I. Introduction.

This research was initiated to improve the knowledge about organophosphorus-induced delayed neuropathy by evaluating the effects of protection and promotion on the delayed polyneuropathy. Techniques such as perfusion fixation, plastic embedding, light microscopy and ultrastructural examination have been used in the past to study nervous system lesions, including the characteristic lesions of organophosphorus induced delayed neuropathy (OPIDN) (Jortner and Ehrich, 1987; Jortner et al., 1989; Dyer et al., 1991; El-Fawal et al., 1988; El-Fawal et al., 1990; Shell et al., 1988; Ehrich et al., 1993). Axonal and myelinated fiber degeneration has previously been seen in peripheral nerve and spinal cord tracts of animals with OPIDN (Jortner and Ehrich, 1987; Dyer et al., 1991; Krinke et al., 1979; Pope et al., 1992). However, specific data relating to the lesions observed in promoted OPIDN in peripheral nerves of adult animals, and their comparison to the characteristic lesions of OPIDN, were not reported at the time this study was begun. The hypotheses tested are that axonal degeneration of promoted OPIDN is more profound but qualitatively similar compared to such changes in organophosphate induced delayed neuropathy alone, and that the severity of clinical signs observed directly corresponds to the degree of morphologic changes.

Experimental protocols used in this study attempted to elucidate the still unknown mechanisms involved in the development of OPIDN. OPIDN is known to follow inhibition of neurotoxic esterase (NTE), also called neuropathy target esterase, by phosphorylation of its active site (Ehrich, 1996; Johnson, 1975a,b, 1982). Although many organophosphorus compounds (OPs) also inhibit acetylcholinesterase (AChE), inhibition of this esterase is unrelated to the appearance of OPIDN. This study was designed to

demonstrate the changes observed in hens with promotion of OPIDN by using enzymatic profiles, clinical signs and neuropathological methods of evaluation.

II. Literature review

A. Axonopathy and neurons.

1. Synthetic activities of perikaryal and dendritic structures.

The fundamental structure of a neuron can be divided into a soma (perinuclear perikaryon and nucleus), a dentritic zone, and an axon. The perikaryon may vary in shape, and can be round, pyramidal or polyhedral, and contains a central ovoid nucleus with a prominent nucleus (Summers et al., 1995). A series of arborized perikaryal processes called the dentritic zone is defined as the receptive portion of the neuron, where an internal or external stimuli is converted to an electrical impulse in the neuron. These electrical stimuli are transported centrifugally along the axon. The latter is the cell process that courses from the axon hillock, near the cell body, to the pre-synaptic terminal of a neuron. Neuronal perikarya are rich in ribosomes, which can be arranged along stacks of membrane in parallel array forming Nissl bodies. Basophilic Nissl bodies are found in the cytoplasm of all neurons except in very small ones. The neuron has no Nissl bodies in the region of the axon hillock and in the axon. Transmission electron microscopy reveals that the perikaryal cytoplasm contains neuroflilaments, microtubules, a few lysosomes, and a rich complement of rough endoplasmic reticulum and polyribosomes, Golgi and mitochondria. (Summers et al., 1995).

Information controlling the synthesis of proteins is encoded in the DNA of the chromosomes within the cell's nucleus (Kandel et al., 1991). In the mature neurons, cell division is no longer possible, and the chromosomes are responsible only for gene expression. The nucleolus contains the specific portion of DNA encoding the RNA (rRNA) for future ribosomal synthesis. In addition to the ribosomal genes of the nucleolus, many other genes are also actively transcribed into the nuclear precursors of mRNA. The resulting mRNA gives rise to three classes of proteins: (1) cytosolic proteins, (2) nuclear, mitochondral and peroxisomal proteins, (3) cell membrane and secretory proteins.

The cytosolic proteins constitute the most abundant group of proteins in the neuronal cell. Cytosolic proteins include: (1) fibrillar components that make up the cytoskeleton (neurofilaments, tubulins, actins, and their associated proteins), (2) the enzymes that catalyze the metabolic reactions of the cell (e.g. proteases, choline acetyltransferase in spinal motor neurons). Messenger RNA molecules for cytosolic proteins emerge through the nuclear pores, and become associated with ribosomes, forming free polyribosomes in the cytoplasm of neurons. The cytosolic proteins can be slightly modified by cotranslational processes (changes during polypeptide synthesis as N-acetylation), or post translational processes (modifications after the polypeptide chain has been completed, such as phosphorylation of serine, threonine, or thyrosine by specific protein kinases) (Kandel et al., 1991).

The nuclear, mitochondrial and peroxisomal proteins are encoded and transcribed in the cell's nucleus and translated on free polyribosomes. They are directed to their proper organelles by post translational importation soon after their synthesis has been completed on free ribosomes (Kandel et al., 1991). In contrast, most membrane and secretory proteins reach their destination by cotranslational transfer (see below). Furthermore, the

distribution of these proteins to the various membrane compartments of the cell depends on amino acid sequences located at the N-terminal end of the protein. Nuclear re-uptake also depends on the sequence of basic amino acid residues that remain on mature proteins.

The cell membrane proteins and secretory proteins are formed on polysomes attached to the endoplasmic reticulum (rough endoplasmic reticulum) in the cytoplasm. Signal sequences of proteins (N-terminal portion), destinated for the cell's major membrane system, differ in their secondary structure from the presequences of proteins targeted to the nucleus, mitochondria, or peroxisomes (Kandel et al., 1991). The peptides can be transported through the lipid bilayer into the lumen of the endoplasmic reticulum by energetic mechanisms and proteolytic cleavage catalyzed by signal peptidase enzymes. Transportation via cotranslational transfer through the membrane continues until a "stop" transfer segment is reached within the polypeptide chain. Axonal transport mechanisms control the distribution of mature proteins produced by cotranslational transfer (membrane and secretory proteins). Such axonal transportation mechanisms are described in subsequent sections.

2. Axonal structures.

The components of an axon can be divided into the axolemma, the axoplasm, and axonoplasmic elements (Waxman, 1978; Peters et al., 1991). The axolemma is the excitable membrane of the nerve fibers. The axoplasm constitutes the fluid compartment, in which bidirectional axonoplasmic flows take place at several velocities (described in the subsequent section). The formed axonoplasmic elements include the mitochondria, smooth endoplasmic reticulum, neurotubules, axonal filaments, miscellaneous membrane-bond bodies, ribosomes and axonal inclusions (glycogen).

In the axon hillock, organelles predominating in the axonoplasm include free ribosomes, mitochondria, neurofilaments and microtubules. Ribosomes are generally absent from the remainder of the axon (Peters et al., 1991). The axon beyond the initial segment contains mitochondria, microtubules, neurofilaments, agranular endoplasmic reticulum, and multivesicular bodies, but is devoid of granular endoplasmic reticulum and free ribosomes. The mitochondria are axonoplasmic elements several microns in length. They are continuously generated in the perikaryon and enter the axon, where they are propagated distally. Axonal mitochondria have been observed in association with microtubules via microtubule-associated proteins (MAP 1), and the latter are responsible for energy coupling between microtubules, mitochondria and other membranous organelles (Peters et al., 1991). Neurofilaments are macromolecular filaments of indefinite length and approximately 10 nm in diameter (Peters et al., 1991). They are responsible for the stabilization of the axon and ensure its radial growth (Fried et al., 1970; Lee et al., 1994). Vertebrate neurofilaments are made up of three protein subunits of approximately 68, 150 and 200 KD (Hoffman et al., 1975; Liem et al., 1978). Phosphorylation of these neurofilaments by protein kinase is crucial for the maintenance of cytoskeletal structure (Nixon et al., 1991). Microtubules consist of tubular structures larger than the neurofilaments. In transverse sections, microtubules measure approximately 20-26 nm in diameter (Peters et al., 1991). They are essentially tubes of a globular polypeptide known as tubulin. Tubulin is found as a dimer of approximately 100 KD, in which each dimer contains two globular polypeptides of about 50 KD, alpha and beta-tubulin. As tubulin is assembled into microtubules, dimers line-up to form 13 protofilaments arranged parallel to each other, forming the wall of the microtubule. The density of microtubules in axons increases as the diameters of the axon decreases. There are approximately 150 microtubules/ μm^2 in unmyelinated axons and less than 15 microtubules/ μm^2 in large myelinated ones (Fadic et al., 1985; Pannese et al., 1984). The function of microtubules

is to maintain the shape of anisotropic cells. In addition, they are partly responsible for axonal transportation and axoplasmic flow (Stephens et al., 1976; Kandel et al., 1991), especially that associated with synaptic vesicles (Gray, 1975, Kandel et al., 1991). Microtubules also participate in subaxolemmal arrangements at the initial axon segment (Westrum, 1976), and are implicated in cell motility and cell division (Peters et al., 1991).

The membranous components of the axoplasm consist of the endoplasmic reticulum, mitochondria, vesicles (multivesicular bodies) and lysosomes. The smooth endoplasmic reticulum (SER) of the axon extends continuously from the axon hillock the axon terminal. In the presynaptic terminals, a primary SER system runs just below the plasma membrane, and extends into the terminal (towards the presynaptic grids). The second SER system occupies the core of the axon and breaks up into spherical synaptic vesicles. SER is implicated in bidirectional fast transport of proteins, glycoproteins, neurotransmitters, mucopolysaccharides, gangliosides and phospholipids, all needed to maintain the axonal and nerve terminals (Droz, 1976; Goodrum et al., 1991).

3. Mechanisms of axonal transportation.

The long axons spanning the large separation between the cell body and the nerve terminal require specialized axonal transportation systems. Axonal transport controls the distribution of membrane and secretory protein in the neuron, and is characterized by fast anterograde, fast retrograde, and slow axoplasmic systems (Kandel et al., 1991). Five discrete rates of anterograde transport and one of retrograde transport have been recognized.

Rapid movement of material (rate between 200-400 mm/day) down the axon is defined as fast axonal anterograde transport (Goodrum et al., 1991). This transport mechanism is thought to involve several cytologic structures including vesicles and agranular (smooth) endoplasmic reticulum. Proteins, glycoproteins, phospholipids, glycolipids, neurotransmitters and associated enzymes, and several other organelles are moved down the axon by fast anterograde transport. The latter provides the proteins and lipids essential to maintain the axonal and nerve terminal membranes. The motor molecule for anterograde movement is thought to be kinesin, an ATPase consisting of two large subunits (alpha), and two small subunits (beta). Kinesin forms cross-bridges between the moving membranous organelles. The fast anterograde transport system depends on oxidative metabolism, and is independent of the cell body (Kandel et al., 1991).

Movement of material in axons at a rate less than 5 mm/day in mammals is considered "slow" axonal transport (Goodrum et al., 1991). Slow transportation is associated with 2 protein groups, termed slow components a and b, which move at different rates and have different compositions. Slow component a (rate between 0.20-1.0 mm/day) essentially contains tubulin, microtubule associated proteins, and neurofilament triplet proteins. Slow component b (rate between 2-5 mm/day) is associated with many proteins including actin and a number of soluble enzymes. Transport mechanisms involve microtubules and neurofilaments for group a proteins or microfilaments and axoplasmic matrix for group b proteins. As an example, the cytosol (cytoskeletal elements and soluble proteins) is transported down the axon by slow axonoplasmic flow. Neurofilaments and microtubules, fibrillar elements of the cytoskeleton, are transported via the slow component a of the slow axonal transport. Calmodulin, neural myosin protein, and clathrin are transported via the faster component b of the slow axoplasmic system (Kandel et al., 1991).

In addition to fast and slow anterograde axonal transport, two other intermediate rates have also been identified (10 mm/day and 100 mm/day) (Goodrum et al., 1991). The faster of these (100 mm/day) is associated with mitochondrial fractions. The slower rate (10 mm/day) is distributed throughout several subcellular fractions and one of its components has been partially characterized as myosin-like actin binding proteins (Williard, 1977). However, most of the proteins involved in these two intermediate transport mechanisms remain unidentified.

Fast retrograde transport is responsible for the return of particles from axonal terminals to the cell body, where they are either degraded or recycled. The motor molecule for fast retrograde transport is a dynein, which is also a microtubule associated ATPase (Goodrum et al., 1991; Hammerschlag et al., 1994). Extracellular components can be transported via this route after endocytosis. Some of these materials, destinated to return to the cell body, are concentrated in large membrane bound organelles that are part of the lysosomal system. Retrograde transport has a role in "informing" the cell body (the site of macromolecular synthesis) about injuries or neuronal changes occurring at more distal levels of the axon.

4. Physiology of the neuron.

The neuron generates membrane axon potentials, in order to carry a message, as an electrical signal along the axon. An appropriate ionic intra and extracellular environment around the cell membrane is essential for the neuron to execute its function. At rest, a driving force exists for moving ions across the membrane. This driving force is defined as the resting membrane potential. The resting membrane potential is present because intra and extracellular ionic concentrations differ on both sides of the membrane, with high

concentrations of potassium ions and negatively charged protein molecules inside, and sodium, chloride, and ions calcium outside the neuron. At rest, the voltage potential is more negative inside the neuron (Vp=-65 mV). In general, the value of resting potential is closer to the equilibrium potential for which the membrane is more permeable (Epot).

Two types of electrical signals exist in neurons. Post-synaptic potentials are observed in cell bodies and axons. They are known as excitatory post-synaptic potentials (EPSP) and inhibitory post-synaptic potentials (IPSP). EPSP and IPSP are additive and the values are determined by the size of input from other neurons. If the spatiotemporal summation of EPSP exceed IPSP, threshold can be reached, followed by depolarization, known as the action potential, along the axon. The action potential is regenerative, and its size is independent of the input. The PSP's are not regenerative and decay over certain distances and time, and are controlled by the release of neurotransmitters at the pre-synaptic terminals. Post-synaptic chemical-gated channels open as the concentration of neurotransmitters increase and bind to these channels. The chemical-gated channels activated by neurotransmitters are not gated by voltage, and are permeable to both sodium and potassium. Since the surface area of dendrites is greater than the cell body, these processes receive most of the input from the pre-synaptic terminal of other neurons.

The action potential is triggered at the axon hillock, where a high density of voltage-gated channels are encountered. The channels that contribute to the action potential are voltage-gated, and selective for different cations. As PSP's are added, sodium channels open first, allowing sodium ions to enter the axon, and consequently, its interior becomes more positive (depolarization if Vm=52 mV). Then potassium-gated channels open, with release of potassium outside the nerve cell, causing repolarization/hyperpolarization. The molecular structure of the sodium and potassium channels differ, accounting for the

variation of ion permeability, eventually being responsible for the temporal differences in voltage and ionic movement across the membrane. This permits the axon potential to be propagated along the axon, in the direction opposite to the hyperpolarization.

Increased conduction velocities can be achieved by larger diameter axons, especially myelinated ones. Schwann cells are present around both myelinated and non-myelinated peripheral nerve and oligodendrocyte processes are seen around myelinated brain and spinal cord axons. These cells are responsible for the synthesis of the myelin sheath, which consists of 75% lipids (phospholipids, glycoproteins, and cholesterol), and 25% proteins. Ultrastructure of myelin shows that it consists of compact multiple lamella (~12 nm wide) surrounding the axon concentrically and segmentally as series of internodes (segments). The node of Ranvier marks the gaps between adjoining Schwann cells or oligodendrocyte processes; it is deprived of myelin. In myelinated axons, the current goes from one node of Ranvier to the next (saltatory potential), decreasing the time constant and increasing the length constant of the impulse.

As the action potential reaches the pre-synaptic terminals, calcium-gated voltage channels open in the pre-synaptic terminal. As more calcium enters the cell, more neurotransmitters are released in the synaptic cleft. The neurotransmitters free in the synaptic cleft generate PSP's on the post-synaptic membrane, after binding with chemical-gated channels, as noted above. The effect observed will depends greatly upon the nature of neurotransmitters released.

5. Axonal injuries and Wallerian degeneration.

Since ribosomes and other organelles essential in protein synthesis are absent from the axons, the integrity of these neurites and their terminals depends on the neuronal cell body for the supply of all structural elements. Thus, the neuronal cell body must synthesize and deliver the needs of the axon, and recycle extraneous products. Wallerian degeneration is a process characterized by fragmentation and dissolution of the distal part of an axon, with digestion and removal of the collapsed myelin tube, secondary to nerve fiber transection or injury (figure 1). In Wallerian degeneration, dissolution of the distal part of the axon is an active process that follows the loss of contact with the perikaryon. In the peripheral nerve, within the first 3-4 days after the fiber injury, the axon swells, initially occurring near one or more nodes of Ranvier in the distal part of the axon. Proximally, this paranodal swelling moves up towards the cell body in a saltatory manner. The associated myelin sheath slowly retracts away from the nodal region as the axon enlarges. The myelin degenerates simultaneously with, but secondary to the axon. The fiber degeneration leads to formation of swellings along the internodes called ellipsoids, and fragmentation of the myelin sheath into chains of myelin ovoids that are slowly removed by phagocytic cells. There also is degeneration over a few internodes proximal to the injury, similar to the changes distal to the site of injury. Furthermore, a reaction is observed in the cell body following axonal injury that is termed the axon reaction. This is characterized by swelling of the cell body, displacement of the nucleus toward the periphery of the soma, and partial dispersion of Nissl substances in the region around the nucleus. This reaction is also referred to as chromatolysis (De Lahunta, 1983). Following the distal axonal disintegration, Schwann cells proliferate within their basal lamina endoneurial covering, and fill the space previously occupied by the axon and myelin forming the band of Buengner. This structure produces a microenvironment which enhances subsequent axonal regeneration.

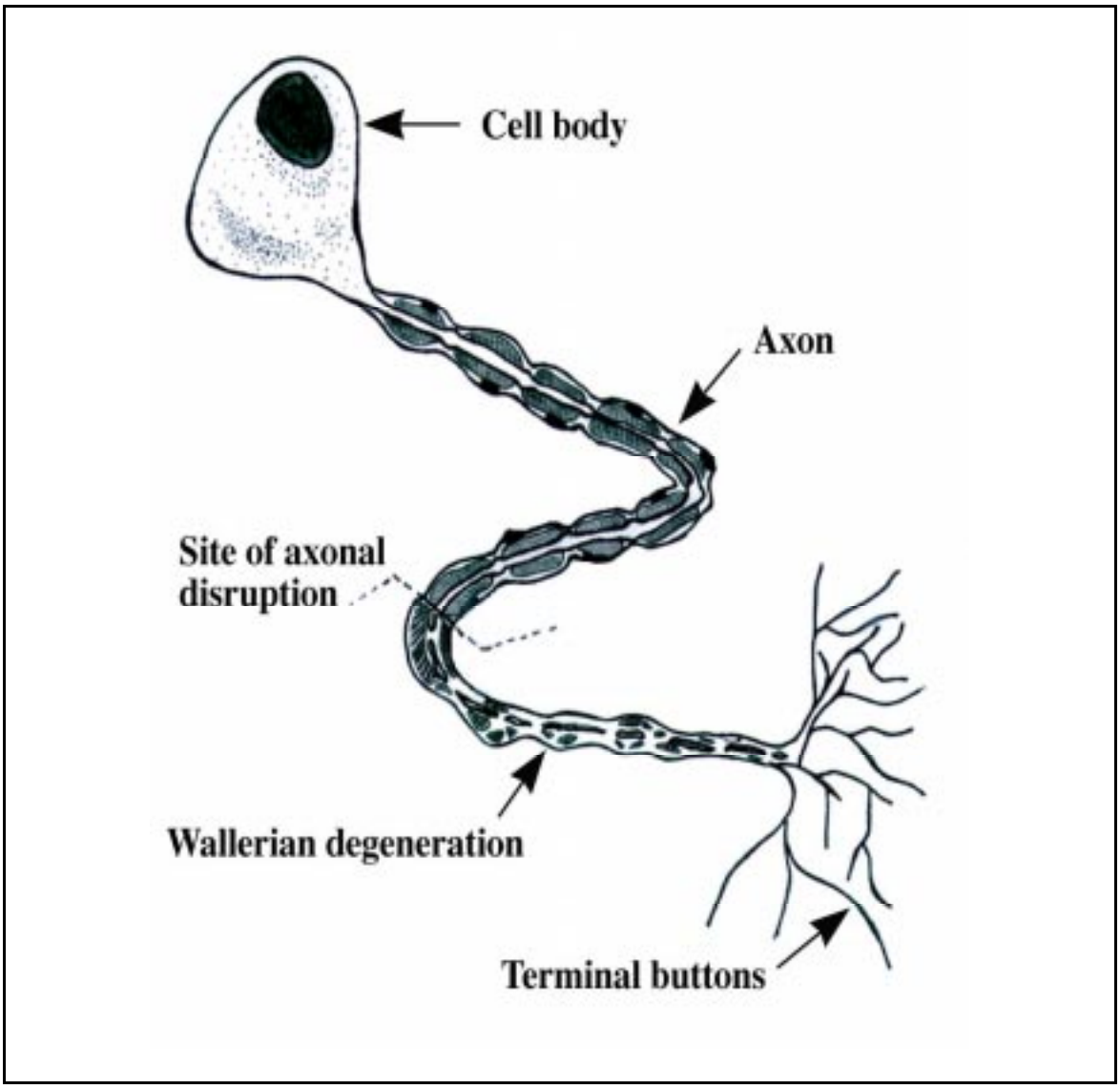


Figure 1: Wallerian degeneration of a neuron showing fragmentation and dissolution of the distal part of the axon, caudal to the site of axonal injury (dotted line).

6. Toxic axonal injuries.

The neuron is susceptible to many insults, including ischemia, hypoxia, infections, intoxications and nutritional deficiencies. A few examples of lesions due to toxic neuronal injury include neurofilamentous aggregates (caused by hexacarbon), and Wallerian-like degeneration (OPIDN, BPAU). Distal axonopathies can result from damage to a cell body, so it is unable to support its axon, or failure of the axonal transport system, with subsequent accumulation of debris at the terminus (Bianchi et al., 1986; Moretto et al., 1987). With some toxic axonal injuries (including OPIDN), the distal (not necessarily terminal) part of the axon degenerates, and the degeneration extends both in the anterograde direction and back up toward the neuronal cell body (Bouldin and Cavanagh, 1979). Within the first 3-4 days after the nerve injury, swelling of the axon with subsequent disintegration can occur. The initial morphological changes occur near one or more Ranvier nodes in the distal part of the axon.

Toxicants can induce disorders of axonal transport, due to local axonal "injury", or major alterations to neuron metabolism. One hypothetical reason for axonal transport disruption is the presence of an interaction of the toxicant or its metabolites, with some steps of the axonal transport metabolic mechanism. A second possible cause is a direct effect upon the movement of some specific transported component. In addition, toxicant mediated defects in neuronal energy metabolism could also alter transport mechanisms (Goodrum et al., 1991).

A variety of axonal toxic injuries lead to focal distention of these neurites known as spheroids (Garman et al., 1990; Wells et al., 1989). They are observed in a variety of diseases, and their distribution, as well as their ultrastructural composition, differ

depending upon the cause of injury. For an example, neuroaxonal dystrophies (NAD) are a group of inherited or acquired neurodegenerative disorders of humans and animals characterized by disseminated axonal swellings (spheroids) in preterminal portions of axons and in synaptic terminals. Ultrastructurally, the swollen axons contain a mixture of neurofilaments, tubulovesicular structures, mitochondria, lysosomes, and membranous bodies. These spheroids may be found in myelinated or unmyelinated fibers (Walkley et al., 1981; Fenoglio-Preiser, 1987), and are considered the hallmark of neuroaxonal dystrophy.

The Schwann cells play an important role in sequestering and removing degenerated axoplasmic organelles which accumulate in spheroids in peripheral nerve fibers. The macrophages participate in the process of demyelination as well, phagocytosing vesiculated and degenerated myelin (Griffin et al., 1993). The most common axonal organelles to be sequestered by the Schwann cells are vesicles, mitochondria and dense membranous bodies (Dahme et al., 1976; Goodrum et al., 1996; Griffin et al., 1993). It is believed that a positive signal originating from degenerating axons induces myelin sheath breakdown. The resulting ellipsoids, referred to as digestion chambers, contain axon fragments, with ovoids of degenerated myelin. Axonal degeneration reduces the production of myelin, and therefore, Schwann cells undergo mitotic proliferation, and macrophage-processed myelin basic protein might contribute to the Schwann cell proliferation seen in Wallerian degeneration (Griffin et al., 1993). This proliferation of Schwann cells forms longitudinal cords called Buengner's bands is similar to those seen in Wallerian degeneration (Anderson et al., 1989). After the axonal reaction is initiated, peripheral nerve axonal regeneration can take place from the proximal stump, where normal axonal structures are preserved. Regenerating axons grow into the distal cords of proliferated Schwann cells (Buengner's band), which provides a directed growth pathway. If the Buengner bands are absent or access to them is blocked, regenerating axons grow in randomly, and ineffectively.

B. Organophosphorus compounds.

1. General considerations.

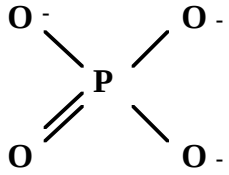
Organophosphorus ester (OP) compounds have been widely used in industry and agriculture. The extent of their use, mainly as synthetic pesticides, but also in plasticizers, hydraulic fluids, lubricants, and petroleum additives reflects utilitarian features of these compounds. Potentially, over 20 million toxic organophosphorus esters are possible, using Schrader's (1963) generalized formula. It has been estimated that over 200 such toxic compounds, out of 500,000 synthesized organophosphorus pesticides, have been used commercially throughout the world (Spencer, 1973). Two major types of OP toxicities have been recognized. The first type of toxicity is related to the inhibition of acetylcholinesterase, resulting in cholinergic crisis. Cholinergic crisis related to OP compounds has been well described in many species (Chambers and Levi, 1992; Abou-Donia and Lapadula, 1990). One other problem associated with exposure to certain organophosphorus compounds is the potential for the development of delayed neuropathy. OP toxicities have been reported in different species, including humans, domestic and wild animals (Johnson, 1975a,b; Abou-Donia and Lapadula, 1990; Abou-Donia et al., 1986; Veronesi et al., 1984; Lapadula et al., 1985; Soliman et al., 1984; Stumpf, 1989; Soliman et al., 1983; Smith et al., 1959; Abou-Donia, 1981; Ehrich, 1996). Of these species, the domestic chicken (hens) has proven to be the most reliable animal model to study the delayed effects of organophosphate intoxications, since it readily shows clinical signs and lesions. Thus, the delayed neuropathic potential of OP compounds are usually extrapolated from studies in this species (Dudek and Richardson, 1979; Dudek et al, 1980; Johnson et al., 1981; Johnson, 1982; Lotti et al., 1978; Lotti et al., 1981).

2. Chemical structure.

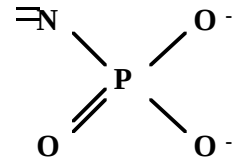
Organophosphorus (OP) compounds are a large and diverse family of chemicals. They are organic compounds containing phosphorus-carbon (P-C) bonds. The phosphorus can be trivalent or pentavalent, depending if three or five outer orbital electrons are present, respectively. The trivalent phosphorus configuration is pyramidal and such compounds are considered to be derivatives of phosphines, PH3. The pentavalent phosphorus compounds are tetrahedral (phosphine oxide, phosphinic acid, phosphonic acid). The pyramidal conformation is unstable, and thus undergoes tautomeric changes to a tetrahedral conformation (Abou-Donia and Lapadula, 1990).

OP compounds may be classified as anhydrides, aliphatics, aromatics, and heterocyclics. The majority of OP insecticides are derivatives of phosphoric acids. The partial structures of these 14 types of OP insecticides known are shown in figure 2 (Chambers and Levi, 1992). Triesters of phosphoric acids (phosphates) are considered the prototype of the entire family of OP compounds, in which all 4 atoms surrounding the phosphorus are oxygen. Sulfur-containing OP compounds also exist. Phosphorothionates (parathion, diazinon, chlorpyrifos) and phosphorothioates contain a P=S bond. The phosphorothionothiolates (e.g. phosphorodithioates) constitute a subclass, where one S atom is as P=S, and the other as a thioester. Thioesters can be linked to an alkyl subtituent, or the leaving group can be attached via S atom (e.g., malathion, dimethoate). Phosphoramides constitute another class of OP compounds, where amides are formed instead of esters. Of the seven phosphoramides used as insecticides today, three are phosphoramidates (fenamiphos, phospholan and mephospholan), two are phosphoramidothionates (propetamphos and isofenphos) and two are phosphoramidothiolates (metamidophos, acephate) (Chambers and Levi, 1992). OP

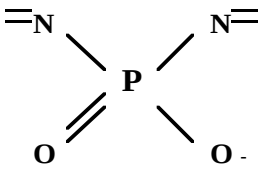
compounds that have one substituent attached by a phosphorus-carbon bond are classified as phosphonates (e.g., trichlorfon), phosphonothionothiolates (fonophos) and phosphonothionates. Phosphinothionates and phosphinates have two P-C bonds.



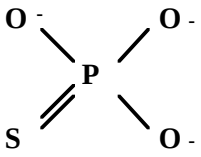
Phosphates



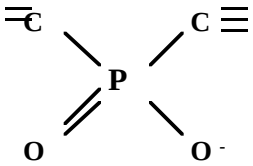
Phosphoramides
Phosphoramidates



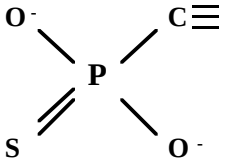
Phosphorodiamidates



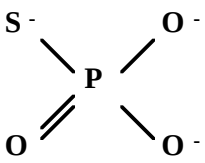
Phosphorothioates
Phosphorothionates



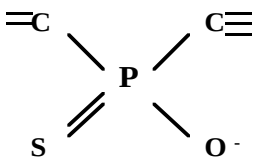
Phosphinates



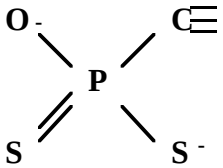
Phosphonothionates



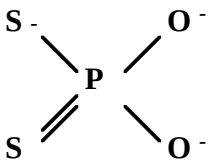
Phosphorothiolates



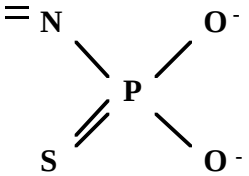
Phosphinothionates



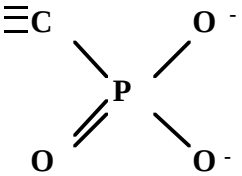
Phosphonothionotiolates



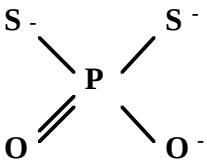
Phosphorodithioates
Phosphorothionothiolates



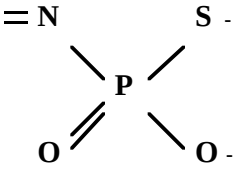
Phosphoramidothionates



Phosphonates



Phosphorothiolates



Phosphoramidothiolates

Figure 2: Structural classification of organophosphorus compounds (Chambers H, 1992).

3. Chemical classification related to toxicological effects of OP compounds.

An important deleterious effect of OP compounds is acute toxicity, related to their ability to inhibit acetylcholinesterase (AChE) by phosphorylation. The potency of OP compounds as anti-AChE agents depends on the resulting degree of AChE phosphorylation. As a consequence, exposure to these OP compounds leads to accumulation of ACh in the cholinergic nerve synapses and neuromuscular junctions. The latter results in junctional hyperexcitabilily, and multiple post synaptic impulses generated by a single presynaptic stimulus in both somatic and autonomic nervous systems. The clinical signs of acute neurotoxicity of OP compounds are described in a subsequent section. A variety of different type of OP classes have the potential to produce acute neurotoxicity (phosphates, phosphorothionates, phosphorothiolates, phosphorothiono-thiolates, phosphorodithiolates, phosphorodithioates, phosphoroamidates, phosphonates, phosphorodiamidates, phosphinates, phosphinothionates, phosphonothionothiolates, phosphoroamidothiolates, phosphoroamidithionates) (Chambers and Levi, 1992). Specific organophosphorus compounds capable of inhibiting acetylcholinesterase and producing acute neurotoxicity include: diisopropyl-fluoro-phosphate (DFP), dichlorvos, malathion, parathion, paraoxon (Abou-Donia and Lapadula, 1990; Hern, 1971; Johnson, 1975a,b; Koelle et al., 1946; Majno et al., 1961; Smith et al., 1930; Smith et al., 1959).

Some OP compounds can induce OPIDN, a toxic effect that is not related to AChE inhibition. These OP compounds are known to inhibit another enzyme called neurotoxic esterase (NTE). Inhibition of NTE is a prerequisite to OPIDN (Johnson, 1974). The mechanism of NTE inhibition is discussed below. Inhibitors of NTE can be divided in two categories depending upon their biological effects. The first group consists of phosphates,

phosphonate, and phosphoramidates, compounds which essentially irreversibly inhibit NTE, and induce delayed neuropathy (OPIDN). Carbamate, sulfonyl fluorides, and phosphinates reversibly inhibit NTE, and do not cause delayed neuropathy (Fig. 2). Among specific organophosphorus products capable of producing delayed neuropathy after NTE inhibition include: tri-ortho-tolyl phosphate (TOTP, also known as tri-o-cresyl phosphate, TOCP), cyclic saligenin phosphates such as phenyl saligenin phosphate (PSP), and tolyl saligenin phosphates (TSP), diisopropyl-fluorophosphate (DFP), mipafox, and methamidophos.

