# EFFECTS OF ORGANOPHOSPHATE ESTERS ON BLOOD VESSELS: A PHYSIOLOGICAL, PHARMACOLOGICAL, AND HISTOLOGICAL ASSESSMENT OF INVOLVEMENT IN ORGANOPHOSPHORUS-INDUCED DELAYED NEUROPATHY (OPIDN)

by

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(ABSTRACT)

The contribution of the cardiovascular system to organophosphate-induced delayed neuropathy (OPIDN) was examined using *in situ* and *in vitro* models for demonstration of response to vasoactive agents (e.g., the cholinergic agonist, acetylcholine; the $\alpha_1$ agonist, phenylephrine; and the $\beta_2$ agonist, salbutamol). These responses were compared before and 1, 3, 7, and 21 days after hens were administered cyclic phenyl saligenin phosphate (PSP, 2.5 mg/kg i.m.), an OP that induces OPIDN but does not significantly inhibit acetylcholinesterase activity, and paraoxon (PXN, 0.1 mg/kg i.m.), an OP that inhibits acetylcholinesterase activity but does not induce OPIDN. The capability of verapamil, a calcium channel blocker, to attenuate these responses was examined, as this agent ameliorates OPIDN. For the *in situ* study, the ischiadic artery was cannulated and alterations in pressure measured at a constant flow used to indicate changes in vascular resistance. Changes in vascular resistance in response to acetylcholine,

phenylephrine, and salbutamol that were different from those in control and PXN-treated hens were noted 1 and 3 days after administration of PSP. These changes were attenuated in hens given PSP and verapamil. Vascular segments from the ischiadic artery were used to provide an *in vitro* model to determine if OPs caused direct vascular damage that was responsible for effects seen in the *in situ* model. In the *in vitro* model, however, responses of PSP and PXN were similar and not modified in vascular segments from hens given verapamil as well as the OPs. This indicated that the contribution of the cardiovascular system to OPIDN was due to more than a direct effect on relatively large caliber vessels. The contribution of the cardiovascular system to OPIDN also did not appear to relate to morphological changes induced by administration of OPs, as no changes in vascular morphology were noted. An OP-induced effect that could contribute to vascular effects noted are levels of plasma catecholamines. These levels were altered in hens given PSP or PXN, with increases seen after administration of PSP and decreases seen after administration of PXN. These alterations in plasma catecholamine levels were attenuated in hens given both verapamil and OP.

I dedicate this work to
my family:
My wife, Carolyn, and my daughters,
Kathy and Jennifer.

Without their love, support, and understanding,
this work would not have been possible.

and to
my friends:
The faculty and staff
of the
VA-MD Regional College of Veterinary Medicine.

They, like a family, supported my efforts.

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I was hired by Dr. John C. Lee as a technician in the physiology / pharmacology research laboratory, which was under his directorship in 1985. Dr. Lee taught me the technical as well as the theoretical aspects of cardiovascular research and allowed to perform several research projects on my own. Dr. Lee has been my friend, supervisor, and unofficial advisor for almost nine years. I value his

counsel and admire his abilities as a scientist, teacher, and administrator.

Dr. Jeff Wilcke has also been my friend and advisor for many years. Dr. Wilcke is one of the few people that is knowledgeable in almost all areas from computer science to medical ethics. His intelligence, wit, and attitude allows him to comfortably interact with people on any level. He has frequently snatched me from the "jaws of death" at committee meetings. I appreciate the time that Dr. Wilcke has taken to advise me and I appreciate his friendship.

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Mr. Ronnie Long also deserves mention. Ronnie has taken time to fax letters and papers for me (that were not sent on time by surface mail) in order for me to meet deadlines. Ronnie produced copies of my thesis and dissertation the day that they were submitted; an awesome task. In fact, all the material that I submitted to reprographics was of the highest quality and returned long before the time that it was needed.

The list of individuals that I have worked with and learned from at this institution would be too long to place in the acknowledgement section of this document. I would like to thank all of the investigators who have allowed me to work on their projects as well as the technicians with which I have worked. I thank you and I will miss you.

I would like to thank The VA-MD Regional College of Veterinary Medicine in general and the Department of Biomedical Science in particular for their support. They have supported me financially through scholarships and assistantships as well as provided me with some of the most modern research tools available.

I feel that the opportunities that they have provided will make me a better scientist.

Finally, I would like to thank my parents whose love, guidance, encouragement, and support has always been there when it was needed.

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ABBREVIATIONS

| | |
|---|---|
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase |
| ACTCh | Acetylthiocholine |
| ANOVA | Analysis of variance |
| BuTCh | Butyrylthiocholine |
| $Ca^{++}$ | Calcium ion |
| $Cd^{++}$ | Cadmium ion |
| CPK | Creatinine phosphokinase |
| CVP | Central venous pressure |
| DAG | Diacyl glycerol |
| DMSO | Dimethyl sulfoxide |
| DP | Diastolic blood pressure |
| EDRF | Endothelium derived relaxing factor |
| IP3 | Inositol trisphosphate |
| K-H | Kreb's Henseleitt phosphate buffer solution |
| LPH | Luxol fast green, periodic acid/Schiff, and hematoxylin |
| LRU | Limb resistance unit ([MABP - CVP] / FLOW) |
| MLCK | Myosin light chain kinase |
| MABP | Mean arterial blood pressure |
| NE | Norepinephrine |
| NTE | Neurotoxic esterase |
| OP | Organophosphorus compound |
| OPIDN | Organophosphorus-induced delayed neuropathy |

| | |
|---|---|
| PE | Phenylephrine |
| PIP | Phosphoenolpyruvate |
| PLC | Phospholipase-C |
| POC | Potential operated channel |
| pS | Picosemins |
| PSP | Phenyl saligenin phosphate |
| PV | Phenyl valerate |
| PXN | Paraoxon |
| ROC | Receptor operated channel |
| SAL | Salbutamol |
| SP | Systolic blood pressure |
| TOCP | Tri-o-cresyl phosphates |
| ωCgTx | Omega conotoxin |

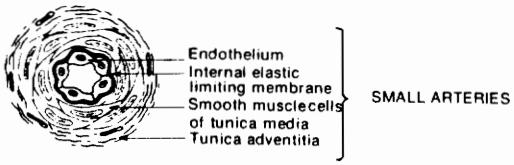
# PART I:

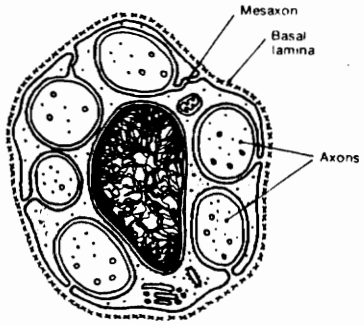
# HYPOTHESIS AND JUSTIFICATION

## STATEMENT OF HYPOTHESIS:

The hypothesis to be tested was that exposure to organophosphate esters which may cause delayed neuropathy cause alterations within the vascular system. These alterations may occur in blood vessels which supply muscles of affected limbs or in blood vessels which supply the peripheral nerves of affected limbs during the time between exposure and clinical manifestations of organophosphorus-induced delayed neuropathy (OPIDN). Alterations may result in structural or functional changes to these vessels and could be demonstrated as alterations in response to vasoactive agents or morphological changes of vessels within affected tissues. Support of this hypothesis by experimentation means that prevention of these vascular changes could ameliorate or attenuate the severity of organophosphorus-induced delayed neuropathy (OPIDN). Since calcium channel blockers, which were first used as cardiovascular drugs, have been demonstrated to ameliorate OPIDN, their beneficial effect may be due to prevention of damage to blood vessels supplying nerves and / or muscles affected in OPIDN.

# OP-INDUCED ALTERATIONS WITHIN THE VASCULAR SYSTEM




1. Damage to sympathetic vasomotor nerves:

   Possible damage to sympathetic nerves and blood vessels could occur concurrent with damage to somatic nerves.

2. Alteration of vascular response at the receptor level:

   Possible changes in response to neurotransmitters may occur at cell surface receptors either through up or down regulation or decreased affinity of the receptors for the transmitter.

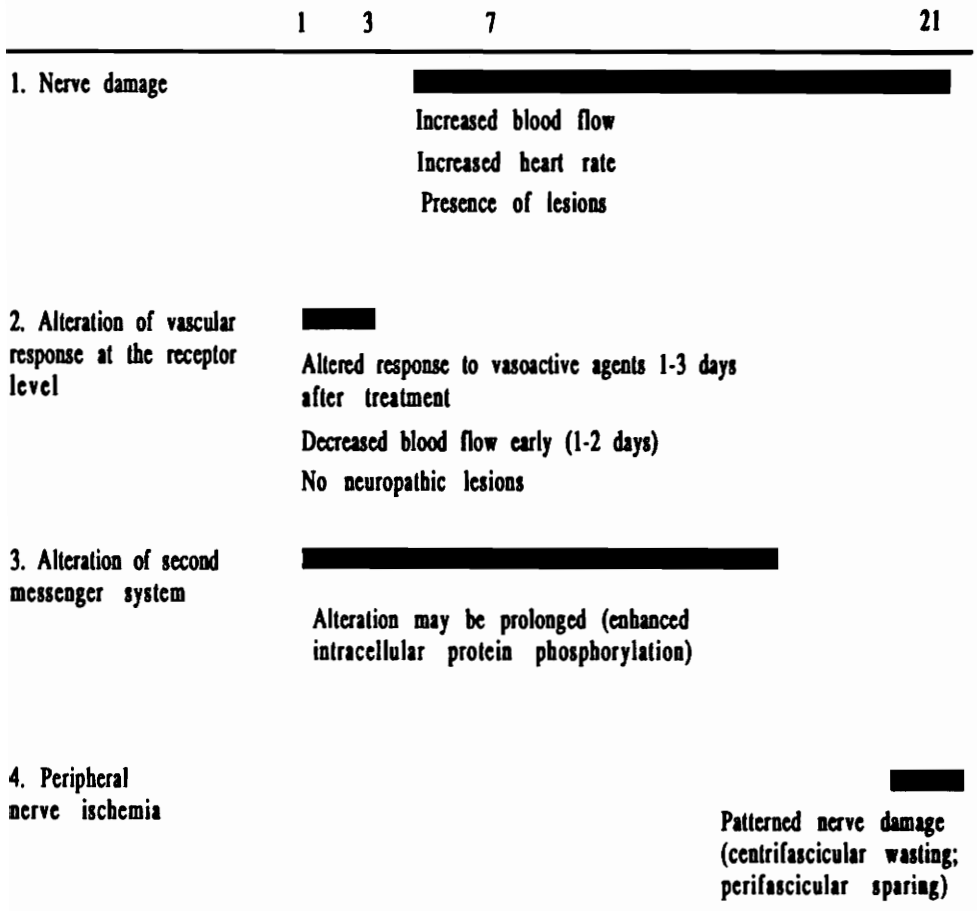
3. Alteration of second messenger systems:

   Changes in nerve cell functions following treatment with neuropathic OPs have been associated with either the inositol trisphosphate pathway and the cyclic adenosine monophosphate pathway.

