been suggested to be associated with increased CANP activity. Nifedipine, a dihydropyridine blocker, acts at this site (Miller, 1987).

An early transient rise in CANP also occured in muscle. This may be due to the early inhibition of muscle cholinesterase that followed administration of PSP, an effect independent of OPIDN. Some OPs have previously been reported to cause a transient increase in sarcoplasmic Ca^{++} and elevated CANP activity (Toth et al., 1981; 1983). The later rise in muscle CANP activity following return to nearly normal levels may reflect the muscle degeneration which occurs secondary to denervation.

Increases in AChE in avian muscle typical of physical denervation and OPIDN occurred late after PSP administration. Ca^{++} blocker treatment prevented such an increase. This, too, may imply that Ca^{++} blockers inhibit activity of proteases, as their inhibition has been reported to prevent this particular biochemical change associated with denervation (Fernandez and Duell, 1980).

CONCLUSIONS

The hypothesis put forth for testing suggested that, as a consequence of an event triggered by OP compounds, possibly the inhibition and aging of membrane-bound protein, NTE, peripheral nerves may be predisposed to increases in Ca^{++} mobilization and the intraneuronal accumulation of this cation. This increase in intracellular Ca^{++} could thereby initiate a cascade of events in both nerve and muscle that may account for some of the detrimental changes occuring during OPIDN.

Previous studies indicated that the use of Ca^{++} channel blockers or reduction in the intracellular Ca^{++} could modify neuropathies and myopathies not caused by OPs. The activation of a Ca^{++}-dependent proteolytic enzyme, CANP, was implicated in the production of dying-back, Wallerian degeneration in some of these studies. The research done for this dissertation demonstrated amelioration of OPIDN in hens given these blockers and neuropathy-inducing OPs. CANP activity increased in both neuronal and muscle tissue following OP exposure. These increases coincided with progression of the neuropathy, but began prior to the appearance of clinical and detectable morphological deficits. The Ca^{++} channel blockers, nifedipine and verapamil, significantly attenuated the activity of CANP, and, in turn, the development of OPIDN. This improvement in a biochemical parameter was substantiated by clinical signs, electrophysiological measurement of nerve function *in vivo* and *in vitro*, and by

histological examinations. The ability of these pharmacological agents to modify OPIDN and CANP activation strongly suggests that Ca^{++} is involved in the development of this neuropathy.

The events secondary to axonopathy, myelin and muscle degeneration, may also be due to increases in Ca^{++}. Muscle, for example, becomes hypersensitive to its neurotransmitter following denervation due to the de novo synthesis of ACh receptors and the incorporation of these receptors into the muscle membrane. This hypersensitivity was shown by others to be enhanced by Ca^{++} accumulation, and demonstrated in our study to be abolished when Ca^{++} channel blockers were used to modify OPIDN. Release of Ca^{++} and its accumulation, which leads to activation of CANP, could also contribute to muscle degeneration, depletion of muscle CPK and an attendant increase in muscle AChE which occur during OPIDN. These events were demonstrated to be abolished by reducing Ca^{++} using Ca^{++} channel blockers.

The modification provided by Ca^{++} channel blockers during the course of OPIDN did not involve protection of NTE, the suspect initiation target in this neuropathy. The relationship of NTE, Ca^{++} and CANP remains to be examined, however.

The present study provides impetus for further investigations into the role of Ca^{++} and CANP during OPIDN. Such invistigations may include the study of intra-axonal Ca^{++} movement and accumulation as OPIDN develops. To date,

increases in intraaxonal Ca^{++} have been demonstrated only during a stage when clinical and pathological changes were evident. Future investigations could also include localization of CANP intra-axonally and modification of OPIDN under conditions when specific inhibitors of CANP are used.

PART VI

GENERAL REFERENCES

REFERENCES

ABOU-DONIA, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21: 511-148.

ABOU-DONIA, M. B. (1984). Toxicokinetics and metabolism of delayed neurotoxic organophosphorus esters. In Delayed Neurotoxicity. (J. M. Cranmer and E. J. Hixson, Eds.) pp. 87-103. Intox Press, Little Rock.

ABOU-DONIA, M. B. (1985). Biochemical toxicology of organophosphorus compounds. In Neurotoxicology (K. Blum and L. Manzo, Eds.), pp. 423-444 Marcel Dekker, Inc. New York.

ABOU-DONIA, M. B., JENSEN, D. N. AND LAPADULA, D. M. (1983). Neurologic manifestations of tri-o-cresyl phosphate delayed neurotoxicity in cats. Neurobehav. Toxicol. Teratol. 5: 431-442.

ABOU-DONIA, M. B., PATTON, S. E. AND LAPADULA, D. M. (1984). Possible endogenous protein phosphorylation in organophosphorus compound-induced delayed neurotoxicity. In Cellular and Molecular Neurobiology (T. Narahashi, Ed.), pp. 265-283 Raven Press, New York.

ABOU-DONIA, M. B., ABDO, K. M., TIMMONS, P. R. AND PROCTOR, J. E. (1986a). Brain acetylcholinesterase, acid phosphatase, and 2′, 3′-cyclic nucleotide-3′-phosphohydrolase and plasma butyrylcholinesterase activities in hens treated with a single dermal neurotoxic dose of S,S,S-tri-n-butyl phosphorotrithioate. Toxicol. Appl. Pharmacol. 82: 461-473.

ABOU-DONIA, M. B., TROFATTER, L. P., GRAHAM, D. G. AND LAPADULA, D. M. (1986). Electromyographic, neuropathologic, and functional correlates in the cat as a result of tri-o-cresyl phosphate delayed neurotoxicity. Toxicol. Appl. Pharmacol. 83: 126-141.

ALDRIDGE, W. N., BARNES, J. M. AND JOHNSON, M. K. (1969). Studies on delayed neurotoxicity produced by some organophosphorus ompounds compounds. Ann. N. Y. Acad. Sci. 160: 314-322.

ALMON, R. R. AND APPEL, S. H. (1976). Cholinergic sites in skeletal muscle. I. Denervation effects. Biochem. 13: 3662-3667.

ALMON, R. R., ANDREW, C. G. AND APPEL, S. H. (1974). Acetylcholine receptor in normal and denervated slow and fast muscle. Biochem. 13: 5522-5528.

ANAND, R. AND EMERY, A. E. H. (1982). Verapamil and calcium-stimulated enzyme efflux from skeletal muscle. Clin. Chem. 28: 1482-1484.

ANDERSON, D. J. (1987). Molecular biology of the acetylcholine receptor: structure and regulation of biogenesis. In The Vertebrate Neuromuscular Junction (M. M. Salpeter, Ed.), pp. 285-315. Alan R. Liss, Inc., New York.

ANDERSON, R. J., ROBERTSON, D. G., HENDERSON, J. D. AND WILSON, B. W. (1988). DFP-induced elevation of strength-duration threshold in hen peripheral nerve. NeuroToxicology. 9: 47-52.