Delayed neuropathy induced by OP compounds has been divided into 2 categories; type I and type II (Abou-Donia and Lapadula, 1990). The major difference between type I and type II OPIDN - inducing compounds is that type I compounds have a pentavalent phosphorus atom, whereas type II compounds have a trivalent phosphorus atom. Type I compounds include derivatives of phosphoric, phosphonic and phosphoramide acids, and phosphorofluoridates, with their sulfur analogs. The type II category compounds consist of phosphorus acid derivatives (triphosphites) and their sulfur analogs (Abou-Donia and Lapadula, 1990). Structural differences exist between type I and type II OP compounds. The ortho-methyl group of OP compounds producing type I neuropathy is responsible for the neurotoxic effect of these aromatic chemicals. The ortho-methyl group of type I compounds must be metabolized to saligenin o-tolyl cyclic phosphate, which is considered the potent neurotoxic metabolite. On the contrary, type II aromatic compounds are neurotoxic without the need of metabolic transformation.

Approximately 60% of the aliphatic OP compounds tested produced delayed neurotoxicity. The descending order of neurotoxic potential as regards the 12 subclasses known to produce OPIDN are: phosphonates = phosphorofluoridates =

phosphonofluoridates = phophorodiaminodofluoridates = phosphoroamidofluoridates > phosphates > phosphorotrithioates > phosphorothioates = phosphonothioates = phosphinofluoridates > phosphorochloridates (Chambers and Levi, 1992). Forty-six percent of the aliphatic aromatic compound group tested have the potential to produce OPIDN and the neurotoxic effect descending order is : phophorodiamidofluoridates > phosphonates > phophonothioates > phosphates > phosphorothioates > phosphinates. Delayed neurotoxicity is observed in 35 % of triarylphosphates evaluated (Chambers and Levi, 1992). Most triarylphosphate esters with one or more phenyl rings substituted in the ortho position or an ethyl group in the para position produce OPIDN. Saligenin cyclic phosphorus compounds are the more neurotoxic product with 62 % producing OPIDN. Compared to type I, few type II chemical products has been tested. Delayed neurotoxicity have been reported in hens with all type II compounds (4) tested (triphenyl phosphite, tri-o-cresyl phosphite, tri-m-cresyl phosphite, and tri-p-cresyl phosphite) (Abou-Donia and Lapadula, 1990).

4. Cyclic Phenyl Saligenin Phosphate (PSP).

Phenyl saligenin phosphate (PSP), an active congener of tri-ortho-tolyl-phosphate (TOTP) (Eto et al., 1961), has been well studied for its ability to produce delayed neurotoxicity in hens (Abou-Donia and Lapadula, 1990; Jortner and Ehrich, 1987). A proposed pathway showing the transformation of TOTP to PSP is presented in Figure 3 and 4. In experimental studies, advantages of PSP related to its capability to produce clinical signs and lesions of delayed neuropathy with few acute cholinergic effects and its ability to induce OPIDN without a need for metabolic activation (Aldridge et al., 1981). PSP is thus considered an excellent compound for experimental evaluation of the clinico-pathological course of OPIDN, hence its choice for this study.

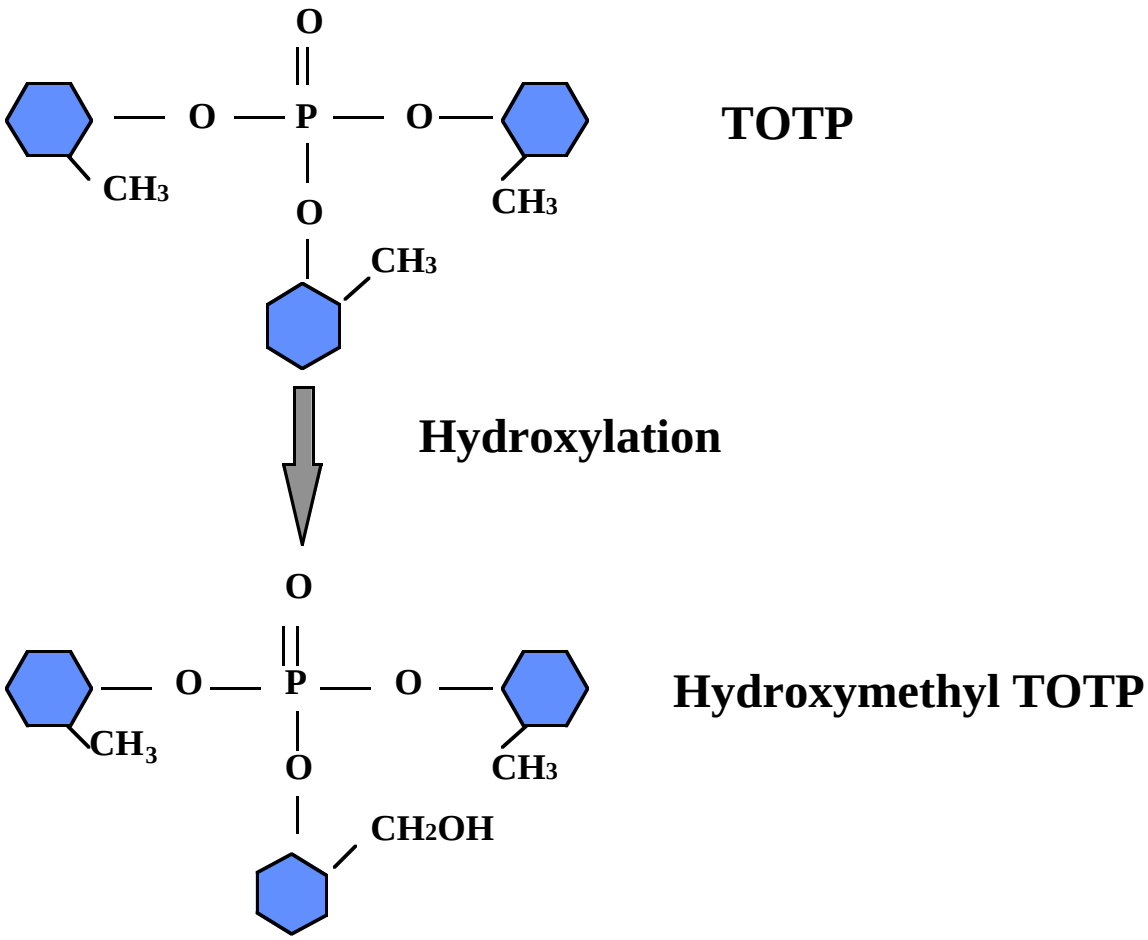


Figure 3: Proposed pathway for hydroxylation of TOTP (Eto et al., 1961).

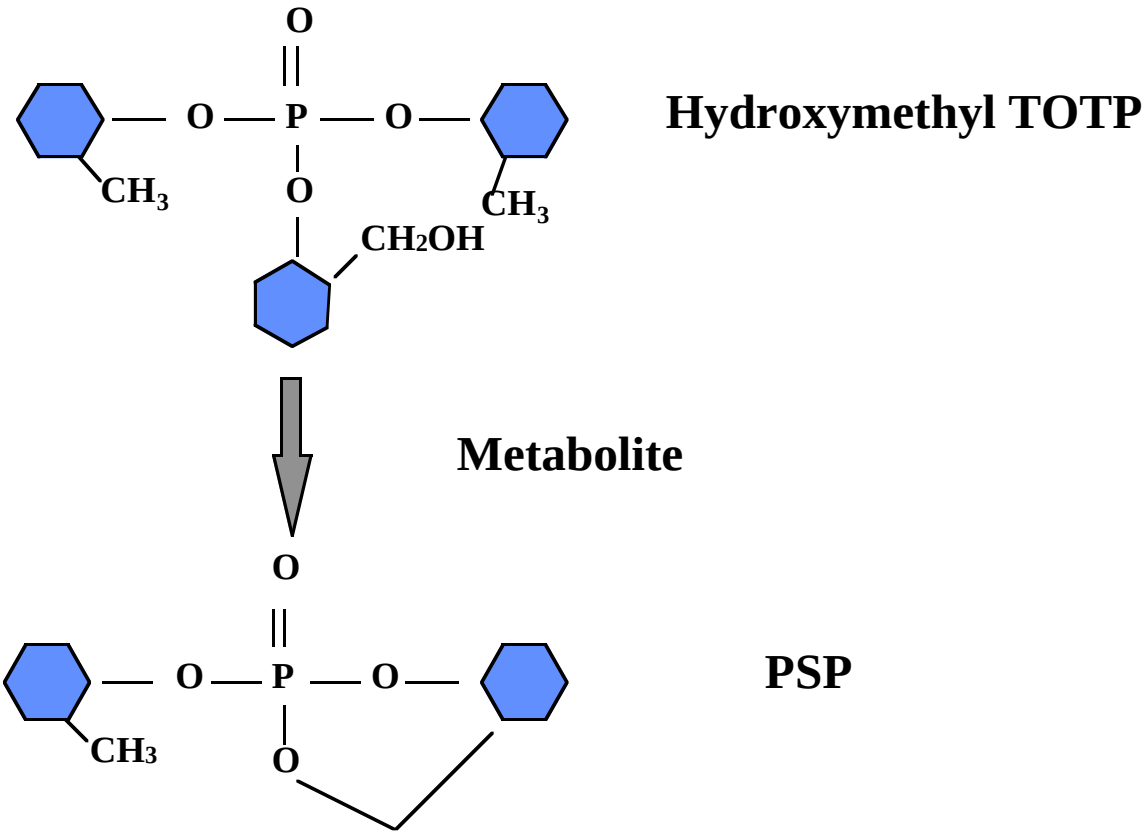


Figure 4: Proposed pathway for cyclization reactions of TOTP, continued from Figure 3 (Eto et al., 1961).

C. Effects of organophosphorus compounds on acetylcholinesterase activity.

Organophosphorus ester (OP) compounds induce neurotoxicity since they can inhibit esterase enzymes by phosphorylation of their active sites (Ehrich, 1996; Farage-Elawar et al., 1988; Aldridge et al., 1972; Johnson, 1969). Among affected enzymes are acetylcholinesterase (AChE) and carboxylesterases. The former (AChE) is of significance, since it is necessary for degradation of acetylcholine, one of the principal neurotransmitters of the nervous system. Where activity of this enzyme is diminished, the synaptic accumulation of acetylcholine results in cholinergic crises, affecting smooth and skeletal muscles, autonomic ganglia, and the central nervous system by overstimulation of acetylcholine receptors (Ecobichon, 1991; Chambers and Levi, 1992; Ballantyne et al., 1992; Sultatos, 1994). Cholinergic poisoning may occur rapidly after sufficient organophosphate exposure, and is thus termed acute OP neurotoxicity. The clinical signs are characterized by vomiting, diarrhea, respiratory distress, excessive salivation, generalized muscle weakness associated with ventral neck flexion, tremors and convulsions (Oliver et al., 1993; Chrisman, 1991). In cases of acute OP intoxication, the degree of AChE inhibition measured in both erythrocytes and central nervous system tissue is directly related to the extent of cholinergic effects observed (Padilla et al., 1996). Clinical signs in these acute neurotoxic episodes occur shortly after exposure, and their intensity and duration varies with the nature of organophosphates and the dosage received during the exposure.

This acute toxicity of organophosporus compounds results from the electrophilicity of the P atom that attacks a neutrophilic receptor site, the serine hydroxyl of the enzyme acetylcholinesterase (AChE). For an example, the phosphotionates (P=S) are considered poorly reactive. However, these OP compounds undergo metabolic activation in vivo by microsomal oxidase enzymes, and are converted in P=O compounds, where the phosphorus is strongly electrophilic. Thus this oxidative reaction increases the acute toxic effects, with the reaction at the target site being directly related to the electrophilic nature of the P-atom. In order for OP compounds to produce cholinergic effects, approximately 85% of AChE activity must be inhibited. AChE activity levels will generally return to normal values within a month after OP intoxication (McCain et al., 1995). Following appropriate exposure, this activity level may be decreased within minutes (Ecobichon, 1991) and can remain lower than normal for 2-3 months (in human erythrocytes).

D. Organophosphate - induced delayed neuropathy (OPIDN)

1. Outbreaks of OPIDN in humans and animals.

In addition to acute (cholinergic) poisoning, another neurotoxic condition has been observed with exposures to some, but not all, organophosphorus esters. This neurotoxic syndrome was eventually characterized as distal myelinated nerve fiber degeneration occurring several days following exposure to certain organophosphates (Cavanagh, 1954). The common term used to describe the resulting syndrome is organophosphorus-induced delayed neuropathy (OPIDN). This toxic neuropathy sporadically affects humans exposed to the OP compounds. The syndrome was originally noticed following treatment of tubercular patients with phosphocresote, a mixture of esters formed from phosphoric acid and coal-tar phenols (Davis et al., 1980; Lorot, 1944). A widespread human outbreak of organophosphorus induced delayed neuropathy (OPIDN) was observed in mid and southern United States in the late 1920s and early 1930s, characterized by ataxia/weakness, and paresis/paralysis, occurring 10-21 days after ingestion of ginger extracts. The causative agent, tri-cresyl-tolyl phosphate (TOCP), was a contaminant in some batches of the extracts of Jamaican ginger, hence the name "Ginger Jake syndrome". At least 20,000 human victims suffered from this syndrome to diverse degrees (Smith et al., 1930). In 1959, another serious epidemic of TOCP induced neuropathy occurred in North Africa, afflicting more than 10,000 persons (Davis, 1963). Other organophosphorus compounds have also been implicated in OPIDN induction in humans: mipafox, leptophos, trichlorfon, parathion, fenthion (Bidstrup, 1953; Johnson et al., 1981; Metcalf, 1984; 1985; 1975; Seneyake et al., 1981). Due to their neurotoxic effects, the production and sale of many of these compounds has ended. There are other less definitive reports suggesting that OPIDN in humans has come from consumption of or occupational exposures to OP neurotoxicants, but the full extent of these events cannot be ascertained.

OPIDN has also been seen in domestic animals exposed to appropriate toxicants. As an example, in 1971, leptophos was responsible for the death of 1300 water buffalo in Egypt. Clinical signs were manifested by ascending paralysis, with exposure following massive application of this product to control cotton leaf-worms (Abou-Donia et al., 1974).

2. Effects of organophosphorus compounds on neurotoxic esterase (NTE)

Johnson (1969, 1970) noted an association of organophosphorus-induced delayed neuropathy (OPIDN), with an antecedent acute, reversible inhibition of a nervous system esterase which he termed "neurotoxic esterase" (NTE). NTE is a carboxylesterase whose inhibition is now commonly measured to predict the potential of OP compounds to cause OPIDN (Johnson, 1982; Aldridge, 1993; Ehrich, 1996; Ehrich et al., 1995; Silbergeld et al., 1993). The function of other carboxylesterases is hydrolyzation of carboxyesters, thioesters, and aromatic amides (Maxwell et al., 1992); however, that of NTE is unknown. As regards its role in OPIDN, two specific reactions seem essential for the neuropathy to occur (Figure 5). The first step, called phosphorylation of the target protein (NTE), must be followed by a process known as "aging" (Richardson, 1995; Lotti et al., 1992; Johnson, 1975a,b; 1982). This can be demonstrated if a radio-labeled organophosphorus ester is used to inhibit the esterase by phosphorylation. In this case, the protein becomes labeled at its active site, where the esteratic activity occurs, and the inhibition of the catalytic activity of NTE can be measured. Aging is the term used when a bond, not easily reversible, is formed between the target protein and the OP, by cleavage of one R group, leaving a negative charge attached to the target protein (Figure 6) (Johnson et al., 1969; 1970). Overall, phosphates, phosphonates, and phosphoramidates have the potential to cause OPIDN since they have R-groups attached to phosphorus by labile -O- or -NH- linkages, which have the potential for aging (Figure 5).

It is believed that 70 % or more inhibition of NTE in brain and spinal cord within 1-48 hours after exposure to an appropriate OP compound is necessary in order for OPIDN to subsequently occur (Johnson, 1975a,b; Johnson, 1982). As noted above, this biological response also requires that aging occurs. There are occasional exceptions to the latter. Mipafox and methamidophos appear to produce neuropathy by inhibition of NTE without the need for the traditional aging process (Lotti et al., 1993; Lotti et al., 1995). A dose-response relationships between NTE inhibition and the neuropathy has been observed (Ehrich et al., 1993, 1995).

As alluded to above, not all OP compounds elicit OPIDN, as demonstrated by their effects on NTE. The inhibitors of NTE can be divided in two categories, depending upon their biological effects (Johnson, 1974). As noted above, phosphates, phosphonates, and phosphoramidates are neurotoxic and form the first group. Carbamates and phosphinates are non-neurotoxic, and constitute the second group (Figure 5). Pretreatment with NTE inhibitors in the second group can be used to protect certain species from the delayed neurotoxic effects of OP compounds in the first group (Johnson, 1974). This alteration of sensitivity to OPIDN in animals pretreated with compounds in the second group will be discussed subsequently.

OP compounds can inhibit NTE within the entire nervous system: brain, spinal cord, and peripheral nerves (Tormo et al., 1993; Ehrich, 1996). Inhibition in brain is usually slightly higher than in spinal cord or peripheral nerve. Furthermore, when whole homogenates of these three separate parts of the nervous system are used to determine NTE activity, baseline levels are slightly greater in the brain (100%) than in the spinal cord(~75%), and much greater than activity in sciatic nerves (~10 %) (Correll and Ehrich,

1991; Johnson, 1982; Ehrich, 1996; Jokanovic et al., 1993). Despite the fact that NTE activity is the greatest within the brain, lesions are less extensive in this tissue than in spinal cord and peripheral nerves. In addition, the NTE activity is similar in all regions of the spinal cord (Correll and Ehrich, 1991) and peripheral nerves despite the fact that restricted regions of the spinal cord (selected cervical and lumbar levels of long tracts) and distal portions of the peripheral nerves show the most severe lesions (Carrera et al., 1992; Carrera et al., 1994; Moretto et al., 1989; Ehrich et al., 1988; Ehrich, 1996). Thus, the relationship between regional levels of NTE and specific localization of lesions in OPIDN is not well understood.

Despite its name, NTE is not restricted to the nervous system, and can also be found in lymphocytes, platelets, spleen, small intestine, placenta, adrenal medulla, and in human neuroblastoma cell lines (Johnson, 1982; Dudek et al., 1982; Maroni et al., 1986; Gubra et al., 1983; Sogorb et al., 1994; Ehrich et al., 1994; Ehrich et al., 1995; Ehrich, 1996; Nostrandt and Ehrich, 1993). There has been interest in evaluation of NTE inhibition within lymphocytes because blood samples can be easily obtained and might be useful in screening for toxicant exposure. However, too many individual variations have been noted to permit determination of any relationship between inhibition of NTE in lymphocytes and subsequent development of clinical neuropathy (Bertocin et al., 1985; Ehrich, 1996).

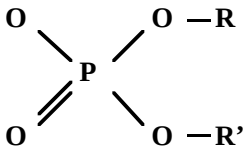
After inhibition of nervous tissue NTE following a single dose of an OP compound to hens, activity levels return with a half-life of 4-6 days. By post-dosing day 21, NTE levels are close to normal (Maxwell, 1992; El - Fawal et al., 1990). Since the clinical signs develop 10-14 days after dosing and the lowest point of NTE activity occurs during the first 48 hours after exposure (Lotti et al., 1980), there appears to be no temporal correlation between NTE inhibition and the OP - induced ataxia. Indeed, clinical signs get

progressively worse while NTE levels return to normal. Thus, the relationship between alteration of NTE levels and the onset of clinical signs and lesions remains unexplained.

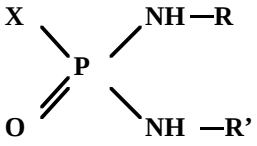
In chronic intoxications (multiple but smaller doses), a higher cumulative dosage of OP compounds needs to be given before OPIDN symptoms occur (Henderson et al., 1992). In the case of multiple exposures to OP compound, clinical signs occur even though less pronounced effects on NTE activity are observed (Lotti et al., 1980; Henderson et al., 1992). The reasons for the weaker correlation between NTE inhibition and clinical severity in multiple dosing paradigms is not clear.

Although there is wide acceptance of the relationship of an appropriate level of NTE inhibition and aging with subsequent OPIDN, several inconsistencies exist. For example, no relation has been established between constant basal NTE inhibition throughout the nervous system and the restricted location of neurologic lesions in OPIDN (Correll and Ehrich, 1991; Johnson, 1982; Ehrich et al., 1995; Ehrich, 1996). Also, correlations between levels of NTE inhibition, and clinical and morphological alterations are not well established. In some studies, no progressive and irreversible neurologic deficits were reported with more than 70% enzyme inhibition (Moretto et al., 1991; Farage-Elawar et al., 1988), whereas in others, 70 % NTE inhibition resulted in neuropathy (Johnson, 1975a,b; 1982). Another reason why NTE may not be the only target responsible for initiation of OPIDN is based on the findings that the same reversible NTE inhibitors can either protect or promote OPIDN lesions and clinical signs, depending upon the sequence of administration in relation to the neuropathic-inducing OP (Johnson, 1982; Pope et al., 1992; Cardoli et al., 1994; Lotti et al., 1991; Moretto et al., 1992; Veronesi et al., 1985).

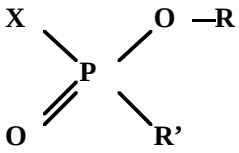
Group A :



Phosphates

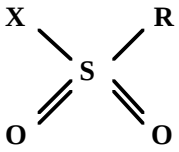


Phosphoramidates

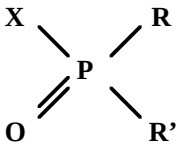


Phosphonates

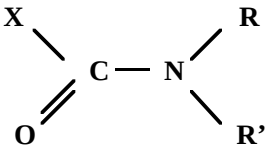
Group B :



Sulphonates



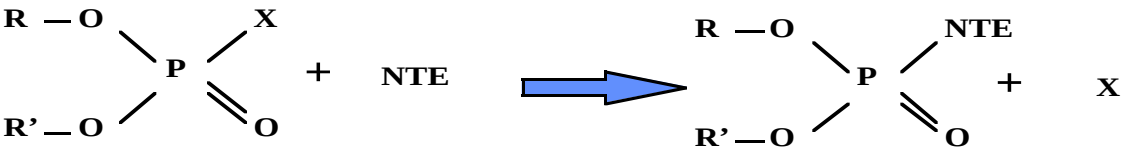
Phosphinates



Carbamates

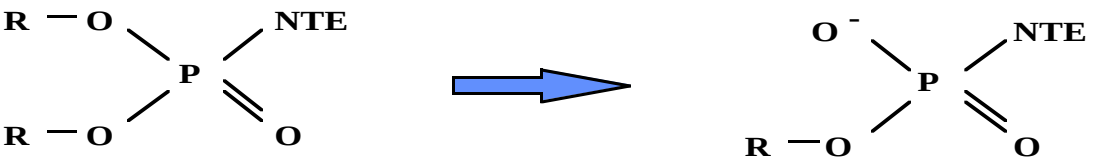
Figure 5: Structural comparison of inhibitors of NTE.

Step 1 :



Active esterase **Inhibited esterase**

Step 2 :



Inhibited esterase **"Aged" inhibited esterase**

Figure 6 : Steps in the interaction of NTE and OP inhibitor (aging). (Johnson, 1975b).

Despite the fact that effects of OP compounds on NTE and AChE have been well documented, little is known about early biochemical pathogenic events in OPIDN. Because of many reported discordances between NTE inhibition and OPIDN, alteration of some hypothesized trophic factors in the progression of the neuropathy has been suggested. These trophic factors include 3H-DFP-binding protein within the active subunit site of NTE (Carrington et al., 1985; Pope et al., 1992; Pope et al., 1993; Escurdo et al., 1995), and ornithine decarboxylase since activity of this enzyme within the spinal cord was dramatically reduced after DFP exposure (Pope et al., 1995).

3. Clinical manifestations associated with OPIDN.

Not all animal species are susceptible to clinical manifestations of OPIDN. The condition has been observed in humans and other primates, cattle, sheep, water buffaloes, cats, chickens, ferrets, and turkeys. Other species such as quail, rats, mice, rabbits, guinea pigs, hamsters and gerbils are more resistant, or show inconsistent delayed neurotoxicity (Abou-Donia, 1981; 1982; Bursian et al., 1983; Jortner et al., 1983; Larsen et al., 1986; Veronesi et al., 1991). Due to its reliability and clarity of responses, the domestic hen is the animal utilized in regulatory testing of chemicals for their ability to elicit OPIDN (US EPA, 1991). Age also influences the susceptibility of a subject to OPIDN, with younger animals less affected (Moretto et al., 1991; Funk et al., 1994). In addition, high social stress enhances the clinical severity of TOCP-induced neuropathy (Ehrich et al., 1983). The general progression of clinical signs in susceptible species consists of incoordination, ataxia and weakness, with progression to complete flaccid paralysis. In hens, for example, ataxia develops 6-14 days after exposure to a neurotoxic dose of PSP (2.5 mg/kg), a type I OPIDN-inducing agent. Clinical signs progress to complete paralysis and prostration

within a 3 week period (El-Fawal et al., 1990). Variation in dosages of delayed neurotoxicant also causes different degrees of clinical signs (Dyer et al., 1992).

Two forms of OPIDN have been described, type I and type II, as noted previously. There are different OPs which produce type I and type II OPIDN. Tri-ortho-tolyl phosphate (TOTP) and triphenyl phosphite (TPPi) are representative of types I and II, respectively. No differences in neurologic signs were noted between hens treated with type I or type II OPIDN-inducing compounds (Abou-Donia and Lapadula, 1990), but location of the lesions differed in the spinal cord.

There are many OP compounds that can induce OPIDN, but not all will be mentioned here. Some of these can also inhibit acetylcholinesterase, resulting in acute cholinergic effects which differ greatly from the clinical effects observed in OPIDN. Indeed, the majority of commercially available pesticides affect AChE more than NTE. On the other hand, exposure to certain OP compounds results mainly in delayed neuropathy, with few or no signs of acute neurotoxicity (Aldridge, 1981; Padilla et al., 1996).

4. Neuropathologic effects of organophosphorus compounds.

a. Light microscopic lesions of type 1 OPIDN

Many pathological studies on different animal species (rats, cats, monkey, sheep, and chickens) have been conducted, generally using TOTP, diisopropyl-fluoro-phosphate (DFP) or mipafox as the toxicant (Carboni et al., 1992; Bishoff, 1967; Bouldin and Cavanagh, 1979; Veronesi et al., 1984; Drakontides et al., 1982; Stumpf et al., 1989; Abou-Donia et al., 1981-1990; Jortner et al., 1983; Jortner, 1984; Boyes et al., 1994). All OP compounds that induce delayed neurotoxicity cause lesions in both the central and peripheral nervous systems. Morphological characteristics and distribution of the damage depends on several factors including the type or chemical structure of the OP compound, species, age, dosages, frequency and duration of exposure, but major alterations occur in distal levels of long myelinated fibers. In type I OPIDN, Wallerian-type degeneration of specific ascending and descending tracts of sensorimotor pathways of the brain stem, spinal cord and peripheral nerves were observed without changes in nerve cell bodies. Light microscopy of central nervous system lesions revealed swollen degenerating argyrophilic myelinated axons, progressing to destruction of the affected fibers distally. Distal axonopathy affecting larger myelinated fibers of peripheral nerves is also a common feature (Lapadula et al., 1992; Krinke et al., 1979). Breakdown of the fiber proceeds, accumulation of axonal debris, formation of multiple myelin-rich ovoids, and increased number of microglia, macrophages, and reactive astrocytes, fibroblasts or Schwann cells (depending on whether peripheral or central fibers are involved) (Jortner, 1982, 1984; Jortner and Ehrich 1987; Cavanagh, 1964; Pressing et al., 1978). The concept of "chemical transection" of distal axons is suggested as the term to describe the pathogenesis of OPIDN (Bouldin and Cavanagh, 1979a,b). In this, the Wallerian-like degeneration

occurs distally to the site of a putative distal non - terminal axonal injury, the "chemical transection". This nerve fiber alteration (Wallerian-like) resembles classical Wallerian degeneration, which is a trophic degeneration that occurs in the neurons distal to a site of traumatic axonal injury (De Lahunta, 1983), in that axonal degeneration and secondary demyelination are observed simultaneously, resulting in axonal swelling along the internodes (ellipsoids), and fiber fragmentation into myelin-rich ovoids (Bouldin and Cavanagh, 1979a). In peripheral nerve Wallerian degeneration, Schwann cells are thought to initiate myelin degradation by sequestering myelin fragments into ovoids and by beginning degradation of the myelin. Later on, macrophages phagocytose and further degrade the myelin (Goodrum et al., 1996; Griffin et al., 1993).

Prior to 1979, "Dying Back" neuropathy was the name given to describe the characteristic axonal degenerating pattern produced by certain organophosphorus compounds. Erroneously, the selective degeneration was thought to begin at the axon's terminus, progressing in the direction of the cell body (Tanaka et al., 1989). In 1979, Bouldin and Cavanagh studied the spatio-temporal spread of axonal degeneration in the neuropathy induced by DFP, by means of the peripheral nerve teased-fiber technique. This technique offers several advantages including assessment of proximo-distal lengths individual fibers. Results of this study showed that focal axonal degeneration was localized distally along the axon, but these lesions were not terminal. Therefore, 'chemical transection' of distal axons was suggested as the term to describe the pathogenesis of OPIDN (Bouldin and Cavanagh, 1979a,b).

Chickens are considered the most appropriate animal for the study of OPIDN because they exhibit locomotor deficits associated with distinct well defined bilateral lesions in the spinal cord (mainly long tracts), brain stem, cerebellum, and peripheral nerves.

The primary site of injuries has been reported to be in the distal, non-terminal axon, affecting longer and large myelinated fibers in the spinal cord and cerebellar medullary white matter and peripheral nerves, with subsequent fiber degeneration distally. Thus, the cervical regions of ascending spinocerebellar tracts and fasciculus gracilis, as well as the lumbosacral area of the descending corticospinal (medial pontine spinal tract), reticulospinal and ventromedial tracts are more affected since these regions constitute the distal levels of these tracts (Beresford et al., 1963; Cavanagh, 1954, 1964; Bouldin and Cavanagh, 1979a,b). Degenerating patterns in the hen's central nervous system induced by tri-ortho-tolyl phosphate (TOTP), diisopropyl-fluoro-phosphate (DFP), and phenyl saligenin phosphate (PSP) have been well studied (Olajos et al., 1978; Tanaka et al., 1989; Jortner and Ehrich, 1987).

Tanaka and Bursian (1989), using thicker sections and silver impregnation techniques, also noticed more central nervous system extensive lesions in hens, involving spinal gray matter, brainstem, and cerebellum. For an example, TOTP (Type I) intoxication induced distal axonal and nerve terminal degeneration involving the spinal cord, medulla and cerebellum. Spinal cord axonal lesions were observed in the cervical level of the fasciculus gracilis and the dorsal and ventral spinocerebellar tracts, in the lumbar region of the medial pontine-spinal tracts, and in the lumbar ventral horns (heavy axonal degeneration). Distal axonopathies and terminal degeneration also were present in the medulla at the level of the lateral vestibular, gracile, external cuneate, lateral cervical nuclei, both dorsal and ventral spinocerebellar tract, and spinal lemniscus. The deep cerebellar nuclei and the cerebellar folia of the anterior lobe were also damaged (Tanaka and Bursian, 1989). With DFP, axonal degeneration was seen mostly within the spinal cord white matter and the brain stem, with extensive lesions in the cervical ascending homolateral, gracilis, vestibulospinal and tectospinal tracts. (Tanaka et al., 1990). In chicken peripheral nerves, the most sensitive regions are the tibial nerve branches to the

lateral head of the gastronemius muscle (Krinke et al., 1979) and the nerve to the biventer cervicis muscle (El-Fawal et al., 1988).