4. Peripheral nerve ischemia:

   Reduction in blood flow has been observed early after exposure to neuropathic OPs. This reduction may produce ischemia and impair nerve function.

# EXPECTED CARDIOVASCULAR RESPONSES TO OP TREATMENT



## JUSTIFICATION FOR THE STUDIES:

Certain organophosphorus esters (OPs) are capable of producing a polyneuropathy some seven to fourteen days after exposure to a threshold amount in susceptible species. Although the most noticeable manifestations of this neuropathy involve a loss of motor control to the hind limb, other systems are impaired as well.

The specific aim of these studies was to investigate the possibility that OPs induce functional or morphological damage to nerves which control the vascular system and and to establish whether this OP-induced damage to vessels which supply the peripheral nerves and skeletal muscles was associated with organophosphorus-induced delayed neuropathy.

After administration of a neuropathy-inducing OP, the model for study attempted to identify or determine over time the following: (1) Alterations in the functional response of blood vessels to pharmacologic agents, nerve stimulation, and muscle stimulation *in vivo* and *ex vivo*. (2) Alterations in morphology and innervation of blood vessels in muscle and nerve tissue. (3) Relationship of changes in vascular function to changes in function or morphology of peripheral nerves or muscles, including determining whether vascular changes are a result of damage to autonomic innervation or are a secondary response to nerve damage. (4) Capability of the calcium channel blocker, verapamil, to prevent vascular changes associated with OPIDN.

There are two peripheral areas where nerves interface with the vascular system which were examined in this study. These are: (1) the nerve fibers that innervate the blood vessels, and (2) the blood vessels which supply the peripheral nerves. The sympathetic nervous system provides primary innervation of the peripheral blood vessels, although the vascular bed of skeletal muscles also receives some parasympathetic innervation. Peripheral nerves affected by neuropathy-inducing OPs also contain blood vessels which supply nutrients to support both glial and nerve cells. The effects of organophosphorus compounds on these vessels and their vasomotor nerves have not been determined. The studies were undertaken in order to provide this information.

This research was an extension of a preliminary study which demonstrated cardiovascular changes during the development of OPIDN after hens were exposed to a neuropathy-inducing dose of phenyl saligenin phosphate (McCain *et al*, 1993). These changes included increased resistance to blood flow prior to clinical signs of OPIDN, and elevation of limb venous flow and arterial blood pressure in hens with clinical signs of OPIDN. It was concluded that neuropathy-inducing OPs could alter hemodynamic parameters.

Administration of OPs can decrease blood flow and increase vascular resistance in skeletal muscles; an effect not expected with ganglionic stimulation by cholinesterase inhibition. My preliminary studies (McCain *et al*, 1993), indicating an increase in vascular resistance and a decrease in blood flow to skeletal muscles of the

hind limb, are supported by a previous study in which a decrease in blood flow was observed in hind limb skeletal muscle, kidney, and spleen after administration of OPs (Kristic, 1978). The adrenergic receptors of vessels in skeletal muscle are primarily $\beta_2$ (vasodilator) while $\alpha_1$ (vasoconstrictor) receptors are predominant on most other vessels (Parkinson, 1990). Stimulation of the post-ganglionic sympathetic fibers through the OP-induced inhibition of synaptic acetylcholinesterase should cause dilation of skeletal muscle blood vessels which would increase flow and decrease vascular resistance. Vasoconstriction, however, can occur at a stimulation rate above 4-5 Hz or catecholamine infusion rates above 2-3 mg/Kg/min (Celander, 1954). Because the decreased flow and increased resistance persisted after cholinesterase activity levels had returned to near normal, damage to post-synaptic sympathetic vasodilator fibers was a possibility and was investigated in this study.

An increase in peripheral resistance may also be indicative of denervation hypersensitivity observed in skeletal muscle and blood vessels. The effect observed in my preliminary study was greater seven days after OP administration than it was on the first day. This effect may result in a higher peripheral resistance due to increased muscle activity. Skeletal muscle, which can contribute as much as 25% to vascular resistance (Hudlicka, 1974), could be responsible, in part, for increased resistance in this tissue. Denervation hypersensitivity in larger vessels as a result of neural damage could also contribute to the effect seen (Geffen and Hughes, 1971), but

further studies are needed to investigate this possible mechanism for the changes observed. The effects of various OPs on the innervation of the vasculature was examined both *in vivo* and *ex vivo* as part of this research in order to determine the contribution of vasculature to OPIDN.

Blood vessels which supply the peripheral nerves could also be affected by neuropathy-inducing OPs. Blood vessels are important as they supply nutrients and contribute to the removal of toxicants from tissues (Maxwell *et al*, 1987). Peripheral vasoconstriction following exposure to OPs could reduce the nutrient supply to nerves during a time of increased activity and metabolic demand by peripheral nerve cells. Blood supply is also vital to regeneration of damaged nerves (Koistinaho *et al*, 1991). Koistinaho and his group demonstrated that adrenergic innervation of vessels within the tibial nerve was destroyed following Wallerian degeneration, a process also seen in OPIDN, and sympathetic vascular nerves may not fully regenerate. This could have a detrimental effect on vascular response, tissue perfusion, and nerve regeneration. If similar effects occurred after exposure to neuropathy-inducing OPs, the same mechanism could also contribute to pathological changes observed after OP administration.

The use of calcium channel blocking agents was shown to ameliorate the damage which normally occurs during OPIDN (El-Fawal *et al*, 1989). As calcium channel blocking agents were first developed because of their major physiologic effect on the

cardiovascular system, it is possible that they may act on the vasculature in order to prevent some of the damage which occurs during OPIDN. These agents enhance vasodilation and increase perfusion. These two effects are known to enhance detoxification and clearance of foreign compounds (Maxwell *et al*, 1987) and have also been reported to have contributed to neural regeneration (de la Torre, 1988). Therefore, it could be reasonably expected to observe amelioration of OPIDN when calcium channel blockers are examined for their effects on this chemically-induced nerve damage. This study was designed to assess cardiovascular effects of calcium channel blockers as they are used to ameliorate OPIDN.

The relationship of damage to blood vessels to damage to nerves was determined (1) by time response studies and (2) by comparison of OPIDN with the neurotoxicity caused by an organophosphate that causes an immediate, but nonneuropathic, effect. This was done to help determine if OP-induced cardiovascular effects are a response to changes which occur secondary to OP-induced neural damage or if OPs induce cardiovascular effects that are directly responsible for some of the damage which occurs.

# PART II

# LITERATURE REVIEW

# CHAPTER 1

# LITERATURE REVIEW:
# ORGANOPHOSPHATE TOXICITY

# CHAPTER 1: LITERATURE REVIEW

## 1.1. HISTORICAL PERSPECTIVE:

Although the toxic effects of organophosphorus (OP) compounds in humans were known before 1930, the accidental poisoning of more than 50,000 Americans by a mixture of cresyl phosphates used to extract ginger brought the irreversible, delayed effects of these compounds to the attention of the scientific community (Abou-Donia 1981). The introduction of OP insecticides and nerve agents followed this "Ginger Jake" incident. OPs were found useful as replacements for nicotinic insecticides which were in short supply during World War II (Koelle, 1981). They were also synthesized, but not used, as chemical weapons in that war. Since that time there has been continued exposure of man and animals to the toxic effects of these compounds. More than 40,000 deaths have been attributed to these compounds worldwide. Currently, OP compounds are used primarily in the agricultural, plastics, and petroleum industries. In the United States more than 60,000 tons of OP insecticides are produced annually, which accounts for the largest portion of the OPs produced. In addition to their use as insecticides, certain triaryl phosphates are used as plasticizers and may contribute as much as 50% by weight of the finished product. In the petroleum industry, OPs are used to prevent pre-ignition and plug fouling in gasolines and enhance the viscosity index of lubricating oils (Salem and Olajos, 1988; Abou-Donia, 1989).

## 1.2. EVIDENCE OF TOXICOSIS AFTER EXPOSURE TO OPs:

Organophosphorus compounds (OPs) produce two very different types of toxicoses: one that appears early and another that appears weeks to months after exposure. The latter is termed organophosphorus ester-induced delayed neuropathy (OPIDN) and is produced by some, but not all, OPs. Early clinical signs of exposure to OPs are usually associated with their esterase inhibiting capabilities, which prolong the effects of acetylcholine in cholinergic synapses of the central nervous system, peripheral ganglia, smooth muscle and glandular muscarinic receptors, and neuromuscular junctions. These early responses may also be elicited by compounds that do not cause the neuropathic condition; however, and some compounds which produce the neuropathy are not potent inhibitors of cholinesterase. The development of cholinergic signs following OP exposure is rapid, appearing within a few minutes to a few hours, depending on the route of exposure, lipid solubility, and dose of the OPs used. Signs may persist for one to three days in humans. Among these signs are stimulation of exocrine glands by acetylcholine, causing an increase in lacrimation, bronchial secretion, salivation, and sweating. In addition, an increase in gastrointestinal smooth muscle activity results in nausea, vomiting, abdominal cramps, diarrhea, tenesmus, and involuntary defecation in man and animals exposed to acetylcholinesterase-inhibiting OPs. Involuntary urination may

result from increased bladder tone. Bradycardia often accompanies OP toxicity due to effects of increased acetylcholine actions in cardiac muscle. This effect is sometimes masked by simultaneous central cholinergic stimulation of cardiac centers and stimulation of sympathetic ganglia. Skeletal muscle activity is also increased by excess acetylcholine due to the inhibitory effects of OPs on the acetylcholinesterase at neuromuscular junctions (Gupta and Dettbarn, 1987; Misulis *et al*, 1987). This may cause weakness, which can be followed by cramps and fasciculations. Cholinergic effects on the central nervous system can produce signs ranging from emotional instability, insomnia, and anxiety to apathy, nightmares, and slurred speech. Central control of skeletal muscles through nicotinic action can produce ataxia and depression or paralysis of respiratory muscles. Death following toxic exposure is attributed to respiratory insufficiency brought on by bronchoconstriction, bronchosecretion, and paralysis of the respiratory muscles: diaphram and intercostals (Murphy, 1986).