ANONYMOUS. (1985). Rules and Regulations. Federal Register 50: 39458-39470.

AXELSSON, J. AND THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. 147: 178-193.

BADALAMENTE, M. A., HURST, L. C. AND STRACHER, A. (1987). Localization and inhibition of calcium-activated neutral protease (CANP) in primate skeletal muscle and peripheral nerve. Exp. Neurol. 98: 357-369.

BAKER, J. H. AND MARGOLIS, R.N. (1987). Calcium-activated protease activity in tenotomized muscle. Muscle and Nerve. 10: 34-40.

BAKER, T., GLAZER, E. AND LOWNDES, H. E. (1977). Subacute neuropathy effects of diisopropylfluorophosphate at the cat soleus neuromuscular junction. Neuropath. Appl. Neurobiol. 3: 377-390.

BAKER, T., LOWNDES, H. E., JOHNSON, M. K. AND SANDBORG, I. C. (1980). The effects of phenylmethanesulfonyl fluoride on delayed organophosphorus neuropathy. Arch. Toxicol. 46: 305-311.

BAKER, T. AND LOWNDES, H. E. (1980). Muscle spindle function in organophosphorus neuropathy. Brain Res. 185: 77-84.

BAKER, T., DRAKONTIDES, A. B. AND RIKER, W. F. (1982). Prevention of the organophosphorus neuropathy by glucocorticoids. Exp. Neurol. 78: 397-408.

BAKER, T. AND STANEC, A. (1985). Methylprednisolone treatment of an organophosphorus-induced delayed neuropathy. Toxicol. Appl. Pharmacol. 79: 348-352.

BALENTINE, J. D. (1988). Spinal cord trauma: in search of the meaning of granular axoplasm and vesicular myelin.

J. Neuropathol. Exp. Neurol. 47: 77-92.

BANIK, N. L., HOGAN, E. L. AND McALHANEY, W. W. (1984). A role for calcium and activation of neutral proteinase in myelinolysis? In Experimental Allergic Encephalomyelitis. pp. 145-152 Alan R. Liss, Inc., New York.

BANIK, N. L., McALHANEY, W. W. AND HOGAN, E. L. (1985). Calcium-stimulated proteolysis in myelin: evidence for a Ca^{++}-activated neutral proteinase associated with purified myelin of rat CNS. J. Neurochem. 45: 581-588.

BARON, R. L., BENNET, D. R. AND CASIDA, J. E. (1962). Neurotoxic syndrome produced in chickens by a cyclic phosphate metabolite of tri-o-cresyl phosphate-a clinical and pathological study. Brit. J. Pharmacol. 18: 465-474.

BECK, B.E., WOOD, C. D. AND WHWNHAM, G. R. (1977). Triaryl phosphate poisoning in cattle. Vet. Pathol. 14: 128-137.

BERLET, H. H. (1987). Calcium-dependent neutral protease activity of myelin from bovine spinal cord: evidence for soluble cleavage products of myelin proteins. Neurosci. Lett. 73: 266-270.

BERLET, H. H., ILZENHOFER, H. AND SCHULZ, G. (1984). Cleavage of myelin basic protein by neutral protease activity of human white matter and myelin. J. Neurochem. 43: 627-633.

BETZ, H. AND CHANGEUX, J. P. (1979). Regulation of muscle acetylcholine receptor synthesis in vitro by cyclic nucleotide derivatives. Nature 278: 749-752.

BHATTACHARYA, S. K., PALMIERI, G. M. A., BERTORINI, T. E. AND NUTTING, D. F. (1982). The effect of diltiazem in dystrophic hamsters. Muscle and Nerve. 5: 73-78.

BICKFORD, A. A. (1984). The hen as a test model for delayed neurotoxicity studies: introductory comments. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 24-25 Intox Press, Little Rock.

BIRNBAUM, M., REIS, M. A. AND SHAINBERG, A. (1980). Role of calcium in the regulation of acetylcholine receptor synthesis in cultured muscle cells. Pflugers Arch. 385: 37-43.

BLAUROCK, A. E., YALE, J. L. AND ROOTS, B. I. (1986). Ca-controlled, reversible structural transition in myelin. Neurochem. Res. 11: 1103-1129.

BLAUROCK, A. E. AND YALE, J. L. (1987). Calcium ions trigger the expansion in bistable myelin. Neurosci. Lett. 73: 167-172.

BLUNT, R. J., JONES, R. AND VRBOVA, G. (1975). Inhibition of cell division and the development of denervation hypersensitivity in skeletal muscle. Pflugers Arch. 355: 189-204.

BOULDIN, T. W. AND CAVANAGH, J. B. (1979). Organophosphorus neuropathy I. A teased-fiber study of the spatio-temporal spread of axonal degeneration. Am. J. Pathol. 94: 241-262.

BOWMAN, W. C. AND MARSHALL, I. G. (1971). Muscle. In Physiology and Biochemistry of the Domestic Fowl (D. J. Bell and B. M. Freeman, Eds.), pp. 707-737 Academic Press, New York.

BRADY, S. T., TYTELL, M., HERIOT, K. AND LASEK, R. J. (1980). Calmodulin in axonal transport. Ann. N. Y. Acad. Sci.

BRIMIJOIN, S. (1983). Molecular forms of acetylcholinesterase in brain, nerve and muscle: nature, localization and dynamics. Prog. Neurobiol. 21: 291-322.

BROWN, G. L. (1937). The actions of acetylcholine on denervated mammalian and frog skeletal muscle. J. Physiol. 89: 438-461.

BROWN, G. L. AND HARVEY, A. M. (1938a). Neuromuscular conduction in the fowl. J. Physiol. 93: 285-300.

BROWN, G. L. AND HARVEY, A. M. (1983b). Reactions of avian muscle to acetylcholine and eserine. J. Physiol. 94: 101-117.

CAROLDI, S., LOTTI, M. AND MASUTTI, A. (1984). Intra-arterial injection of diisofluorophosphate or phenylmethanesulphonyl fluoride produces unilateral neuropathy or protection, respectively, in hens. Biochem. Pharmacol. 33: 3213-3217.

CARRINGTON, C. D. AND ABOU-DONIA, M. B. (1983). The time course of protection from delayed neurotoxicity induced by tri-o-cresyl phosphate and o-o-diisopropyl phosphorofluoridate by phenyl methyl sulfonyl fluoride in chickens. Toxicol. Lett. 18: 251-256.

CAVALLERI, A. AND COSI, V. (1980). Polyneuropathy in shoe factory workers. In Advances in Neurotoxicology, (L. Manzo, Ed.), pp. 193-200. Pergamon Press, New York.

CAVANAGH, J. B. (1964). Peripheral nerve changes in ortho-cresyl phosphate poisoning in the cat. J. Pathol. Bact. 87: 365-383.

CAVANAGH, J. B. (1985). Peripheral nervous system toxicity: a morphological approach. In Neurotoxicology (K. Blum and L. Manzo, Eds.), pp. 1-44 Marcel Dekker, Inc., New York.