Features of the lesions of OPIDN as seen by light microscopy are best observed with appropriately stained sections from epoxy resin embedded tissue. In the early stages of axonal degeneration, there are focal regions of poor axoplasmic staining, swelling, and associated thin myelin sheaths. Dark-staining intra-axonal debris accumulates in the axoplasm, especially in paranodal regions. Fragmentation of damaged fibers ensues as the process evolves into the Wallerian-like stage, with axonal and myelin ovoids, debris accumulation, and phagocytosis by Schwann cells. Axons become shrunken with disorganized and altered myelin sheaths (Jortner and Ehrich, 1987; Prineas, 1969). The onset of and severity of lesions correlates clinical neurologic abnormalities (Massicotte et al., 1997). Using standard single neurotoxic dosages, the first lesions are noted approximately on days 4-7, when a few degenerated fibers are observed (Massicotte et al., 1997; El-Fawal et al., 1988; Jortner and Ehrich, 1987). By day 21, severe and extensive degeneration is seen in the majority of subjects exposed to appropriate dosages of the neurotoxicant. In peripheral nerves, weeks after the clinical onset due to single toxic dose, axons of many injured peripheral nerves regenerate (Jortner, 1982; Jortner and Ehrich, 1987).

As regards the toxicant used in the present study, lesions produced by PSP in hens were similar in nature and distribution to those observed with TOTP intoxication, and consisted of distal axonal degeneration involving the peripheral nerves, spinal cord, medulla and cerebellum (Tanaka and Bursian, 1989; Cavanagh, 1954; Jortner and Ehrich, 1987). Spinal cord axonal lesions were observed in the cervical level of the fasciculus gracilis tract, in the cervical area of the dorsal and ventral spinocerebellar tracts, in the lumbar region of the medial pontine-spinal tracts.

b. Electron microscopic studies in OPIDN

Several reports exist on transmission electron microscopic changes observed in OPIDN mainly in axons, myelin and fiber terminals. In distal levels of affected axons, OPs cause early axoplasmic accumulation of branching cisternal membranous structures, resembling agranular reticulum, prior to swelling and further nerve fiber degeneration (Bischoff, 1967,1970; Bouldin and Cavanagh, 1979b; Prineas, 1969). At a later stage of axonal degeneration, changes consisted of loss of neurotubules and neurofilaments, degenerated mitochondria, accumulation of membranous and dense bodies structures, granular transformation and reduction of axoplasm (Bischoff, 1967,1970; Bouldin and Cavanagh, 1979b; Prineas, 1969). End-stage Wallerian-like fiber degeneration revealed the presence of various degrees of axonal and myelin debris in Schwann cells (Prineas, 1969).

Lesions in the myelin sheaths are also seen. In peripheral nerve, intra-myelinic vacuoles can also usually be observed within the extra-cellular space of the intraperiod line of the myelinated sheaths, closest to the axon (i.e., between the inner loop of Schwann cell cytoplasm and the most inner dense line of the myelinated sheaths) (Bouldin and Cavanagh, 1979b). The deterioration of the myelin sheaths and axons form ovoids that are phagocytized by Schwann cells (Bishoff, 1985). With further progression of these lesions, granular degeneration develops associated with complete collapse of axons (Jortner et al., 1984, 1987; Bouldin and Cavanagh, 1979b). Subsequently, Wallerian degeneration occurs with complete loss of the axon and myelin sheath distal to the presumed site of injuries. These are replaced by compact bands of proliferative Schwann cells, the so-called Buengner's bands.

The ultrastructure of axon terminals in degenerating nerve fibers have been studied in the central and peripheral nervous systems (Bischoff, 1967; Bouldin and Cavanagh, 1979a,b; Drakontides et al., 1982; Prineas, 1969). OP compounds causing delayed neuropathy induce the formation of membranous structures and vacuoles, as distal focal axonal alterations, preceding myelinated nerve fiber degeneration. Lesions in the presynaptic endings of the anterior horn of the spinal cord was also described, and consisted of swollen motor terminals and spherical synaptic vesicles (Bischoff, 1970; Prineas, 1969). Accumulation and agglutination of proliferated vesicular elements are common feature in presynaptic endings. These are mingled with crowded circular membrane complexes and myelin figures. In addition, dark presynaptic terminals rich with condensed organelles and tangled filamentous structures are seen (Bishoff, 1970).

The work of Bouldin and Cavanagh (1979a,b) deserves special mention. Their electron microscopy confirms that nerve fiber varicosities precede the degeneration of axons, and the lesions are initially focal, and non-terminal. The ultrastructural modification of the teased-fiber technique, also useful in light microscopy, has greatly improved the quality of information obtained from electron microscopic evaluation of nervous tissue, giving more precise information in regard to the spatio-temporal progression of organophosphorus (DFP) induced axonal degeneration in recurrent laryngeal nerves of cats. In this study, the initial lesions consisted of focal, non-terminal midinternodal and paranodal demyelination. The axons continued to further degenerate with time. Twenty days after OP administration, marked focal axonal non-terminal, mid internodal vacuoles gave rise to swellings (varicosities), and degeneration associated with paranodal demyelination. More details on ultrastructural descriptions of the varicosities observed in OP induced axonal degeneration can be appreciated using this technique. The importance of these varicosities reside in the fact they may play a role in the pathogenesis of OPIDN.

The focal axonal degeneration leads eventually to Wallerian degeneration distally to the initial and focal site of injuries. Therefore, the focal nature of axonal damage seems to play a significant role in OPIDN (Bouldin and Cavanagh, 1979 a,b). The pathophysiology of the formation of intra-axonal and intra-myelinic vacuoles, as well as the association between these vacuoles is unknown, but may be related to altered fluid balance in affected fibers. It has been postulated that OP compounds bind to unknown proteins of the myelin sheath or axonal membrane, causing membrane damage, altering directly or indirectly the regulation of the transmembranic ionic gradient. Passive influx of water accumulates within the area of intra-period gaps, forming vacuoles into the intracellular space. Water would eventually accumulate in the dilated agranular endoplasmic reticulum.

5. Protection and promotion of OPIDN by PMSF.

Certain non-neuropathic NTE inhibitors can either protect against or potentiate the effects of neuropathic organophosphate NTE inhibitors. The four types of non-neuropathic prophylactic agents recognized in the literature include carbamates, sulfonates, phosphinates, and organophosphorus esters. Protective effects observed with non-neuropathic agents against several organophosphate products known to cause OPIDN have been well documented, especially in rats, mice, hens and cats. Phenylmethylsulfonyl fluoride (PMSF) is a serine/ cysteine protease inhibitor sulfonate, known to prevent the development of OPIDN (Johnson, 1970; Baker et al., 1980). These serine/ cysteine proteases have a role in a variety of cellular repair and regeneration processes in many tissues, and are thought to be involved in neurite outgrowth during development and regeneration of the nervous system (Monard et al., 1983; Krystosec et al., 1984).

Protection against OPIDN can be achieved by giving a non-neuropathic inhibitor of NTE prior to a neuropathic organophosphate. The timing of PMSF administration relative to neurotoxic organophosphate (OP) exposure is critical to the degree of protection achieved. For an example, a study showed that PMSF could more efficiently prevent OPIDN caused by the neuropathic OP mipafox when PMSF was used shortly (4-48 hours) before mipafox exposure (Pope et al., 1993). Efficacy of PMSF protection decreased with time, as only a mild degree of protection was seen when mipafox was administered 14 days after PMSF injection (Veronesi et al.,1985).

The basis of protection of PMSF and the other sulfonates noted above is that while these compounds can inhibit NTE, the second step of molecular rearrangement or "aging" cannot occur (Johnson, 1970). Studies report no signs of ataxia during the period when chickens were treated with 90 mg/kg of PMSF (Pope et al., 1992), despite the fact that, at this dosage, PMSF caused at least 85 % NTE inhibition in brain, spinal cord, and peripheral sciatic nerves (Pope et al., 1992). The time limit for protection generally is related to the time required for the NTE activity to recover. PMSF is a long acting NTE inhibitor; therefore, protection against neurotoxic OP compound (DFP) can last up to 6 days. Carbamates are short acting NTE inhibitors, and the prophylactic effect can be observed for only 2 days (Johnson, 1970). The duration of protection obtained with PMSF can be reduced if another non-neuropathic short acting agent such as a carbamate is administered prior to PMSF, indicating that the first agent to bind to the target is responsible for the length of protection initiated (Johnson, 1974).

Intra-arterial injection of PMSF into the sciatic artery followed by systemic injection of DFP showed that if PMSF (1 mg/kg) is given unilaterally in the sciatic artery prior to systemic injection of DFP (1.1 mg/kg- 24 hours after PMSF injection), unilateral ataxia

develops 10-14 days after DFP administration, in the opposite leg not receiving PMSF injection (Caroldi et al., 1984). Hens dosed with PMSF in the sciatic artery showed asymmetrical symptoms more severe on the side not injected with PMSF prior to DFP. At day 15, hens showed a complete paralysis on the vehicle injected side, but partial paralysis on the leg protected by PMSF injection. It has been hypothesized that the protection against DFP in these cases could be explained if both targets for initiation and prophylaxis were localized in degeneration prone axonal segments instead of other parts of the nervous system (Caroldi et al., 1984).

One of the theories proposed concerning protection from OPIDN by non-neuropathic NTE inhibitors is that they shield of the target site from subsequent modification by neuropathic compounds (Johnson et al., 1990). In recent studies, a [3H] DFP-binding protein has been identified as the active site subunit of NTE (Thomas et al., 1989; Ruffer-Turner et al., 1992). Binding of radiolabeled DFP to this protein is prevented by the non-neurotoxic agents suggesting this as an important mechanism in the prevention of OPIDN (Carrigton et al., 1985; Pope et al., 1991; Glynn et al., 1993). Two different [3H] DFP-binding proteins with inhibitor sensitivities for this target site have been isolated (Pope et al., 1993; Escurdo et al., 1995).

Interestingly, while pretreatment with non-neuropathic compounds can prevent clinical signs of OPIDN, the same non-neurotoxic product can potentiate such clinical signs if administered after the known neurotoxic OP (Moretto et al., 1992; 1993). The effect is termed "promotion" of OPIDN (Pope and Padilla, 1990; Richardson, 1995). Thus, treatment with PMSF after exposure to DFP or mipafox exacerbates delayed neurotoxicity. In studies during which PMSF was given 4 hours after these neuropathic agents (mipafox-50 mg/kg; DFP-0.5 mg/kg), hens showed an increase in the severity of ataxia, and an

earlier clinical onset of neurologic deficits than with the use of neurotoxic OP compounds alone (Pope and Padilla, 1990; Pope et al., 1992). As an example in hens, a grade 4 level of ataxia was observed 14 days after exposure to only neurotoxic OP compounds, compared to its recognition only 10 days after OP administration in hens also treated with PMSF 4 hours post-OP dosing (Pope and Padilla, 1990; Pope et al., 1993). The minimal PMSF dosage reported to induce promotion in hens treated with DFP (0.5 mg/kg) is 5 mg/kg subcutaneously. At this dosage, PMSF inhibits 50% of NTE activity. This promotion was also observed when the PMSF was administered as late as 11 days after DFP (0.5 mg/kg) intoxication.

The question whether PMSF-induced promotion takes precedence over protection has not been definitely answered. One study indicates that PMSF given after DFP exposure in hens that have been pretreated with PMSF as a prophylactic agent results in more severe clinical signs than those treated with the OP compound alone (Lotti et al., 1991). In this model, there was no relationship between the PMSF dose and promotion effects (Lotti et al., 1991). However, another study reports that chickens (70 days old) challenged with the same treatment protocol (PMSF-DFP-PMSF) showed no evidence of neurotoxicity for the duration of the observation period, and no axonal degeneration up to 16 days after treatment (PMSF-DFP-PMSF) (Pope et al., 1992).

PMSF also alters sensitivity to organophosphorus induced delayed neurotoxicity in developing animals. Young chickens are known to be more resistant to clinical signs of OPIDN (Johnson and Barnes, 1970; Pope et al., 1992; Funk et al., 1994). However, PMSF post-treatment in these young chicks increased their sensitivity to neuropathic OP compounds. As an example, chickens treated with DFP alone showed an age-related increased in the severity of neurologic deficits observed (Funk et al., 1994). In the latter

study, neuropathological changes of OPIDN within the central and peripheral nervous systems were noted in 10-week-old DFP exposed chicks, using toluidine blue and Bodian's silver stains (Funk et al., 1994). A relatively similar degree of motor dysfunction has been reported among 3 different age groups of chickens (35-49-70 days) in PMSF post treated animals (Pope et al., 1992). With regard to neuropathic lesions within these 3 age groups, the younger chickens (35-49 days) showed only mild degeneration in the fasciculus gracilis and spinocerebellar tract (score ~ 1), after exposure to DFP alone, using a Fink-Heimer silver impregnation known to be sensitive to detect terminal axonal degeneration (Pope et al., 1992). The older chickens (70 days) showed a moderate degeneration in both the fasciculus gracilis and spinocerebellar tracts (score ~ 2). In contrast, PMSF administered to young chicks (35 to 70 days) after DFP produced severe degeneration of the fasciculus gracilis and spinocerebellar tracts, regardless of the age at the time of PMSF administration (score ~ 3) (Pope et al., 1992). In a more recent study evaluating younger chicks (14 days), no clinical signs or lesions of OPIDN were detected after OP (PSP) treatment, and post treatment with PMSF did not alter the progression of delayed neurotoxicity (Harp et al., 1997).

Several hypotheses have been proposed to explain the relation between PMSF and age-related sensitivity to OPIDN. One explanation for the observed exacerbation of toxicity with PMSF could be a variation in the disposition of the neuropathic OP compound, such that more neurotoxicant-target interactions occur over time or that less neurotoxicant is eliminated from sensitive regions of the nervous system (Pope and Padilla, 1990; Pope et al., 1992). The latter theory has been challenge by other studies, suggesting that promotion may affect a site other than NTE (Moretto et al., 1992), and that pharmacokinetic interactions are not likely present (Lotti et al., 1991) Because young chickens showed significant recovery of motor function after DFP exposure, another

theory has been postulated, suggesting that neuropathic agents can also induce neuronal repair processes, and PMSF modifies the neurologic deficits by decreasing the efficacy of these process (Johnson, 1993; Moretto et al., 1992; Pope et al., 1993; Pope et al., 1995). A third theory suggests that potentiation can only occur if prior induction of morphological changes is present. Pretreatment with PMSF prevents the induction stage, therefore potentiation (with post-OP PMSF) is not observed (Pope et al., 1993; Pope et al., 1992).

In a recent study, Pope et al. (1995) proposed that a soluble factor was released in the spinal cord after exposure to a neuropathic OP. This far unidentified factor had dramatic effects on cell growth, i.e., significantly increasing the neurite outgrowth. The use of PMSF as potentiation agent caused an increment in neurite outgrowth. The activity of another spinal cord enzyme, called ornithine decarboxylase (OCD), is also decreased by the use of neuropathic OP agents, suggesting that other biochemical changes exist, and may play a developmental role in OP-induced degenerative neuropathies. This suggests that factors other than NTE inhibition may be involved in OPIDN, especially promotion of OPIDN (Pope et al., 1995; Moretto et al., 1994).

6. Other modifiers of OPIDN.

a. Steroids

These compounds have the capacity to reduce nervous system injury. High dosages of glucocorticoids have been used to facilitate recovery after human and animal acute and chronic spinal cord trauma. The pathogenesis of this injury relates to post traumatic microvascular damage, secondary ischemia and oxygen radical-induced cell membrane lipid peroxidaton occur as early events in injured spinal cord tissue (Braughler et al., 1989).

Glucocorticoids have been shown to possess free radical scavenging and membrane antioxidant properties, to some extent preventing lipid peroxidation. In addition, the use of methylprednisolone at 30 mg/kg i.v. in cats soon after spinal cord injury also provides support of energy metabolism, prevents progressive post-traumatic ischemia, and inhibits neurofilament degradation, and vasoactive prostaglandin F2alpha and thromboxane A2 formation (Hall, 1993). Other studies confirm that high antioxidant doses of methylprednisolone can also improve recovery from chronic spinal cord injury (Young et al., 1988).

With regard to the effects of steroids in OPIDN, a single dose of methylprednisolone or repeated doses of triamcinolone administered immediately after parenteral administration of DFP have been reported to reduce the severity of the clinical and histopathological effects (Drakontides et al., 1982; Baker et al., 1985; Ehrich et al., 1985). Pretreatment of hens with glucocorticoids decreased motor impairment (Glees, 1961; Beyer-Mears et al., 1980). Glucorticoid pretreatment can also diminish loss of neuromuscular transmission at 48 hours following nerve injury (Hall et al., 1993).

Other studies showed that clinical signs in chickens given tri-o-tolyl phosphate (TOTP) were less severe when 15-30 ppm of corticosterone was added to the birds' regular diet (Ehrich and Gross, 1982). Nevertheless, protection against DFP was not as efficiently achieved as protection for TOTP. The differences in the protection observed between DFP and TOTP may be related to the need of TOTP to be activated by hepatic microsomal enzymes to become neurotoxic, whereas DFP is a direct inhibitor of neurotoxic esterase (Ehrich et al., 1985).

Biphasic effects have been reported for both minerals and glucocorticoid administration, depending on the dose, with amelioration clinical signs of OPIDN at low doses, and exacerbation at higher dosages (Ehrich et al., 1986; Ehrich et al., 1988; Lidsky et al., 1990). The mechanism of action of steroids on OPIDN is unknown. As with corticosterone, the beneficial protective effects of low concentrations of triamcinolone on clinical OPIDN induced by TOTP and PSP were more significant than the benefit of this glucocorticoid of DFP induced OPIDN. Detrimental effects in hens was seen when high concentrations of both corticosterone and triamcinolone were used, likely related to the potentiation of nerve injuries, and decrease in muscle mass associated with such dosing paradigms (Sapolsky et al., 1985).

The use of mineralocorticoids such as deoxycortisone also protected hens from OPIDN (Ehrich et al., 1986, 1988). Even though mineralocorticoids could help in reducing the neurotoxic effect of OP compounds, a combination of deoxycorticosterone and triamcinolone did not have additive protective effects against OPIDN, suggesting that these steroids exert their action via similar mechanisms (McDonald, 1980).

b. Calcium-Channel Blockers

An early change that occurs after axonal traumatic injury is the intracellular entry of excess calcium, which may be important in mediating the damaging effects in the neurite (Kandel et al., 1991). Excess calcium has several damaging consequences leading to cytotoxicity and cell death. Mechanisms involved in these processes include activation of calcium-dependent proteases, and of phospholipase A2 pro-inflammatory arachidonic acid metabolites, and the generation of free radicals. Among the latter are superoxides, which

all have toxic actions on neurons (Farber, 1981; Mayer et al., 1989; Aroneski et al., 1992; Kandel et al., 1991).

Increased *de novo* synthesis of ACh receptors in denervated muscle has been observed as concentration of intracellular calcium increased during OPIDN (Almon and Appel, 1976), which may be the basis of hypersensitivity to ACh following OP exposure in such muscles. Furthermore, the elevation or reduction in ACh receptors seem to correlate with the severity of clinical signs of OPIDN observed (maximum increment at 15 days) (Almon and Appel, 1976). The mechanism of action suggested to explain the nerve and muscle necrosis is the activation of a calcium dependent neutral protease. In fact, some OP compound produce severe muscle inactivation for a length of time sufficient to cause release of calcium, resulting in degradation and myopathy (Schlaepfer et al., 1984; Leonard et al., 1979). Decrement of the neurotoxic response to phenyl saligenin phosphate was measured by electrophysiological testing, histopathologic changes, and calcium-activated neutral protease levels in peripheral nerves (El-Fawal et al., 1990). Ataxia was less pronounced in hens given PSP and a calcium channel blocker, than in hens where PSP was administered alone (El-Fawal et al., 1989; El-Fawal et al., 1990).

The role of elevation of calcium in the pathogenesis of OPIDN has been supported by studies using calcium channel blockers has been proposed to decrease the neurotoxic effects (El-Fawal et al, 1990; Vitarius et al., 1994). Two well known calcium channel blockers, verapamil and nifedipine, have been reported to reduce the clinical, electrophysiological and morphologic neuropathic changes induced by PSP administration in hens (El-Fawal et al., 1989; 1990). Verapamil is a slow-channel calcium blocking agent that exerts its action by blocking the transmembranic influx of extracellular calcium ions across membranes of vasculature, smooth muscle and myocardial cells. The mechanism by

where nifedipine inhibits calcium ion influx across the slow calcium channel is different from verapamil. Nifedipine blocks both surface voltage-sensitive calcium channels and acts intracellularly to inhibit release of calcium from its stores (mitochondria, endoplasmic reticulum) instead of blocking only membrane channels as with verapamil (Needleman et al., 1985).

The mechanism by which calcium channel blockers alter OP neurotoxicity is unclear. Because of the effect of calcium channel blockers on smooth and cardiac muscles, the use of verapamil or nefedipine could alter the distribution of administered OP compounds, since cardiac output and blood flow would be increased. Regeneration of axons in OPIDN could also be facilitated by an increased blood flow. A more recent study indicates that cardiovascular response is not related to OPIDN mechanisms, and the axonal degeneration involve intracellular calcium (McCain et al., 1995; 1996). Another study suggests that OP compounds cause an increase in calcium-activated neutral protease; therefore a calcium channel blocker (nifedipine) could be used to decrease OPIDN by reducing the calcium-mediated disruption of axonal homeostasis (El-Fawal et al., 1990). Verapamil has the potential to inhibit activity of hepatic oxidative enzymes (Hunt et al., 1989). Since this enzyme is in part responsible for detoxification of OP compounds, it was thought that the use of this product could worsen the effect observed with OPIDN. However, verapamil did not cause significant inhibition of hepatic enzymes in hens, when treatment was initiated one day prior to OP administration (El-Fawal et al., 1990). Inhibition of hepatic enzymes with verapamil takes at least a week of intensive treatment, and is minimized in the one-day pre-OP dosing model (El-Fawal et al., 1990). Thus, this is not likely to be the mechanism that affects the course of OPIDN. The above noted studies indicate that influx of calcium is implicated in the pathogenesis of OPIDN. Although the mechanism for reduction of OPIDN by calcium channel blockers is yet

unknown, the amelioration may be due to the consequences of variation in intracellular free-calcium in both nerves and muscles (Gupta, 1995; Gupta and Abou-Donia, 1993).

III. Experimental Design and Methods.

A. Experimental animals and study design.

As discussed previously, adult chickens are susceptible to OPIDN and provide an excellent animal model to study clinical and pathological alterations caused by OPIDN, hence its choice in this study. Seventy-nine adult Female White Leghorn chickens (> 8 months of age) were used in the experiment described below. They were lines selected from the Cornell random-bred population obtained from the Department of Poultry Science, Virginia Polytechnic Institute and State University. The chickens were kept in range-type housing with metal flooring from approximately 4 months of age. They received water and food ad libitum. The food consisted of corn-soy poultry crumble diet designed for chickens at Virginia Tech. These chickens were free of apparent diseases and vaccinated against Marek's disease. The chickens were not transported during the experimental period, to avoid stress that could influence some of the results (Ehrich et al., 1983).

This experiment was designed to ultimately study the clinical and neuropathologic effects of promotion and protection on OPIDN. The serine/cysteine protease phosphate inhibitor phenylmethylsulfonyl fluoride (PMSF) has been used both to promote and to protect against neuropathic events of OPIDN in hens, depending upon the sequence of PMSF administration as we explained in previous sections (Lotti et al., 1991; Veronesi et al., 1985; Pope et al., 1993). The intent of this work was to expand upon these studies by correlating clinical and neuropathological findings in these modifications of OPIDN.

The chickens were divided in 8 different groups for the study, as outlined in Table 1. To provide appropriate models of OPIDN and to study dose-effect of phenyl saligenin

phosphate (PSP), single dosages of 0.5, 1.0, or 2.5 mg/kg were administered to adult hens by intra-muscular injection. The first group (n = 9) received a low dose of PSP (0.5 mg/kg). The second group (n = 10) was administered an intermediate dose of PSP at 1 mg/kg. The third group (n = 10) was administered a high dosage of PSP (positive controls - 2.5 mg/kg). To see if PMSF had an effect by itself, a fourth group (n = 10) received phenylmethylsulfonyl fluoride (PMSF - 90 mg/kg) only, via subcutaneous injection.

PMSF was used to study promotion of, and protection from PSP-induced neurotoxicity. For protection, a fifth group of hens (n = 10) received phenyl saligenin phosphate (PSP) at 2.5 mg/kg, a dosage known to cause severe clinical and morphologic changes, 12 hours after PMSF administration (90 mg/kg). To study promotion, PMSF was administered 4 hours after 2 levels of PSP challenge. A sixth group (n = 10) was administered PMSF (90 mg/kg) 4 hours after the low dose injection of PSP (0.5 mg/kg), and a seventh group (n= 8) received the intermediate dose of PSP (1mg/kg) and PMSF (90 mg/kg) 4 hours post PSP dosing. The eighth group consisted of 13 untreated animals used as negative controls. A summary of this protocol is given in Table 1.

Table 1: Animal numbers, group distribution, and schedule of sacrifice.

	NTE-AChE activity (24-hrs post-dosing) (# of animals)	**Peripheral nerves** Immersion fixation (9-10d post-dosing) (# of animals)	Perfusion (15-16d post-dosing) (# of animals)
PSP (0.5 mg/kg)	3	3	3
PSP (1 mg/kg)	3	4	3
PSP (2.5 mg/kg)	3	4	3
PMSF only (90 mg/kg)	3	4	3
PMSF (90 mg/kg) / PSP (2.5 mg/kg) (protection)	3	3	4
PSP (0.5 mg/kg) / PMSF (90 mg/kg) (promotion)	1	4	4
PSP (1 mg/kg)/ PMSF (90 mg/kg) (promotion)	3	2	4
Control	3	7	3

B. PSP synthesis and dosages.

Phenyl saligenin phosphate (PSP), synthesized as described by Jortner and Ehrich, (1987) (Lark Enterprises, Webster MA), was chosen as the organophosphate used to induce delayed neuropathy. A solution of phenyl phosphodichlorate (0.078 mol) in dichloromethane was added to triethylamine (0.079 mol) and o-hydroxybenzyl alcohol (0.075 mol). After evaporation and drying, the product was crystallized and recrystallized in chloroform and petroleum ether. Finally, the PSP product obtained was dissolved in dimethylsulfoxide (DMSO). The PSP concentration was such that the hens received a volume of 0.5 ml/kg of 5 mg/ml (2.5 mg/kg) and 1.0 ml/kg of 1 mg/ml (1 mg/kg).

C. PMSF use and dosages.

The serine/cysteine hydrolase inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Company, St-Louis, MO) is a potent in vivo inhibitor of NTE which does not cause OPIDN. PMSF was used in two roles in this study, for promotion or protection from OPIDN. PMSF was used as a promotor of OPIDN since PMSF has been reported to potentiate the delayed effects of OP compounds when given after OP administration (Moretto et al., 1992; Lotti et al., 1991). Four hours after PSP challenge, PMSF at a dose of 90 mg/kg in dimethyl sulfoxide (DMSO), 1ml/kg s.c. was administered. PMSF at the same dosage was injected without PSP challenge as a control. The protective effect of PMSF was also tested by administrating the same dosage of PMSF 12 hours prior to PSP challenge. Protective effects are explain in details in the literature review section (see Protection and promotion of OPIDN by PMSF, page 40).

D. Evaluation and scoring of clinical signs.

During the course of the experiment, each chicken was examined daily to detect the progression of neurologic deficits. The examiner was unaware of chemicals administered to the hens. Neurologic deficits were graded according to a 8 point scale described as follows (Cavanagh, 1961):

Clinical Scores

Clinical scores	Neurologic deficits
0	No neurologic deficits
1	Minor changes in walking performance
2	Slight, but definite clumsiness or incoordination, bending of hock
3	Does not stand upright
4	Ataxia, greater degree of incoordination (serious incapacitation)
5	Does not move fast or actively
6	Marked ataxia with inability to maintain upright stance for any lenght of time
7	Down, difficulty standing, more down than up
8	Total inability to rise or move, weak limb movement

E. Neuropathologic evaluation.

1. Euthanasia and tissue collection.

In order to monitor the progression of neuropathologic changes over time, biventer cervicis nerves (El-Fawal et al., 1988) from 3-4 chickens in each of the categories described above were collected for light and electron microscopy, using immersion fixation or perfusion. The nerve were collected at 2 different time intervals. The first samples were collected at 9-10 days post-dosing and underwent immersion fixation. Perfusion-fixation technique was reserved for day 15-16 post-dosing samples. Nerve fiber teasing methods (Bouldin et al., 1979; Jortner and Ehrich, 1987; Krinke et al., 1979) were also performed on randomly selected animals, within each different group.

Immersion fixed biventer cervicis nerves were obtain immediately after sacrifice of the hens. Euthanasia was performed by an overdose of pentobarbital administered via the cutanea ulnaris vein. The nerves and overlying muscle were then removed, placed and straightened on a card, and immediately immersed in 3 % gluteraldehyde. They were held in this fixative at 4 degrees Celsius for approximately 48 hours. The nerves were then dissected from the surrounding muscle and adipose tissue, and placed in a fresh 3 % gluteraldehyde solution.

For each perfused animal, 2000 I.U. of heparin was administered in the cutanea ulnaris vein with a 21 gauge butterfly needle, to prevent undesired blood coagulation that could have interfered with the perfusion technique. Following administration of anticoagulant, a deep anesthesia was induced by intravenous administration of pentobarbital. A ventral midline incision was then made, cutting through the skin and the

pectoralis muscles. The sternum was cut with scissors from the xiphoid process all the way cranially, then reflected, paying particular attention not to injure the exposed viscera. The pericardium was localized and cut to allow good visualization of the heart. The left ventricle at the apex of the heart was opened by incision of the cardiac muscles. A 13 gauge gavage needle was then inserted through this opening to the base of the aorta and clamped in position. An incision in the right atrium was then made. The cardiovascular system was immediately flushed with saline solution (0.9% sodium chloride adjusted to pH 7.4) at a pump setting of 100 ml/min for 1-2 minutes, until the liver blanched. The birds were then perfused with at least 1500 ml of fixative, 5% gluteraldehyde in 0.1 M sodium phosphate buffer (pH = 7.4).

After perfusion was complete, the cadavers were placed in a plastic bag, and stored at 4 degree Celsius, for 5 to 24 hours. At that time, the biventer muscle and attached tendon, containing about a 3.5 cm segment of the nerve, were removed and placed in a fresh cold 5 % gluteraldehyde buffered solution for 24-48 hours. Then the biventer cervicis nerves were dissected from the surrounding muscles and stored in a fresh 5 % gluteraldehyde buffered solution at 4 degrees Celsius.

2. Tissue processing for microscopy

Cross-section of nerves were embedded in Polybed® epoxy resin. For this, the gluteraldehyde fixed nerves were post-fixed for 2 hours in 2 % OsO_4 in 0.1 M phosphate buffer at pH 7.4, and washed with 0.1 M phosphate buffer solution. Following post-fixation, the tissues were dehydrated in graded concentrations of ethanol (15%, 30%, 50%, 70%, 95%, 100%), and cleared in propylene oxide. The samples were then infiltrated with 50:50 propylene oxide/epoxy resin for 24 hours, followed by 100% epoxy resin for at least

24 hours. The samples were properly oriented in embedding molds and embedded in an epoxy resin. These tissue blocks were polymerized in a 60 degree Celsius oven for 24-48 hours then removed from the molds and stored.

For samples destinated for light microscopy evaluation, semi-thin cross-sections were cut at a thickness of 1-1.2 μm and collected on glass microscope slides. At least 6 sections were collected per block. The sections were heat-fixed to the slide (hot plate 70 to 90 degree Celsius), and stained for light microscopy with toluidine blue 1%, and safranin 0.5%. Cover slips were placed over the slides using a 3:1 permount: xylene solution. The slides were dried overnight in the exhaust hood.

For transmission electron microscopy evaluation, selected blocks were cut at 80 nm, and then placed on new 200 mesh grids (2-3 grids per each block). These thin sections were first stained with 2% uranyl acetate to enhance contrast of nucleic acid-containing structures and subsequent lead citrate staining. The latter staining agent was to enhance staining of membranes of cellular components. Both of these stains increased the electron scattering power of biological specimens (Hayat, 1970). The grids were rinsed with distilled water, dried, and placed in a grid box, with care taken to record grid identity on a grid box form. These thin sections were evaluated for ultrastructural changes with a JEOL JEM-100CX II transmission electron microscope.

From each of experimental groups described above, glutaraldehyde-fixed myelinated nerve fibers from 3-5 hens were teased onto glass slides for light microscopic study. For these evaluations, segments of biventer cervicis nerve branches of at least 1.5 cm in length were osmicated (2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4), dehydrated in graded alcohols (30%, 50%, 70%, 95%, 100%), and cleared in cedarwood

oil. Separation of the individual myelinated nerve fibers from the nerve was performed under a dissecting microscope. The separated fibers were placed on a microscope slide, and oriented parallel to each other. When 50 or more fibers were obtained, the slides were dried at 50-60 degrees, and covered with coverslips mounted with Permount (Spencer et al., 1970; Greenfield and Gowin, 1997). Two or more internodes in these preparations were evaluated by light microscopy to determine proportions of degenerating fibers.