Some OPs have the ability to produce a polyneuropathy two to four weeks after exposure to a threshold level in susceptible species, including man. This OP-induced delayed neuropathy (OPIDN) displays a strong correlation with the inhibition of a different esterase, brain neuropathy target esterase (NTE, neurotoxic esterase). Significant inhibition (>70%) of this esterase in the brain is associated with the pathologic changes in the long axons of peripheral nerves and leads to impaired metabolic function and transport in affected

neurons (Lotti and Johnson, 1980; Johnson and Lotti, 1980; Ohkawa, 1980; Sprague *et al*, 1981; Moretto *et al*, 1987). A reduction in the neuron's ability to maintain long axons results in a Wallerian type of degeneration with distally accentuated axonal degradation followed by myelin degradation (Davis and Richardson, 1980). The accepted animal model for study of this disorder is the hen, as clinical signs are not obviousl in laboratory rodents (Abou-Donia, 1981).

The accepted treatment of cholinergic poisoning, which is characteristic of acute OP toxicosis, is the administration of atropine which acts as a competitive antagonist at postsynaptic muscarinic receptors. Atropine decreases the capability acetylcholine to bind with the receptor and therefore decreases the effects of excess acetylcholine. Atropine does not easily pass the blood brain barrier and higher doses are required in order to obtain sufficient concentrations in the central nervous system to alleviate these signs of cholinergic poisoning. Atropine, however, has little or no effect on peripheral neuromuscular activity, as it does not compete with acetylcholine at the nicotinic receptors (Gall, 1981; Clinton *et al*, 1988; Taylor, 1990).

For treatment of acute toxicosis associated with exposure to OPs, atropine is most often given in conjunction with cholinesterase reactivators such as praldoxime (2-PAM i.e. pyridine-2-aldoxime methyl iodide) and other oximes. Hydroxylamine and hydroxamic acids may also be used as cholinesterase reactivators (Gall, 1981; Bukowski, 1990; Taylor, 1990). These agents can reactivate

phosphorylated esterase by nucleophilic attack on the phosphorus atom of the phosphorylated enzyme. This reaction occurs at a much faster rate than spontaneous hydrolysis. Once the phosphorylated enzyme is "aged", reactivators are ineffective (Johnson, 1975; Davis *et al*, 1985).

The delayed clinical signs of OPIDN closely follow the progression of peripheral neural degeneration (Davis and Richardson, 1980; Jortner and Ehrich, 1987). Signs usually appear following a latent period of seven to twenty-one days. The neuropathy is usually progressive and irreversible. Although the modification of the outcome of OPIDN has been demonstrated by prophylaxis with corticoids (Ehrich and Gross, 1986; Ehrich *et al*, 1986; Ehrich *et al*, 1985; 1988), carbamates (Deyi *et al*, 1981), and calcium channel blockers (Dretchen *et al*, 1985; El-Fawal *et al*, 1989, 1990b), specific treatments for OPIDN that are effective after OP exposure have not been described.

In humans, the early signs of the OP-induced neuropathy are cramping of the calves, paresthesis of the feet and occasionally paresthesis of the hands (Hopkins, 1975; Murphy, 1986). These signs are rapidly followed by muscle weakness, bilateral foot drop, and, in some cases, loss of balance. Depending on the severity of neuropathy, weakness of the knee or hip may also be experienced. Wrist-drop and weakness of the hands and elbow have also been reported. Sensory loss is slight or absent (Hopkins, 1975), although some reports indicate that it may progress to the same degree as

motor loss. Severity of these signs depends on the compound used, the dose administered, the route of administration, and the age and health of the patient (Francis, 1983). Signs are also indicative of a general myopathy of the affected limbs. Muscle fiber diameter can be reduced by as much as fifty percent in severe intoxication. There is also an increase in the level of plasma creatinine phosphokinase (CPK), a probable indicator of muscle injury. Recovery is often incomplete, with muscle fibers being replaced by connective tissue (Abou-Donia, 1981; Cisson and Wilson, 1982). Spasticity of the legs and footdrop was noted in patients ten to twelve years following the 1930 outbreak (Aring, 1942). Specific antidotes for the delayed effects of OPs have not been identified.

## 1.3. CHEMISTRY AND BIOCHEMISTRY OF OPs:

The principal toxic effects of OP compounds are due to their ability to inhibit esterases. One class of enzymes inhibited by OPs are the cholinesterases, such as acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine (ACh) into choline and acetate. The first step in the hydrolysis reaction is the combination of enzyme and substrate. The phosphorus atom of the OP binds to the active, esteratic site of the cholinesterase molecule, which contains the exposed hydroxyl group of a serine molecule. The OP, therefore, substitutes for the carbonyl carbon atom of acetylcholine in the enzyme-substrate reaction (Bakry *et al*, 1988).

The attack of the serine hydroxyl by the OP produces a phosphorylated enzyme that is no longer capable of hydrolyzing acetylcholine. Deacylation and regeneration of the acetylcholinesterase occur when acetylcholine combines with the enzyme and is hydrolyzed into acetate and choline. A molecule of acetylcholinesterase can hydrolyze approximately 300,000 molecules of acetylcholine per minute (Murphy, 1986). When OPs combine with this enzyme, reactivation is limited (Davis and Richardson, 1980). Regeneration of acetylcholinesterase after exposure to OPs can take several hours if the alkyl groups on the phosphorylated enzyme are either ethyl or methyl (Murphy, 1986). Tertiary alkyl groups on the organophosphorus compounds enhance the stability of this complex and little or no regeneration is observed (Davis and Richardson, 1980; Abou-Donia, 1981; Davis *et al*, 1985). A process called "aging" occurs when an alkyl group is lost from the OP-substrate complex. This further enhances the stability of the phosphorylated enzyme (Johnson, 1975; Davis *et al*, 1985).

The delayed neuropathy associated with some OPs has been correlated with the inhibition of a membrane-bound esterase termed neurotoxic esterase (NTE, neuropathy target esterase) (Johnson, 1975). Johnson suggested that irreversible binding of OPs to NTE initiates events responsible for the delayed neuropathy. A strong correlation exists between the early inhibition of this enzyme and the subsequent development of OPIDN. Lotti and Johnson (1980) have further demonstrated that a 70-80% inhibition of NTE 1 to 48

hours following exposure to a suspect OP compound is predictive of ensuing neuropathy. Inhibition of NTE is currently used as a screen to predict the capability of OPs to cause OPIDN (Anonymous, 1985). Although carbamates and sulfonates can also inhibit NTE activity, the reaction is reversible and reactivation of the enzyme occurs. In fact, pretreatment with carbamates has been shown to have a protective effect against OPIDN probably by competitive reversibl mechanism (Gall, 1981; Deyi *et al*, 1981).

## 1.4. PATHOLOGY OF OPIDN:

The pathological signs of OPIDN can be seen in nerve, muscle, and neuromuscular junctions. The lesion most commonly associated with this neuropathy is a distal to proximal axonopathy which proceeds to Wallerian degeneration, a degeneration and disintegration of the axon and, secondarily, destruction of the myelin sheath distal to a lesion dividing it. This Wallerian degeneration preferentially affects long, large diameter myelinated fibers of the peripheral and central nervous system. In the central nervous system, long pathways such as the spinocerebellar, fasciculus gracilis, tectospinal, and cerebellospinal tracts of the spinal cord are affected (Jortner and Ehrich, 1987). Neurons with high axonal volume are more susceptible than smaller fibers or unmyelinated fibers.

Early signs of neural damage in the limb of the hen following exposure to PSP include focal regions of axoplasmic staining, axonal swelling, and an accumulation of dense staining debris which could

be seen in a few fibers seven days after administration (Jortner and Ehrich, 1987; El-Fawal *et al*, 1990a). The most pronounced neuropathological damage followed the onset of clinical signs. Two weeks following exposure, when clinical evidence of OPIDN was significant, peripheral nerves displayed extensive Wallerian degeneration as well as some bands of Bünger (bands formed from the union of sheath cells during the regeneration of peripheral neurons). Ultrastructural examination revealed early signs to include invaginations of Schwann cells on the axonal surface and intra-axonal aggregation of mitochondria, dense bodies and, to some degree, membranous figures composed of myelin. Advanced signs include disruption of cytoskeletal elements as evidenced by focal clearing which evolved to include the entire axonal profile. Dense bodies, flocculent material associated with the breakdown of mitochondria, and evidence of phagocytosis have been demonstrated in damaged nerve (Bouldin and Cavanagh, 1979; Jortner, 1984; Jortner and Ehrich, 1987).

Muscle necrosis has also been observed following exposure to OP compounds. Fiber damage has been reported for compounds that produce OPIDN as well as those that do not. Parathion, a compound that does not produce OPIDN, has been shown to produce muscle necrosis in exposed rats. New lesions appeared for five to eight days following daily injections, however, less than five percent of the fibers were affected (Kibler, 1972). Histological examination revealed that necrotic fibers displayed a disruption of sarcoplasmic

reticulum and infiltration by macrophages and monocytes (Dettbarn, 1984; Patterson *et al*, 1988). Replacement of these fibers by connective tissue and fat has been reported (Abou-Donia, 1981). In muscles of hens treated with TOCP, an OP that causes delayed neuropathy without significant inhibition of neural acetylcholinesterase activity, fibers became more rounded and some stained more darkly, relative to unaffected fibers, with hematoxylin and eosin (Wecker *et al*, 1978). There was also a decrease in muscle fiber diameter which was proportional to body weight loss (Cisson and Wilson, 1982).