CAVERO, I., AND SPEDDING, M. (1983). Calcium antagonists: a class of drugs with a bright future. Life Sci. 33: 2571-2581.

CHAN, S. Y., OCHS, S. AND JERSILD, R. A. (1984). Localization of calcium in nerve fibers. J. Neurobiol. 15: 89-108.

CHANG, C. C. AND TANG,S. S. (1974). Differentiation between intrinsic and extrensic acetylcholine receptors of the chick biventer cervicis muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. 282: 379-388.

CHANG, C. C. AND TUNG, L. H. (1974). Inhibition by actinomycin D of the generation of acetylcholine receptors induced by denervation of skeletal muscle. Eur. J. Pharmacol. 26: 386-388.

CHERNIACK, M. G. (1986). Organophosphorus esters and polyneuropathy. Ann. Int. Med. 104: 264-266.

CISSON, C. M. AND WILSON, B. W. (1982). Degenerative changes in skeletal muscle of hens with tri-ortho-cresyl phosphate-induced delayed neurotoxicity: altered acetylcholinesterase molecular forms and increased plasma creatine phosphokinase activity. Toxicol. Appl. Pharmacol. 64: 289-305.

CISSON, C. M. AND WILSON, B. W. (1983). Percutaneous toxicity and delayed neurotoxicity of organophosphates in the scaleless hen. Toxicol. Appl. Pharmacol. 67: 310-321.

CLARK, C. R., MACLUSKY, N. J. AND NAFTOLIN, F. (1981). Glucocorticoid receptors in the spinal cord. Brain Res. 217: 412-415.

CLOW, D. W. AND BOEGMAN, R. J. (1987). Effect of denervation on adenine nucleotides in skeletal muscle from normal and dystrophic mice. Exp. Neurol. 96: 334-343.

COHEN, S. D. AND EHRICH, M. (1976). Cholinesterase and carboxylesterase inhibition by dichlorvos and interactions with malathion and triorthotolyl phosphate. Toxicol. Appl. Pharmacol. 37: 39-48.

CORRELL, L. AND EHRICH, M. (1987). Comparative sensitivities of avian neural esterases to in vitro inhibition by organophosphorus compounds. Toxicol. Lett. 36: 197-204.

D'AMOUR, F. E. AND BLOOD, F. R. (1954). Muscle-Nerve physiology. In Manual for Laboratory Work in Mammalian Physiology, experiment 49. University of Chicago Press, Illinois.

DAVIS, C. S. AND RICHARDSON, R. J. (1980). Organophosohorus compounds. In Experimental and Clinical Neurotoxicology (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527-544 Williams and Wilkins, Baltimore.

DAVIS, C. S., JOHNSON, M. K. AND RICHARDSON, R. J. (1985). Organophosphorus compounds. In Neurotoxicity of Industrial and Commercial Chemicals (J. L. O'Donoghue, Ed.), pp. 1-23 CRC Press, Fl.

DAVIS, H. L. (1983). Trophic action of nerve extract on denervated skeletal muscle in vivo: dose dependency, species specificity, and timing of treatment. Exp. Neurol. 80: 383-394.

DEL CASTILLO, J. AND KATZ, B. (1957). The identity of intrinsic and extrinsic acetylcholine receptors in the motor end plate. Proc. Roy. Soc. London (B). 146: 357-361.

DELORENZO, R. J. (1982). Calmodulin in neurotransmitter release and synaptic function. Fed. Proc. 41: 2265-2272.

DETTBARN, W. D., PATTERSON, G. T., GUPTA, R. C. AND MISULIS, K. E. (1987). Acetylcholinesterase activity in use and disuse of muscle. In Neurobiology of Acetylcholine (N. J. Dun and R. L. Perlman, Eds.), pp. 103-110. Plenum Press, New York.

DOWBEN, R. M. (1980). Contractility. In Medical Physiology, Vol. 1, 14th ed. (V. B. Mountcastle, Ed.), pp. 82-119. C. V. Mosby Co., St. Louis.

DRAKONTIDES, A. B., BAKER, T. AND RIKER, W. F. (1982). A morphological study of the effect of glucocorticoid treatment on delayed organophosphorus neuropathy. NeuroToxicology. 3: 165-178.

DRAKONTIDES, A. B. AND BAKER, T. (1983). An electrophysiologic and ultrastructural study of the phenylmethanesulfonyl fluoride protection against a delayed organophosphorus neuropathy. Toxicol. Appl. Pharmacol. 74:

411-422.

DRETCHEN, K. L., BOWLES, A. M. AND RAINES, A. (1986). Protection by phentoin and calcium blocking agents against the toxicity of diisopropylfluorophosphate. Toxicol. Appl. Pharmacol. 83: 584-589.

DUBOIS, D. C. AND ALMON, R. R. (1980). Disuse atrophy of skeletal muscle is associated with an increase in number of glucocorticoid receptors. Endocrinol. 107: 1649-1651.

DUBOIS, D. C. AND ALMON, R. R. (1981). A possible role for glucocorticoids in denervation atrophy. Muscle and Nerve 4: 370-373.

DUNCAN, C. J. (1978). Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. Experientia. 34: 1531-1672.

DURHAM, H. D. AND ECOBICHON, D. J. (1984). The function of motor nerves innervating slow tonic skeletal muscle in hens with delayed neuropathy induced by tri-o-tolyl phosphate. Can. J. Physiol. Pharmacol. 62: 1268-1273.

DURHAM, H. D. AND ECHOBICHON, D. J. (1986). An assessment of the neurotoxic potential of fenitrothion in the hen. Toxicology. 4: 319-332.

EBASHI, S. AND SUGITA, H. (1979). The role of calcium in physiological and pathological processes of skeletal muscle. In Current Topics in Nerve and Muscle Research (A. J. Aguayo and G. Karpati, Eds.), pp.73-84 Excerpta Medica, Amsterdam.

EHRICH, M. AND GROSS, W. B. (1982). Effect of corticosterone on toxicity of malathion and TOTP in hens. Neurobehav. Toxicol. Teratol. 4: 789-792.

EHRICH, M. AND GROSS, W. B. (1983). Modification of triorthotolyl phosphate toxicity in chickens by stress. Toxicol. Appl. Pharmacol. 70: 249-254.

EHRICH, M., JORTNER, B. S. AND GROSS, W. B. (1985). Absence of a protective effect of corticosterone on o-o-diisopropyl phosphofluoridate (DFP) induced delayed neuropathy in chickens. NeuroToxicology. 6: 87-92.

EHRICH, M., JORTNER, B. S. AND GROSS, W. B. (1986a). Dose-related beneficial and adverse effects of dietary corticosterone on organophosphorus-induced delayed neuropathy in chickens. Toxicol. Appl. Pharmacol. 83: 250-260.