3. Determination of lesion intensity (scoring scale).

Each cross section of the epoxy-resin embedded biventer nerve was examined by light microscopy. For each slide, photomicrographs (250X) of one entire cross section were taken. Projection of these micrographs on a plane surface allowed good resolution and enlargement to distinguish individual fiber integrity. Counts of the number of normal, degenerating, and regenerating fibers, and general qualitative changes were recorded for each cross-section. When the integrity of a nerve fiber was questionable, the particular tissue sections were reviewed with the light microscope for better resolution and magnification. Percentages of degenerated and regenerated nerve fibers were calculated for each hen. Teased nerve fiber preparations were also evaluated by light microscopy to determine qualitative and quantitative pathology changes.

A five category neuropathologic intensity scoring system was established, based upon percentages of degenerated fibers observed for both cross sections and teased nerve fiber techniques. The lower and higher values of the score scale were determined according to the lowest and highest number of degenerated fibers, respectively. Less than five percent nerve fiber degeneration was considered normal variation according to the age of the experimental subjects. This neuropathologic scale score is as follows:

Lesion score - percent of degenerating fibers.

Pathology scores	Percentage of degenerating fibers
0	< 5 %
1	≥ 5 % and < 20 %
2	≥ 20 % and < 40 %
3	≥ 40 % and < 60 %
4	≥ 60 %

F. NTE activities.

1. Tissue collection for NTE.

Fresh brain and cervical spinal (C1-C6 segment) cord were removed to analyze for neurotoxic esterase 36 to 48 hours after dosing the hens. These samples were taken from all experimental groups described previously. Three animals per group were used for NTE determinations. Brain and spinal cord fresh specimens were placed in a separate containers, kept on ice for no longer than three hours, and then frozen at -70 degree Celsius until the NTE analyses were performed.

2. Determination of neurotoxic esterase activity.

Activities of neurotoxic esterase in brain and spinal cord were measured based on the method described for hens by Sprague et al., 1981, and modified for microassay by Correll and Ehrich (1991). In this method, paraoxon (13.76 mg/ml acetone) and mipafox (1.166 mg/ml acetone) solutions were diluted 1:50 in Tris-EDTA (50 mM Tris 0.2 mM EDTA; pH 8.00) buffer just prior to use, and were added to a 25 µl volume to microtiter well containing 50 µl of tissue homogenate [0.33mg brain and 0.45 mg spinal cord, prepared in 50 mM Tris, 0.2 mM EDTA (pH=8.0)]. The volume in the well was adjusted to a volume of 150 µl total for the initial preincubation. After addition of each reagent, the plate was mixed using the automix function of the microplate reader, and incubated at 37 degrees Celsius for 20 minutes. Fifty µl of a solution containing 53 µg of phenyl valerate dissolved in 0.03 % triton-X in saline was added to the previous reagents of the plate, and incubated at 37 degrees Celsius for an additional 15 minutes. This hydrolyzing reaction was stopped 15 minutes after incubation by a solution containing 10 % SDS and 0.04 % 4-aminoantipyridine in 0.5 M Tris. Fifty µl of 0.4 % potassium ferricyanide solution was then added, and the reaction products were read at 510 nm (10-45 min). NTE activity was determined as the difference in enzyme activities between samples incubated with paraoxon and samples incubated with both paraoxon and mipafox. This calculation was based on a phenol standard curve (NTE activity = [abs/mg protein] / slope of standard curve / 15 min). Activity was expressed as nmol of phenol formed / min / mg of protein. Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). Two hundred and fifty µl of Bio-Rad reagent was mixed with 10 µl sample or protein standard (bovine serum albumin in water; 0.0187-0.8 mg/ml), and read spectrophotometrically at 595 nm.

G. Statistical methods.

For NTE activities in brain and cervical spinal cord, the variance was analyzed using ANOVA statistical test. Evaluation of means and variance were analyzed using GLM statistical analysis procedures, to account for the unbalanced data between the groups. Significance of relationships between clinical and neuropathology lesion scores was determined by linear regression using MANOVA statistical analysis (SAS/ STAT User's Guide, 1989).

Experimental results.

A. Neurotoxic esterase (NTE) inhibition (% in brain and spinal cord).

The degree of brain and spinal cord NTE inhibition 24 hours post - dosing is given in Table 2 for all experimental groups. The neurotoxic esterase activity was very sensitive to all doses (2.5, 1.0, or 0.5 mg/kg) of PSP alone, resulting in a very high percent of NTE inhibition (Table 2, Figure 7,8), without statistical differences among them. NTE inhibition in hens which were treated with 90 mg/kg PMSF alone (NTE inhibition in brain = 77% and spinal cord = 37 %) and the negative controls (0 % inhibition) were different from NTE activities in PSP-treated hens ($p<0.0001$; $\propto = 0.05$). NTE activities in the birds challenged with PMSF 90 mg/kg alone were also statistically different from NTE activities in the negative control animals. The mean percentage of NTE inhibition in the brain was significantly higher than in the spinal cord for the positive control birds (PSP 2.5 mg/kg), the hens challenged with PSP 0.5 mg/kg followed by PMSF 90 mg/kg (promoted) and the ones treated with PMSF 90 mg/kg prior to PSP 2.5 mg/kg administration (protected). Figures 7 and 8 show the % of NTE inhibition for the brain and spinal cord in each group. The means of the percentages of NTE inhibition within the brain and spinal cord are reported in Table 2.

Table 2 : Activity and % of NTE inhibition 24 hours post-dosing (brain and spinal cord).

DRUGS	Activity Brain [a]	Activity Sp Cord [a]	% Inhibition Brain [b]	% Inhibition Sp Cord [b]
PSP 0.5 mg/ kg	0.817 C/ min	0.193 C/ min	92.3 ± 0.3 %	92.3 ± 0.4 %
PSP 1.0 mg/ kg	1.704 C/ min	0.193 C/ min	84.0 ± 0.2 %	92.3 ± 1.2 %
PSP 2.5 mg/ kg	0.817 C/ min	0.353 C/ min	92.3 ± 0.7 %	86.0 ± 1.2 %
PSP 0.5 mg/ kg & PMSF	0.479 C/ min	0.870 C/ min	95.5 ± 0.2 %	65.5 ± 2.1 %
PSP 1.0 mg/ kg & PMSF	0.571 C/ min	0.034 C/ min	94.7 ± 0.2 %	98.7 ± 0.2 %
PMSF 90 mg/ kg	2.449 C/ min	1.589 C/ min	77.0 ± 0.6 %	37.0 ± 2.8 %
PMSF & PSP 2.5 mg/ kg	1.065 C/ min	0.441 C/ mmin	90.0 ± 0.3 %	82.5 ± 0.1 %
Control	10.648 C/ min	2.522 C/ min		

[a] Neuropathy target esterase (NTE) activity expressed as nmol product formed/min/mg brain or spinal cord protein.

[b] Mean neuropathy target esterase (NTE) inhibition ± SD, expressed as percentages.

Percentage of NTE inhibition 24 hours post dosing-brain

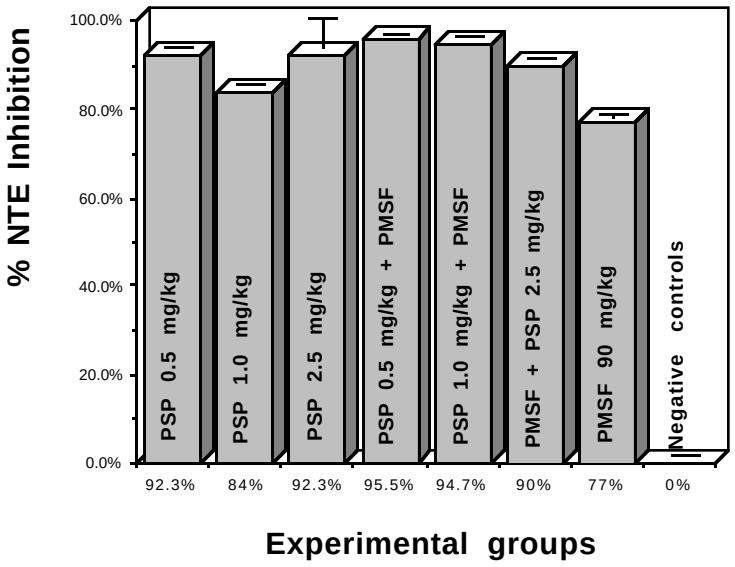


Figure 7 : Percentage of neuropathy target esterase (NTE) inhibition in the brain 24 hours post-dosing, in all groups (mean ± SD). The X numbers represent the exact percentage of NTE inhibition in each groups. The percentages of NTE inhibition were high in all groups, except for negative controls. PSP and PMSF represent phenyl saligenin phosphate and phenylmethylsulfonyl fluoride respectively.

Percentage of NTE inhibition 24 hours post dosing-spinal cord

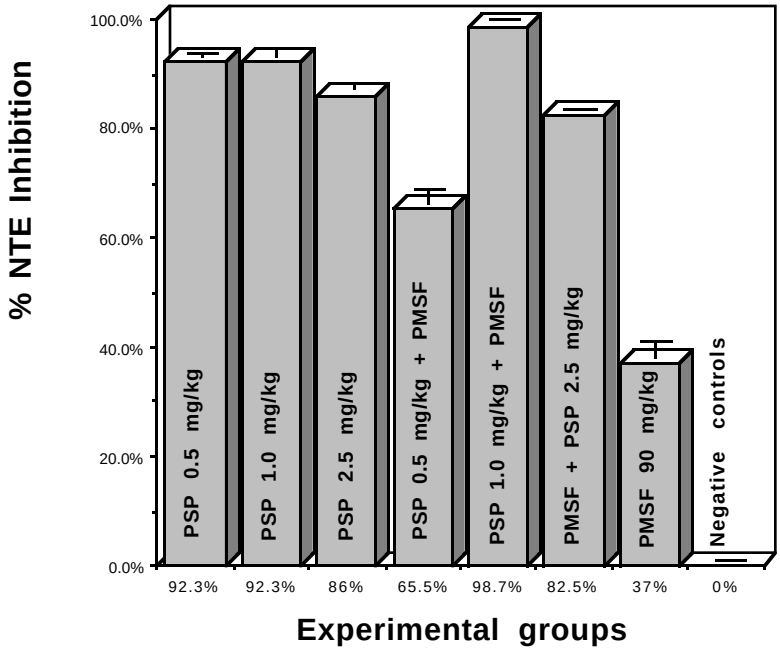


Figure 8 : Percentage of neuropathy target esterase (NTE) inhibition in the cervical spinal cord 24 hours post-dosing, in all groups (mean ± SD).). The X numbers represent the exact percentage of NTE inhibition in each groups. The percentages of NTE inhibition were high in all groups except for PMSF use alone and the negative controls. PSP and PMSF represent phenyl saligenin phosphate and phenylmethylsulfonyl fluoride respectively.

B. Clinical scores.

In the study the positive controls (1.0 - 2.5 mg/kg of PSP) and the hens given PMSF as a promotor of PSP-induced delayed neuropathy developed significant clinical signs. In affected hens, there was a post-dosing period of approximately 8 days, in which no evidence of neuropathy was noted. This was followed by signs encountered among the test groups. Clinical signs started with mild incoordination, progressing with time to flexion of the hock and difficulty in standing upright. At time of sacrifice, severely affected birds showed marked ataxia and total inability to rise or move. There was a dose-effect observed as the concentration of PSP increased, since more severe clinical signs were present at higher PSP dosages for both PSP used alone, and also when PMSF was used concomitantly as a promotor (see below and Tables 3-10, Figures 9-20).

1. Control and PMSF (90 mg/kg) groups.

In the control group, no clinical signs were observed at any time for any birds (mean clinical score = 0; n = 4-8), as reported in Table 7 and Figure 13. In addition, PMSF did not cause clinical signs by itself. The mean clinical scores for the PMSF 90 mg/kg dosed hens were minimal (< 0.5) until day 8 post-dosing (n=4-8). After this time, no clinical signs were observed in any birds until sacrifice (mean clinical score = 0; n=8 or n=4), as shown in Table 6 and Figure 12. The clinical scores of the control group and the PMSF 90 mg/kg group were not statistically different during the course of this study (day 2 - day 15-16).

2. Effects of PSP administration (0.5; 1.0; 2.5 mg/kg) on clinical scores.

In hens receiving the 3 doses of PSP, there was a post-dosing preclinical period of 8 days (2.5 mg/kg [high dose] group), following which there was a divergence of mean clinical scores (Tables 3-5, Figures 9-11,17). This separation was most marked between the high and mid-dose groups (2.5 and 1.0 mg/kg) as compared to low dose and controls (0.5 and 0 mg/kg). Significant differences of the mean clinical scores between PSP 1.0 mg/kg (mean clinical score = 2.5 ± 1.0; n=4) and PSP 2.5 mg/kg (mean clinical score = 3.8 ± 1.0; n=4) occurred at day 11 post-dosing ($p<0.0001$; alpha = 0.05). As a general observation, the clinical scores for PSP 1.0 mg/kg and for PSP 2.5 mg/kg progressed similarly over time (Figures 9,10). The progression of clinical score are shown for PSP 0.5 mg/kg (Table 4; Figure 11), for PSP 1.0 mg/kg (Table 4; Figure 10), and for PSP 2.5 mg/kg (Table 3; Figure 9). Comparison of mean clinical scores over time between the three treatment groups (0,5 - 1.0 - 2.5 mg/kg) is presented in Figure 17.

By the end of the study (15-16 days post PSP dosing), the clinical scores among the 3 groups of hens given PSP (0.5; 1.0; 2.5 mg/kg) were significantly different ($p< 0.0001$; $\propto = 0.05$). At day 15 post-dosing, hens given the highest dosage of PSP alone (2.5 mg/kg) had the most profound clinical deficits, with marked difficulty or total inability to move (mean clinical scores = 7.5 ± 1.0), as reported in Table 3. At this dosage, the clinical scores were almost maximal on the 0-8 clinical scale score we employed (see Clinical Score Scale - Experimental Design and Methods). At the end of this study, the hens dosed with 1.0 mg/kg PSP had a mean clinical score of 5.5, which was significantly higher ($p< 0.0001$) than the control group (mean clinical score = 0; $p< 0.0001$) as shown

in Table 4. No clinical effects were observed when PSP was administered at 0.5 mg/kg, since hens of this group had a mean clinical score of 0 (no clinical effect) at the time of sacrifice (Table 5). Since no clinical signs were reported with the PSP 0.5 mg/kg dosage, its mean clinical score was similar to the negative controls (mean clinical score = 0).

3. Protection - PMSF 90 mg/kg followed by PSP 2.5 mg/kg

In general, the birds given PMSF 12 hours prior to 2.5 mg/kg of PSP were protected from the clinical signs previously shown to be induced by that dosage of the neurotoxicant alone ($p<0.0001$; alpha = 0.05). Specifically, there were no significant differences between the negative controls and this group protected by PMSF 90 mg/kg at any time, and at the end of the study (day 16 post-dosing), the mean clinical score for these was 0 (Tables 7,8, Figures 13,14). On the other hand, comparison of terminal mean clinical scores between the PMSF 90 mg/kg + PSP 2.5 mg/kg group (mean clinical score = 0; n=5), and PSP 2.5 mg/kg (unprotected) group (mean clinical score = 7.5; n=4) showed significant statistical differences ($p<0.0001$; alpha = 0.05) (Tables 3,8, Figures 9,14). This was also true at day 9 of the study when the protected group had a mean clinical score of 0.125 (n=8) compared to 1.125 (n=8) ($p<0.0001$; alpha = 0.05) in hens given 2.5 mg/kg PSP alone. Temporal comparison between the hens dosed with PSP at 2.5 mg/kg alone and the related protected group are presented in Figure 18. At day 15-16, no significant difference between clinical scores were noted between groups of hens given PMSF 90 mg/kg only, hens protected from PSP (2.5 mg/kg) with PMSF (90 mg/kg), and negative controls (Tables 6-8, Figures 12-14).

4. Potentiation - PSP 0.5 or 1.0 mg/kg followed by PMSF 90 mg/kg.

The use of PMSF 90 mg/kg 4 hours following PSP administration significantly enhanced or promoted the clinical signs, compared to the use of PSP alone at the same dosage. At the end of this study (day 15), the mean clinical score of the PSP 0.5 mg/kg + PMSF 90 mg/kg group was 4.0 (n=4), which was statistically higher ($p<0.0001$; alpha = 0.05) than when PSP 0.5 mg/kg was used alone (mean clinical score = 0; n=4) (Tables 5,10, Figures 11,16). Hens given PSP 1.0 mg/kg + PMSF 90 mg/kg had a terminal mean clinical score of 7.0 (n=4) which was statistically higher ($p<0.0001$; alpha = 0.05) than hens given only PSP 1.0 mg/kg (mean clinical score = 5.5; n=4) (Tables 4,9, Figures 10,15). Potentiation of clinical signs seen with PSP (0.5 mg/kg and 1.0 mg/kg) by subsequent administration of PMSF was initially noted on day 8 (Figure 20). At day 15-16 post-dosing, there was no statistical difference between the hens given PSP 2.5 mg/kg (mean clinical score = 7.5; n=4), where lesions were considered maximal, and the hens challenged with PSP at 1.0 mg/kg followed by PMSF 90 mg/kg (mean clinical score = 7.0 ; n=4) (Tables 3,9). Furthermore, the mean clinical score at the time of terminal sacrifice for the hens given PSP 0.5 mg/kg + PMSF 90 mg/kg was significantly lower than the score of hens given PSP 1.0 mg/kg + PMSF 90 mg/kg ($p<0.0001$; alpha = 0.05) (Tables 9,10). Comparisons of mean clinical scores over time, between hens challenged with either PSP 0.5 mg/kg or PSP 1.0 mg/kg followed by PMSF 90 mg/kg are presented in Figure 20.

Table 3: *Clinical scores* vs *day post-dosing* (PSP 2.5 mg/kg).

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 5	0;0;0;0;0	0.0
3	0.0	n = 5	0;0;0;0;0	0.0
4	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
5	0.1	n = 8	0;0;0;0;0;0;1;0	0.4
6	0.1	n = 8	0;0;0;0;0;1;0;0	0.4
7	0.4	n = 8	0;0;0;1;0;2;0;0	0.7
8	0.4	n = 8	0;0;0;1;0;2;0;0	0.7
9	1.1	n = 8	0;1;0;2;2;2;0;2	1.0
10	2.5	n = 4	1;3;2;4	1.3
11	3.8	n = 4	3;4;3;5	1.0
12	4.8	n = 4	7;4;3;5	1.7
13	7.0	n = 4	8;6;7;7	0.8
14	7.3	n = 4	8;6;7;8	1.0
15	7.5	n = 4	8;6;8;8	1.0

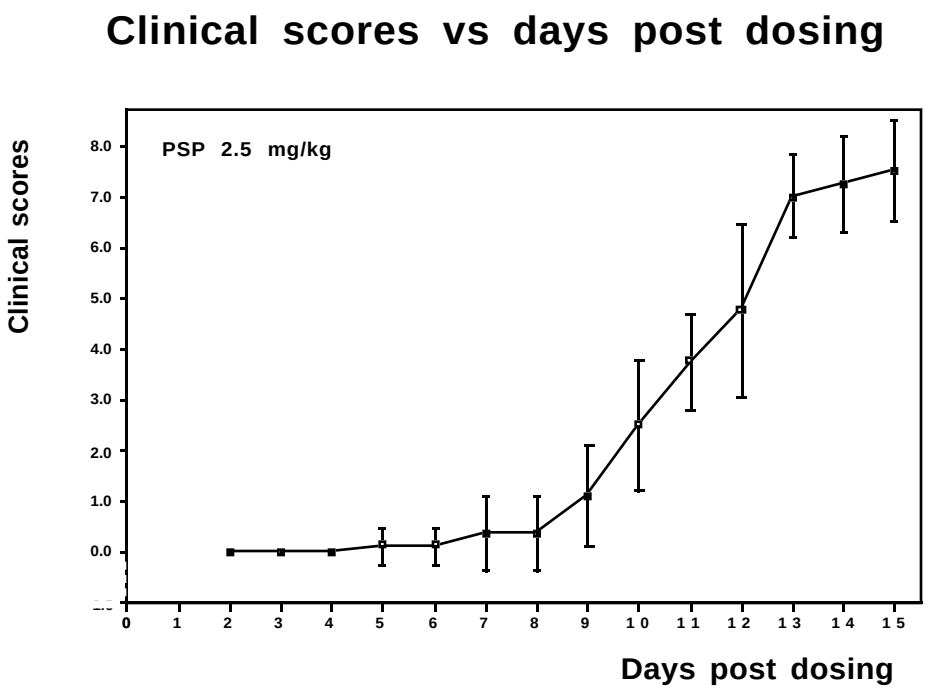


Figure 9: Mean clinical scores (0-8) after administration of phenyl saligenin phosphate (PSP) 2.5 mg/kg. At this dosage, there was a post-dosing preclinical period of 8 days, emphasizing the delayed nature of this neuropathy, then the clinical score increased with time (mean ± SD; n=4-8). At the end of the study, the mean terminal clinical score was almost maximal (7.5 ±1.0). Individual scores are shown in table 3.

Table 4 : ***Clinical scores*** **vs** ***day post-dosing*** **(PSP 1.0 mg/kg).**

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 4	0;0;0;0	0.0
3	0.0	n = 4	0;0;0;0	0.0
4	0.3	n = 8	0;2;0;0;0;0;0;0	0.7
5	0.3	n = 8	0;2;0;0;0;0;0;0	0.7
6	0.3	n = 8	0;2;0;0;0;0;0;0	0.7
7	0.5	n = 8	0;2;0;2;0;0;0;0	0.9
8	0.5	n = 8	0;2;0;2;0;0;0;0	0.9
9	1.1	n = 8	0;2;0;3;0;2;2;0	1.0
10	2.3	n = 4	1;3;3;2	1.0
11	2.5	n = 4	2;3;3;2	1.0
12	2.5	n = 4	2;3;3;2	1.0
13	3.0	n = 4	3;4;3;2	1.0
14	4.5	n = 4	4;4;7;3	2.0
15	5.5	n = 4	6;5;7;4	2.0

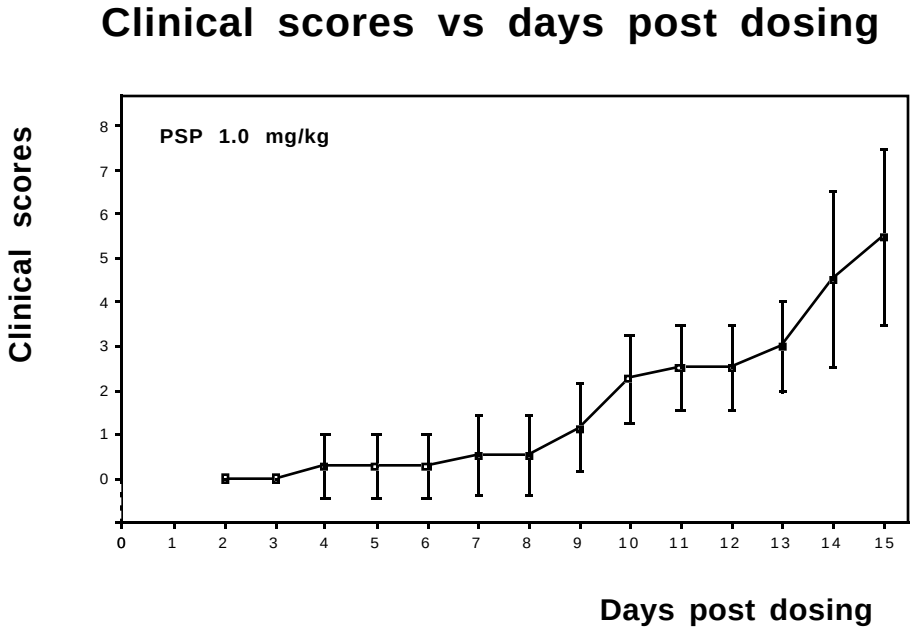


Figure 10 : Mean clinical scores (0-8) after administration of phenyl saligenin phosphate (PSP) 1.0 mg/kg. At this dosage, there was a post-dosing preclinical period of 8 days, emphasizing the delayed nature of this neuropathy, then the clinical score increased with time (mean ± SD; n=4-8). The mean terminal clinical score was 5.5 ± 2.0 at day 15 post-dosing. Individual scores are shown in table 3.

Table 5 : *Clinical scores* vs *day post-dosing* (PSP 0.5 mg/kg).

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
3	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
4	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
5	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
6	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
7	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
8	0.3	n = 8	0;1;1;0;0;0;0;0	0.0
9	0.3	n = 8	0;1;1;0;0;0;0;0	0.5
10	0.0	n = 4	0;0;0;0	0.0
11	0.0	n = 4	0;0;0;0	0.0
12	0.0	n = 4	0;0;0;0	0.0
13	0.0	n = 4	0;0;0;0	0.0
14	0.0	n = 4	0;0;0;0	0.0
15	0.0	n = 4	0;0;0;0	0.0

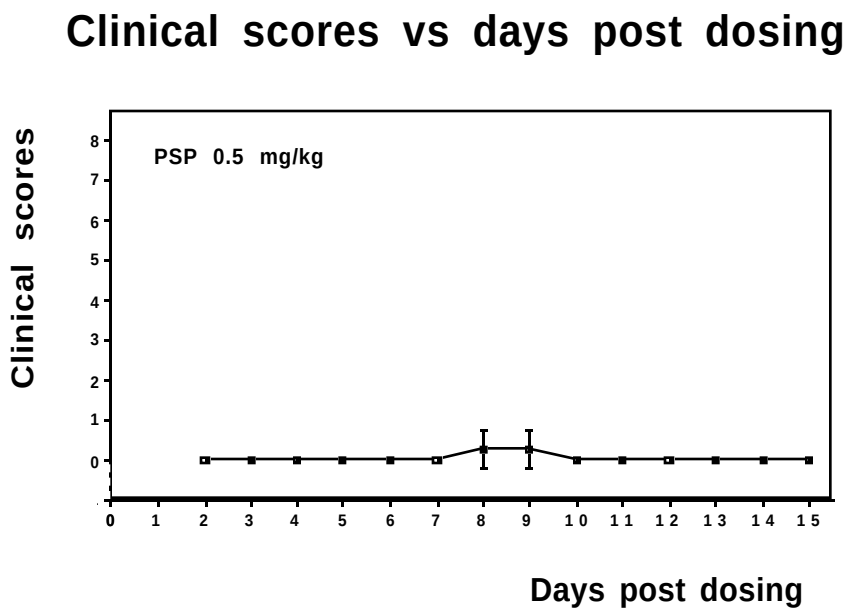


Figure 11 : Mean clinical scores (0-8) after administration of phenyl saligenin phosphate (PSP) 0.5 mg/kg. No clinical effects were observed when this dosage of PSP was administered (mean ± SD). Thus, PSP 0.5 mg/kg was the no observed effect level. Mean individual scores are shown in table 5.

Table 6 : *Clinical scores* vs day(s) *post-dosing* (PMSF 90 mg/kg)

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 4	0;0;0;0	0.0
3	0.5	n = 4	0;0;0;2	1.0
4	0.3	n = 8	0;0;0;0;0;0;0;2	0.7
5	0.3	n = 8	0;0;0;0;0;0;0;2	0.7
6	0.1	n = 8	0;0;0;0;0;0;0;1	0.4
7	0.3	n = 8	0;1;0;0;0;0;0;1	0.5
8	0.3	n = 8	0;1;0;0;0;0;0;1	0.5
9	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
10	0.0	n = 4	0;0;0;0	0.0
11	0.0	n = 4	0;0;0;0	0.0
12	0.0	n = 4	0;0;0;0	0.0
13	0.0	n = 4	0;0;0;0	0.0
14	0.0	n = 4	0;0;0;0	0.0
15	0.0	n = 4	0;0;0;0	0.0
16	0.0	n = 4	0;0;0;0	0.0

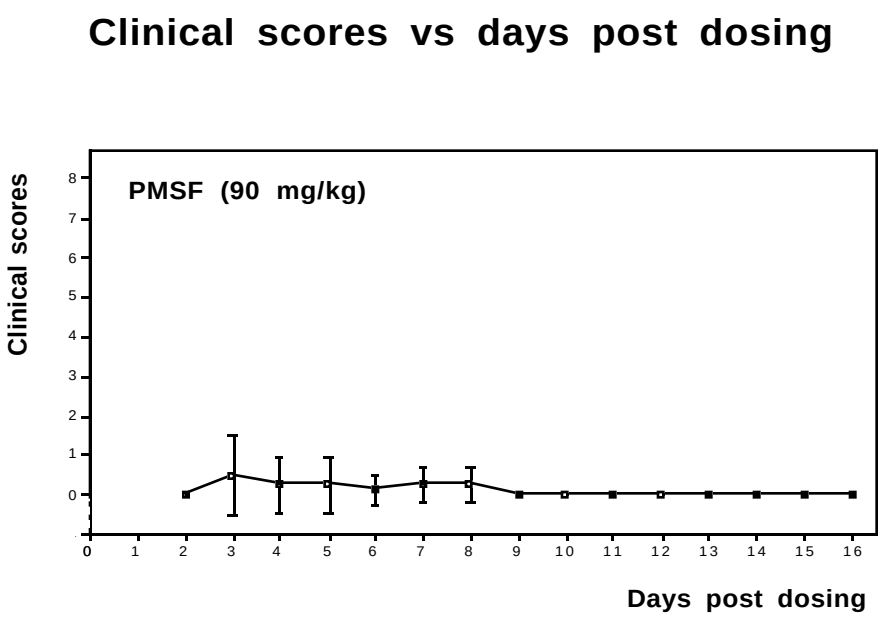


Figure 12 : Mean clinical scores (0-8) after administration of phenylmethylsulfonyl fluoride (PMSF) 90 mg/kg. The mean clinical scores were minimal (< 0.5) until day 8 post-dosing (mean ± SD; n=4-8). After this time, no clinical signs were observed in any hens until the day of sacrifice (n=4). Individual scores are shown in table 6.

Table 7 : *Clinical scores* vs *day post-dosing* (negative controls)

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0	n = 8	0;0;0;0;0;0;0;0	0
3	0	n = 8	0;0;0;0;0;0;0;0	0
4	0	n = 8	0;0;0;0;0;0;0;0	0
5	0	n = 8	0;0;0;0;0;0;0;0	0
6	0	n = 8	0;0;0;0;0;0;0;0	0
7	0	n = 8	0;0;0;0;0;0;0;0	0
8	0	n = 8	0;0;0;0;0;0;0;0	0
9	0	n = 8	0;0;0;0;0;0;0;0	0
10	0	n = 4	0;0;0;0	0
11	0	n = 4	0;0;0;0	0
12	0	n = 4	0;0;0;0	0
13	0	n = 4	0;0;0;0	0
14	0	n = 4	0;0;0;0	0
15	0	n = 4	0;0;0;0	0
16	0	n = 4	0;0;0;0	0

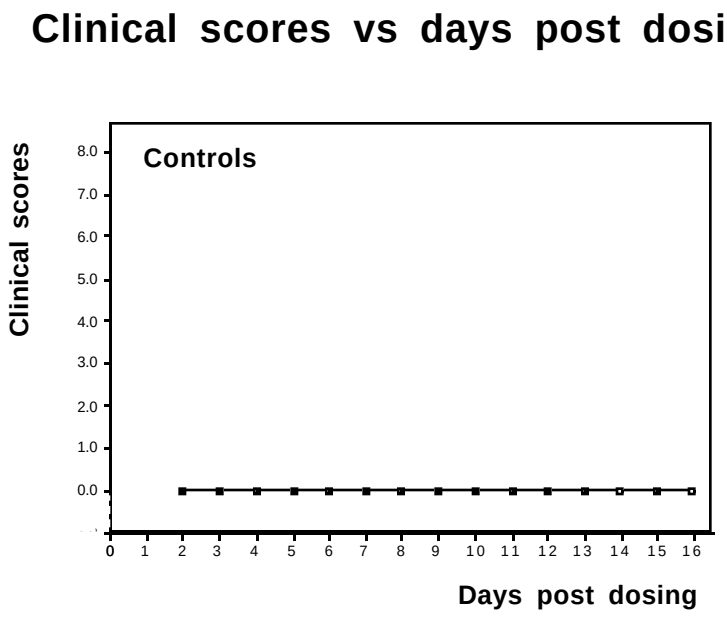


Figure 13 : Mean clinical scores (0-8) for the negative control group. In the negative control group, no clinical signs were observed at any time, in any hens (mean clinical score = 0). Individual scores are shown in table 7.