# CHAPTER 2

# THE NEURAL / VASCULAR INTERFACE AND ITS POTENTIAL ROLE IN OP TOXICITY

# CHAPTER 2: THE NEURAL / VASCULAR INTERFACE AND ITS POTENTIAL ROLE IN OP TOXICITY

## 2.1. PERIPHERAL INNERVATION AND NERVOUS CONTROL OF THE VASCULATURE:

The peripheral nerves are the element that are most affected by OPIDN. These nerves are not only served by the vasculature but also play a role in vasomotor tone and blood flow. Thus, in this way, they affect their own nutrient supply. The peripheral vasculature that supplies nerves affected in OPIDN is controlled especially by the autonomic nervous system (ANS); primarily by vasomotor fibers of the sympathetic division of the ANS. Vasomotor fibers emerge from the lateral horn of the spinal cord via the ventral roots, branch off and form the rami communicants albi (Gaskill 1885), and terminate in the ganglia of the sympathetic chain. From there, they continue as postganglionic fibers and enter the muscle with the peripheral nerves. Some vasomotor fibers, however, do not pass through the ganglia of the sympathetic trunk but have their ganglia in the ventral root, which explains why some vasomotor activity persists following lumbar sympathectomy (Randall, 1950). These fibers again branch off and terminate in the adventitia of the vessel. Axons of vasomotor fibers come into contact with the external muscle layer of peripheral blood vessels and form a network of unmyelinated processes approximately 1um in diameter with varicosities of 3μm to 5μm forming at intervals between 80μm and 100μm (Akester, 1971). These varicosities come into close contact with the smooth muscle at

the adventital-medial border in arterioles (dia.=100μm to 50μm) and terminal arterioles (dia.=50μm to 15μm) and actually penetrate the media of veins and larger arteries (Fuxe and Sedvall, 1965). The mesh of different types of vasculature is greater in resistance vessels (arterioles, metarterioles, terminal arterioles, precapillary sphincters, and, to some degree, postcapillary venules) than in capacitance vessels (the major veins). Innervation of the larger muscle blood vessels (dia. > 100μm) is of the multiunit type where the transmitter is released from varicosities in close contact with muscle fibers. Single unit or visceral innervation is found in smaller vessels (dia. < 100μm) whereby electrical activity is conducted throughout the muscle layer by numerous gap junctions between adjacent muscle fibers (Bozler, 1948; Folkow, 1964).

Postganglionic fibers of the sympathetic nervous system may either adrenergic or cholinergic according to the type of transmitter released (Alquist, 1948). Adrenergic fibers of the $\alpha_1$ type elicit a vasoconstrictor response when stimulated while cholinergic fibers generally evoke a vasodilator response (Bell, 1969; Bell and Vought, 1971; Lee *et al*, 1976; Su, 1977; Eglin and Whiting, 1985). The release of transmitters from sympathetic terminals occurs at stimulation rates approximately ten times lower than that of somatic motor nerve fibers (Folkow, 1952). *Ex vivo* work indicates that the frequency of stimulation of somatic fibers under resting conditions is about 50hz while that of autonomic fibers is 1-3hz. At 6hz, 80-90% of maximal constriction is attained and maximal constriction is

obtained at a frequency of 10-15hz. At this rate of stimulation, blood flow is reduced to 10% of its resting value. Maximum constriction of the vessels occurs at four to six hertz and flow is reduced by only 30% by stimulation at 10-15hz (Mellander, 1960). Stimulation of vasoconstrictor fibers not only increases resistance, but small arterioles and precapillary sphincters may actually be occluded. This reduces the area for diffusion and transcapillary transport (Eriksson and Lisander,1964; Gray, 1971).

Sympathetic vasodilator fibers, which utilize acetylcholine as a postganglionic neurotransmitter and are stimulated at lower frequencies than their adrenergic counterparts, have also been identified (Uvnas, 1971). Vasodilation may be evoked when these fibers are stimulated at a frequency of 1hz and is maximal when they are stimulated at 12hz. At frequencies above 15hz vasoconstriction occurs and masks the effect of vasodilation. A decrease in oxygen consumption and an increase in blood flow following stimulation of vasodilator fibers indicates that the precapillary sphincters are not involved (Folkow, 1964). The response to stimulation of these fibers diminishes over time and has been reported to be due to a reduced release of transmitter at the nerve terminals (Mauskopf *et al*, 1969; Amenta, 1988).

Studies performed on vessels isolated from skeletal muscle indicate that, in the absence of sympathetic stimulation, low levels of acetylcholine ($10^{-9}$ to $10^{-8}$ M) caused relaxation of these vessels while higher concentrations ($10^{-7}$ to $10^{-5}$ M) cause contracture

(Vanhoutte, 1974). Following norepinephrine contracture, acetylcholine causes further contracture in the veins and relaxation of the arteries. These studies indicate that there are both excitatory and inhibitory acetylcholine receptors. The action of these receptors depends on the concentration of acetylcholine (Vanhoutte, 1974). The mechanism of these effects has been postulated and includes three possible pathways. Circulating acetylcholine could bind to muscarinic receptors on the endothelial wall causing the release of endothelium derived relaxing factor (EDRF) which can activate guanylate cyclase within the vascular smooth muscle. This in turn inhibits cyclic GMP and causes relaxation (Simionescu and Simionescu, 1986). Relaxation can also be obtained when acetylcholine binds to presynaptic receptors, inhibiting the release of norepinephrine. Acetylcholine can cause contracture by directly binding to muscarinic receptors of the smooth muscle (Furchgott and Zawadzki, 1980; Meraji *et al*, 1987; Duckles, 1988; Duckles and Garcia-Villalon, 1990).

Nerve fibers not associated with the autonomic nervous system can also affect the cardiovascular system. The stimulation of sensory neurons, particularly group III and IV fibers, can produce either a pressor or depressor response depending on the frequency of stimulation (Coote and Perez-Gonzales, 1970), intensity of stimulation, and duration of stimulation (Karashing, *et al*, 1981). These studies demonstrated that sensory stimulation composed of higher voltages and frequency and a longer pulse duration was

necessary to produce a pressor response indicating that thinner high threshold fibers are affected (Coote and Perez-Gonales, 1970). The increase in blood pressure during muscular activity is also caused by sensory stimulation of sensory mechanical and chemical receptors (Wenzel *et al*, 1965).

## 2.2. CENTRAL NERVOUS SYSTEM CONTROL OF THE VASCULATURE:

The role of supraspinal centers in the control of blood flow has been known for over 100 years. Neurons in the medulla adjust their activity in response to the afferent input from peripheral cardiovascular receptors and use this input to control blood vessels via sympathetic fibers (Folkow, 1964; Folkow and Neil, 1971). The pressor center, which is responsible for the tonic activity of the blood vessels, is located in the lateral reticular formation in the rostral two-thirds of the medulla while the depressor center is in the reticular formation caudal and medial to the pressor center (Dowman, 1972). The resting vascular tone is maintained by a sympathetic stimulation rate of 1 to 2 hz. of the pressor center. Vasodilation primarily occurs by inhibition of vasocomotor tone (Celander, 1954).

Neurons from both pressor and depressor centers have afferent and efferent connections in the hypothalamus and other supramedullary structures. Experimental evidence has

demonstrated central inhibition of cholinesterase following proximal microinjections of echothiophate into the posterior hypothalamic nucleus, with a dose related increase in blood pressure was observed. The peripheral mechanism involved was the sympathetic nervous system since intravascular injections of phentolamine abolished this response. Intracerebrovascular injections of atropine also inhibited the response, indicating the involvement of central muscarinic receptors (Buccafusco and Brezenoff, 1979). Proximal injections of carbachol, a cholinergic agonist, also evoked a pressor response from the ventromedial hypothalmic nucleus and a depressor response from the dorsomedial and premammallary hypothalamic nuclei (Brezenoff, 1972).

Central nervous system control of the vasculature includes areas other than the medulla. Cortical stimulation of the motor cortex, orbital cortex, gyrus cinguli, and temporal lobe can produce either pressor or depressor effects depending on frequency of stimulation. Emotional arousal, which can alter blood pressure and flow, indicates involvement of the bulbar area of the limbic center (Peiss, 1965). Fibers pass from the cortex to the paryamidal tract or substantia reticularis to terminate in the lateral spinal horns (Uvnas, 1960).

## 2.3. VASCULAR SUPPLY OF THE PERIPHERAL NERVES:

Nutrients as well as toxic agents are transported to the peripheral nerves through the *vasa nervorum*. This network of

blood vessels is formed from nutrient arteries arising from larger adjacent vessels and which, by anastamoses, form an epineural and perineural vascular plexus. Many of these vessels penetrate the perineurium, which is the connective tissue surrounding the nerve, and form an endoneural network comprised primarily of capillaries. The capillary network is most extensive in the gray matter of the dorsal root ganglia. The venous plexus is similar to that of the arterial network (Olsson, 1975).

The permeability of the vessels supplying the peripheral nerves has been studied using protein tracers (Washman, 1966; Olsson, 1967). Intravenous injections of trypan blue or Evans blue caused blue staining of all perenchymatous tissues except brain and spinal cord. The lack of staining in the brain and spinal cord is due to the presence of the blood-brain barrier. In the peripheral nervous system, dorsal root ganglia and epineurium were stained while the endoneurium, which includes cells that are in the closest contact with the nerves, was either unstained or slightly stained. Experiments in which fluorescent labels were attached to albumen or gamma globulin showed that these proteins can migrate to the innermost border of the perineurium but not beyond into the endoneurium (Olsson, 1975). However, recent evidence indicates that plasma proteins are found in damaged neurons as early as five minutes following injury (Loberg and Torvik, 1991). Fibers which pass through the lesioned area also take up plasma proteins through retrograde axonal transport but the nucleus is spared.