EHRICH, M., BRILES, R. W., BRILES, W. E., DUNNINGTON, E. A., MARTIN, A., SIEGEL, P. B. AND GROSS, W. B. (1986b). Neurotoxicity of triorthotolyl phosphate in chickens of different genotypes in the presence and absence of deoxycorticosterone. Poultry Sci. 65: 375-379.

EHRICH, M. AND GROSS, W. B. (1986). Effect of supplemental corticosterone and social stress on organophosphorus-induced delayed neuropathy in chickens. Toxicol. Lett. 31: 9-13.

EHRICH, M., JORTNER, B. S. AND GROSS, W. B. (1988). Types of adrenocorticoids and their effect on organophosphorus-induced delayed neuropathy in chickens. Toxicol. Appl. Pharmacol. 92: 214-223.

ELLISON, G. W. (1984). Corticosteroids in neurologic diseases. Hosp. Prac. 19: 105-113.

ELLMAN, G. L., COURTNEY, K. D., ANDRES, V. AND FEATHERSTONE, R. M. (1961). A new rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1: 88-95.

EL-SEBAE, A. H., SOLIMAN, S. A., ABO EL-AMAYEM, M. AND AHMED, N. S. (1977). Neurotoxicity of organophosphorus insecticides leptophos and EPN. J. Environ. Sci Hlth. B12: 269-288.

ETO, M., CASIDA, J. E. AND ETO, T. (1962). Hydroxylation and cyclization reactions in the metabolism of tri-o-cresyl phosphate. Biochem. Pharmacol. 11: 337-352.

EYRE, P. AND GOFF, D. (1968). A simple myograph for recording hind limb muscle contractions in anaesthetized rabbits. J. Inst. Sci. Technol. 14: 56.

FAMBROUGH, D. M. (1970). Acetylcholine sensitivity of muscle fiber membranes: mechanism of regulation by motoneurons. Science 168: 372-373.

FAMBROUGH, D. M. (1979). Control of acetylcholine receptors in skeletal muscle. Physiol. Rev. 59: 165-227.

FARBER, J. L. (1981). The role of calcium in cell death. Life Sci. 29: 1289-1295.

FENICHEL, G. M., KIBLER, W. B., OLSON, W. H. AND DETTBARN, W. D. (1972). Chronic inhibition of cholinesterase as a cause of myopathy. Neurol. 22: 1026-1033.

FERNANDEZ, H. L. AND DUELL, M. J. (1980). Protease inhibitors reduce effects of denervation on muscle endplate acetylcholinesterase. J. Neurochem. 35: 1166-1171.

FLORINI, J. R. (1987). Hormonal control of muscle growth. Muscle and Nerve 10: 577-598.

FRANCIS, B. M. (1983). Effects of dosing regimens and routes of administration on organophosphorus ester induced delayed neurotoxicity. NeuroToxicology. 4: 139-146.

FRANCIS, B. M., METCALF, R. L. AND FISHER, S. W. (1983). Responses of laboratory rodents to selected avian neurotoxicants. Arch. Environ. Contam. Toxicol. 12: 731-738.

FRIEDLI, W. G. AND MEYER, M. (1984). Strength-duration curve: a measure for assessing sensory deficit in peripheral neuropathy. J. Neurol. Neurosurg. Psychiat. 47: 184-189.

GINSBORG, B. L. AND WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. Br. J. Pharmacol. 15: 410-411.

GLAZER, E. J., BAKER, T. AND RIKER, W. F. (1978). The neuropathology of DFP at cat soleus neuromuscular junction. J. Neurocyt. 7: 741-758.

GODFRAIND, T., MILLER, R. AND WIBO, M. (1986). Calcium antagonism and calcium entry blockade. Pharmacol. Rev. 38: 321-416.

GOLDBERG, A. L. (1969). Protein turnover in skeletal muscle: effects of denervation and cortisone on protein catabolism in skeletal muscle. J. Biol. Chem. 244: 3223-3229.

GOLDSTEIN, D. A., McGUIGAN, M. A. AND RIPLEY, B. D. (1988). Acute tricresylphosphate intoxication in childhood. Human Toxicol. 7: 179-182.

GORDON, T., JONES, R, AND VRBOVA, G. (1976). Changes in chemosensitivity of skeletal muscle as related to endplate formation. Prog. Neurobiol. 6: 103-136.

GRAMPP, W., HARRIS, J. B. AND THESLEFF, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221: 743-754.

GUPTA, R. C. AND DETTBARN, W. D. (1987). Alterations of high-energy phosphate compounds in skeletal muscles of rats intoxicated with diisopropylphosphorofluoridate (DFP) and soman. Fund. and Appl. Toxicol. 8: 400-407.

GUYTON, A. C. (1986). Contraction of skeletal muscle. In Textbook of Medical Physiology, pp. 120-135. W. B. Saunders, Philadelphia.

HANSEN, L. G. (1984). Biotransformation of organophosphorus compounds relative to delayed neurotoxicity. In Delayed Neurotoxicity. (J. M. Cranmer and E. J. Hixson, Eds.) pp. 72-86. Intox Press, Little Rock.

HARBORNE, A. J., SMITH, M. E. AND JONES, R. (1978). The effect of hydrolytic enzymes on the acetylcholine sensitivity of the skeletal muscle cell membrane. Pflugers Arch. 377: 147-153.

HARRIS, R. (1971). Chronaxy. In Electrodiadnosis and Electromyography (S. Licht, Ed.), pp. 218-239. Elizabeth Licht, New Haven.

HARTZELL, H. C. AND FAMBROUGH, D. (1972). Acetylcholine receptors: distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J. Gen. Physiol. 60: 248-262.

HAYNES, R. AND MURAD, F. (1985). Adrenocorticotrophic hormones, adrenocorticoid steroids and their synthetic analogs, inhibitors of adrenocortical steroid biosynthesis. In Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th ed. (A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad, Eds.), pp. 1459-1489. MacMillan Publishing, New York.

HECKMAN, J. R. (1972). Excitability curve: a new technique for assessing human peripheral nerve excitability in vivo. Neurology 22: 224-230.

HESS, J. W., MURDOCK, K. J. AND NATHO, G. J. W. (1968). Creatine phosphokinase: a spectrophotometric method with improved sensitivity. Am. J. Clin. Pathol. 50: 89-97.

HOYLE, G. (1983). Excitation-contraction coupling. In Muscles and Their Neural Control, pp. 193-225. John Wiley and Sons, New York.

HOFF, H. E. AND GEDDES, L. A. (1962). Properties of skeletal muscle. In Physiology Laboratory Manual, pp. 10-16. Baylor University, Texas.

HOFFMAN, P. N. (1986). The biology of neurofilaments in health and disease. Discussions Neurosci. 3: 28-32.

HUDECKI, M. S., POLLINA, C. M. AND HEFFNER, R. R. (1984). In vivo effects of three calcium blockers on chickens with inherited muscular dystrophy. Exp. Neurol. 84:

512-523.