Table 8 : *Clinical score* **vs** *day(s) post-dosing*
(PMSF 90 mg/kg + PSP 2.5 mg/kg)

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 4	0;0;0;0	0.0
3	0.0	n = 4	0;0;0;0	0.0
4	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
5	0.4	n = 8	0;0;0;0;1;1;0;1	0.5
6	0.4	n = 8	0;0;0;0;1;1;0;1	0.5
7	0.4	n = 8	1;0;0;0;0;1;0;1	0.5
8	0.1	n = 8	1;0;0;0;0;0;0;0	0.4
9	0.1	n = 8	1;0;0;0;0;0;0;0	0.4
10	0.2	n = 5	1;0;0;0;0	0.4
11	0.2	n = 5	1;0;0;0;0	0.4
12	0.0	n = 5	0;0;0;0;0	0.0
13	0.2	n = 5	0;1;0;0;0	0.4
14	0.0	n = 5	0;0;0;0;0	0.0
15	0.0	n = 5	0;0;0;0;0	0.0

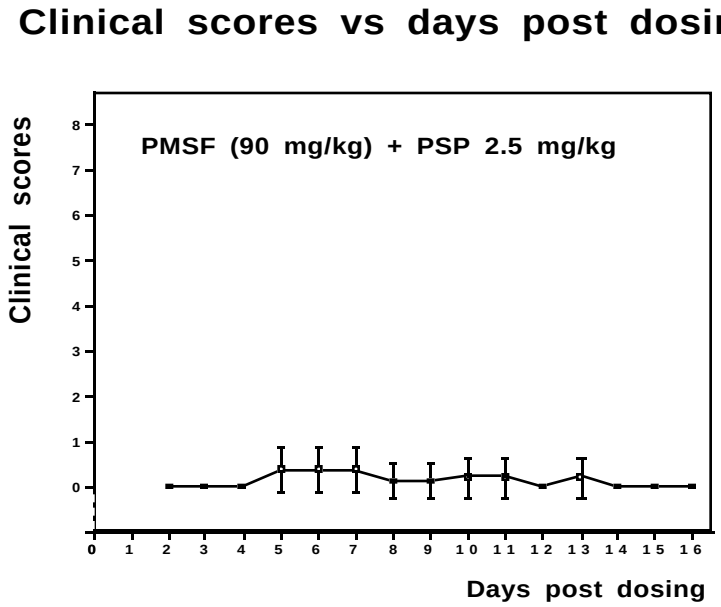


Figure 14 : Mean clinical scores (0-8) for the hens where phenylmethylsulfonyl fluoride (PMSF) 90 mg/kg was administered followed by a dose of phenyl saligenin phosphate (PSP) at 2.5 mg/kg (mean ± SD). The hens given PMSF 12 hours prior to 2.5 mg/kg of PSP were protected from the clinical signs previously shown to be induced by PSP 2.5 mg/kg. The mean terminal clinical score was 0 at day 15 post-dosing (n=5). Individual scores are shown in table 8.

Table 9 : *Clinical scores* **vs** *day(s) post-dosing*
(PSP 1.0 mg/kg + PMSF 90 mg/kg)

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 3	0;0;0	0.0
3	0.0	n = 3	0;0;0	0.0
4	0.1	n = 7	0;0;0;0;0;1	0.4
5	0.1	n = 7	0;0;0;0;0;1	0.4
6	0.3	n = 7	1;0;0;0;1;0	0.5
7	0.7	n = 7	2;1;1;0;0;0	0.8
8	1.6	n = 7	2;3;1;0;0;0	1.2
9	2.4	n = 7	3;3;2;2;3;2	0.5
10	2.5	n = 4	3;2;3;2	0.6
11	4.8	n = 4	6;6;3;4	1.5
12	5.0	n = 4	7;6;3;4	1.8
13	6.0	n = 4	7;7;5;5	1.2
14	6.5	n = 4	7;7;6;6	0.6
15	7.0	n = 4	7;7;7;7	0.0
16	7.5	n = 4	7;7;8;8	0.6

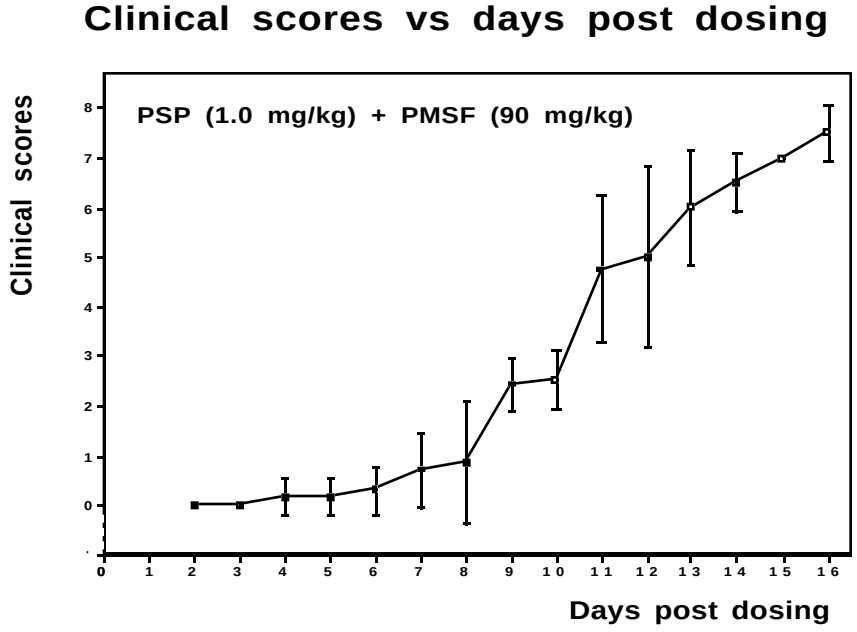


Figure 15 : Mean clinical scores (0-8) when phenylmethylsulfonyl fluoride (PMSF) was administered 4 hours after a dose of phenyl saligenin phosphate (PSP) 1.0 mg/kg. At this dosage, there was a post-dosing preclinical period of 7 days, emphasizing the delayed nature of this neuropathy, then the clinical score increased with time (mean ± SD; n=4-8). At the end of the study, the mean terminal clinical score was almost maximal (7.5 ± 0.5). Individual clinical scores are shown in table 9.

Table 10 : *Clinical scores* **vs** *day(s) post-dosing*
(PSP 0.5 mg/kg + PMSF 90 mg/kg)

Day post-dosing	Mean clinical scores	Number of hens(n)	Individual scores	Standard deviation
2	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
3	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
4	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
5	0.0	n = 8	0;0;0;0;0;0;0;0	0.0
6	0.4	n = 8	0;2;0;0;0;0;0;0	0.7
7	0.4	n = 8	0;2;0;0;0;0;0;0	0.7
8	1.6	n = 8	2;3;2;2;1;1;1;1	0.7
9	2.4	n = 8	2;3;2;2;2;1;1;1	0.7
10	1.5	n = 4	2;2;1;1	0.6
11	2.0	n = 4	2;3;2;1	0.8
12	3.0	n = 4	3;3;4;2	0.8
13	3.3	n = 4	3;3;4;3	0.5
14	3.8	n = 4	4;4;4;3	0.5
15	4.0	n = 4	4;4;4;4	0.0

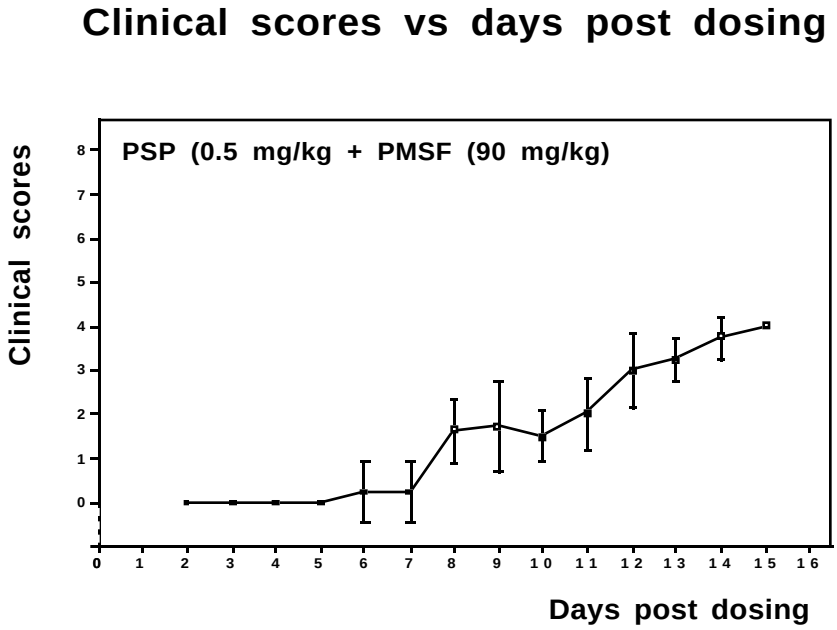


Figure 16 : Mean clinical scores (0-8) when phenylmethylsulfonyl fluoride (PMSF) was administered 4 hours after a dose of phenyl saligenin phosphate (PSP) 0.5 mg/kg. At this dosage, there was a post-dosing preclinical period of 8 days, emphasizing the delayed nature of this neuropathy, then the clinical score increased with time (mean ± SD; n=4-8). The mean terminal clinical score was 4.0 at day 15 post-dosing. Individual clinical scores are shown in table 10.

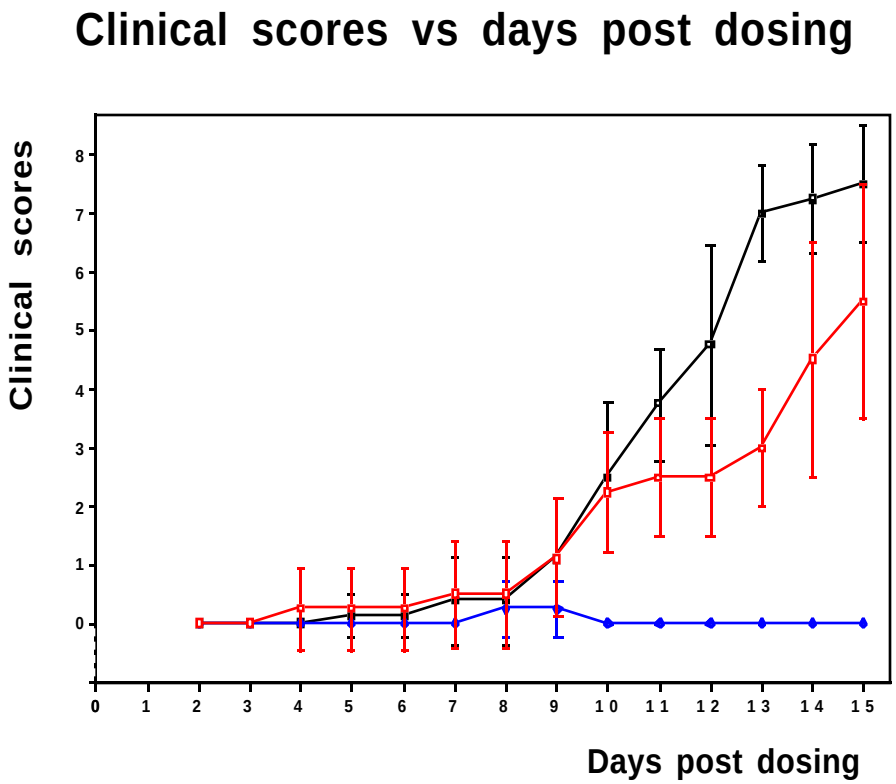


Figure 17 : Comparison between the clinical scores (0-8) after phenyl saligenin phosphate (PSP) 0.5 mg/kg, 1.0 mg/kg, or 2.5 mg/kg. The terminal clinical score increased, relative to the dosage of PSP (mean ± SD). There was a post-dosing period of 8 days, following which there was divergence of mean clinical scores. This initial separation was most marked between the PSP 1.0 mg/kg and 2.5 mg/kg as compared to the low dose of PSP (0.5 mg/kg). Significant differences of the mean clinical scores between PSP 1.0 mg/kg and PSP 2.5 mg/kg occurred at day 11 post-dosing. The lowest dose of PSP (0.5 mg/kg) had no discernible clinical effect (mean terminal clinical score = 0). The number of hens in each groups at specific times are given in Tables 3, 4, and 5.

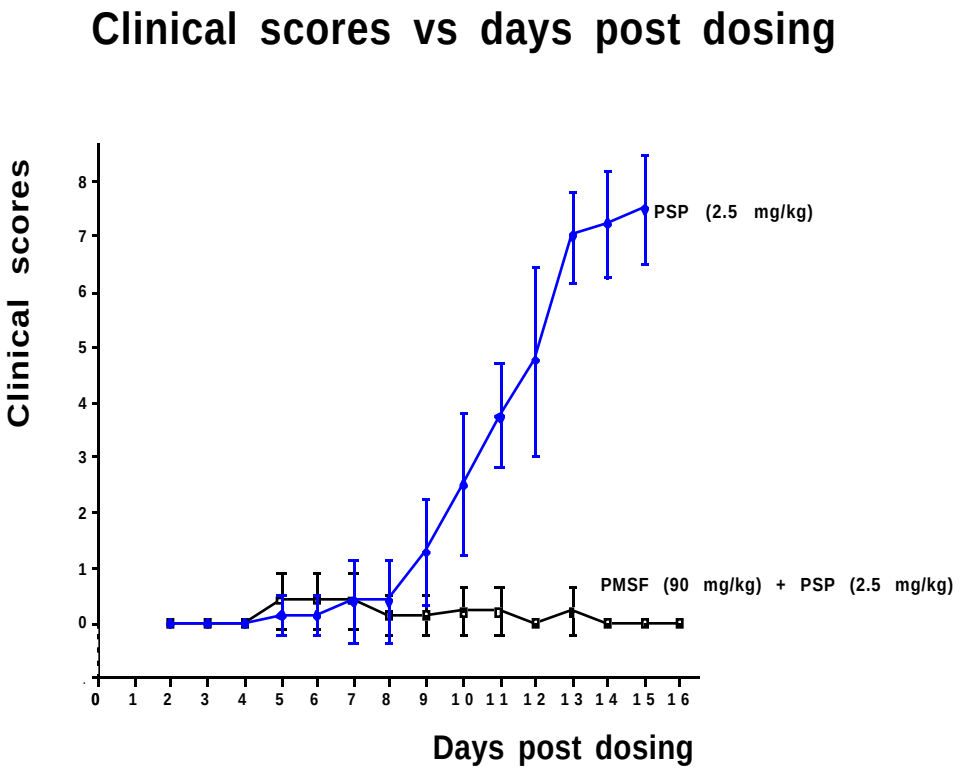


Figure 18 : Comparison between the clinical scores (0-8) obtained with phenyl saligenin phosphate (PSP) 2.5 mg/kg used alone, and PSP 2.5 mg/kg following phenylmethylsulfonyl fluoride (PMSF) 90 mg/kg administration. Significant differences were noted from day 9 post-dosing ($p = 0.0001$; $\alpha = 0.05$). These results shown significant protection against clinical signs commonly induced by PSP 2.5 mg/kg alone (mean ± SD). The number of hens in each groups at specific times are given in Tables 3, and 8.

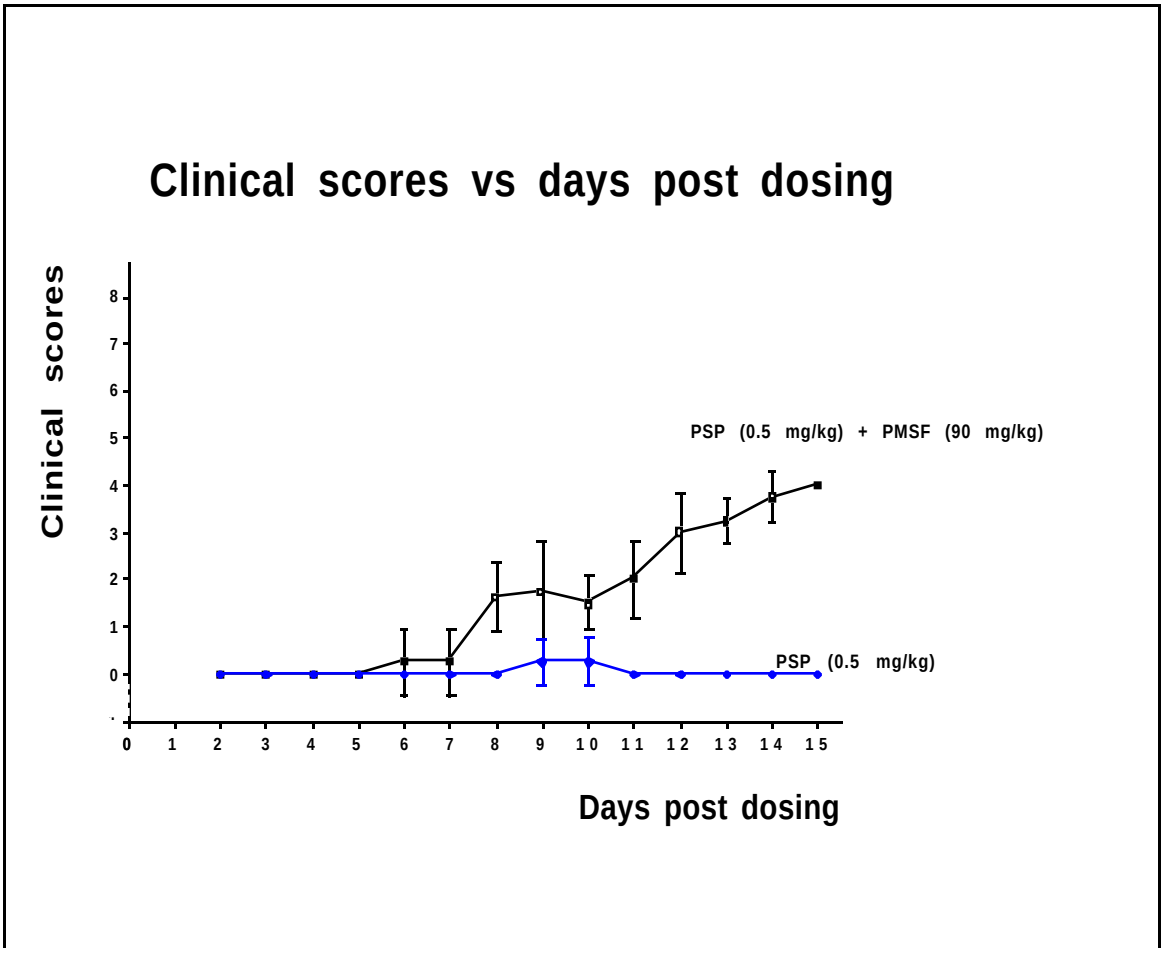


Figure 19 : Comparison between the clinical scores (0-8) obtained with phenyl saligenin phosphate (PSP) 0.5 mg/kg used alone, and when PSP 0.5 mg/kg was followed by administration of phenylmethylsulfonyl fluoride (PMSF) 90 mg/kg (mean ± SD). Clinical signs of OPIDN was elicited when PMSF was administered 4 hours after the subclinical dose of PSP 0.5 mg/kg. Potentiation of clinical signs seen with PSP 0.5 mg/kg by subsequential administration of PMSF was initially noted on day 8 post-dosing. At the end of the study, the mean clinical score of the promoted group (mean clinical score = 4.0; n=4) was statistically different ($p = 0.0001$; $\propto = 0.05$) than when PSP 0.5 mg/kg was used alone (mean clinical score = 0; n = 4). The number of hens in each groups at specific times are given in Tables 5, and 10.

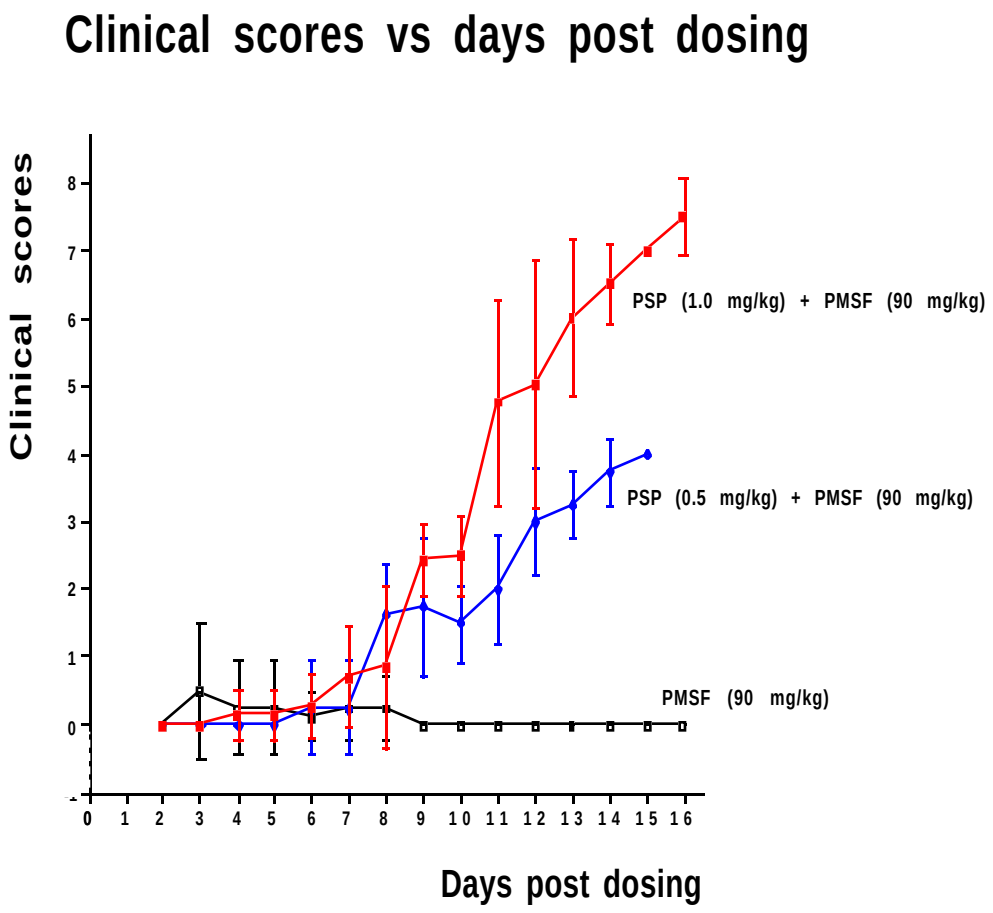


Figure 20 : Comparison between the mean clinical scores of promoted groups (PSP 0.5 mg/kg or PSP 1.0 mg/kg, followed by PMSF 90 mg/kg). Potentiation of clinical signs seen with phenyl saligenin phosphate (PSP) 0.5 or 1.0 mg/kg, by subsequential administration of phenylmethylsulfonyl fluoride (PMSF), was initially noted on day 8 post-dosing. At day 15 post-dosing, the mean clinical scores for hens given PSP 0.5 mg/kg + PMSF was significantly lower than the scores of hens given PSP 1.0 mg/kg followed by PMSF. These values indicate that the terminal clinical scores were related to the initial PSP dosages (mean ± SD). No significant clinical effect was seen in hens dosed only with PMSF. The number of hens in each groups at specific times are given in Tables 6, 9, and 10.

C. Qualitative morphologic evaluation.

1. Light microscopic evaluation in cross sections.

Light microscopic evaluation of cross sections of distal regions of biventer cervicis nerves revealed a range of degenerative changes observed in treated hens on post - dosing days 9 and 15-16, when compared to a normal biventer cervicis nerve obtained from a negative control (Figure 21). As typical for OPIDN, distal axonopathy associated with Wallerian-like degeneration was the most consistent morphological change detected. These changes were exemplified in the PSP positive controls (PSP 1.0 or 2.5 mg/kg), and included large swollen myelinated axons associated with pallor of staining and intra-axonal debris to complete replacement of degenerated myelinated fibers by columns of proliferating Schwann cells known as bands of Buengner. Thinning and fragmentation of the myelin sheath, with formation of myelin ovoids and axonal debris in surrounding Schwann cells were noted in sections where axons were undergoing Wallerian-like degeneration (Figures 22,23,24). Large phagocytic macrophages were apparent, surrounding more advanced degenerating fibers (Figures 23,24). These changes did not differ between affected groups.

Another interesting histopathological feature consisted of thick endoneurial collagen layers surrounding large degenerated myelinated axons (Figure 25). These collagen layers were observed around affected myelinated nerve fibers in birds treated with the high doses of PSP (1.0 mg/kg and 2.5 mg/kg), and in birds of the promoted groups (PSP 0.5 mg/kg + PMSF 90 mg/kg; PSP 1.0 mg/kg + PMSF 90 mg/kg). Development of such collagen structures was minimal with the low dose of PSP (0.5 mg/kg), in the group protected from OPIDN effects (PMSF 90 mg/kg + PSP 2.5 mg/kg), and when PMSF was used alone. The negative control group had none of these collagen structures. The amount of collagen

around affected fibers tended to increase with time, as the overall degeneration in the biventer cervicis nerves became more widespread and severe.

On post-dosing day 15-16, a few regenerating fibers were present in some nerves, where the original lesions were more severe (PSP 0.5 mg/kg + PMSF 90 mg/kg; PSP 1.0 mg/kg + PMSF 90 mg/kg). By light microscopy, these appeared as small non (pre)-or thinly-myelinated neurites. The exact characterization of the regeneration process was difficult to evaluate with light microscopy, because regenerating fibers could be confused with later stage of axonal degeneration (bands of Buengner), and normal small thinly-myelinated axons. At day 9 post-dosing, regenerative fibers were minimal to absent.

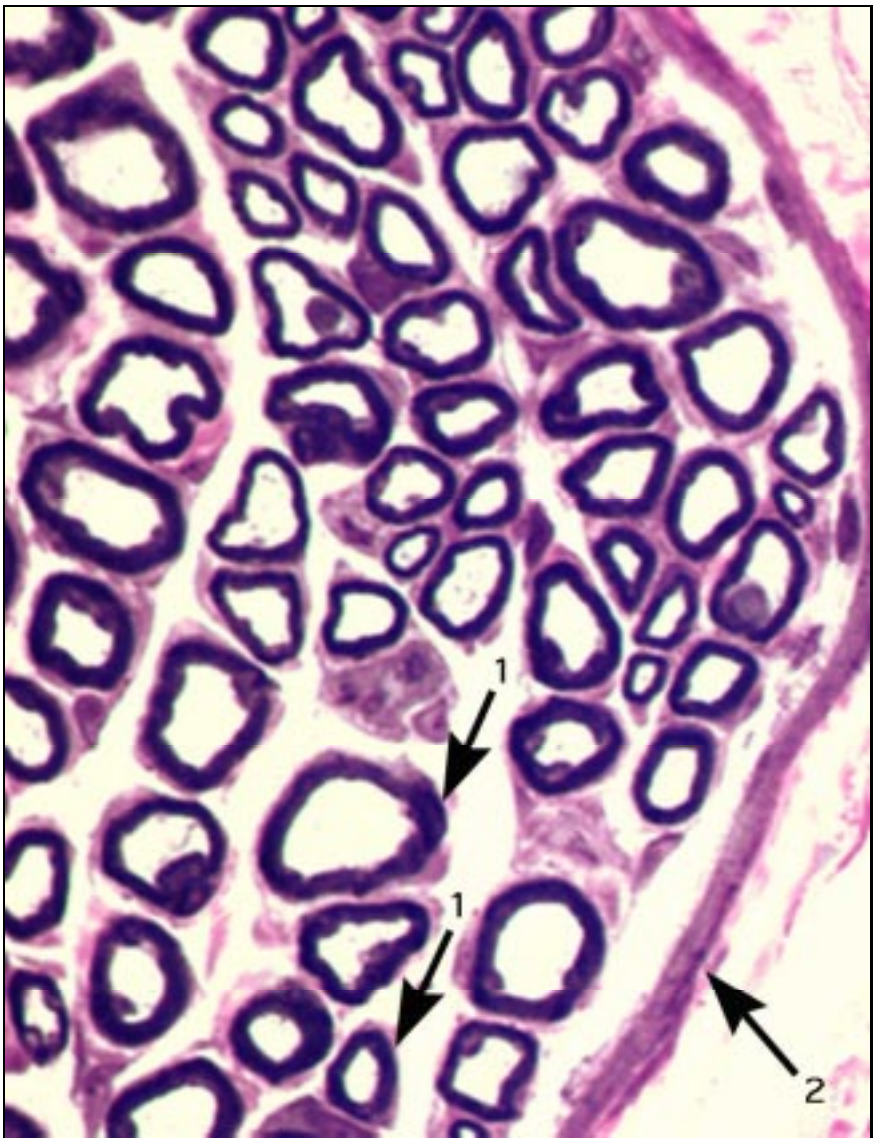


Figure 21: Cross section of the biventer cervicis nerve from a negative control hen, showing normal myelinated nerve fibers (1), and perineurium (2) (Toluidine blue - safranin stain - 250X).

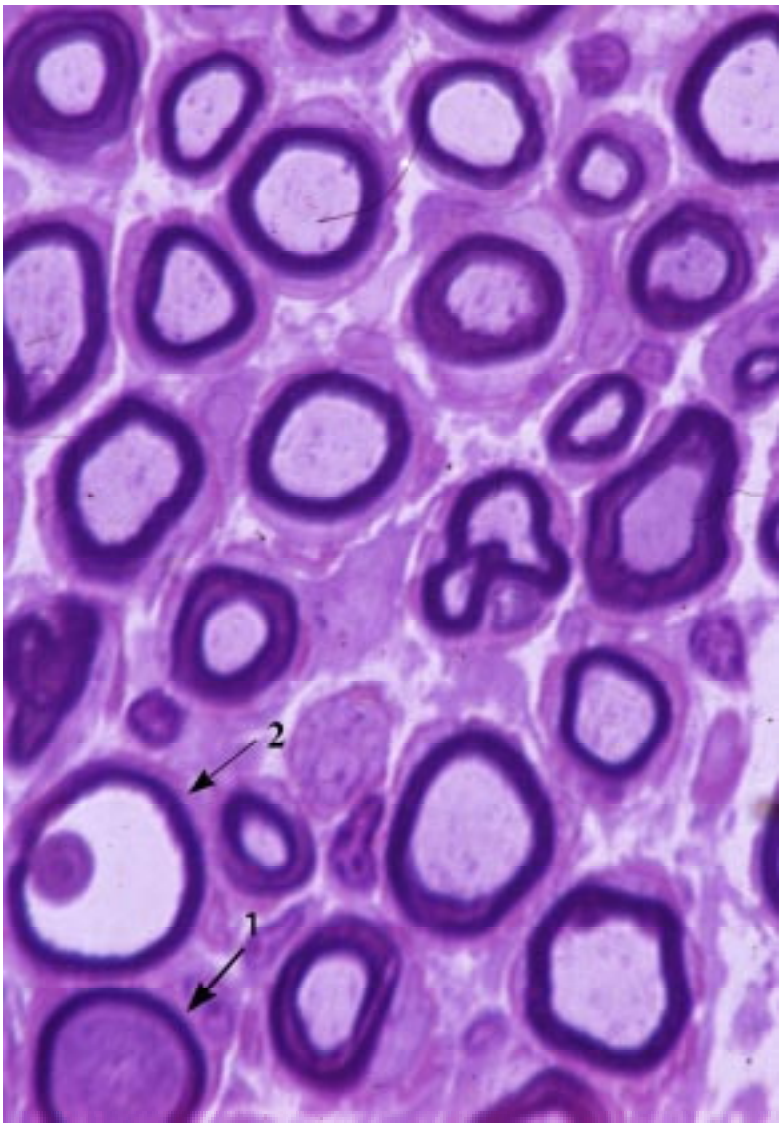


Figure 22 : Early stages of myelinated fiber degeneration in a hen dosed with PSP 1.0 mg/kg 9 days earlier. These include myelinated fibers with dark (1) or pale (2) stained swollen axons (Toluidine blue- safranin stain - 250X).