When neuropathy follows trauma or mechanical damages, there is an immediate exudation of albumin at the site of the lesion for the first day. Distal involvement is evident after the first day. A second period of extravasation of protein, which causes endoneural edema, occurs about two weeks after the injury and usually involves all vessels distal to the lesion (Olsson, 1975). This second influx of protein into the nerve has been associated with Wallerian degeneration. Toxic and metabolic conditions, such as ischemia, which cause peripheral nerve disease, cause a similar protein influx and endoneural edema (Lundborg, 1970; Olsson, 1975)

## 2.4. EFFECTS OF OPs ON CARDIOVASCULAR PARAMETERS:

Although investigations into the cardiovascular effects of exposure to OPs have been conducted (Batillard *et al*, 1990; Maxwell *et al*, 1987; Vitterlein and Hasse, 1979; Vanhoutte, 1974; ), the investigations conducted to date have examined the relatively short term effects of potent cholinesterase inhibitors and there are few papers on the effects of OPIDN on cardiovascular parameters. The inhibition of acetylcholine by soman (pinacolyl methylphosphonofluoridate) caused severe respiratory depression and a transient hypertensive effect but no changes in heart rate or cardiac output. The distribution of blood was altered following exposure and an increase of flow to the brain, heart, and lungs was observed. Decreases in blood flow to the muscle, skin, and kidneys was observed (Maxwell *et al*, 1987). Multiple linear regression

indicated that there was a positive correlation between blood flow and the alleviation of effects that was not due to esterase activity alone. Vitterlein and Hasse (1979) obtained similar results.

More recently, the effects of parathion and paraoxon on lung vasculature has been examined in the isolated, perfused rabbit heart-lung preparation (Delaunois, *et al*, 1992). This study demonstrated that capillary endothelium becomes more permeable and induces pulmonary edema without altering hemodynamic parameters at any level of the vascular bed. It was further determined that this effect was mediated through muscarinic receptors as atropine given prior to paraoxon completly abolished the response observed in lungs treated with paraoxon only.

Recently, an investigation was conducted which followed the alteration of heart rate and body temperature following DFP administration in the rat. (Gordon, 1993). DFP is an OP that causes both acute AChE inhibition and OPIDN. A significant increase in heart rate was observed 3 days after the administration of the OP, a result similar to that obtained in our laboratory using PSP treated hens (McCain *et al*, 1993). This indicates a similarity of response to neurotoxic OPs in both the hen and the rat.

# CHAPTER 3

# THE ROLE OF CALCIUM IONS IN NERVES AND BLOOD VESSELS AND THEIR CONTRIBUTION TO OPIDN.

## CHAPTER 3: THE ROLE OF CALCIUM IONS IN NERVES AND BLOOD VESSELS AND THEIR CONTRIBUTION TO OPIDN:

### 3.1. THE CALCIUM CHANNEL:

Calcium channels are not uniform and can be subdivided into three (or four) major categories: (1.) T-type channels (transient), (2.) N-type channels (neuronal), and (3.) L-type channels (long lasting, large capacitance). A fourth type of channel, P-type, has recently been identified on the perikaryon of nerve cells (Kostyuk, 1989). These channels have been differentiated based on their sensitivity to organic calcium channel antagonists and the inorganic calcium channel antagonist, cadmium ($Cd^{++}$). T-type channels are insensitive to organic calcium channel antagonists (i.e., verapamil, nifedipine) and the inorganic calcium channel antagonist, $Cd^{++}$. They are activated by weak depolarizations which reduce membrane potential to approximately -70 mV and, once activated, conductance through these channels is quite low (approximately 9 picosiemens [pS]). The inactivation of T-type channels appears to be voltage dependent in a manner similar to that of sodium ($Na^{+}$) channels. T-type channels also resemble $Na^{+}$ channels in that they are more persistant in patch clamp techniques than are N-type or L-type $Ca^{++}$ channels (Nayler, 1988).

L-type channels are strongly blocked by organic calcium channel antagonists and $Cd^{++}$. These channels are activated by strong depolarizations which reduce the membrane potential to -10 mV (-20 mV to -5 mV). Once activated, calcium conductance is quite high (approximately 25 pS) and displays intermittent conductance

with periods of sustained conductance throughout the depolarization period. L-type channels can be further subdivided based on their sensitivity to omega conotoxin (ωCgTx). One subtype, $L_n$, is found in the nerve terminal and is inhibited by ωCgTx. Another subtype, $L_m$, is found predominantly in skeletal muscle and is insensitive to blockade by ωCgTx.

N-type calcium channels activate near the beginning of the depolarization cycle and are inactive near the end of the cycle. Conductance through these channels is moderate (about 13 pS). N-type channels are insensitive to the organic calcium channel blockers but are sensitive to $Cd^{++}$ and ωCgTx. These channels require strong depolarizations in order to operate (to -10 mV). N-type calcium channels are similar to the L-type channels in that they can use Barium as a charge carrier although, unlike the L-type channels, $Ca^{++}$ is the preferred charge carrier (Nayler, 1988). These channels are believed to be limited to neuronal membranes (Spedding, 1987).

## 3.2. CALCIUM AND NERVES:

Calcium ions ($Ca^{++}$) are an important component of synaptic neurotransmission. It was demonstrated that some $Ca^{++}$ moves into the neuron during the action potential through potential operated calcium channels (Hodgkin and Baker, 1978). These channels are found primarily in the axon terminal. It was proposed that $Ca^{++}$ acts as an intracellular second messenger associated with transmitter release. This concept was based on evidence that when $Na^{+}$ and $K^{+}$

channels are blocked (tetrodotoxin and tetraethyl ammonium, respectively), $Ca^{++}$ influx produces a secretory potential. This secretory potential can be blocked by the inorganic $Ca^{++}$ channel blocker, $Mg^{++}$. $Ca^{++}$ entry has been related to transmitter release in voltage clamp studies which revealed that graded depolarization caused a graded entry of $Ca^{++}$ into the nerve terminal and a graded release of transmitter (Llineas *et al*, 1977). Hauser and Reese (1977) demonstrated that in cholinergic nerve terminals, $Ca^{++}$ channels are in close proximity to dense bodies which are located on the inner face of the synaptic membrane. Calcium entry initiated the fusion of the neurotransmitter vesicles to the membrane and the subsequent extrusion of vesicular contents into the synaptic cleft.

Calcium ions are also important in the manufacture and degradation of structural elements of the neuron (see section 3.4).

## 3.3. CALCIUM AND VASCULAR SMOOTH MUSCLE:

Contraction of vascular smooth muscle, like that of skeletal and cardiac muscle, is dependent on the influx of extracellular calcium ions ($Ca^{++}$). Unlike skeletal muscle, which stores large amounts of $Ca^{++}$ in the sarcoplasmic reticulum, most of the activator $Ca^{++}$ for vascular smooth muscle is derived from extracellular stores. Some studies have demonstrated that although small arteries can contract in calcium free solutions, $^{45}Ca^{++}$ release from small arteries is less than that for large arteries and may indicate a greater dependence of these arteries on extracellular $Ca^{++}$.

The movement of $Ca^{++}$ into vascular smooth muscle cells occurs through potential operated $Ca^{++}$ channels (POC) and receptor operated $Ca^{++}$ channels (ROC). Evidence for potential operated channels has been obtained primarily from patch clamp techniques. When the membrane is clamped at approximately -45 mV, force develops and suggests the presence of POCs. Two types of potential dependent $Ca^{++}$ currents have been identified. A transient current evoked by small depolarizations more negative than -40 mV and a maintained current at membrane potentials greater than -40 mV. It has been demonstrated that the maintained current is ATP dependent and that the transient current is ATP independent (Ohya and Spercles, 1989).

The presence of receptor operated channels (ROC) on vascular smooth muscle cells is supported by the observation that norepinephrine (NE), when added to rat mesentry arteries exposed to high potassium solutions, caused an increase in tone without further depolarization and an accompanying increase in $^{45}Ca^{++}$ influx (Cauvin *et al*, 1988). Other studies have demonstrated that when the $Ca^{++}$ channels are blocked by calcium channel blocking agents in concentrations that completely suppress the potassium induced response, NE can still elicit a maintained contractile response even when intracellular stores of $Ca^{++}$ have been depleted (Nyborg and Mulvaney, 1984). Direct evidence of an ATP activated channel has been demonstrated in the rabbit ear artery. The $Ca^{++}$ influx through this channel could increase the cytosolic $Ca^{++}$ level by approximately

500 nM, which was sufficient to induce contraction (Ohya and Sperelakis, 1989).

Calcium also contributes to stimulation of excitation-contraction coupling in vascular smooth muscle. This can be done as adrenergic agonists (i.e. epinephrine, norepinephrine) stimulate $\alpha_1$ adrenergic receptors. The $\alpha_1$ adrenergic receptors were linked to phospholipase C (PLC) through an a proposed guanine nucleotide-binding protein (G protein). PLC initiates the hydrolysis of phosphatidyl-inositol-4,5-bisphosphate ($PIP_2$) into inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ is important in the contraction of smooth muscle as it causes the release of $Ca^{++}$ from intracellular stores. Four $Ca^{++}$ bind to the regulatory protein, calmodulin, forming a $Ca^{++}$ / calmodulin complex which activates the calmodulin sensitive enzyme, myosin light chain kinase (MLCK), a phosphorylating enzyme. MLCK phosphorylates the regulatory chain (light chain) of each myosin head causing myosin to bind to actin and undergo a conformational change which results in contraction. When $Ca^{++}$ falls below a critical level, myosin phosphatase splits the phosphate from the myosin molecule and contraction ceases (Guyton, 1991).

In contrast to stimulation of the $\alpha_1$ receptors in vascular smooth muscle just described, stimulation of skeletal muscle blood vessels by the sympathetic nervous system results in vasodilation, a component of the fight or flight response. Neurogenic sympathetic vasodilation occurs primarily through the action of NE on $ß_2$ adrenergic receptors. Stimulation of these receptors uses another

type of G protein ($G_S$) to link receptor binding to stimulate adenylate cyclase. This enzyme is responsible for the synthesis of the second messenger, cyclic adenosine monophosphate (cAMP) from ATP. It has been suggested that cAMP in vascular smooth muscle enhances the activity of some membrane bound $Ca^{++}$ pumps and through these, lowers the intracellular $Ca^{++}$ level and hyperpolarizes the cell (Kamm amd Stull, 1985).