IQBAL, Z. AND OCHS, S. (1980). Fast axoplasmic transport of calmodulin in mammalian nerve: possible involvement in axoplasmic transport. Ann. N. Y. Acad. Sci. : 389-390.

ISHIURA, S. (1981). Calcium-dependent proteolysis in living cells. Life Sci. 29: 1079-1087.

ISHIURA, S. (1981). Studies of a calcium activated neutral protease from chicken skeletal muscle. J. Biochem. 84: 225-230.

ITOH, H., KISHIDA, H., TADOKORO, M. AND OIKAWA, K. (1984). Studies on the delayed neurotoxicity of organophosphorus compounds (II). J. Toxicol. Sci. 9: 37-50.

ITOH, H., KISHIDA, H., TAKEUCHI, E., TADOKORO, M., UCHIKOSHI, T. AND OIKAWA, K. (1985). Studies on the delayed neurotoxicity of organophosphorus compounds (III). J. Toxicol. Sci. 10: 67-82.

JANSCO, G., KARSCU, S., KIRALY, E., SZEBENI, A., TOTIF, L., BACSY, E., JOO, F. AND PARDUCZ, A. (1984). Neurotoxin induced nerve cell degeneration: possible involvement of calcium. Brain Res. 295: 211-216.

JOHNSON, M. K. (1975). Organophosphorus esters causing delayed neurotoxic effects. Arch. Toxicol. 34: 259-288.

JOHNSON, M. K. (1980). Organophosphate neuropathy progress in understanding. In Advances in Neurotoxicology (L. Manzo, Ed.), pp. 223-235 Pergamon Press, New York.

JOHNSON, M. K. (1987). Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. Trends Pharmacol. Sci. 8: 174-179.

JOLESZ, F. AND SRETER, F. A. (1981). Development, innervation, and activity pattern induced changes in skeletal muscle. Ann. Rev. Physiol. 43: 531-552.

JONES, R. AND VRBOVA, G. (1974). Two factors for the development of denervation hypersensitivity. J. Physiol. 236: 517-538.

JORTNER, B. S. (1984a). Pathology of organophosphorus-induced delayed neurotoxicity. In Proceedings of the Fourteenth Conference on Environmental Toxicology (J. D. MacEwan and E. H. Vernot, Eds.), pp. 106-117. National Information Service, Springfield, Va.

JORTNER, B. S. (1984b). Selected aspects of the anatomy and response to injury of the chicken (Gallus domesticus) nervous system. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 38-49. Intox Press, Little Rock.

JORTNER, B. S. AND EHRICH, M. (1987). Neuropathological effects of phenyl saligenin phosphate in chickens. NeuroToxicology 8: 97-108.

JORTNER, B. S., SHELL, L., EL-FAWAL, H. AND EHRICH, M. (1988). Regeneration of peripheral nerve in organophosphorus ester-induced neuropathy. J. Neuropathol. Exp. Neurol. 47: 313.

JULIAN, R. J., GALT, D. E. AND BUTLER, D. (1975). Diagnosis of triorthocresyl phosphate poisoning in cattle. 18th Annual Proceedings, American Association of Veterinary Laboratory Diagnosticians: 407-418.

KALLO, J. R. AND STEINHARDT, R. A. (1983). The regulation of extrajunctional acetylcholine receptors in the denervated rat diaphragm muscle in culture. J. Physiol. 344: 433-452.

KAMAKURUA, K., ISHIURA, S., SUGITA, H. AND TOYOKURA, Y. (1982). Identification of Ca^{++}-activated neutral protease (CANP) in the rat peripheral nerve. Biomed. Res. 3: 91-94.

KAMAKURA, K., ISHIURA, S., SUGITA, H. AND TOYOKURA, Y. (1983). Identification of Ca^{++}-activated neutral protease in peripheral nerve and its effects on neurofilament degeneration. J. Neurochem. 40: 908-913.

KAMAKURA, K., ISHIURA, S., SUZUKI, K., SUGITA, H. AND TOYOKURA, Y. (1985). Calcium-activated neutral protease in peripheral nerve, which requires uM order Ca^{++}, and its effect on the neurofilament triplet. J. Neurosci. Res. 13: 391-403.

KAMAKURA, K., ISHIURA, S., FUJITA, T., NONAKA, I. AND SUGITA, H. (1985). Localization of calcium-activated neutral protease (CANP) in the peripheral nerve. Muscle and Nerve. 8: 380-382.

KANDEL, E. R. (1985a). Factors controlling transmitter release In Principles of Neural Science (E. R. Kandel and J. H. Schwartz, Eds.), pp. 120-131 Elsevier, New York.

KANDEL, E. R. (1985b). Synaptic formation, trophic interactions between neurons, and the development of behavior. In Principles of Neural Science (E. R. Kandel and J.

H. Schwartz, Eds.), pp. 743-756 Elsevier, New York.

KAWAMOTO, R. M. AND BASKIN, R. J. (1986). Isolation and characterization of sarcoplasmic reticulum from normal and dystrophic chicken. Muscle and Nerve 9: 248-256.

KELLY, F. J., McGRATH, J. A., GOLDSPINK, D. F. AND CULLEN, M. J. (1986). A morphological/biochemical study on the actions of corticosteroids on rat skeletal muscle. Muscle and Nerve 9: 1-10.

KOMULAINEN, H. AND BONDY, S. C. (1987). Increased free intrasynaptosomal Ca^{++} by neurotoxic organometals: distinctive mechanisms. Toxicol. Appl. Pharmacol. 88: 77-86.

KOMULAINEN, H. AND BONDY, S. C. (1988). Increased free intracellular Ca^{2+} by toxic agents: an index of potential neurotoxicity? Trends Pharmacol. Sci. 9: 154-156.

KONAGAYA, M., BERNARD, P. A. AND MAX, S. R. (1986). Blockade of glucocorticoid receptor binding and inhibition of dexamethasone-induced muscle atrophy in the rat by RU38486, a potent glucocorticoid antagonist. Endocrinol. 119: 375-380.

KRAIKITPANITCH, S., HAYGOOD, C. C., BAXTER, D. J., YUNIS, A. A. AND LINDEMAN, R. D. (1976). Effects of acetylsalicyclic acid, dipyridamole, and hydrocortisone on epinephrine-induced myocardial injury in dogs. Am. Heart J. 92: 615-622.

KRINKE, G., ULLMAN, L., SACHSSE, K. AND HESS, R. (1979). Differential susceptibility of peripheral nerves of the hen to triorthocresyl phosphate and to trauma. Agents and Actions 9: 227-231.

LAPADULA, D. M., KINNES, C. G., SOMJEN, G. G. AND ABOU-DONIA, M. B. (1982). Monosynaptic reflex depression in cats with organophosphorus neuropathy: effects of tri-o-cresyl phosphate. NeuroToxicology. 3: 51-62.

LASKOWSKI, M. B., OLSON, W. H. AND DETTBARN, W. D. (1975). Ultrastructural changes at the motor end plate produced by an irreversible cholinesterase inhibitor. Exp. Neurol. 47: 290-306.