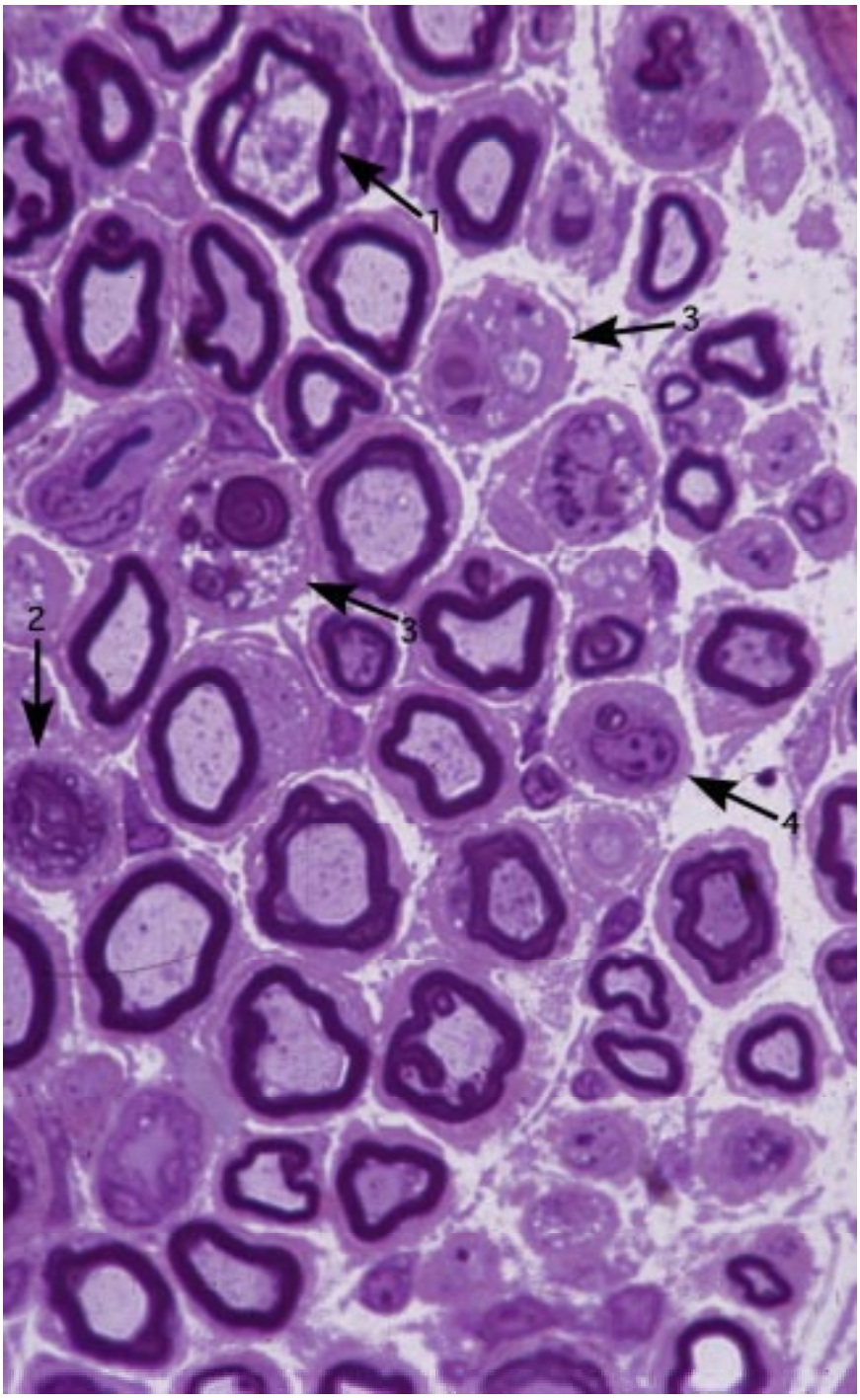


Figure 23 : Cross section of the biventer cervicis nerve from a positive control (PSP 2.5 mg/kg) at 15 days post - dosing, showing severe nerve fiber degeneration. Swollen axons (1), myelin ovoids (2), more advanced Wallerian-like degeneration (3), and bands of Buengner are seen (Toluidine blue- safranin stain - 250X).

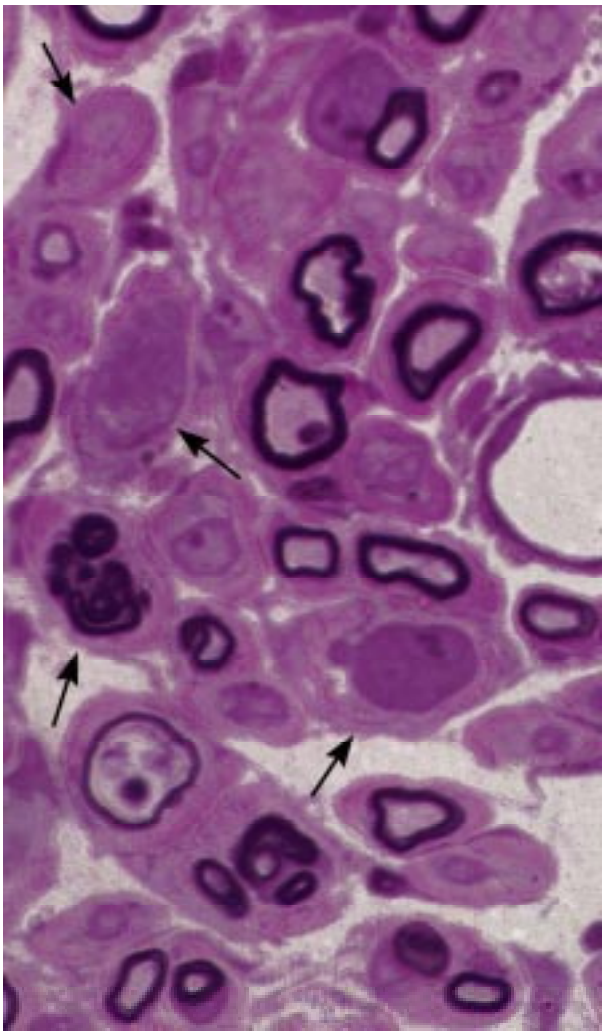


Figure 24: Promotion (0.5 mg/kg PSP followed by 90 mg/kg PMSF) at day 15 post-dosing showing extensive, often advanced myelinated fiber degeneration (arrows). Only a few (mainly small) myelinated fibers are intact (Toluidine blue - safranin stain - 250X).

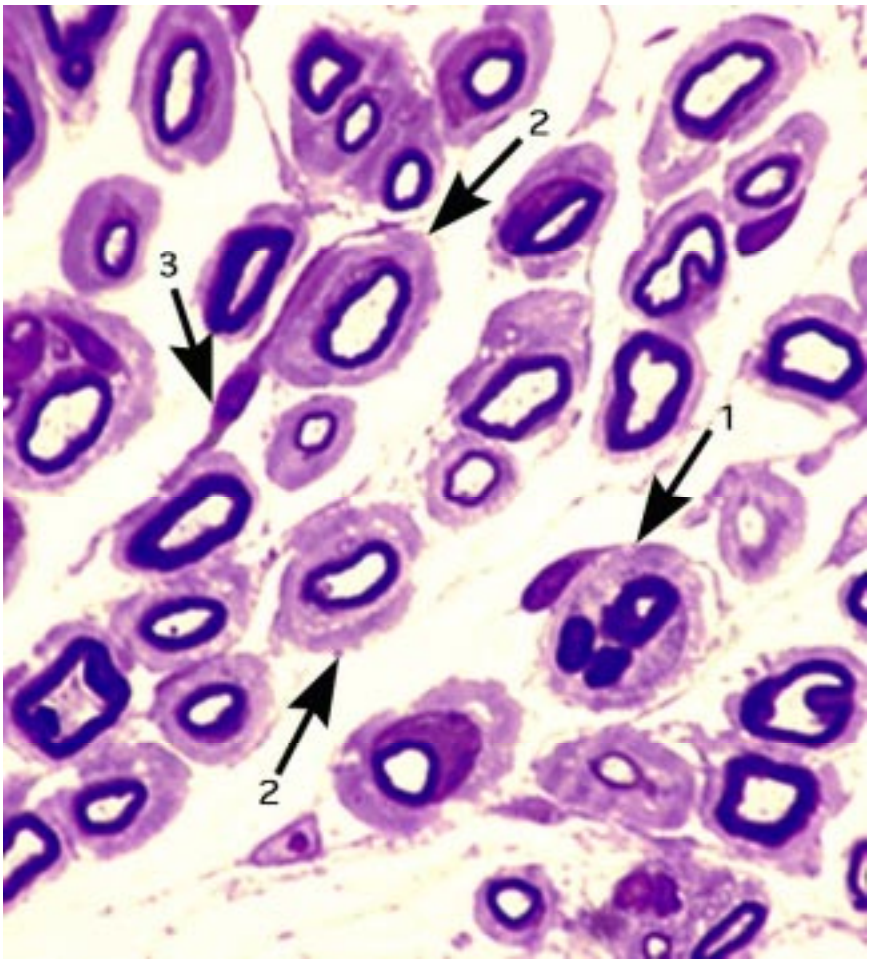


Figure 25: Nerve from a hen dosed with PSP 2.5 mg/kg 9 days earlier, showing occasional degenerating myelinated nerve fibers (1). Note the prominent pink-staining collars of collagen around many fibers two of which are indicated (2) and related hypertrophic endoneurial fibroblasts (3) (Toluidine blue - safranin stain - 250X).

2. Light microscopic evaluation of teased fibers.

Pathologic abnormalities of teased myelinated fibers were described by assessing the integrity of the myelin sheath, axonal swelling and myelin degenerative products. The pathologic changes observed were compared to negative controls in order to evaluate whether the teased fiber abnormalities exceeded what would be expected in the biventer cervicis nerve of adult hens. Internodal length and diameter were not measured.

At day 9 post dosing, only few degenerative changes were observed in the nerve fibers. A few affected fibers were observed in the positive controls, and the promoted groups. No degenerative fibers were detected in the negative controls. At day 15-`6 post dosing, qualitative degenerative changes were present more frequently than at day 9 post dosing. Nerve fiber degeneration was most severe in both the promoted groups and the positive control (PSP 2.5 mg/kg), absent in the negative controls (Figure 26), and minimal in the other groups (PMSF 90 mg/kg, protected, PSP 0.5 mg/kg). These pathologic abnormalities consisted of paranodal or intersegmental demyelination, axonal swelling, fragmentation of the fibers, and myelin ovoids (Figures 27,28,29). Some fiber segments were completely replaced by the ovoids. No qualitative differences in fiber degeneration among affected groups were noted.

Artifacts recognized during teased fiber evaluation included excessive stretching of the nerves during biopsy causing separation at the nodes of Ranvier, and splitting of the nerve fiber during teasing.

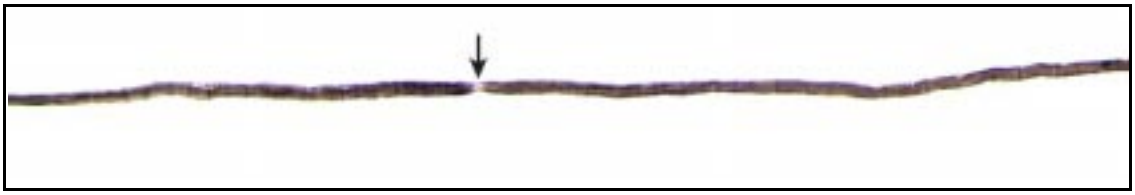


Figure 26 : Segment of a normal teased nerve fiber of the biventer cervicis nerve from a negative control hen at 15 days post - dosing. The arrow shows a node of Ranvier (50X).

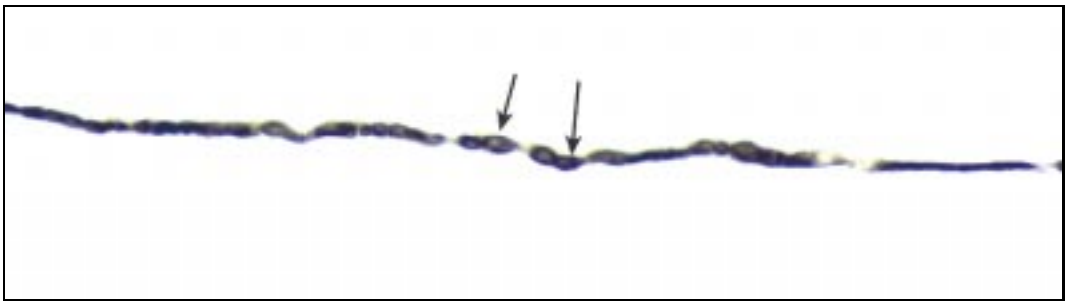


Figure 27 : Portion of a teased nerve fiber of the biventer cervicis nerve from a promoted hen (PSP 0.5 mg/kg followed by PMSF) at 15 day post-dosing. These pathologic abnormalities consisted of myelin ovoids, and axonal swelling (50X).

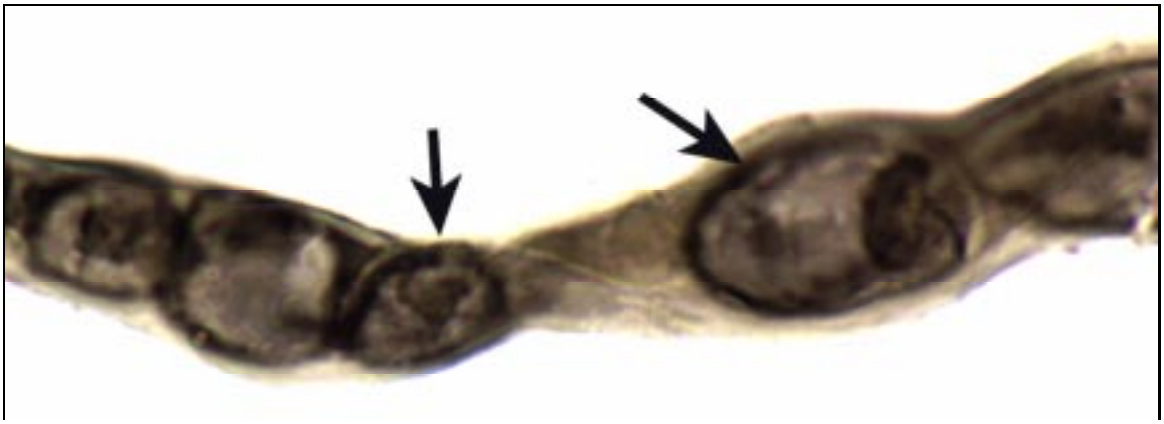


Figure 28: Advanced nerve fiber degeneration with myelin ovoids, axonal swelling and fiber breakdown observed in a teased nerve fiber of the biventer cervicis nerve from a hen of a promoted group (PSP 0.5 mg/kg followed by PMSF) at 15 days post - dosing (250X).

Figure 29 : Axonal swelling observed in a teased nerve fiber of the biventer cervicis nerve from a positive control (2.5 mg/kg) at 15 days post-dosing (250 X).

3. Electron microscopy.

As seen by transmission electron microscopy, an early stage of neuropathy in affected fibers of the biventer cervicis nerve consisted of striking hypertrophy and distortion of agranular-like reticulum with excess accumulation of branching cisternal membranous structures in affected axons (Figure 30). In addition, there was an increase in number of axonal mitochondria which subsequently underwent swelling and degeneration (Figure 31). As the neuropathologic changes progressed in the affected fibers, axons became swollen and contained concentrically arranged membranes, sometimes enclosing vesicles and degenerating mitochondria, dark lamellated osmiophilic inclusions, and small electron dense granules (Figures 31, 32). The remaining axoplasm was either rarefied and swollen, or coarse-granular and shrunken (Figures 30,31). Fibers then underwent fragmentation of axons and myelin sheaths within Schwann cells, forming myelin ovoids (Figures 32, 33). Proliferation of the Schwann basal membrane was detected in fibers undergoing Wallerian-like degeneration. Excess collagen was visualized around degenerating fibers (Figures 32, 33). Identification and differentiation among regenerating fibers, collapsed vessels, and bands of Buengner was also possible using electron microscopy. These changes were seen in both PSP-induced OPIDN and in promotion of that condition, with no qualitative differences among them.

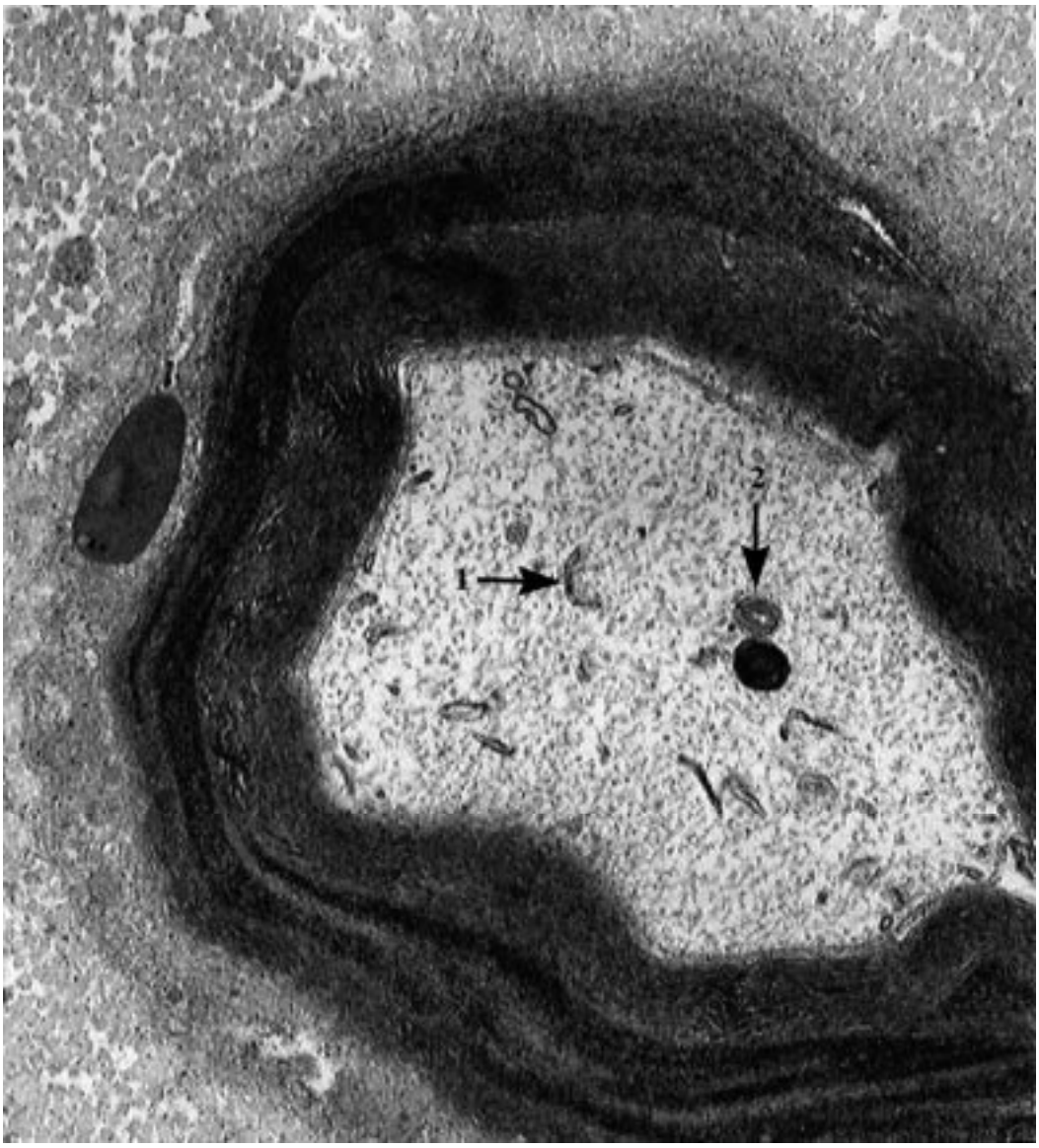


Figure 30: Cross section of a myelinated fiber in a promoted group (PSP 1.0 mg/kg + PMSF) at 16 days post-dosing showing proliferation of smooth endoplasmic reticulum-like tubules (1) and multilamellar bodies (2) within the axon (36,400X).

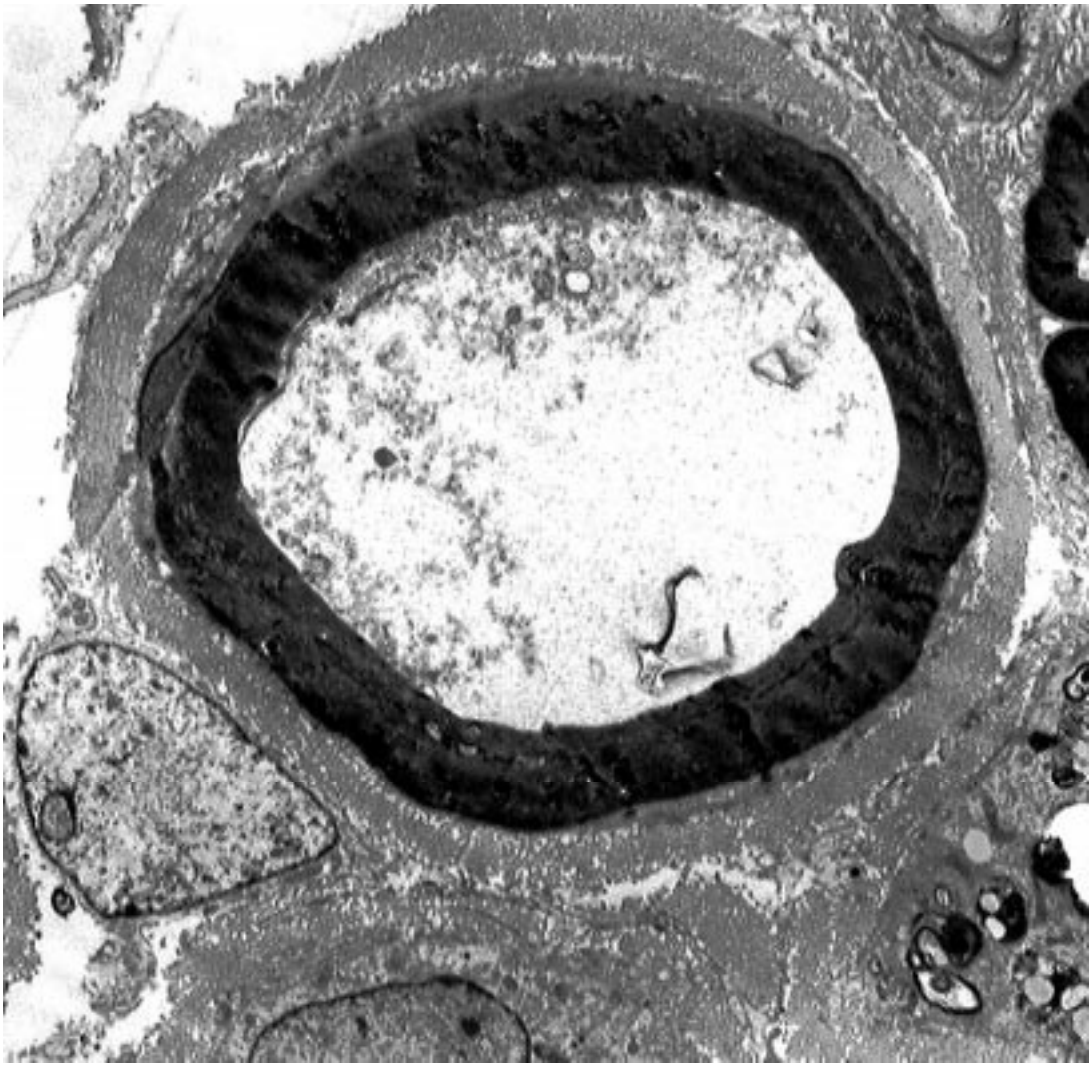


Figure 31: Cross section of a myelinated fiber in a promoted group at 16 days, showing axonal swelling with granular degeneration of its content (7,830X).

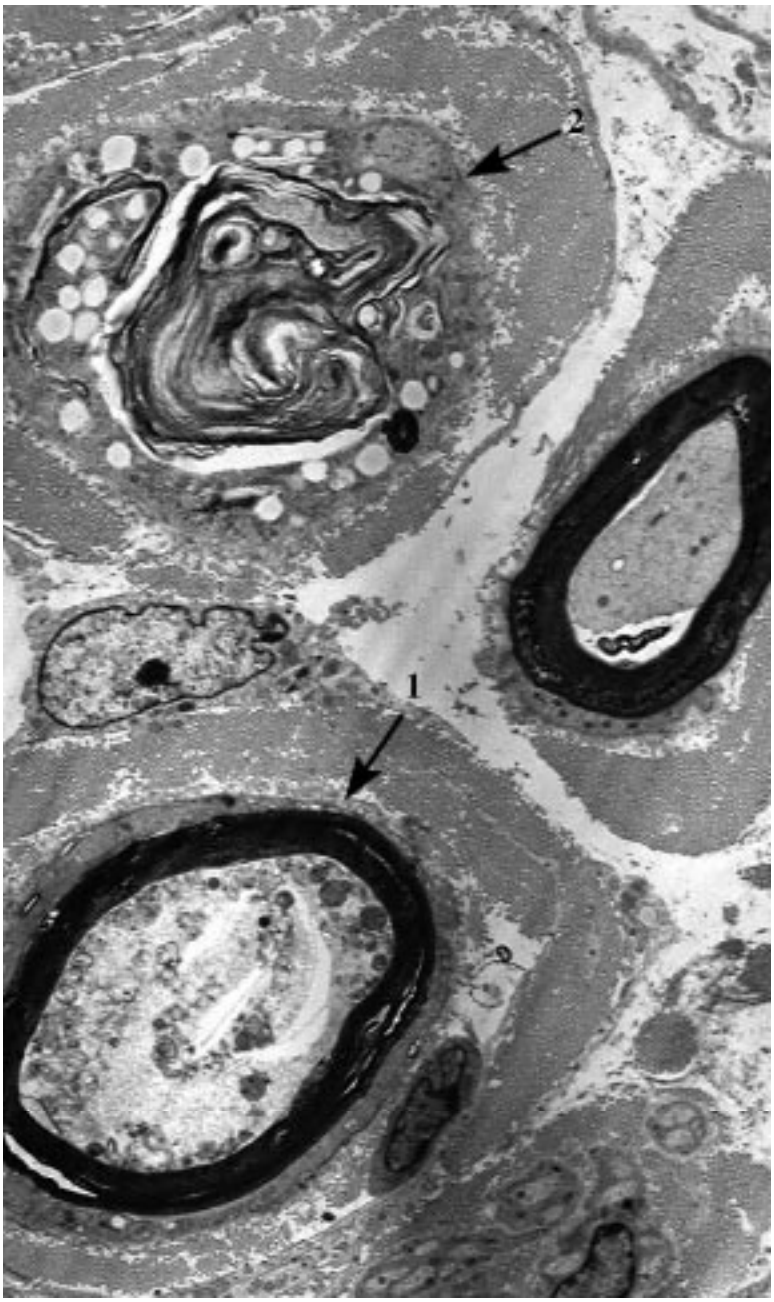


Figure 32: Cross section of myelinated fibers in the promoted hen at 16 days. There is nerve fiber degeneration showing axonal swelling with disorganized masses of altered mitochondria, and membranous multilamellar bodies (1). An advanced stage of myelinated nerve fiber degeneration, showing fragmentation of the axon and myelin sheath, within a phagocytic cell is noted (2) (7,540X).

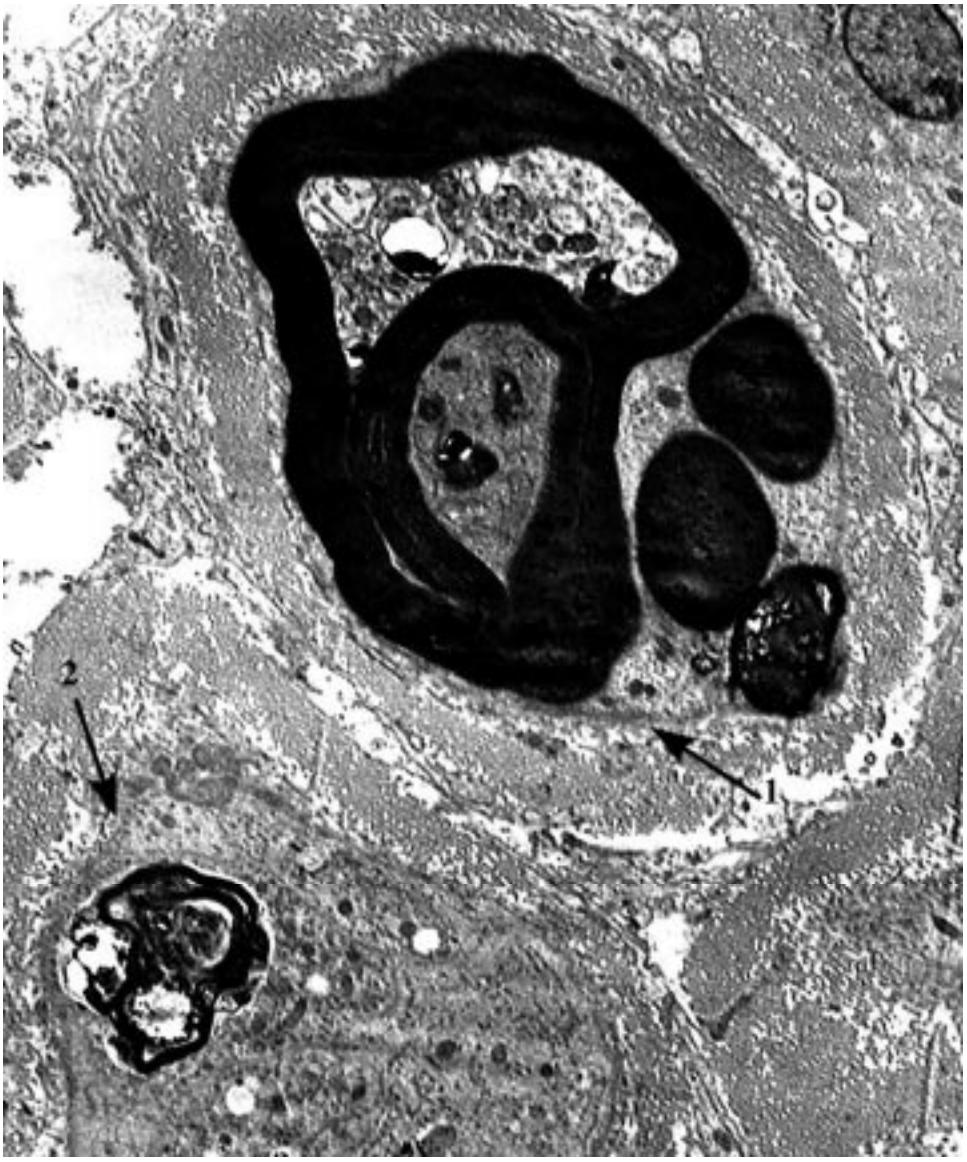


Figure 33: Cross section of degenerated myelinating fibers from a promoted hen at 16 days post-dosing. Axonal collapse and fragmentation of myelin sheaths forming aggregation of membranous masses are seen (1). Advanced degeneration of a myelinated nerve fiber within a macrophage or Schwann cell is also noted (2) (7,960X).

D. Neuropathy lesion scores.

Cross sections of Polybed® resin embedded nerve preparations and teased myelinated fibers were assessed to determine the degree of nerve fiber degeneration. For the cross-sections and the teased nerve fibers, the intensity of pathologic alterations of myelinated fibers or their degenerative products were graded from 0 - 4 (see Lesion score - Experimental Design and Methods). The results obtained in cross sections were similar to those using the teased fiber technique. As a general observation, the pathologic scores obtained with cross sections or the teased fiber techniques were significantly higher at day 15-16 post-dosing, compared to day 9 post-dosing. These results showed a progression in lesion intensity, similar to the observation previously made in regard to the development of clinical signs (see the clinical score section).

1. Nine days post-dosing.

At day 9 post-dosing, the pathologic scores obtained with cross sections of biventer cervicis nerves were all 1.0 or below (Table 11). At that time, only the promoted group PSP 1.0 mg/kg + PMSF 90 mg/kg was statistically different than the others (mean pathology score = 1.0; $p = 0.1$; alpha = 0.05). The mean neuropathy lesion scores of the other groups in decreasing order were 0.75 for the PSP (0.5 mg/kg) + PMSF (90 mg/kg) group, followed by 0.25 for both PSP (2.5 mg/kg) and PSP (1.0 mg/kg) groups (Table 11, Figure 34). For the other groups including PMSF (90mg/kg), PMSF (90mg/kg) + PSP (2.5 mg/kg), PSP (0.5 mg/kg), and the controls, the mean neuropathy lesion score was zero (Table 11, Figure 34). Therefore, at nine day post-dosing, the lesion are still minimal in all groups, including the positive control hens where PSP (0.5, 1.0 or 2.5 mg/kg) was used as the only agent. It is interesting to notice that even at day 9 post-dosing,

both promoted groups [PSP (1.0 mg/kg) + PMSF (90 mg/kg) and PSP (0.5 mg/kg) + PMSF (90 mg/kg)] had the two highest neuropathy lesion scores. The pathological scores from each group are also shown in association with the corresponding clinical score obtained at the time of sacrifice (Figure 34).

The severity of nerve fiber degeneration in the biventer cervicis at day 9 post-dosing was also evaluated with the teased fiber technique (Table 12). The data were similar to that collected from cross section samples. The mean teased fiber neuropathy lesion scores in decreasing order were 1.0 for the PSP (2.5 mg/kg) and both promoted groups [PSP (1.0 mg/kg) + PMSF (90 mg/kg) and PSP (0.5 mg/kg) + PMSF (90 mg/kg)], followed by 0.75 for the PSP (1.0 mg/kg) group and 0.25 for the PMSF (90mg/kg) group (Table 12, Figure 35). For the other groups including PMSF (90mg/kg) + PSP (2.5 mg/kg), PSP (0.5 mg/kg), and the negative controls, the mean neuropathy lesion score was zero (Table 12, Figure 35). The teased fiber mean neuropathology scores in each group teased fiber are shown in association with the clinical score obtained at the time of sacrifice (Table 12, Figure 35).