## 3.4. CALCIUM AND NEUROPATHIES WITH EMPHASIS ON OPIDN.

Calcium homeostasis in the neuronal cell is maintained by several intracellular mechanisms. These mechanisms include membrane bound channels and pumps such as the sodium / calcium exchange mechanism, receptor operated calcium channels, and voltage operated calcium channels. Intracellular calcium is also sequestered and released by intracellular organelles such as calcisomes, endoplasmic reticulum, mitochondria, and synaptic vesicles. Increases in intracellular calcium have been associated with a number of neuropathic disorders including those caused by trauma and by toxicants through the activation of calcium dependent enzyme systems. Some of these enzyme systems, when activated by abnormally high concentrations of calcium, can cause cellular damage. Calcium activated mechanisms which have been identified as mediators of neuronal damage include phospholipase $A_2$, phospholipase C, phosphokinase C, calpain 1 and 2, calmodulin-

dependent kinase activation, calcium-dependent endonuclease activation, and c-fos activation. Neuronal cell mitochondria can also be detrimentally affected by uptake of high amounts of intracellular calcium (Kauppinen *et al*, 1989; Verity *et al*, 1990).

There is also evidence that the use of calcium channel blocking agents can reduce the toxic effects of exposure to OPs. Dretchen *et al* (1985) investigated the possibility that repetitive discharges from the nerve terminal could be inhibited by calcium channel blocking agents and demonstrated that verapamil could protect against the toxic effects of DFP in mice. Pretreatment of mice with verapamil significantly increased the $LD_{50}$ of DFP. It was indicated that verapamil was protective in doses between 2.5 and 6.0 mg/kg and that, interestingly, at higher doses, (7.8 mg/kg and above) verapamil lowered the $LD_{50}$.

Several investigators have examined the role of calcium-mediated phosphorylation after treatment with neuropathic OPs (Patton *et al*, 1983; 1985; 1986; Abou-Donia *et al*, 1984; 1988; 1990; Suwita *et al*, 1986). These studies examined the role of enhanced phosphorylation of cytoskeletal proteins (microtubules, neurofilaments, and microtubule-associated protein-2 [MAP-2]) following treatment with TOCP. Phosphorylation of these structures was increased in brain, spinal cord, and sciatic nerve of hens following treatment with TOCP. This increase in phosphorylation is thought to occur through the action of calcium / calmodulin-dependent kinases (Abou-Donia *et al*, 1988). The specific proteins

which caused increased phosphorylation in this study were a-tubulin, b-tubulin, MAP-2, and neurofilament proteins (70kDa, 160 kDa, and 210kDa). Dr. Abou-Donia suggests that the enhanced activity of protein kinase II leads to the increased phosphorylation and eventual degradation of cytoskeletal elements. This causes an increase in the rate of fast retrograde axonal transport resulting in the accumulation of these degradation products in the distal portion of the axon. Neurofilaments and tubulin polymerize, condense, and dissolve. Endoplasmic reticulum and mitochondria trapped in the distal portion of the axon degenerate and release $Ca^{++}$ ions which enhances $Ca^{++}$-activated proteolysis (probably through calpain 1 and 2) of the cytoskeleton. This results in membrane disruption, ionic imbalance, water uptake and axonal swelling (Abou-Donia and Lapadula, 1990).

Further evidence for calcium involvement in OPIDN has been demonstrated in our laboratory through the use of calcium channel antagonists (El-Fawal *et al*, 1989; 1990; Nostrandt *et al*, 1993; McCain *et al*, 1993) both *in vivo* and *ex vivo*. In these studies, verapamil (and nifedepine in the El-Fawal studies) decreased the neurotoxic effects associated with exposure to neurotoxic OPs, cyclic phenyl saligenin phosphate. The effects of calcium channel antagonism included a decrease in the binding of OPs to neurotoxic esterases, a decrease in the onset and severity of clinical signs, a decrease in rheobase and chronaxie, and a decrease in the number of lesions observed peripheral nerves.

# PART III

# OBJECTIVES AND INVESTIGATIVE PROCEDURES

# CHAPTER 4

# SPECIFIC OBJECTIVES

## CHAPTER 4: SPECIFIC OBJECTIVES:

The general objectives of this study were:

1. To document the role of the circulatory system in the development of OPIDN.

2. To evaluate the effect of various treatments, including the administration of a neuropathy-inducing OP, on vascular response to various vasoactive agents both *in vivo* and *ex vivo* in order to separate local response from actual vascular damage.

3. To correlate these effects with morphological changes in muscle and nerve blood vessels.

4. To determine whether the calcium channel blocking agent, verapamil, could alter the vascular response and determine whether this could contribute to its ability to ameliorate OPIDN.

The specific objectives of the research proposed were as follows:

1. To determine the time related effects of a neuropathy-inducing OP (phenyl saligenin phosphate, PSP), an OP that does not produce OPIDN (paraoxon), and nerve crush on limb vascular resistance *in vivo*.

The isolated perfused hind limb preparation was used for such studies, with changes that followed OP administration or nerve crush noted. This procedure indicated alterations in vascular performance in response to nerve and muscle stimulation following OP administration and nerve crush. The procedure also supplied *in vivo* dose-response data for adrenergic and cholinergic agonists among the treatment groups and evaluated whole body response to vasoactive agents normally released during sympathetic stimulation (or ganglionic esterase inhibition) as well as determined the extent to which differences in response occurred with the different treatment groups.

2. To examine the time-related response of vessels *ex vivo* to adrenergic and cholinergic vasoactive agents ($\alpha_1$, $\beta_2$, and muscarinic agonists) and transmural electrical stimulation following exposure to organophosphorus compounds which do and do not cause delayed neuropathy. These vasoactive agents are primarily responsible for the neurogenic control of vascular caliber and the control of blood flow.

This procedure gave an indication of direct damage to vessels which could be compared to *in vivo* data to separate local responses (i.e. alterations of the microenvironment) from actual vascular damage.

3. To determine if time-related changes in vascular architecture occurred in hind limb skeletal muscle and peripheral nerve following exposure to OPIDN producing compounds. These parameters included muscle fiber to blood vessel ratio, blood vessel wall to lumen ratio, and capillary tortuosity in both skeletal muscle and peripheral nerve.

Changes in the function of skeletal muscles and peripheral nerves can cause alterations in morphology of their associated vascular supply. These data were correlated with the data from the *in vivo* and *ex vivo* studies.

4. To assess time-related changes in adrenergic vascular innervation in muscle and nerve blood vessels after administration of OPs. Luxol fast green, periodic acid-Schiff, and hemotoxylin (LPH) staining was used to determine the damage to nerves supplying the blood vessels of skeletal muscle. Quantification of nerve fiber to blood vessel ratio in tissue cross sections was determined with this method.

This study was designed to investigate possible damage which could occur to sympathetic nerves which innervate blood vessels of skeletal muscle and peripheral nerves. It also added morphological support for the physiological data obtained from the *in vivo* and *ex vivo* studies (see objectives 1 and 2).

5. To determine the effects of verapamil co-treatment on specific objectives 1-5.

This was done because verapamil was shown to ameliorate the effects of PSP treatment in hens. Since verapamil also has a profound effect on the cardiovascular system, the reduction of pathologic effects, esterase inhibition, and clinical signs could have been due to their effect on the cardiovascular parameters.

# CHAPTER 5

# INVESTIGATIVE PROCEDURES

## CHAPTER 5: INVESTIGATIVE PROCEDURES

### 5.1. Experimental protocol:

5.1.1. ANIMALS:

Adult hens have been used by the Environmental Protection Agency as models to screen for neuropathic effects of OPs. Therefore hens were used in the present studies. They were group housed in wire bottom cages with 4 birds per cage. They were placed on a 12 hour light / dark cycle. Commercial feed and water were administered *ad libitum.*

5.1.2. TREATMENT GROUPS:

Hens were separated into several groups based on treatment. Each study had 4 treatment groups, which consisted of 10 hens. Each group was further subdivided into two smaller groups (5 hens each), one receiving the calcium channel blocker, verapamil (7 mg/kg, i.m.), and the other acting as a control. The treatments were as follows:

1. Negative control: This group received the vehicle, DMSO only.
2. Experimental Group # 1: This group received phenyl saligenin phosphate (PSP), an active metabolite of tri-o-cresyl phosphate (TOCP) and an OP that produces OPIDN at dosages that do not cause clinically significant depression of AChE. The dose used (2.5 mg/kg i.m.) was within the range previously demonstrated to cause OPIDN (2-10 mg/kg i.m. [Ehrich and Jortner,1987]).

3. Experimental Group # 2: This group received paraoxon (PXN), a cholinesterase-inhibiting organophosphate that does not cause OPIDN (0.1 mg/kg i.m., a dose that produces a >70% inhibition of brain acetylcholinesterase as determined by preliminary experimentation).

4. Experimental Group # 3: This group was subjected to unilateral nerve crush of the sciatic nerve. This procedure abolished the nerve supply to one limb without affecting the other. This provided immediate nerve damage unrelated to vascular changes.

5.1.3. EXPERIMENTAL PROCEDURE:

Hens were given intramuscular injections of toxicants and/or vehicle on day 0. They were examined on days 1, 3, 7, and 21, according to the procedures listed below.

Hens were evaluated for clinical signs every other day following treatment using the method developed by Sprague *et al* (1980). This method rates the walking behavior of hens on a scale of 0 (no walking impairment) to 5 (paralysis of both hindlimbs and upper limb involvement).

## 5.2. Experimental methods:

5.2.1. STUDY # 1: The perfused hind limb preparation.

This study used the isolated perfused hen hind limb preparation as described by Polucha, Ringer, and Scott (1981) to determine the temporal changes in hind limb vascular resistance (objective 1) and the effect of skeletal muscle and vasoactive agents (acetylcholine, phenylephrine, and salbutamol) on vascular resistance (objective 2).