LEONARD, J. P. AND SALPETER, M. M. (1979). Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. Cell Biol. 82: 811-819.

LINDSLEY, D. F. AND HOLMES, J. E. (1984). Basic Human Neurophysiology. pp. 128-129 Elsevier, New York.

LIDSKY, T. I., MANETTO, C. AND EHRICH, M. (1988). Electrophysiological studies in chickens given organophosphorus compounds and corticosterone. J. Toxicol, Environ. Hlth (in press).

LINKHART, T. A. AND WILSON, B. W. (1975). Appearance of acetylcholinesterase and creatine kinase in plasma of normal chickens after denervation. J. Neurol. Sci. 26: 193-201.

LOMO, T. AND ROSENTHAL, J. (1972). Control of ACh sensitivity by muscle activity in the rat. J. Physiol. 221: 493-513.

LONG, J. P. AND CHIOU, C. Y. (1970). Pharmacological testing methods for drugs acting on the peripheral nervous system. J. Pharmaceu. Sci. 59: 133-148.

LOPACHIN, R. M., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1988). Organophosphate intoxication alters distribution of elements in chicken periperal axons. Neuroscience Abstracts 14: 775.

LOTTI, M. (1987). Organophosphate-induced delayed polyneuropathy in humans: perspectives for biomonitoring. Trends Pharmacol. Sci. 8: 176-177.

LOWNDES, H. E., BAKER, T. AND RIKER, W. F. (1974). Motor nerve dysfunction in delayed DFP neuropathy. Eur. J. Pharmacol. 29: 66-73.

LLADOS, F. T. (1985). Neurogenic origin of an experimental myopathy induced by the ionophore A23187. J. Neurol. Sci. 69: 171-182.

LUCO, J. V. AND EYZAGUIRRE, C. (1955). Fibrillation and hypersensitivity to ACh in denervated muscle; effect of length of degenerating nerve fibers. J. Neurophysiol. 18: 65-73.

MANSOUR, N. A., EL-FEEL, E. A. AND SHERBY, S. M. (1986). Comparitive enzyme activities in hen neural tissues after treatment with organophosphates. The Toxicologist 6: 19.

MARSHALL, I. G. (1971). Actions of acetylcholine and carbachol on the chick biventer cervicis muscle. Br. J. Pharmacol. 42: 462-472.

MATA, M., STAPLE, J. AND FINK, D. J. (1986). Changes in intra-axonal calcium distribution following nerve crush. J. Neurobiol. 17: 449-467.

McARDLE, J. J. (1983). Molecular aspects of the trophic influence of nerve on muscle. Prog. Neurobiol. 21: 135-198.

McGILVERY, R. W. (1983). Use of high-energy phosphate: muscular contraction. In Biochemistry, A Functional Approach, pp. 370-389. W. B. Saunders, Philadelphia.

McLAUGHLIN, J., ABOOD, L. G. AND BOSMAN, H. B. (1974). Early elevation of glycosides, acid phosphatase, and acid proteolytic enzyme activity in denervated skeletal muscle. Exp. Neurol. 42: 541-554.

McMANAMAN, J. L., BLOSSER, J. C. AND APPEL, S. (1981). The effects of calcium on acetylcholine receptor synthesis. J. Neurosci. 1: 771-776.

MEDINACELLI, L. AND CHURCH, A. C. (1984). Peripheral nerve reconnection: inhibition of early degenerative processes through the us of a novel fluid medium. Exp. Neurol. 84: 396-408.

METAFORA, S., FELSANI, A., COTRUFO, R., TAJANA, G. F., DI IORIO, D., DEL RIO, A., DE PRISCO, P. P. AND ESPOSITO, V. (1980). Neural control of gene expression in the skeletal muscle fiber: the nature of the lesion in the muscular protein-synthesizing machinery following denervation. Proc. R. Soc. Lond. B. 209: 239-255.

METCALF, R. L. (1984). Historical perspective of organophosphorus ester-induced delayed neurotoxicity. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 7-22 Intox Press, Little Rock.

MEZEI, C., MEZEI, M. AND HAWKINS, A. (1974). 2′, 3′-cyclic AMP 3′-phosphohydrolase activity during Wallerian degeneration. J. Neurochem. 22: 457-458.

MILEDI, R. (1960). Junctional and extrajunctional acetylcholine receptors in skeletal muscle fibers. J. Physiol. 151: 24-30.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. J. Physiol. 151: 1-23.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. Science 235: 46-235.

MORETTO, A., LOTTI, M., SABRI, M. I. AND SPENCER, P. S. (1987). Progressive deficit of retrograde axonal transport is associated with the pathogenesis of di-n-butyl dichlorvos axonopathy. J. Neurochem. 49: 1515-1522.

NIXON, R. A., BROWN, B. A. AND MAROTTA, C. A. (1983). Limited proteolytic modification of a neurofilament protein involves a proteinase activated by endogenous levels of calcium. Brain Res. 275: 384-388.

OCHS, S. AND IQBAL, Z. (1982). The role of calcium in axoplasmic transport in nerve. In Calcium and Cell Function, vol 3. (W. Y. Cheung, Ed.), pp. 325-355 Academic Press, New York.

OCHS, S. (1987). The action of neurotoxins in relation to axoplasmic transport. NeuroToxicology 8: 155-166.

OLAJOS, E. J. (1987). Biochemical evaluation of organophosphorus-ester induced delayed neurotoxicity (OPIDN): the role of 2',3'-cyclic nucleotide 3'-phosphohydrolase. NeuroToxicology 8: 211-222.

OLAJOS, E. J., SHOPP, G. AND ROSENBLUM, I. (1982). Effect of acute organophosphorus exposure on 2', 3'- cyclic nucleotide 3'-phosphohydrolase activity in hen neural tissue. NeuroToxicology 3: 146-150.

OLIVER, I. T. (1955). A spectrophotometric method for the determination of creatine phosphokinase and myokinase. Biochem. J. 61: 116-122.

OTTO, D., UNSICKER, K. AND GROTHE, C. (1987). Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transectioned sciatic nerve on neuron death in adult rat dorsal root ganglion. Neurosci. Lett. 83: 156-160.

PATTON, S. E., LAPADULA, D. M., O'CALLAGHAN, J. P., MILLER, D. B. AND ABOU-DONIA, M. B. (1985). Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-o-cresyl phosphate to hens. J. Neurochem. 45: 1567-1577.

PATTON, S. E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986). Relationship of tri-o-cresyl phosphate-induced delayed neurotoxicity to enhancement of in vitro phosphorylation of hen brain and spinal cord proteins. J. Pharmacol. Exp. Therap. 239: 597-605.

PERDRIZET, J. A., CUMMINGS, J. E. AND LAHUNTA, A. (1985). Presumptive organophosphate-induced delayed neurotoxicity in a paralyzed bull. Cornell Vet. 75: 401-410.