Table 11 : *Pathological* vs *Clinical Scores* - Day 9 Sacrifice (cross section).

Groups	Number of hens	Mean clinical scores (0-8) [a]	Mean lesion scores (0-4) [a]
Control	n = 7	0.0 ± 0.0	00 ± 0.0
PMSF(90mg/kg)	n = 4	0.0 ± 0.0	0.0 ± 0.0
PMSF(90mg/kg)+PSP(2.5mg/kg)	n = 3	0.3 ± 0.5	0.0 ± 0.0
PSP(0.5mg/kg)	n = 3	0.6 ± 0.5	0.0 ± 0.0
PSP(1.0mg/kg)	n = 4	1.0 ± 1.0	0.3 ± 0.5
PSP(2.5mg/kg)	n = 4	1.3 ± 0.9	0.3 ± 0.5
PSP(0.5mg/kg)+PMSF(90mg/kg)	n = 4	2.3 ± 0.5	0.8 ± 0.9
PSP(1.0mg/kg)+PMSF(90mg/kg)	n = 2	3.0 ± 0.7	1.0 ± 0.0

[a] Results on clinical scores and pathology scores are express as mean ± SD (see Tables 3-10 for individual clinical scores; individual pathology scores are in Appendix 3).

Pathology vs Last Clinical Scores - Day 9 (Cross sections)

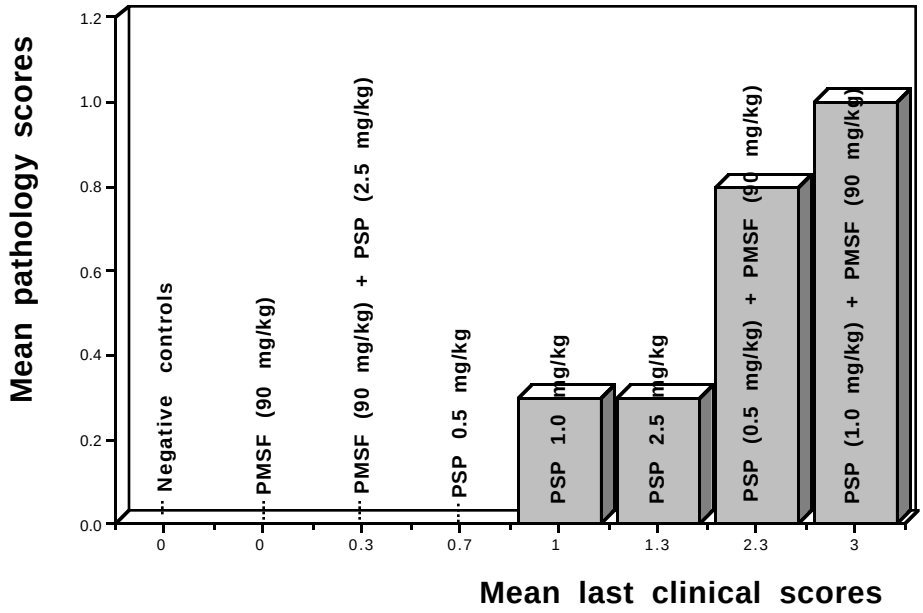


Figure 34 : Cross sectional mean neuropathological scores (0-4 scale) obtained from each group. In axis, the mean clinical scores at the time of sacrifice are reported. At 9 days post-dosing, the neuropathologic damage is still minimal.

Table 12 : *Pathological* vs *Clinical Scores* - Day 9 Sacrifice (teased fibers).

Groups	Number of hens	Mean clinical scores (0-8) [a]	Mean pathology scores (0-4) [a]
Control	n = 5	0.0 ± 0.0	0.0 ± 0.0
PMSF(90mg/kg)	n = 4	0.0 ± 0.0	0.0 ± 0.0
PMSF(90mg/kg)+PSP(2.5mg/k	n = 3	0.3 ± 0.6	0.0 ± 0.0
PSP(0.5mg/kg)	n = 1	0.0 ± 0.0	0.0 ± 0.0
PSP(1.0mg/kg)	n = 4	1.0 ± 1.2	0.8 ± 0.5
PSP(2.5mg/kg)	n = 2	1.5 ± 0.7	1.0 ± 0.0
PSP(0.5mg/kg)+PMSF(90mg/k	n = 3	2.0 ± 0.0	1.0 ± 0.0
PSP(1.0mg/kg)+PMSF(90mg/k	n = 2	2.5 ± 0.7	1.0 ± 0.0

[a] Results on clinical scores and pathology scores are express as mean ± SD (see Table 3-10 for individual clinical scores; individual pathology scores are in Appendix 5). For this table mean clinical scores were calculated only for the hens where the teased fiber method was used

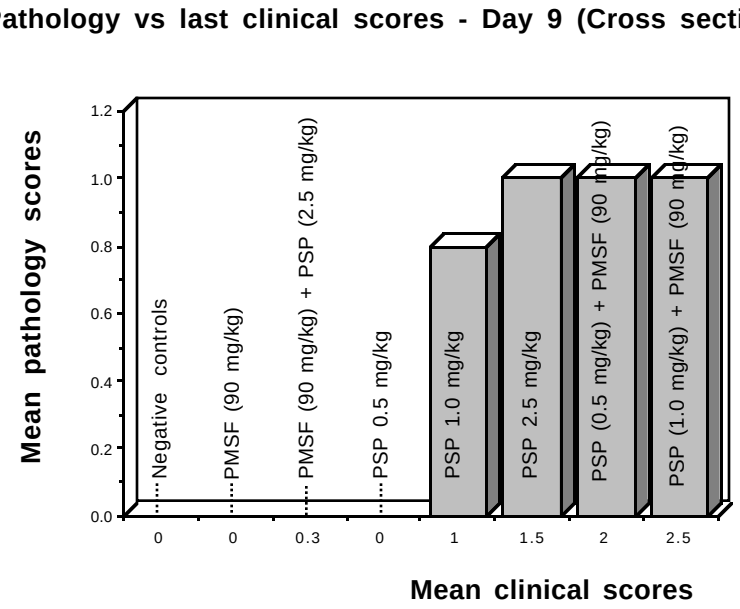


Figure 35 : Teased fiber mean neuropathological scores (0-4 scale) obtained from each group. In axis, the mean clinical scores at the time of sacrifice are reported. At 9 days post-dosing, the neuropathologic damage is still minimal.

2. Fifteen days post-dosing.

Comparison of clinical and lesion scores obtained in cross sections are given in Table 13 and Figure 36, and for teased fibers in Table 14, Figure 37. Both techniques gave similar results. There was a statistically significant correlation of r = 0.61 (p = 0.0001) between the last clinical scores (clinical score at time of sacrifice), and the neuropathological scores obtained in cross section. The degree of correlation between the last clinical scores (clinical score at time of sacrifice), and the neuropathological scores obtained with the teased fiber technique was similarly significant (r = 0.63; p = 0.0001), and is shown in Figure 39. Therefore, the degree of clinical signs predicts the degree of myelinated fiber degeneration observed in the biventer cervicis nerve. The correlation between the clinical scores and the neuropathological scores obtained in cross section can be visualized in Figure 38. Minimal or no pathological effects were detected in the negative controls, PSP 0.5 mg/kg, PMSF (90 mg/kg), and the PMSF + PSP (2.5 mg/kg) (protected) groups. However, moderate to severe clinical and neuropathologic alterations were seen in both potentiated groups [(0.5 or 1.0 mg/kg) PSP followed by PMSF], and in positive control groups (PSP at 1.0 or 2.5 mg/kg). Neuropathy lesion scores at day 15-16 post-dosing obtained in cross section and with teased fiber preparations are further detailed below.

Cross section: At day 15-16 post-dosing, there were significant differences between both promoted groups [PSP (0.5 mg/kg) + PMSF (90 mg/kg); PSP (1.0 mg/kg) + PMSF (90 mg/kg)] and the positive higher controls [PSP (2.5 mg/kg); PSP (1.0 mg/kg)], when compared to the other groups such as PMSF (90mg/kg) + PSP (2.5 mg/kg) (protected), PMSF (90mg/kg), PSP (0.5 mg/kg), and negative controls. Even though the

mean neuropathological score was different between the promoted groups (mean neuropathology score for both promoted groups = 3.5) and the positive control groups (mean neuropathology score for both positive control groups = 2.67), there were no statistical differences between these groups. At termination of the study, there were no statistical differences between the PMSF (90mg/kg) + PSP (2.5 mg/kg), the PMSF (90mg/kg), the PSP (0.5 mg/kg), and the negative control groups (table 13). In general, the axonal damage was severe in both promoted groups, moderate in the positive control groups (PSP 1.0 mg/kg and 2,5 mg/kg), and minimal to absent in the other groups including the low dose of PSP (0.5 mg/kg), and the negative controls as reported in Table 13. The pathological scores obtained in each group are also shown in association with the clinical scores obtained at the time of sacrifice (Figure 36).

Teased fibers: At day 15-16 post-dosing, there were significant differences between the promoted groups [PSP (0.5 mg/kg) + PMSF (90 mg/kg); PSP (1.0 mg/kg) + PMSF (90 mg/kg)] and the positive controls [PSP (2.5 mg/kg); PSP (1.0 mg/kg)], when compared to the others groups including, PMSF (90mg/kg) + PSP (2.5 mg/kg), PMSF (90mg/kg), PSP (0.5 mg/kg), and negative controls (Table 14). In addition, there were no statistical differences between the PMSF (90mg/kg) + PSP (2.5 mg/kg) (promoted), the PMSF (90mg/kg), the PSP (0.5 mg/kg), and the negative control groups (Table 14). In general, the axonal damage was severe in both promoted groups and positive controls (PSP 2.5 mg/kg), and minimal to absent in the other groups including the negative controls as reported in Table 14. The mean pathology scores obtained in each group with the teased fiber technique are also shown in association with the clinical scores obtained at the time of sacrifice (Figure 37).

Table 13 : *Pathological* vs *Clinical Scores* - Day 15-16 (cross section)

GROUPS	Number of hens	Mean clinical score (0-8) [a]	Mean pathological score (0-4) [a]
Controls	n = 3	0.0 ± 0.0	0.0 ± 0.0
PMSF (90mg/kg)	n = 3	0.0 ± 0.0	0.0 ± 0.0
PMSF (90mg/kg)+PSP (2.5mg/k	n = 4	0.0 ± 0.0	0.3 ± 0.5
PSP (0.5mg/kg)	n = 3	0.0 ± 0.0	0.0 ± 0.0
PSP (1mg/kg)	n = 3	5.5 ± 1.5	2.7 ± 0.5
PSP (2.5mg/kg)	n = 3	7.3 ±1.1	2.7 ± 0.5
PSP (0.5mg/kg)+PMSF (90mg/k	n = 4	4.0 ± 0.0	3.5 ± 0.5
PSP (1mg/kg)+PMSF(90mg/kg)	n = 4	7.5 ± 0.5	3.5 ± 0.5

[a] Results on clinical scores and pathology scores are express as mean ± SD (see Table 3-10 for individual clinical scores; individual pathology scores are in Appendix 4).

Pathology vs Clinical Scores - Day 15-16 (Cross sections)

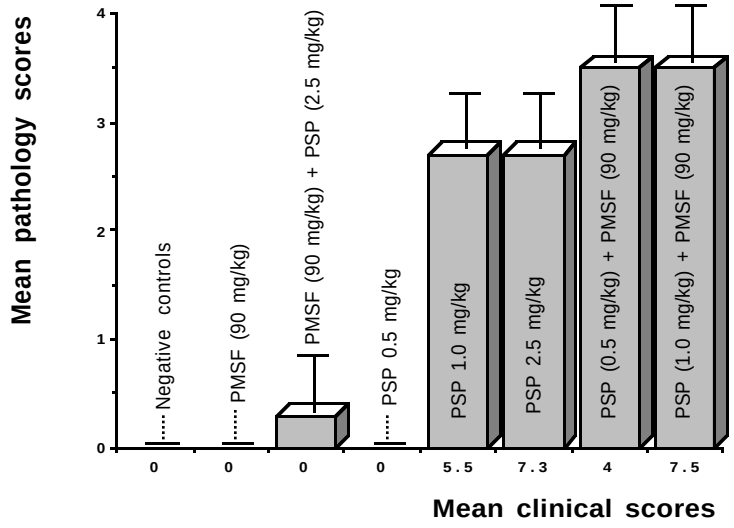


Figure 36 : Mean neuropathological scores (0-4 scale) from each group. In the axis, the clinical scores at the time of sacrifice are reported (0-8 scale). At 15-16 days post-dosing, the neuropathologic damage is almost maximal for the promoted groups, intermediate for the positive controls, and minimal for the other groups.

Table 14 : *Pathological* vs *Clinical Scores* - Day 15-16 (teased fibers)

Groups	Number of hens	Mean clinical scores (0-8) [a]	Mean pathology scores (0-4) [a]
Control	n = 3	0.0 ± 0.0	0.0 ± 0.0
PMSF(90mg/kg)	n = 2	0.0 ± 0.0	0.0 ± 0.0
PMSF(90mg/kg)+PSP(2.5mg/kg)	n = 2	0.0 ± 0.0	0.5 ± 0.7
PSP(0.5mg/kg)	n = 3	0.0 ± 0.0	0.0 ±0.0
PSP(1.0mg/kg)	n = 3	5.7 ± 1.5	2.3 ± 0.6
PSP(2.5mg/kg)	n = 2	8.0 ± 0.0	2.0 ± 0.0
PSP(0.5mg/kg)+PMSF(90mg/kg)	n = 4	4.0 ± 0.0	2.0 ± 0.8
PSP(1.0mg/kg)+PMSF(90mg/kg)	n = 3	7.7 ± 0.6	2.7 ± 0.6

[a] Results on clinical scores and pathology scores are express as mean ± SD (see Table 3-10 for individual clinical scores; individual pathology scores are in Appendix 6).
[b] For this table mean clinical scores were calculated only for the hens where the teased fiber method was used.

Pathology vs Clinical Scores - Day 15-16 (Teased fibers)

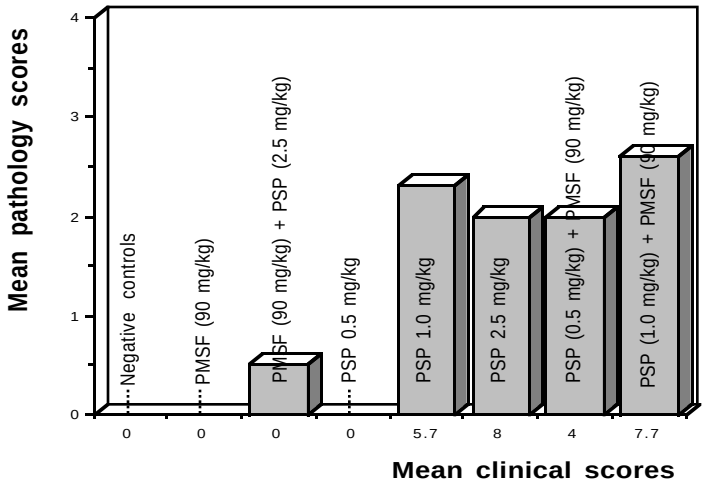


Figure 37 : Mean nerve fiber degeneration (%) from each group with the teased fiber technique. In the axis, the clinical scores at the time of sacrifice are reported (0-8 scale). At 15-16 days post-dosing, the neuropathologic damage is almost maximal for the promoted groups and positive controls (PSP 1.0 mg/kg; PSP 2.5 mg/kg), and minimal for the other groups.

Correlation between clinical and neuropathological scores. (Cross section)

Pathology scores vs clinical scores (cross sections)

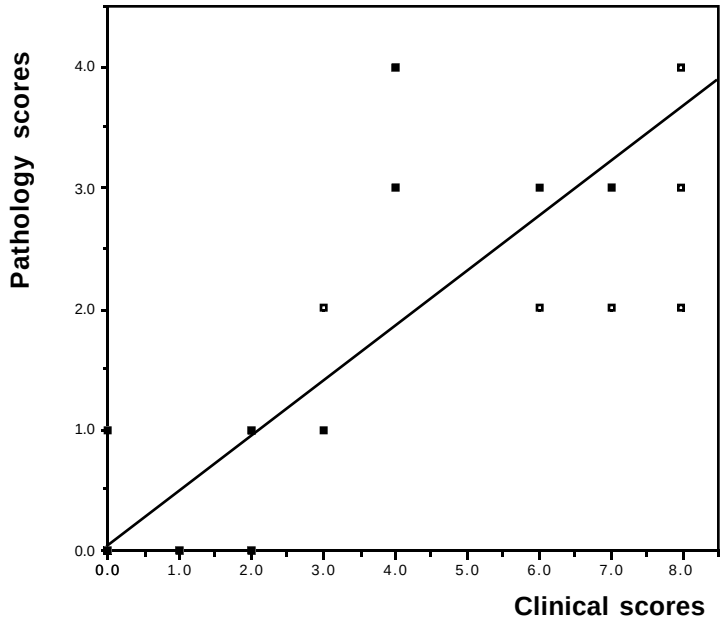


Figure 38 : Correlation between the clinical scores and the pathology scores in cross sections (r = 0.61). This graph represents the last clinical scores (X) obtain prior to euthanasia, and the pathology scores determined on the biventer cervicis nerve. The significance of relationships between clinical and pathology scores was determined by linear regression using a modified analysis of variance (MANOVA) (n = 61; $p < 0.0001$).

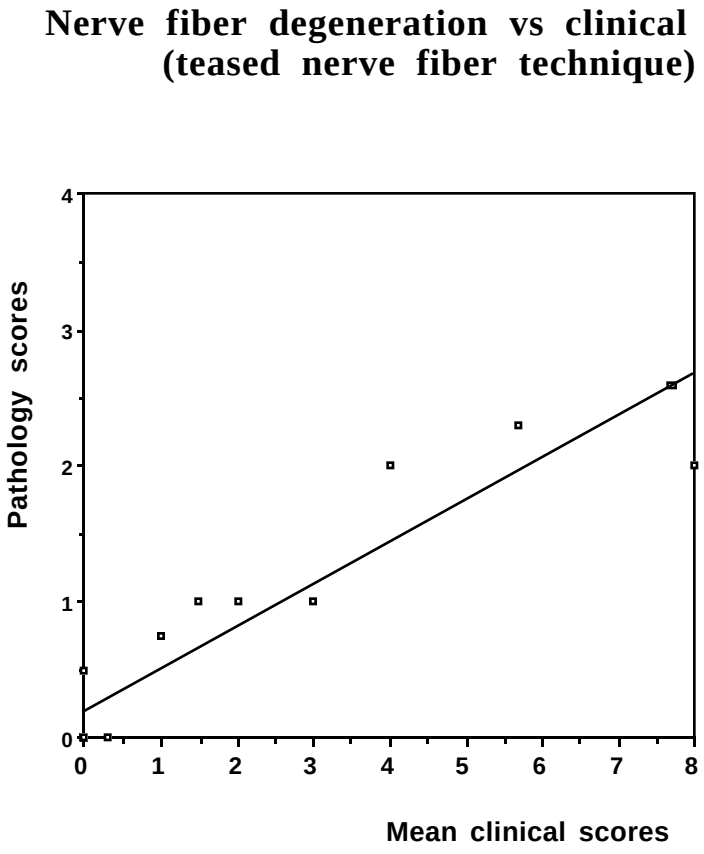


Figure 39 : Correlation between the clinical scores and pathology scores obtained with the teased fiber technique ($r = 0.63$). This graph represents the last clinical scores (X) obtain prior to euthanasia, and the pathology scores determined on the biventer cervicis nerve. The significance of relationships between clinical and pathology scores was determined by linear regression using a modified analysis of variance (MANOVA) ($n = 52$; $p < 0.0001$).

V. Discussion.

This study revealed that the serine/cysteine protease inhibitor PMSF has a marked effect on the incidence and severity of OPIDN induced by PSP in hens. These effects vary depending upon the sequence of administration of these compounds. Thus, PMSF given prior to PSP had a protective effect, in that neurotoxic doses of the OP are prevented from expressing the neuropathy. When administered after PSP, PMSF enhanced the effect of a subneurotoxic (0.5 mg/kg) dose of PSP, with neuropathy equivalent to a highly toxic dose (2.5 mg/kg). These effects were manifested clinically and neuropathologically. The inhibition of NTE was less clear, reflecting the difficulty in establishing its relationship to OPIDN in previous studies (Pope et al., 1990; Lotti et al., 1991; Lotti et al., 1993; Moretto et al., 1994; Moretto et al., 1992; Johnson et al., 1993; Ehrich, 1996).

A. Significance of neurotoxic esterase inhibition.

A determination of the percentage of brain NTE inhibition within hours of exposure of hens to OP compounds (24-48 hours) is required by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) before new OP insecticides can be registered for use, to predict the potential for developing OPIDN in susceptible animals (US EPA, 1991). It has been previously reported that delayed neuropathy can develop if more than 70-80 % NTE inhibition occurs, and if the process of aging followed NTE inhibition (Johnson, 1993). In the present studies, clinical signs of delayed neurotoxicity and associated neuropathological changes did not occur in hens dosed with PMSF 90 mg/kg, although the percentage of NTE inhibition observed in that group (>70 %) was above the level reported to lead to the development of OP delayed neuropathy (Johnson, 1975; 1982; 1990). The absence of

clinical signs despite this level of NTE inhibition suggests that aging of the enzyme does not occur with PMSF, and that it is a reversible NTE inhibitor (Pope et al., 1992, 1993). Thus NTE inhibition alone as a biomarker is not accurate in evaluating the likelihood of OPIDN.

In this study, brain NTE and spinal cord NTE were very sensitive to the phenyl saligenin phosphate (PSP) organophosphorus compound, therefore a relationship between doses of PSP and percentage of enzyme inhibition could not be established. The high percentage of NTE inhibition which we obtained with PSP alone, regardless of dose (0.5, 1.0, 2.5 mg/kg), were similar to other studies (El-Fawal et al., 1990; Jortner and Ehrich, 1987). As an example, Jortner and Ehrich (1987) found 85-93 % inhibition with 2 mg/kg, 3 mg/kg and 10 mg/kg, whereas in the present study, the dosage used (0.5, 1.0, 2.5 mg/kg) caused 86-92 % inhibition. The basal NTE activity (control) was slightly lower in the present study (NTE activity = 10.6 nmol of product formed/min/mg brain protein) compared Jortner and Ehrich study (1987) (NTE activity = 22.4 nmol of product formed/min/mg brain protein), but this did not seem to affect sensitivity to PSP-induced inhibition.

PSP, the test compound used in this study, is known for its high neuropathic potential, and is, therefore, thought to easily phosphorylate NTE, and cause aging of this phosphorylated enzyme (Johnson, 1982). The fact that NTE of hen brain was markedly inhibited (>90%) at a dosage of 0.5 mg/kg without development of clinical signs or neuropathologic effects suggests that aging may not be related to the occurrence of OPIDN (Lotti et al., 1992; Johnson, 1993), or that inhibition of brain NTE may not be indicative of PSP induced delayed neuropathy which is primarily manifest in the spinal cord and peripheral nerves, rather than in the brain (Ehrich, 1996). Brain NTE, however, is

generally recognized as the most sensitive and useful indicator of potential to develop OPIDN (Ehrich, 1996). Many hypotheses have been proposed to explain the equivocal relationship between NTE inhibition and delayed neurotoxicity. While until recently all known neuropathic OP compounds were thought to undergo an aging reaction, a more recent study (Johnson and Safi, 1992) suggested that this second critical step following NTE inhibition in the initiation of OPIDN may be achieved through alternative molecular processes (see below).

In the present study, it was also difficult to establish a relationship between NTE inhibition and delayed neurotoxicity when the reversible NTE inhibitor PMSF was administered after phenyl saligenin phosphate (PSP). The latter combination increased locomotor deficits and neuropathologic changes in hens, compared to PSP alone. NTE was already maximally inhibited by the PSP, and therefore, no further inhibition was measured. This suggests that NTE inhibition is not the only mechanism contributing to the development of delayed neuropathy, and supports other reports that also suggest promotion of delayed neurotoxic agents was unrelated to NTE inhibition (Pope et al., 1992; Moretto et al., 1994). Therefore, the present study, similar to other reports (Pope et al., 1992; Lotti et al., 1991), suggests that NTE is not the site for promotion and potentiation of OPIDN. These studies report that clinical signs and neuropathologic changes were more severe when a reversible NTE inhibitor followed dosing with OP compounds, even when the OP dosage already inhibited NTE more than 90 %. Another reason why NTE may not be the only target responsible for initiation of OPIDN is based on the findings that the same reversible NTE inhibitors (PMSF) can either protect or promote OPIDN lesions and clinical signs, depending upon the sequence of administration in relation to the neuropathic-inducing OP (Cardoli et al., 1994; Johnson, 1982; Lotti et al., 1991; Moretto et al., 1992; Pope et al., 1992 ; Veronesi and Padilla, 1985). In support of this, Aldridge (1993)

suggested that the promotion site, although other than NTE, could be similar to and/ or "linked" with that enzyme, facilitating the ability of aging near its catalytic center (Osman et al., 1996). Other trophic factors are also thought to be involved in the progression of OPIDN, and could explain part of the neuropathic mechanism. These hypothesized trophic factors include ornithine decarboxylase enzymes (Pope et al., 1995) and a 3H-DFP-binding protein within the active subunit site of NTE (Carrington et al., 1985; Pope et al., 1992; Pope et al., 1993; Escurdo et al., 1995).

Many other findings further challenge the relationship of NTE inhibition to OPIDN. For example, no correlation has been established between NTE inhibition in the nervous system and specific location of neurologic lesions produced by OPIDN (Correll and Ehrich, 1991; Johnson, 1982; Ehrich, 1996). Also, correlations between NTE activities, and clinical and morphological signs are not well established. In some studies, no neurologic deficits were reported with more than 70% inhibition (Moretto et al., 1991; Farage-Elawar et al., 1988), whereas in others, 70 % NTE inhibition resulted in neuropathy (Johnson, 1975b; 1982). The disconcordance between NTE inhibition and delayed neurotoxicity has caused some to question the use of NTE inhibition as a biomarker for OPIDN. However, it remains the best biomarker available at this time (Ehrich, 1996). Further studies need to be conducted to better understand the early biochemical events that occur in OPIDN in order to identify more precise biomarkers of OPIDN.

B. Clinical signs and morphologic changes in OPIDN.

Clinical signs and neuropathology evaluations are also required by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) before new OP insecticides can be registered for use (US EPA, 1991). This report has demonstrated that clinical scores are an excellent and easy way to evaluate the degree of severity of OPIDN caused by administration of PSP. This present study is unique in that it reports a dose-response statistical correlation between terminal clinical signs and neuropathologic scores for OPIDN, and for protection and promotion of this neuropathy. In this study, the correlation between clinical signs and neuropathological lesions of OPIDN obtained in the biventer cervicis nerve (r^2 = 0.61) was much higher than the previous weak correlation coefficient reported by Prentice and Roberts, 1982 (r^2 = 0.26-0.36). The clinical scores were significantly different between groups in which PSP was used alone at doses of 0.5, 1.0 and 2.5 mg/kg, even though NTE inhibition was not statistically different. More severe clinical signs were associated with increased dosages of PSP. Similar findings were also observed in previous studies (Ehrich et al., 1993, 1995). At the time of sacrifice, the clinical scores were elevated in hens receiving PSP at 1.0 mg/kg and 2.5 mg/kg, but absent in hens given 0.5 mg/kg. Thus 0.5 mg/kg was the no observed effect level (NOEL). The clinical scores in the 1.0 and 2.5 mg/kg groups increased with time in each group, until day-15,16 post-dosing was reached (the end of the study), although no significant differences were seen between these two groups. It is possible that the terminal clinical scores for PSP 1.0 mg/kg and 2.5 mg/kg would have been greater (or maximal score in both groups) with a more prolonged study, since clinical signs are known to progress at least up to 21 days after administration of PSP (Jortner and Ehrich, 1993; Jortner et al., 1989). Most of these PSP-dosed birds were also free of clinical signs until day-9 post-

dosing, emphasizing the delayed nature of this neuropathy. A post-dosing preclinical period for development of neurotoxic events was also noted in other studies when PSP was used at 2.5 mg/kg (Dyer et al., 1992; Jortner and Ehrich, 1987).

The lesions in the biventer cervicis nerve in OPIDN induced by PSP alone and in PSP neuropathy promoted by PMSF were qualitatively similar. They resembled previously reported light microscopic changes such as: axonal swelling with attenuation of the myelin sheaths, formation of myelin-rich ovoids, accumulation of axonal debris, and increased number of macrophages, and reactive Schwann cells (Jortner et al., 1982; Jortner, 1984; Jortner and Ehrich, 1987; Cavanagh, 1964; Pressig et al., 1978; Bouldin and Cavanagh, 1979a, b). Quantitative neuropathological changes in hens receiving 1.0 or 2.5 mg/kg of PSP were prominent, and did not significantly differ between these two groups. Thus, this was less discriminative than the clinical scores (see page 112). Paralleling the clinical effects, 0.5 mg/kg did not elicit lesions.

PMSF 90 mg/kg administered prior to a neurotoxic dose of PSP (2.5 mg/kg) protected hens from the clinical signs and neuropathological changes of OPIDN, as this group was not significantly different from the control group. This protection was also noted in other studies with adult hens (Pope et al., 1993; Lotti et al., 1991; Pope and Padilla, 1990; Randall et al., 1997). The protective effect of PMSF against delayed neurotoxicants has also been observed in rats (Veronesi and Padilla, 1985) exposed to mipafox, and in cats exposed to DFP (Baker et al., 1980). These findings can be helpful in differentiating initial biochemical events associated with the development of delayed neuropathy. PMSF's protection against OP compounds capable of inducing delayed neuropathy is thought to result from making the NTE target unavailable to neuropathic compounds. Consequently "aging" at the active site cannot occur, and thus neuropathic

consequences are not observed (Pope et al., 1990; Johnson et al., 1990). In the present study, the presence of clinical signs when PSP 2.5 mg/kg was given alone and the absence of clinical signs when PMSF was given prior to PSP 2.5 mg/kg administration, supports the concept that "aging" is a necessary event, after NTE inhibition, leading to later development of OPIDN (Veronesi and Padilla, 1985). Therefore, a non-toxic reversible NTE inhibitor could protect susceptible species against the delayed neurotoxic effects of OPIDN, and could be potentially useful when such an exposure is anticipated.

In the present study, at time of sacrifice (day-15,16), there was no statistical clinical difference between the 2 promoted groups [PSP (1.0 mg/kg) + PMSF (90 mg/kg); PSP (0.5 mg/kg) + PMSF (90 mg/kg)], and lesion scores were similar and close to maximal in both. PSP used alone at 1.0 mg/kg already caused severe axonal damage, therefore no statistically significant differences were present by adding the promotor PMSF 90 mg/kg after PSP (1.0 mg/kg) exposure. In contrast, there were statistically significant differences between PSP alone at 0.5 mg/kg compared to the group where PMSF 90 mg/kg was administered as a promotor 4 hours after that PSP (0.5 mg/kg) exposure. The neuropathology scores were not statistically different between the PSP 0.5 mg/kg group and the control hens. As previously discussed, lesion scores were close to maximal when PMSF 90 mg/kg was administered to hens 4 hours after PSP (0.5 mg/kg) exposure. The mean neuropathology scores obtained for this promoted group was even higher than the positive control group (PSP 2.5 mg/kg). This feature clearly indicate that PMSF at 90 mg/kg administered 4 hours after PSP exposure causes quantitative promotion of morphologic changes in the biventer cervicis nerve of adult hens at least after a single exposure. Other reports support the promoting effect of PMSF on clinical signs caused by different delayed neurotoxicants such as mipafox, di-butyl 2,2-dichlorovinyl phosphate or DFP (Pope and Padilla, 1990; Moretto et al., 1992; Osman et al., 1996; Lotti et al., 1991)

and on neuropathological changes in young chicks (Pope et al., 1992; Peraica et al., 1993; Harp et al., 1997) and adult hens (Randall et al., 1997) .

The order of PMSF administration is critical to protect hens from OPIDN, since PMSF administered 4 hours after PSP potentiates rather than protects against clinical signs of OPIDN. It is difficult to establish a unifying hypothesis that would explain both protection and potentiation caused by the same PMSF compound. A variation in the disposition of the neuropathic OP, such that more neurotoxicant-target interactions occur over time or that less neurotoxicant is eliminated from sensitive regions of the nervous system could explain the exacerbation of clinical signs (Pope et al., 1992). PMSF could have a higher affinity than the OP for some population of nontarget esterase, with consequent displacement of the OP from these non neuropathic sites, freeing the OP for subsequent binding to neuropathic sites (Pope and Padilla, 1990). PMSF was also capable of potentiating neurologic deficits when administered after a neurotoxicant that was not an OP compound, further challenging the relationship of NTE to OPIDN (Moretto et al., 1992). Moretto et al. (1992) proposed that a variation in the intrinsic activities of inhibitors, described as partial agonists, full agonists and antagonists, may contribute to promotion mechanisms. Even though many hypotheses have been postulated to explain the mechanism of protection and potentiation of OPIDN (see above) (Johnson, 1982; Lotti et al., 1991; Moretto et al., 1992; Pope et al., 1992), no hypothesis can explain the complete mechanism of OPIDN at this time. Potentiation is a phenomenon of environmental relevance, since chemicals similar to PMSF can be concomitantly found with OP compounds, suggesting that more severe signs of OPIDN could be observed in such mixed exposures (Pope and Padilla, 1990). Further studies regarding potentiation and protection of OP compounds are needed to better understand the pathogenesis of OPIDN.