This procedure determined the functional response of the hind limb vasculature to vasoactive agents, direct nerve stimulation, and indirect muscle stimulation.

Isolated perfused hind limb of the hen:

For the procedure, hens were anesthetized with sodium pentobarbital (30 mg/kg i.v.) or ketamine (0.1 mg/kg i.p.). The brachial artery and vein were cannulated. The arterial catheter was coupled to a pressure transducer (Statham P-23ID) and a physiograph (Grass 7D) to determine systemic arterial pressure while the venous catheter was used for the infusion of maintenance drugs (ketamine and heparin). A catheter was inserted cranially in the ischiadic artery and connected to a variable speed peristaltic pump. Another catheter was placed caudally in the same artery and connected to the pump outflow. Pressure was monitored in the hind limb with a Statham (P-23ID) pressure transducer at a point proximal to the insertion of the caudal catheter. Flow through the pump was sufficient to produce a mean pressure of 125 mmHg (control level as determined by preliminary observation). The flow

was maintained at this constant rate throughout the experiment and alterations of pressure will reflect changes in limb resistance.

An electrode holder was placed around the ischiadic nerve and connected to a square wave stimulator (Grass S-44, Quincy, MA). Pin electrodes were placed in the lateral and medial head of the gastrocnemius muscle. These electrodes were also connected to the stimulator.

Vasoactive agents were delivered in cumulative stepped logarithmic doses into the pump tubing at a point prior to the pressure transducer and changes in pressure were monitored thus creating dose-response curves. The following agents and treatments were used to assess vascular performance:

a. Adrenergic response:

1. $\alpha_1$: Phenylephrine (PE; selective $\alpha$-1 agonist; $10^{-8}$ to $10^{-3}$ moles/kg).
2. $\beta_2$:Salbutamol (SAL; selective ß-2 agonist; $10^{-8}$ to $10^{-3}$ moles/kg).

b. Cholinergic response:

1. acetylcholine (ACh; $10^{-8}$ to $10^{-3}$ moles/kg).

c. Transmural and direct electrical stimulation:

The sciatic nerve was stimulated with electrical current under the following conditions: rectangular pulses at 8-10 volts, 5 msec, 8 Hz for 60 for autonomic nerves and 40 Hz for somatic motor nerves. The preparation was allowed to recover until it is judged

to be in a resting state (approximately 5-10 min) before further testing.

To determine the effect of muscle activity on vascular resistance, the sciatic nerve was directly stimulated at 40 Hz and the muscle was transmurally stimulated at 40 Hz. Current differences were determined for each treatment group.

The above procedure yielded dose-response data for a number of vasoactive agents as well as *in vivo* response to nerve and muscle stimulation. The data obtained were statistically analyzed using multivariate analysis for comparison of effects of phenylephrine, salbutamol, acetylcholine, verapamil, and stimulation on resistance vessels of the hind limb.

5.2.2. STUDY # 2: Isolated vessel procedure.

This study utilized the isolated vessel procedure to assess alterations in contractile response of the ischiadic artery to pharmacologic agents and transmural stimulation (objective 3). Tissues were removed at this time for histological examination (part of objectives 4 and 5 [see study # 3]) and included brain, gastrocnemeus muscle, sciatic and tibial nerve.

This procedure determined vascular response *ex vivo* which indicated alterations in vascular function caused either directly by OPs or secondary to neural damage. Hens examined prior to signs of

neural damage (4-6 days) indicated whether functional vascular changes occured prior to neural damage.

Isolated vessel preparation: The 2 cm segment of the ischiadic artery was quickly removed from hens (euthanized by cervical dislocation) and placed in a petri dish containing warm (37°C), aerated (95% $O_2$ - 5% $CO_2$) Krebs-Henseleit (K-H) buffer solution (composition in g/l: NaCl 6.9; $MgSO_4 \cdot 7H_2O$, 0.29; $KH_2PO_4$, 0.15; glucose, 2.0; $NaHCO_3$, 2.1; KCl, 0.35; $CaCl_2$, 0.28). The vessel was then cleaned of extraneous tissue and muscular arterioles emanating from the ischiadic artery were ligated with silk suture (5-0, Ethicon, Somerville, NY). Arterial segments were then trimmed to 1.5 cm. The vessel was suspended between two glass cannulae and tied in place. The tips of the cannulae, which were fitted to the luminal diameter of the vessel (1.5 - 2.0 mm), were separated by 1 cm. The preparation was transferred to an organ bath (Radnoti Glass Technologies Inc., Monrovia, CA) containing K-H buffer. A pressure transducer (Cobe Laboratories Inc, Lakewood, CO) was placed in the inflow cannula and connected to a physiographic recorder (TA-240, Gould instrument Co., Valley View, OH). The cannula were connected to a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Chicago, IL) via silicone tubing (size 14, Masterflex, Cole-Parmer Instrument Co., Chicago, IL) and the lumen of the vessel was perfused with oxygenated K-H buffer at a flow rate of 6.0 ml/min. The volume of the luminal circuit was 10 ml. Bath solution and

luminal solutions were changed every 15 minutes. The vessel preparation was allowed to recover for 30 minutes prior to treatment with vasoactive agents.

Log concentration-response curves in response to phenylephrine (PE), salbutamol (SAL) and acetylcholine chloride (ACh) ($10^{-8}$ to $10^{-3}$ moles/l) (Sigma Chemical Co., St. Louis, MO) were generated by plotting cumulative increments of agent concentration against pressure change (expressed as a % of potassium contraction) within the luminal circuit. The vessels were precontracted with potassium chloride ($3 \times 10^{-3}$ M) prior to administration of salbutamol and acetylcholine (the vasodilating agents). Agents were injected (0.1 ml aliquot) into the luminal tubing prior to its entry into the pump. After the final injection of each agent, fresh K-H buffer was perfused through the luminal circuit for 5 minutes. The vessel preparation was allowed to recover for 15 minutes, the luminal circuit and bath were refilled with fresh K-H buffer, and the next agent was administered. When the final agent had been tested, the vessel was removed and measured. Representative vessels from each group were processed for histological examination.

5.2.3. STUDY # 3: Biochemical and histological evaluation of tissue.

This study used tissue specimens obtained during studies 1 and 2 for morphometric analysis of nerve and muscle vasculature and quantitative and qualitative assessment of innervation of the vasculature in these tissues. Biochemical analyses of frozen tissue

(nerve and brain) were also conducted at this time (objectives 4 and 5).

Histological evaluation using the LPH technique was used to determine if differences exist in the microvasculature of muscle and nerve of the hind limb among treatment groups. Cytochemical staining of nerve fibers was used to indicate the extent of damage to vasomotor fibers of muscle blood vessels and nerve tissue. The biochemical evaluation was used to determine differences in the activity levels for brain neurotoxic esterase (NTE), brain acetylcholinesterase (AChE), epinephrine, and norepinephrine among the various treatment groups.

5.2.3.1. Morphometric evaluation of vascular supply to muscle and nerve:

Luxol fast blue - periodic acid Schiff - hematoxylin (LPH) staining was used to examine blood vessel density in both nerve and muscle as described by Bell and Scarrow (1984).

5.2.3.2. Assessment of innervation and vascularization in muscle and nerve:

Tissues selected for examination in this study included the ischiadic artery, vein, and nerve as well as a section of the gastrocnemius muscle. The artery, vein and nerve were taken from the mid femoral region and the section of gastrocnemius muscle examined was taken from the medial head at a point 1-2 mm distal to the point of entry of the tibial nerve, which has displayed

evidence of neuropathological changes following PSP treatment (Jortner and Ehrich, 1987). Cross sections of the muscle would, therefore, include a section of the sural nerve and its associated artery and vein. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded and 10 mm sections were cut and stained. Tissues were stained for general histological purposes with Luxol fast green, periodic acid-Schiff, and hematoxylin (LPH). This staining process, which differentiates neural elements, allowed the examination of muscle fiber diameter and vessel to fiber ratio (Goto, 1988). Nerve cross sections were examined for the presence and structure of endoneurial blood vessels and to assess nerve fiber damage associated with OPIDN.

This staining technique resulted in qualitative data (vascular tortuosity) and was analyzed using the Kruskal-Wallis method for nonparametric data. The number of fibers surrounding a vessel can be counted, therefore, decreases in the number of fibers would indicate a decrease in the innervation. The difference in the means was determined using analysis of variance and least square difference.

5.2.3.3. Biochemical evaluation of nerve, and blood:

1. NTE: The ability of neurotoxic esterase to hydrolyze phenyl valerate was determined spectrophotometrically in brain tissue as described by Sprague *et al* (1981) and modified as a microassay (Correll and Ehrich, 1991) to increase assay efficiency. This method

determines the sensitivity of NTE to inhibition by mipafox after carboxylesterases have been inactivated by paraoxon.

2. AChE: Acetylcholinesterase activity was determined spectrophotometrically as described by Ellman *et al* (1961). This method utilizes acetylthiocholine iodide as a substrate for AChE; the enzyme hydrolyzes it into acetate and thiocholine. Thiocholine reacts with dithiobisnitrobenzoate (DNB) to produce a yellow anion which can be measured.

3. Catecholamines: The level of circulating catecholamines was determined using a BAS electrochemical / liquid chromatography detector with a catecholamine column. Heparinized blood samples are collected and centrifuged. A 1 ml aliquot of plasma is placed in a tube containing 0.1 ml EGTA-glutathione solution and frozen at -70°C for later analysis.

4. Calcium: The concentration of calcium in the blood was measured using an ionized calcium analyzer (Radiometer ICA-1). A heparinized sample of blood is injected into the analyzer and ion concentration corrected to pH 7.4 is determined.

These methods are quantitative and were compared using analysis of variance and least square difference among treatment groups.

## 5.3. Statistical Methods

Data were analyzed using analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences among control and experimental groups, with $p<0.05$ considered significant. All data are expressed as mean $\pm$ SEM, except where otherwise indicated.