PHILIPS, T. E. AND BOYNE, A. F. (1984). Liquid nitrogen-based quick freezing: experiences with bounce-free delivery of cholinergic nerve terminals to a metal surface. J. Electron Microsc. Tech. 1: 9-29.

PRICE, D. L. AND GRIFFIN, J.W. (1980). Neurons and ensheathing cells as targets of disease processes. In Experimental and Clinical Neurotoxicity (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 2-23. Williams and Wilkins, Baltimore.

PRINEAS, J. (1969). Triorthocresyl phosphate myopathy. Arch. Neurol. 21: 150-151.

RAFTERY, M. A. (1986). Structural and functional aspects of the nucotinic acetylcholine receptor. Discussions Neurosci. 3: 22-28.

RANDALL, V. L., LOPACHIN, R. M. AND SAUBERMANN, A. J. (1988). Distribution of elements in axotomized nerves determined by microprobe analysis. The Toxicologist 8: 246.

REDDY, M. K., ETLINGER, J. D., RABINOWITZ, M., FISCHMAN, D. A. AND ZAK, R. (1975). Removal of Z-lines and a-actinin from isolated myofibrils by a calcium-activated neutral protease. J. Biol. Chem. 250: 4278-4284.

RIBERA, A. B. AND NASTUK, W. L. (1988). Effects of chronic prednisolone treatment on postjunctional membrane responses to agonists. Muscle and Nerve 11: 265-269.

RICHARDSON, R. J. (1984). Neurotoxic esterase: normal and pathogenic roles. In Cellular and Molecular Neurotoxicology (T. Narahashi, Ed.), pp. 285-295 Raven Press, New York.

RIKER, W. F., BAKER, T. AND SASTRE, A. (1982). Electrophysiologic and clinical aspects of glucocorticoids on certain neural systems. In Current Topics in Neuroendocrinology (D. Ganten and D. Pfuff, Eds.), pp. 69-105. Springer-Verlag, New York.

RIOS, E. AND BRUM, G. (1987). Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature 325: 717-720.

RITCHIE, J. M. (1986). Ion channels in neural membranes. Discussions Neurosci. 4: 11-55.

ROBERTS, D. V. (1977). A longitudinal electromyographic study of six men occupationally exposed to organophosphorus compounds. Int. Arch. Occup. Environ. Hlth. 38: 221-229.

ROBERTS, N. L., FAIRLY, C. AND PHILLIPS, C. (1984). Screening, acute delayed and subchronic neurotoxicity studies in the hen: measurements and evaluation of clinical signs following administration of TOCP. In Delayed

Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 131-138. Intox Press, Little Rock.

ROBERTSON, D. G., SCHWAB, B. W., SILLS, R. D., RICHARDSON, R, J. AND ANDERSON, R. J. (1987). Electrophysiologic changes following treatment with organophosphorus-induced delayed neuropathy-producing agents in the adult hen. Toxicol. Appl. Pharmacol. 87: 420-429.

ROBERTSON, D. G., MATTSON, A. M., BESTERVELT, L. L., RICHARDSON, R. J. AND ANDERSON, R. J. (1988). Time course of electrophysiologic effects induced by di-n-butyl-2,2-dichlorovinyl phosphate (DBVC) in the adult hen. J. Toxicol. Enviorn. Hlth. 23: 283-294.

ROOMANS, G. M. (1988). Introduction to X-ray microanalysis in biology. J. Electron Microsc. Tech. 9: 3-18.

ROTUNDO, R. L. (1987). Biogenesis and regulation of acetylcholinesterase. In The Vertebrate Neuromuscular Junction (M. M. Salpeter, Ed.), pp. 247-284. Alan R. Liss, Inc., New York.

ROULEAU, G., KARPATI, G., CARPENTER, S., SOZA, M., PRESCOTT, S. AND HOLLAND, P. (1987). Glucocorticoid excess induces preferential depletion of myosin in denervated skeletal muscle fibers. Muscle and Nerve 10: 428-438.

ROWLAND, L. P. (1985). Diseases of the motor unit: the motor neuron, peripheral nerve, and muscle. In Principles of Neural SCience (E. R. Kandel and J. H. Schwartz, Eds.), pp. 196-208 Elsevier, New York.

RUBIN, L. L. (1985). Increases in muscle Ca^{2+} mediate changes in acetylcholinesterase and acetylcholine receptors caused by muscle contraction. Proc. Natl. Acad. Sci. 82: 7121-7125.

SALPETER, M. M. (1987). Development and neural control of the neuromuscular junction and the junctional acetylcholine receptor. In The Vertebrate Neuromuscular Junction (M. M. Salpeter, Ed.), pp. 55-115. Alan R, Liss, New York.

SANDERS, D. E., LAHUNTA, D. E., CUMMINGS, J. F. AND SANDERS, J. A. (1985). Progressive paresis in sheep due to delayed neurotoxicity of triaryl phosphates. Cornell Vet. 75: 493-504.

SAUBERMANN, A. J., ECHLIN, P., PETERS, P. D. AND BEEUWKES, R. (1981). Application of scanning electron microscopy to X-ray analysis of frozen-hydrated sections I. Specimen handling techniques. J. Cell Biol. 88: 257-267.

SAUBERMANN, A. J. AND HEYMAN, R. V. (1987). Quantitative digital X-ray imaging using frozen hydrated and frozen dried tissue sections. J. Microsc. 146: 169-182.

SCHANNE, F. A. X., KANE, A. B., YOUNG, E. E. AND FARBER, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science 206: 700-702.

SCHLAEPFER, W. W. (1971). Experimental alterations of neurofilaments and neurotubules by calcium and other ions. Exp. Cell Res. 67. 73-80.

SCHLAEPFER, W. W. (1977). Vesicular disruption of myelin simulated by exposure of nerve to calcium ionophore. Nature. 265: 734-736.

SCHLAEPFER, W. W. (1987). Neurofilaments: structure, metabolism and implication in disease. J. Neuropath. Exp. Neurol. 46: 117-129.

SCHLAEPFER, W. W. AND BUNGE, R. P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J. Cell. Biol. 59: 456-470.

SCHLAEPFER, W. W. AND HASLER, M. B. (1979). Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. Brain Res. 168: 299-309.

SCHLAEPFER, W. W. AND ZIMMERMAN, U.P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. In Axonal Transport in Neuronal Growth and Regeneration (J. S. Elam and P. Cancalon, Eds.), pp. 261-273 Plenum Press, New York.

SCHLAGER, M. K., PLEBAN, P. A. AND OLAJOS, E. J. (1985). The effect of tri-o-cresyl phosphate (TOCP) on 2', 3'-cyclic nucleotide 3'-phosphohydrolase activity in rat neural and non-neural tissue. The Toxicologist 5: 196.