Morphologic changes when OPIDN was promoted were previously studied using the cervical spinal cord of 35-70 day-old chicks treated with PMSF following OP exposure (Pope et al., 1992). That study used silver impregnation of degenerative nerve fibers to show that the extent of axonal degeneration was increased by the reversible NTE inhibitor PMSF, given after a dose of a neuropathy-inducing OP (DFP), known to be insufficient to cause ataxia when given alone. Chicks of this age are known to be more resistant to clinical signs of OPIDN (Moretto et al., 1991; Funk et al., 1994). The distribution of axonal lesions associated with potentiation of OPIDN in the central and peripheral nervous system of adult hens has also been recently described (Randall et al., 1997). In the latter study, six μm thick sections of sciatic, tibial and peroneal nerves, and portions of the cerebrum, midbrain cerebellum, medulla oblongata and spinal cord were paraffin embedded and stained with Luxol fast blue/ periodic acid Shiff/ cresyl echt (Randall et al., 1997). The pattern of lesions produced by the OP compound (DFP) alone or in combination with PMSF was consistent with a central - peripheral distal axonopathy, affecting ascending and descending spinal cord tracts, as well as peripheral nerves (Randall et al., 1997). The present study is the first time that perfusion-fixation, and plastic (epoxy resin) embedding were used to demonstrate lesions in the biventer cervicis nerve when OPIDN was promoted in adult hens. This procedure provided greater resolution of peripheral nerve lesions than that of Randall et al., 1997. This biventer cervicis nerve was especially sensitive to OPIDN (El-Fawal et al., 1988, 1990), allowing both qualitative and quantitative morphological assessments. Clinical and neuropathological findings reported by Randall et al., 1997, were similar to those in the present study, even though the former did not include statistical correlation between the clinical signs and the neuropathological changes. This present work was also stronger in that the promotion was obtained from a NOEL of OP compound (PSP 0.5 mg/kg), and therefore, showed that lesions could appear even with a non-toxic dose. The study by Randall et al. only showed promotion of pre-existing lesions (Randall

et al., 1997). This present work is also the first study to examine neuropathological changes of promotion in OPIDN under conditions of dose-response.

Light microscopic evaluation was helpful to further characterize the nature of peripheral nerve damage observed in this particular neuropathy. The use of resin embedding allowed thin (1 μm thick) sections to be cut for light microscopic study, which gave excellent light microscopic resolution, and proved useful in our assessments. The microscopic resolution obtained in the biventer cervicis nerve with resin embedding was greater than in previous studies (Randall et al., 1997; Pope et al., 1992; Peraica et al., 1993; Harp et al., 1997), due to the thinner sections which allowed a more critical evaluation of promoted lesions. Distal axonopathy, affecting large diameter axons was the most consistent morphologic change observed in the biventer cervicis nerve, as also observed in other studies (Jortner and Ehrich, 1987; El-Fawal et al., 1988). The neuropathology scoring (see Methods) was an appropriate way to quantify the damage observed in biventer cervicis nerve sections. According to this technique, most but not all of nerve fibers were counted. However, sufficient nerve fibers were counted in each sections to obtain an accurate quantitative evaluation of nerve fiber degeneration.

Some problems were encountered with neuropathological evaluation of nerves in cross section. Some of the axonal lesions were difficult to characterize when immersion-fixation was used, or when perfusion was incomplete, because bands of Buengner were at times difficult to differentiate from collapsed blood vessels. In some cases, electron microscopy was needed to differentiate these structures. In certain sections, some of the regenerating fibers observed in this study could have been mistaken for small normal myelinated fibers, therefore, this method was not precise enough to determine an accurate regenerative nerve fiber count. Another study has previously shown regenerating fibers

following OP administration (Jortner et al., 1989). It would be useful in the future to evaluate the diameter of nerve fibers in proportion to myelin thickness, to have a better appreciation of early axonal swelling, true regenerating fibers, and mild secondary demyelination. Other problems encountered when nerve fiber degeneration was evaluated included artefactual myelin splits, variation in staining of sections making the axoplasm artefacually more granular, and traumatic nerve manipulation during dissection or processing, resulting in crushed fibers. The latter needed to be reprocessed in order to make accurate evaluation of neuropathologic changes. One needs to be aware of these artifacts in assessing nerve fiber injury in these preparations.

The neuropathologic changes observed in cross sections of the biventer cervicis nerve were quantitatively similar to the lesions obtained with the teased fiber technique. In this procedure, segments of individual myelinated fibers were separated and viewed by light microscopy. The correlation between clinical signs and quantitative neuropathological changes was slightly greater when the teased fiber were used, despite a lower number of nerve fibers evaluated. Other studies have characterized organophosphorus neuropathy by evaluating axonal degeneration with teased fiber techniques (Bouldin and Cavanagh, 1979a,b). Even though teased fibers are a well known method for evaluating nerve fiber degeneration (Greenfield and Godwin, 1997), normal and neuropathological changes have not been yet reported for the hen biventer cervicis nerve in OPIDN. In addition, the results obtained with teased fibers have never been compared with embedded peripheral nerve tissue in cross sections. It is possible that a certain degree of biais could have occured during the nerve teased fiber evaluation due to the observers, and because degenerated teased fibers are more fragile, therefore more predisposed to break prior to evaluation. Even though some problems were encountered with this methode of evaluation, the teased fiber technique was useful in this study because it provided additional qualitative

information of the nerve fibers, since it allowed assessment of individual nerve fibers over long distances. By use of the two neuropathological techniques (cross-section and teased fibers), it was possible to characterize the pathologic changes observed in nerve fibers. No previous studies of promoted OPIDN have used the teased fiber technique.

Electron microscopic evaluation was helpful in some biventer nerve cross sections to further identify the nature of axonal damage observed by light microscopy. As noted above, differentiation between bands of Buengner and collapsed blood vessels was only possible by electron microscopy where perfusion fixation had failed, or in some cases of immersion fixation. This is also the first time ultrastructural changes were examined in peripheral nerve fibers of adult hens in promoted OPIDN. No qualitative difference was not seen between these two methods of eliciting OPIDN. We found the classical myelinated nerve fiber lesion in OPIDN in both promoted and positive control groups, as described in other reports (Bischoff, 1967,1969,1970; Prineas, 1969; Bouldin and Cavanagh, 1979b). This included the presence of early proliferation of axonal agranular endoplasmic reticulum-like cisterns, swelling of axons with accumulation of membranous and mitochondrial debris, granular degeneration of axons and fragmentation of myelinated fibers (Bischoff, 1967,1969,1970; Prineas, 1969; Bouldin and Cavanagh, 1979b; Jortner and Ehrich, 1987).

VI. Conclusions.

This study assessed PMSF-induced promotion and protection of OPIDN in hens using clinical signs, NTE inhibition and neuropathologic scoring. In this report, a known non-reversible NTE inhibitor PSP, administered at 0.5 mg/kg, caused > 80 % NTE inhibition without subsequent clinical signs or lesions of OPIDN in hens. It is thus reasonable to question the specificity of the relationship of NTE inhibition to OPIDN. These findings suggest that NTE might be too sensitive to evaluate the likelihood of development of OPIDN with certain types of OP compounds. In this report, clear demonstration was made that the same reversible inhibitor of NTE (PMSF) could both potentiate or promote clinical signs and neuropathologic changes of OPIDN, depending upon the sequence of PMSF administration. These findings support strongly the hypothesis that NTE is not the specific and only target responsible for initiation of OPIDN. Instead, PMSF may alter some other general process that contributes to neurotoxicity, but which is unrelated to NTE inhibition. Because of serious concerns for public health, the use of reliable biomarkers predicting the likelihood of OPIDN in susceptible species is needed. In the absence of better biomarkers at the moment, premarket testing of OPs for NTE inhibition should continue. It is recognized that further studies need to be conducted to better understand the early biochemical events that occurs in OPIDN, in order to identify more precise biomarkers of OPIDN.

There was a statistically significant correlation of r = 0.61 between the last clinical scores (clinical score at time of sacrifice), and the neuropathological scores. Therefore, clinical signs reflects the severity of lesions observed in the biventer cervicis nerve. Since this correlation exists between clinical scores and neuropathologic scores, this work suggests that clinical studies could be sufficient to predict the likelihood of OPIDN development, and morphologic evaluations might not be necessary in all cases. This would reduce the costs of industrial and regulatory safety studies. Nevertheless, light and electron microscopy morphologic evaluation seem to be essential if we are to eventually identify the mechanism of the development of OPIDN. Protection and promotion bring many questions and give rise to many new hypotheses on OPIDN mechanisms. Further studies with combined teased fiber-transmission electron microscopic studies could certainly help to understand initiating events leading to OPIDN. Since OPIDN is progressive, understanding the mechanism involved in OPIDN could be beneficial in the prevention and treatment of this neuropathy.

VII. References

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Appendix

1. Clinical signs and OPIDN
2. Pathology and OPIDN
3. Pathology and OPIDN, immersion fixation in cross sections
4. Pathology and OPIDN, perfusion fixation in cross sections
5. Pathology and OPIDN, immersion fixation (teased nerve fibers)
6. Pathology and OPIDN, perfusion fixation (teased nerve fibers)

Appendix 1: Clinical signs and OPIDN

DRUGS	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16
PSP (2.5 mg/Kg)			0	0	0	0	0								
PSP (2.5 mg/Kg)			0	0	0	0	0	1							
PSP (2.5 mg/Kg)			0	0	0	0	0	0							
PSP (2.5 mg/Kg)	0	0	0	0	0	1	1	2							
PSP (2.5 mg/Kg)	0	0	0	0	0	0	0	2	1	3	7	8	8	8	
PSP (2.5 mg/Kg)	0	0	0	0	1	2	2	2	3	4	4	6	6	6	
PSP (2.5 mg/Kg)	0	0	0	1	0	0	0	0	2	3	3	7	7	8	
PSP (2.5 mg/Kg)	0	0	0	0	0	0	0	2	4	5	5	7	8	8	
average	0	0	0	0.125	0.125	0.375	0.375	1.286	2.5	3.75	4.75	7	7.25	7.5	
stdev	0	0	0	0.354	0.354	0.744	0.744	0.951	1.291	0.9574	1.7078	0.8165	0.9574	1	
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)			0	0	0	1	1	1							
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)			0	0	0	0	0	0							
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)			0	0	0	0	0	0							
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)			0	0	0	0	0	0	1	1	0	0	0	0	
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	0	0	1	1	0	0	0	0	0	0	1	0	0	
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
average	0	0	0	0.375	0.375	0.375	0.125	0.125	0.2	0.2	0	0.2	0	0	0
stdev	0	0	0	0.518	0.518	0.518	0.354	0.354	0.4472	0.4472	0	0.4472	0	0	0
PSP (1 mg/Kg)			0	0	0	0	0	0							
PSP (1 mg/Kg)			2	2	2	2	2	2							
PSP (1 mg/Kg)			0	0	0	0	0	0							
PSP (1 mg/Kg)			0	0	0	2	2	3							
PSP (1 mg/Kg)	0	0	0	0	0	0	0	0	1	2	2	3	4	6	
PSP (1 mg/Kg)	0	0	0	0	0	0	0	2	3	3	3	4	4	5	
PSP (1 mg/Kg)	0	0	0	0	0	0	0	2	3	3	3	3	7	7	
PSP (1 mg/Kg)	0	0	0	0	0	0	0	0	2	2	2	2	3	4	
average	0	0	0.25	0.25	0.25	0.5	0.5	1.125	2.25	2.5	2.5	3	4.5	5.5	
stdev	0	0	0.707	0.707	0.707	0.926	0.926	1.246	0.9574	0.5774	0.5774	0.8165	1.7321	1.291	
PSP (1 mg/Kg)+PMSF (90 mg/Kg)			0	0	1	2	2	3							
PSP (1 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	1	3	3							
PSP (1 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	1	1	2							
PSP (1 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	1	0	2	3	6	7	7	7	7	7
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	0	0	0	0	0	0	0	2	2	6	6	7	7	7	7
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	0	0	0	0	1	0	0	3	3	3	3	5	6	7	8
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	0	0	1	1	0	0	0	2	2	4	4	5	6	7	8
average	0	0	0.143	0.143	0.286	0.714	0.857	2.429	2.5	4.75	5	6	6.5	7	7.5
stdev	0	0	0.378	0.378	0.488	0.756	1.215	0.535	0.5774	1.5	1.8257	1.1547	0.5774	0	0.5774

Appendix 1: Clinical signs and OPIDN

DRUGS	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16
PMSF (90 mg/Kg)			0	0	0	0	0	0							
PMSF (90 mg/Kg)			0	0	0	1	1	0							
PMSF (90 mg/Kg)			0	0	0	0	0	0							
PMSF (90 mg/Kg)			0	0	0	0	0	0							
PMSF (90 mg/Kg)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PMSF (90 mg/Kg)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PMSF (90 mg/Kg)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PMSF (90 mg/Kg)	0	2	2	2	1	1	1	0	0	0	0	0	0	0	0
average	0	0.5	0.25	0.25	0.125	0.25	0.25	0	0	0	0	0	0	0	0
stdev	0	1	0.707	0.707	0.354	0.463	0.463	0	0	0	0	0	0	0	0
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	2	2							
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	2	2	3	4							
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	2	2							
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	2	2							
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	1	1	2	2	3	3	4	4	
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	1	1	2	3	3	3	4	4	
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	1	1	1	2	4	4	4	4	
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)			0	0	0	0	1	1	1	1	2	3	3	4	
average			0	0	0.25	0.25	1.625	1.75	1.5	2	3	3.25	3.75	4	
stdev			0	0	0.707	0.707	0.744	1.035	0.5774	0.8165	0.8165	0.5	0.5	0	
PSP (0.5 mg/Kg)			0	0	0	0	0	0							
PSP (0.5 mg/Kg)			0	0	0	0	0	1	1						
PSP (0.5 mg/Kg)			0	0	0	0	0	1	1						
PSP (0.5 mg/Kg)			0	0	0	0	0	0	0						
PSP (0.5 mg/Kg)			0	0	0	0	0	0	0	0	0	0	0	0	
PSP (0.5 mg/Kg)			0	0	0	0	0	0	0	0	0	0	0	0	
PSP (0.5 mg/Kg)			0	0	0	0	0	0	0	0	0	0	0	0	
PSP (0.5 mg/Kg)			0	0	0	0	0	0	0	0	0	0	0	0	
PSP (0.5 mg/Kg)			0	0	0	0	0	0.25	0.2857	0	0	0	0	0	
average			0	0	0	0	0	0.463	0.488	0	0	0	0	0	
stdev															
Control (DMSO)			0	0	0	0	0	0	0	0	0				
Control (DMSO)			0	0	0	0	0	0	0	0	0	0	0	0	0
Control (DMSO)			0	0	0	0	0	0	0	0	0	0	0	0	0
Control (DMSO)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control (DMSO)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control (DMSO)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control (DMSO)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
average	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
stdev	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 2: Pathology and OPIDN

DRUGS	I / p f	LAST CS	%NOR F	%DEG F	Pscore
PSP (2.5 mg/Kg)	I	1	100	0	0
PSP (2.5 mg/Kg)	I	0	98.8	1.2	0
PSP (2.5 mg/Kg)	I	2	98.7	0	0
PSP (2.5 mg/Kg)	I	2	91.5	7.6	1
PSP (2.5 mg/Kg)	pf	8	71	29	2
PSP (2.5 mg/Kg)	pf	6	71	26.2	3
PSP (2.5 mg/Kg)	pf	8	46.3	53.7	3
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	I	1	100	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	I	0	95.6	4.4	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	I	0	97.9	2.1	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	I	8	99.3	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	pf	0	98.7	1.3	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	pf	0	100	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	pf	0	96.8	2.6	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	pf	0	87.2	11.1	1
PSP (1 mg/Kg)	I	0	100	0	0
PSP (1 mg/Kg)	I	2	98.3	1.3	0
PSP (1 mg/Kg)	I	0	98.2	0.9	0
PSP (1 mg/Kg)	I	2	85.7	5.5	1
PSP (1 mg/Kg)	pf	6	71	29	2
PSP (1 mg/Kg)	pf	7	62	38	2
PSP (1 mg/Kg)	pf	4	38.3	61.7	4
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	3	93	5.1	1
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	3			
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	7	44.9	52.9	3
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	7	52.7	47.3	3
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	8	34.4	65.6	4
PSP (1 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	8	38.5	61.5	4
PMSF (90 mg/Kg)	I	0	100	0	0
PMSF (90 mg/Kg)	I	0	99.3	0.7	0
PMSF (90 mg/Kg)	I	0	93.9	1.5	0
PMSF (90 mg/Kg)	I	0	84.1	4.7	0
PMSF (90 mg/Kg)	pf	0	96.1	3.9	0
PMSF (90 mg/Kg)	pf	0	99.4	0.6	0
PMSF (90 mg/Kg)	pf	0	96.3	3.7	0
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	3	77.4	22.6	2
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	2	98.8	1.2	0
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	2	88.1	11.9	1
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	I	2	97.8	2.2	0
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	4	27.6	65.5	4
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	4	46.7	52.6	3
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	4	0.9	89.3	4
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)-4 hrs	pf	4	20.7	58	3

Appendix 2: Pathology and OPIDN

DRUGS	I / p f	LAST CS	%NOR F	%DEG F	Pscore
PSP (0.5 mg/Kg)	I	0	100	0	0
PSP (0.5 mg/Kg)	I	1	97.2	2.8	0
PSP (0.5 mg/Kg)	I	1	97	3	0
PSP (0.5 mg/Kg)	pf	0	97.9	2.1	0
PSP (0.5 mg/Kg)	pf	0			
PSP (0.5 mg/Kg)	pf	0	100	0	0
PSP (0.5 mg/Kg)	pf	0	97	1.5	0
Control (DMSO)	I	0	99.2	0	0
Control (DMSO)	I	0	97.1	2.9	0
Control (DMSO)	I	0	100	0	0
Control (DMSO)	I	0	99.5	0.5	0
Control (DMSO)	I	0	100	0	0
Control (DMSO)	I	0	100	0	0
Control (DMSO)	I	0	97.8	1.1	0
Control (DMSO)	pf	0	82.1	4.9	0
Control (DMSO)	pf	0	97.6	2.4	0
Control (DMSO)	pf	0	98.2	1.8	0
Control (DMSO)	pf	0	98.8	0.6	0

I/pf: Immersion/ perfusion
LAST CS: Last clinical sign
%Nor F: Percentage of normal fibers
%DEG F: Percentage of degenerated fibers
Pscore: Pathology score

Appendix 3: Pathology and OPIDN, immersion fixation (cross sections)

DRUGS	LAST CS	%NOR F	%DEG F	%REG F	Pscore
Control (DMSO)	0	99.2	0	0.8	0
Control (DMSO)	0	97.1	2.9	0	0
Control (DMSO)	0	100	0	0	0
Control (DMSO)	0	99.5	0.5	0	0
Control (DMSO)	0	100	0	0	0
Control (DMSO)	0	100	0	0	0
Control (DMSO)	0	97.8	1.1	1.1	0
average	0.000	99.086	0.643	0.271	0.000
stdev	0.000	1.175	1.078	0.472	0.000
PMSF (90 mg/Kg)	0	100	0	0	0
PMSF (90 mg/Kg)	0	99.3	0.7	0	0
PMSF (90 mg/Kg)	0	93.9	1.5	4.6	0
PMSF (90 mg/Kg)	0	84.1	4.7	11.2	0
average	0.000	94.325	1.725	3.950	0.000
stdev	0.000	7.341	2.076	5.297	0.000
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	1	100	0	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	95.6	4.4	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	97.9	2.1	0	0
average	0.333	97.833	2.167	0.000	0.000
stdev	0.577	2.201	2.201	0.000	0.000
PSP (0.5 mg/Kg)	0	100	0	0	0
PSP (0.5 mg/Kg)	1	97.2	2.8	0	0
PSP (0.5 mg/Kg)	1	97	3	0	0
average	0.667	98.067	1.933	0.000	0.000
stdev	0.577	1.677	1.677	0.000	0.000
PSP (1 mg/Kg)	0	100	0	0	0
PSP (1 mg/Kg)	2	98.3	1.3	0.4	0
PSP (1 mg/Kg)	0	98.2	0.9	0.9	0
PSP (1 mg/Kg)	2	85.7	5.5	8.8	1
average	1.000	95.550	1.925	2.525	0.250
stdev	1.155	6.618	2.445	4.200	0.500
PSP (2.5 mg/Kg)	1	100	0	0	0
PSP (2.5 mg/Kg)	0	98.8	1.2	0	0
PSP (2.5 mg/Kg)	2	98.7	0	1.3	0
PSP (2.5 mg/Kg)	2	91.5	7.6	0.9	1
average	1.250	97.250	2.200	0.550	0.250
stdev	0.957	3.879	3.644	0.656	0.500

Appendix 3: Pathology and OPIDN, immersion fixation (cross sections)

DRUGS	LAST CS	%NOR F	%DEG F	%REG F	SCORE
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	3	77.4	22.6	0	2
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	2	98.8	1.2	0	0
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	2	88.1	11.9	0	1
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	2	97.8	2.2	0	0
average	2.250	90.525	9.475	0.000	0.750
stdev	0.500	9.992	9.992	0.000	0.957
PSP (1.0 mg/Kg)+PMSF (90 mg/Kg)	3	93	5.1	1.9	1
PSP (1.0 mg/Kg)+PMSF (90 mg/Kg)	2	90.5	9.5	0	1

LAST CS: Last clinical sign
%NOR F: Percentage of normal fibers
%DEG F: Percentage of degenerated fibers
%REG F: Percentage of regenerated fibers
Pscore: Pathology score

Appendix 4: Pathology and OPIDN, perfusion fixation (cross sections)

DRUGS	LAST CS	%NOR F	%DEG F	%REG F	Pscore
Control (DMSO)	0	82.1	4.9	13	0
Control (DMSO)	0	97.6	2.4	0	0
Control (DMSO)	0	98.2	1.8	0	0
Control (DMSO)	0	98.8	0.6	0.6	0
average	0.000	94.175	2.425	3.400	0.000
stdev	0.000	8.065	1.812	6.406	0.000
PMSF (90 mg/Kg)	0	96.1	3.9	0	0
PMSF (90 mg/Kg)	0	99.4	0.6	0	0
PMSF (90 mg/Kg)	0	96.3	3.7	0	0
average	0.000	97.267	2.733	0.000	0.000
stdev	0.000	1.850	1.850	0.000	0.000
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	98.7	1.3	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	100	0	0	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	96.8	2.6	0.6	0
PMSF (90 mg/Kg)+ PSP (2.5 mg/Kg)	0	87.2	11.1	1.7	1
average	0.000	95.675	3.750	0.575	0.250
stdev	0.000	5.801	5.014	0.802	0.500
PSP (0.5 mg/Kg)	0				
PSP (0.5 mg/Kg)	0	97.9	2.1	0	0
PSP (0.5 mg/Kg)	0	100	0	0	0
PSP (0.5 mg/Kg)	0	97	1.5	1.5	0
average	0.000	98.300	1.200	0.500	0.000
stdev	0.000	1.539	1.082	0.866	0.000
PSP (1 mg/Kg)	5	78.4	21.6	0	2
PSP (1 mg/Kg)	6	71	29	0	2
PSP (1 mg/Kg)	7	62	38	0	2
PSP (1 mg/Kg)	4	38.3	61.7	0	4
average	5.500	57.100	42.900	0.000	2.667
stdev	1.528	16.892	16.892	0.000	1.155
PSP (2.5 mg/Kg)	8	42.9	55.6	1.5	3
PSP (2.5 mg/Kg)	8	71	29	0	2
PSP (2.5 mg/Kg)	6	71	26.2	2.8	3
PSP (2.5 mg/Kg)	8	46.3	53.7	0	3
average	7.333	62.767	36.300	0.933	2.667
stdev	1.155	14.261	15.134	1.617	0.577

Appendix 4: Pathology and OPIDN, perfusion fixation (cross sections)

DRUGS	LAST CS	%NOR F	%DEG F	%REG F	SCORE
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	4	27.6	65.5	6.9	4
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	4	46.7	52.6	0.7	3
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	4	0.9	89.3	9.8	4
PSP (0.5 mg/Kg)+PMSF (90 mg/Kg)	4	20.7	58	21.3	3
average	4.000	23.975	66.350	9.675	3.500
stdev	0.000	18.910	16.189	8.630	0.577
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	7	44.9	52.9	2.2	3
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	7	52.7	47.3	0	3
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	8	34.4	65.6	0	4
PSP (1 mg/Kg)+PMSF (90 mg/Kg)	8	38.5	61.5	0	4
average	7.500	42.625	56.825	0.550	3.500
stdev	0.577	7.986	8.266	1.100	0.577

LAST CS: Last clinical sign
%NOR F: Percentage of normal fibers
%DEG F: Percentage of degenerated fibers
%REG F: Percentage of regenerated fibers
Pscore: Pathology score

Appendix 5: Immersion fixation - Nerve fiber teasing

1. PSP 2.5 mg/kg
2. PMSF + PSP 2.5 mg/kg (protected)
3. PSP 1.0 mg/kg
4. PSP 1.0 mg/kg + PMSF (promoted)
5. PMSF 90 mg/kg
6. PSP 0.5 mg/kg + PSMF (promoted)
7. PSP 0.5 mg/kg
8. Negative controls

Appendix 5: Pathology and OPIDN, immersion fixation (teased nerve fibers)

Groups	Hens	Last CS	% deg.- cross	Pscore-cross	% Deg.- NFT	pscoret
1.0	2191.0	1.0	0.0	0.0	9.0	1.0
1.0	2451.0	2.0	7.6	1.0	14.0	1.0
average		1.5			11.5	1.0
stdev		0.7			3.5	0.0
2.0	2073.0	1.0	0.0	0.0	2.0	0.0
2.0	2530.0	0.0	2.1	1.0	2.0	0.0
2.0	2273.0	0.0	4.4	0.0	0.0	0.0
average		0.3			1.3	0.0
stdev		0.6			1.2	0.0
3.0	2235.0	0.0	0.0	0.0	8.0	1.0
3.0	2036.0	2.0	0.3	0.0	6.0	1.0
3.0	2258.0	0.0	0.9	0.0	2.0	0.0
3.0	1985.0	2.0	5.5	1.0	10.0	1.0
average		1.0			6.5	0.8
stdev		1.2			3.4	0.5
4.0	2542.0	2.0	9.5	1.0	12.0	1.0
4.0	2089.0	3.0	5.1	1.0	13.0	1.0
average		3.0			12.5	1.0
stdev		0.7			0.7	0.0
5.0	2118.0	0.0	0.7	0.0	0.0	0.0
5.0	2115.0	0.0	4.7	0.0	0.0	1.0
5.0	2282.0	0.0	0.0	0.0	0.0	0.0
5.0	1958.0	0.0	1.5	0.0	0.0	0.0
average		0.0			0.0	0.3
stdev		0.0			0.0	0.5
6.0	2242.0	2.0	2.2	0.0	8.0	1.0
6.0	4872.0	2.0	11.9	1.0	13.0	1.0
6.0	2508.0	2.0	1.2	0.0	8.0	1.0
average		2.0			9.7	1.0
stdev		0.0			2.9	0.0
7.0	1984.0	0.0	2.4	0.0	0.0	0.0
7.0	2238.0	0.0	0.0	0.0	0.0	0.0
average		0.0			0.0	0.0
stdev		0.0			0.0	0.0

Appendix 5: Pathology and OPIDN, immersion fixation (teased nerve fibers)

Group	Hens	Last CS	% deg.- cross	Pscore-cross	% Deg.- NFT	pscoret
8.0	2422.0	0.0	2.9	0.0	0.0	0.0
8.0	2408.0	0.0	0.5	0.0	0.0	0.0
8.0	2096.0	0.0	1.1	0.0	0.0	0.0
8.0	2367.0	0.0	0.0	0.0	0.0	0.0
8.0	2320.0	0.0	0.0	0.0	0.0	0.0
average		0.0			0.0	0.0
stdev		0.0			0.0	0.0

Last CS: Last clinical score
% Deg.-cross: Percentage of nerve fiber degeneration (cross sections)
Pscore-cross: Pathology score (cross sections)
% deg.-NTF: Percentage of degeneration (nerve teased fibers)
pscoret: Pathology score (nerve teased fibers)

Appendix 6: Perfusion fixation - Nerve fiber teasing

1. PSP 2.5 mg/kg
2. PMSF + PSP 2.5 mg/kg (protected)
3. PSP 1.0 mg/kg
4. PSP 1.0 mg/kg + PMSF (promoted)
5. PMSF 90 mg/kg
6. PSP 0.5 mg/kg + PSMF (promoted)
7. PSP 0.5 mg/kg
8. Negative controls

Appendix 6: Pathology and OPIDN, perfusion fixation (teased nerve fibers)

Groups	Hens	Last CS	% Deg.-cross	Pscore-cross	% deg.-NFT	pscoret
1.0	2470.0	8.0	29.0	2.0	23.0	2.0
1.0	2278.0	8.0	53.7	3.0	21.0	2.0
average		8.0			22.0	2.0
stdev		0.0			1.4	0.0
2.0	2048.0	0.0	2.6	0.0	0.0	0.0
2.0	2159.0	0.0	1.3	0.0	9.0	1.0
average		0.0			4.5	0.5
stdev		0.0			6.4	0.7
3.0	2482.0	6.0	29.0	2.0	37.0	2.0
3.0	2358.0	7.0	38.0	2.0	41.0	3.0
3.0	2266.0	4.0	61.7	4.0	30.0	2.0
average		5.7			36.0	2.3
stdev		1.5			5.6	0.6
4.0	2354.0	8.0	65.6	4.0	32.0	2.0
4.0	2574.0	8.0	61.5	4.0	57.0	3.0
4.0	2338.0	7.0	47.3	3.0	51.0	3.0
average		7.7			46.7	2.7
stdev		0.6			13.1	0.6
5.0	2210.0	0.0	3.9	0.0	0.0	0.0
5.0	2211.0	0.0	0.6	0.0	0.0	0.0
average		0.0			0.0	0.0
stdev		0.0			0.0	0.0
6.0	2099.0	4.0	65.5	4.0	29.0	2.0
6.0	2424.0	4.0	52.6	3.0	43.0	3.0
6.0	0.0	4.0	89.3	4.0	33.0	2.0
6.0	2105.0	4.0	58.0	3.0	10.0	1.0
average		4.0			28.8	2.0
stdev		0.0			13.8	0.8
7.0	2170.0	0.0	2.1	0.0	2.0	0.0
7.0	2379.0	0.0	1.5	0.0	0.0	0.0
7.0	2516.0	0.0	1.5	0.0	0.0	0.0
average		0.0			0.7	0.0
stdev		0.0			1.2	0.0

Appendix 6: Pathology and OPIDN, perfusion fixation (teased nerve fibers)

Groups	Hens	Last CS	% Deg.-cross	Pscore-cross	% deg.-NFT	pscoret
8.0	2022.0	0.0	0.6	0.0	0.0	0.0
8.0	2183.0	0.0	4.9	0.0	0.0	0.0
8.0	2276.0	0.0	2.4	0.0	0.0	0.0
average		0.0			0.0	0.0
stdev		0.0			0.0	0.0

Last CS: Last clinical score
% Deg.-cross: Percentage of nerve fiber degeneration (cross sections)
Pscore-cross: Pathology score (cross sections)
% deg.-NTF: Percentage of degeneration (nerve teased fibers)
pscoret: Pathology score (nerve teased fibers)

Vita

Christiane Massicotte was born on June 3, 1970 in Montreal, Canada. After learning piano for 15 years, she obtained a Post-Laureat in piano interpretation in 1989. She then taught piano interpretation for 2 years at Vincent d'Indy (University of Montreal). She graduated from Jean de Brebeuf in May 1990 with an International Baccalaureate Degree in Science, Major in Biochemistry. Christiane then attended University of Montreal to pursue the Doctor in Veterinary Medicine program, and graduated in May, 1994. In 1994-1995 she was an intern at the Veterinary Teaching Hospital of the University of Minnesota. Following this internship (in 1995), she began a neurology residency at the Virginia-Maryland College of Veterinary Medicine.