SCHMID, A., KAZAZOGLOU, T., RENAUD, J. F. AND LAZDUNSKI, M. (1984). Comparitive changes of levels of nitrendipine Ca^{++} channels, of tetrodotoxin-sensitive Na^{+} channels and oubain-sensitive $(Na^{+}+K^{+})$-ATPase following denervation of rat and chick skeletal muscle. FEBS 172: 114-118.

SEIFERT, J. AND CASIDA, J. E. (1982). Possible role of microtubules and associated proteases in organophosphorus ester-induced delayed neurotoxicity. Biochem. Pharmacol. 31: 2065-2070.

SHELL, L., JORTNER, B. S. AND EHRICH, M. (1988). Assessment of organophosphorus-induced delayed neuropathy in

chickens using needle electromyography. Toxicol. Environ. Hlth. 24: 21-33.

SIMS, N. R. AND CARNEGIE, P. R. (1978). 2',3'-cyclic nucleotide 3'-phosphodiesterase. In Advances in Neurochemistry, Vol. 3 (B. W. Agranoff and M. H. Aprison, Eds.), pp. 1-41. Plenum Press, New York.

SMITH, K. J., HALL, S. M. AND SCHAUF, C. L. (1985). Vesicular demyelination induced by raised intracellular calcium. J. Neurol. Sci. 71: 19-37.

SOMKUTI, S. G., TILSON, H. A., BROWN, H. R., CAMPBELL, G. S., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1988). Lack of delayed neurotoxic effect after tri-o-cresyl phosphate treatment in male Fischer 344 rats: biochemical, neurobehavioral, and neuropathological studies. Fundam. Appl. Toxicol. 10: 199-205.

SOZA, M., KARPATI, G. AND CARPENTER, S. (1986). Calcium paradox in skeletal muscles: physiologic and morphologic observations. Muscle and Nerve 9: 222-232.

SPALLA, M., KUO, T. H. AND WIENER, J. (1987). Calcium-activated protease in hamster cardiomyopathy. Muscle and Nerve. 10: 54-59.

SPRAGUE, G. L., SANDVIK, L. L., BICKFORD, A. A. AND CASTLES, T. R. (1980). Evaluation of a sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. Life Sci. 27: 2523-2528.

SPRAGUE, G. L., BICKFORD, A. A. AND CASTLES, T. R. (1984). Neurohistological and clinical recovery in hens after administration of tri-o-tolyl phosphate. The Toxicologist 4: 26.

STEFANI, E. AND CHIARANDINI, D. J. (1982). Ionic channels in skeletal muscle. Ann. Rev. Physiol. 44: 357-372.

STUART, L. D. AND OEHME, F. W. (1982). Organophosphorus delayed neurotoxicity: a neuromyelopathy of animals and man. Vet. Hum. Toxicol. 24: 107-117.

SUWITA, E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986a). Calcium and calmodulin stimulated in vitro phosphorylation of rooster brain tubulin and MAP-2 following a single oral dose of tri-o-cresyl phosphate. Brain Res. 374: 199-203>

SUWITA, E., LAPADULA, D. M. AND ABOU-DONIA, M. B. (1986b). Calcium and calmodulin-enhanced in vitro phosphorylation of hen brain cold-stable microtubules and spinal cord neurofilament triplet proteins after a single oral

dose of tri-o-cresyl phosphate. Proc. Natl. Acad. Sci. 83: 6174-6178.

TANAKA, J. C. AND BARCHI, R. L. (1987). Voltage-dependent cation channels in skeletal muscle. In Sarcolemmal Biochemistry, Vol. 2 (A. M. Kidwai, Ed.), pp. 39-65. CRC Press, Florida.

TAYLOR, J. D. (1967). A neurotoxic syndrome produced in cats by a cyclic phosphate metabolite of tri-o-cresyl phosphate. Toxicol. Appl. Pharmacol. 11: 538-545.

TIPNIS, U. R. AND MALHOTRA, S. K. (1979). α-Bungarotoxin binding sites (acetylcholine receptors) in denervated mammalian sarcolemma. J. Supramolec. Struct. 12: 321-334.

TOTH, L., KARSCU, S., POBERAI, M. AND SAVAY, G. (1983). Histochemical evidence for the role of Ca^{2+} and neutral protease in the development of the subacute myopathy induced by organophosphorus compounds. Acta Histochem. 72: 71-75.

TOTH, L., KARSCU, S., POBERAI, M. AND SAVAY, G. (1981). A light and electron microscopic histochemical study on the mechanism of DFP-induced acute and subacute myopathy. J. Neuropathol. Appl. Neurobiol. 7: 399-410.

VASILESCU, C. AND FLORESCU, A. (1980). Clinical and electrophysiological study of neuropathy after organophosphorus compounds poisoning. Arch. Toxicol. 43: 305-315.

VASILESCU, C., ALEXIANU, M. AND DAN, A. (1984). Delayed neuropathy after organophosphorus insecticide (Dipterex) poisoning: a clinical, electrophysiological and nerve biopsy study. J. Neurol. Neurosurg. Neuropsych. 47: 543-548.

VERONESI, B. (1984). A rodent model of organophosphorus-induced delayed neuropathy: distribution of central (spinal cord) and peripheral nerve damage. Neuropathol. Appl. Neurobiol. 10: 357-368.

VERONESI, B. AND PADILLA, S. (1985). Phenylmethylsulfonyl fluoride protects rats from mipafox-induced delayed neuropathy. Toxicol. Appl. Pharmacol. 81: 258-264.

WATSON, D. F. AND GRIFFIN, J. W. (1987). Vacor neuropathy: ultrastructural and axonal transport studies. J. Neuropathol. Expt. Neurol. 46: 96-108.

WAXMAN, L. (1981). Calcium-activated proteases in mammalian tissue. Methods Enzymol. 80: 664-680.

WECKER, L., KIAUTA, T. AND DETTBARN, W. D. (1978). Relationship between acetylcholinesterase inhibition and the development of a myopathy. J. Pharmacol. Exp. Therap. 206: 97-104.

WECKER, L., LASKOWSKI, M. B. AND DETTBARN, W. D. (1978). Neuromuscular dysfunction induced by acetylcholinesterase inhibition. Fed. Proc. 37: 2818-2822.

WILSON, B. W., ISHIKAWA, Y., CHOW, E. AND CISSON, C. M. (1984). Multilevel studies of organophosphate toxicity. In Delayed Neurotoxicity (J. M. Cranmer and E. J. Hixson, Eds.), pp. 253-265 Intox Press, Little Rock.

WROGEMAN, K. AND PENA, S. D. J. (1976). Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle disease. Lancet 7961: 672-673.

WYNN PARRY, C. B. (1971). Strength-duration curves. In Electrodiagnosis and Electromyography (S. Licht, Ed.), pp. 241-271. Elizabeth Licht, New Haven.

ZECH, R. AND CHEMNITIUS, J. M. (1987). Neurotoxicant sensitive esterase. Enzymology and pathophysiology of organophosphorus ester-induced delayed neuropathy. Prog. Neurobiol. 29: 193-218.

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