# PART IV

# RESULTS

## INTRODUCTION TO RESULTS

The two manuscripts contained in the results section of this document represent the major findings of the research described in the methods section. The research documented in the first manuscript examined the response of hind limb blood vessels specifically, and the cardiovascular system in general 1, 3, 7, and 21 days after administration of PSP and PXN. Another aspect of this study was to determine the effect of OPs on vascular response to vasoactive agents and to correlate these responses to the onset and progression of OPIDN. This study gave an indication of the events which occur *in vivo* after administration of these OPs and validates observations made in our preliminary study.

The second manuscript continues the investigation of hind limb vascular response to vasoactive agents by examining these responses *ex vivo* which eliminated the effects of endogenous mediators, skeletal muscle and microvasculature on the vascular response. Measurements of these responses were taken at the same time points as those of the first study. The second study also examined the histological changes that occur in muscle, nerve and vascular tissue as a result of OP administration and determined the levels of catecholamines and calcium in the blood of treated hens.

A third section has been added to the results section in order to document data that were collected but not integrated into the two manuscripts. This data primarily consist of observations made on

hens which had undergone unilateral nerve crush. The data were not included in the manuscripts because the onset and mechanism of the neuropathy was so different from that of the chemical neuropathy induced by PSP. The examination of these animals was concurrent with those examined in the first two studies.

# CHAPTER 6

# THE EFFECT OF CYCLIC PHENYL SALIGENIN PHOSPHATE AND PARAOXON TREATMENT ON VASCULAR RESPONSE TO ADRENERGIC AND CHOLINERGIC AGENTS IN HENS

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# THE EFFECT OF CYCLIC PHENYL SALIGENIN PHOSPHATE AND PARAOXON TREATMENT ON VASCULAR RESPONSE TO ADRENERGIC AND CHOLINERGIC AGENTS IN HENS

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**ABSTRACT:**

Response of peripheral blood vessels to adrenergic and cholinergic agonists were examined 1, 3, 7, and 21 days after hens were treated with a single 2.5 mg/kg intramuscular injection of cyclic phenyl saligenin phosphate (PSP) or 0.10 mg/kg paraoxon (PXN). These two organophosphates (OPs) cause different clinical effects in exposed animals, as PSP causes organophosphate-induced delayed neuropathy (OPIDN) and PXN causes acute poisoning through inhibition of acetylcholinesterase. Half of the hens in each group were given ths calciun channel antagonist, verapamil (7 mg/kg, i.m.) as this agent ameliorated the neuropathic effect of OPIDN. For these studies, the ischiadic artery was cannulated both prograde and retrograde and the blood shunted through a pump to maintain a constant flow. Alterations in pressure measured at the pump outflow were used to indicate changes in limb vascular resistance. Dose-response curves were generated for the response to intravenous administration of acetylcholine (ACh), phenylephrine (PE) and salbutamol (SAL) ($10^{-8}$ to $10^{-4}$ moles/ kg). Acetylcholine ($10^{-8}$ to $10^{-7}$ moles/kg), caused an increase in vascular resistance whereas concentrations of $10^{-6}$ to $10^{-4}$ moles/kg caused a decrease in vascular resistance in hens given PSP 1 and 3 days previously. The response of PXN treated hens to ACh was not significantly altered from that of vehicle-treated hens. The resistance generated in response to PE, an $\alpha_1$ agonist, in PSP treated hens was greater than levels in vehicle-treated hens on days 1 and 3 and greater than the

response seen in hens treated with PXN. Salbutamol, a $\beta_2$ agonist, in concentrations of $10^{-7}$ to $10^{-4}$ moles/kg caused an increase in resistance 1 and 3 days after PSP and a decrease on day 7. These responses to SAL were different in PXN treated hens, in which there was a lesser increase in resistance at concentrations of $10^{-8}$ to $10^{-7}$ moles/kg and a decrease in resistance at $10^{-5}$ to $10^{-4}$ moles/kg after administration of PXN. These observations indicate that responses to vasoactive agents are altered in OP treated hens and that responses also differ between a compound capable of causing OPIDN (PSP) and a compound that only causes acute effects (PXN).

## INTRODUCTION:

Organophosphorus compounds (OPs) are used in a wide variety of settings in our society. Many of today's most potent insecticides and nerve gases are OPs. They are used by the petroleum and plastics industries as product additives and in medicine to treat glaucoma. OPs are capable of causing at least two distinct clinical syndromes, acute cholinergic poisoning and organophosphate-induced delayed neuropathy (OPIDN). Exposure to OPs that results in cholinergic poisoning cause the inhibition of a number of esterases, including the acetylcholinesterase (AChE) which is primarily responsible for the clinical signs seen shortly after exposure (Abou-Donia, 1985). In addition, humans, as well as other mammalian and avian species, may develop an irreversible neuropathy one to three weeks after exposure to a single toxic dose of certain OPs (Lotti, 1992; Abou-Donia, 1981; Johnson, 1975). The neuropathy follows early inhibition of neurotoxic esterase (NTE). The inhibition of both central and peripheral esterases as well as the neuropathy induced by OPs may cause changes in many major organ systems.

Several studies have indicated that OPs affect the cardiovascular system. Dowman (1972) demonstrated that inhibition of cholinesterase in the hypothalamus and medulla contributed to peripheral vasoconstriction by increasing sympathetic outflow. Cardiovascular effects of cholinesterase-inhibiting compounds and cholinergic agonists that have been observed include a pressor response after proximal injection of the OP, DFP (diisopropyl

phosphorofluoridate), in the medulla and midbrain of dogs (Brezenoff and Giuliano, 1982; Kriwic 1978). These studies, however, identified effects which occurred shortly after administration of potent cholinesterase inhibitors. A preliminary study performed in our laboratory demonstrated that there were significant cardiovascular changes which occur in the hen following a single exposure to a neuropathy-inducing OP that was not a potent cholinesterase inhibitor. These changes occurred both before and after the onset of clinical signs. Administration of the neuropathy-inducing OP was shown to decrease blood flow and increase vascular resistance of skeletal muscles of the hind limb of hens, an effect not expected if there had been ganglionic stimulation by cholinesterase inhibition (McCain, *et al*, 1993). The above observations supported a previous study in which a decrease in blood flow was observed in skeletal muscle, kidney, and spleen after administration of paraoxon to rats (Vetterlein and Haase, 1979). The effects observed in these two studies contrast with effects that would be expected following stimulation of the post-ganglionic sympathetic fibers through the OP-induced inhibition of synaptic acetylcholinesterase. This should cause dilation of skeletal muscle blood vessels through $\beta_2$ adrenergic receptors, which should increase flow and decrease vascular resistance (Parkinson, 1990).

This study examines the temporal effect of a neuropathy-inducing OP on vascular response to adrenergic and cholinergic agents as OP-induced changes in blood flow could contribute to tissue

damage seen after administration of these toxicants (Patterson, *et al*, 1988; Cisson and Wilson, 1982). Such OP-induced changes in blood flow could be due to alteration of the response to adrenergic and / or cholinergic stimulation. The study compared responses in hens given a neuropathy-inducing OP (cyclic phenyl saligenin phosphate, PSP) with responses in hens given an OP that did not cause OPIDN (paraoxon). As previous studies indicated that OPIDN could be ameliorated by treating hens with the cardiovascular drug, verapamil, a calcium channel blocker, the effect of treatment with verapamil on these responses was also examined at this time.

## METHODS AND MATERIALS:

**ANIMALS:** Experiments were conducted on adult white leghorn hens obtained from the Department of Poultry Science at Virginia Polytechnic Institute and State University. Hens were placed in wire bottom cages (24" X 22" X 22") with four birds per cage. Hens were housed in a temperature controlled building with a 12 hour day / night cycle. Water and commercial chicken feed were provided *ad libitum*.

**TREATMENT:** The hens were weighed and randomly divided into three groups with 40 hens in each group. One group of 40 hens was given 2.5 mg/kg phenyl saligenin cyclic phosphate (PSP, 2-phenoxy-4H-1-1-,3-2-benzodioxaphosphorin-2-oxide [Lark Enterprises, Webster, MA]) by intramuscular injection. PSP was dissolved in dimethyl sulfoxide (DMSO) to yield a 5 mg/ml solution. A second group of 40 hens was given paraoxon (PXN, O-O-diethyl,O-p-nitrophenyl phosphate), 0.10 mg/kg by intramuscular injection. A stock solution of paraoxon (13.76 mg/ml in acetone) was diluted with saline to yield a 0.20 mg/ml solution immediately prior to injection. The control group of 40 hens was given an equivalent volume (0.5 ml/kg) of the vehicle. One half of the hens in each group (20) were given the calcium channel antagonist, verapamil (7 mg/kg, i.m.) the day before, the day of, and daily for two days after OP treatment. Hens were examined for effects on the cardiovascular system 1, 3, 7, and 21 days after OP treatment. Days 1 and 3 represent time points prior to histological evidence of neuropathy and day 7 is a time point

prior to clinical signs of neuropathy but after histological signs are evident (El-Fawal,*et al*, 1990). Day 21 represents a time point when clinical signs of OPIDN are maximal.

**INDICES OF NEUROTOXIC EFFECTS:** Clinical signs of neuropathy were assessed every other day following treatment according to the scale developed by Sprague *et al* (1981). Using this system, normal hens were given a score of 0. Hens with minor alterations of gait were given scores of 1. Scores of 2 were given to hens that had some difficulty walking; scores of 3 were given when definite ataxia was evident. A score of 4 was assigned to hens with paralysis of the hind limbs. Hens received a score of 5 when wing droop, a sign of upper limb involvement, was observed in addition to hind limb paralysis.

**ESTERASE DETERMINATIONS:** Neurotoxic esterase activity was determined spectrophotometrically in brain tissue using the method of Sprague *et al* (1981), in which the capacity to hydrolyze phenyl valerate is assessed in the presence of mipafox and / or PXN. Acetylcholinesterase (AChE) activities were determined spectrophotometrically in brain samples by measuring the yellow anion produced by hydrolysis of acetylthiocholine (Ellman, *et al*,1961).

**EXPERIMENTAL PROCEDURE:** On the day of data collection, hens were weighed and restrained in lateral recumbency. A local anesthetic (0.5 to 0.7 cc of 2% lidocaine [Butler, Columbus, OH]) was injected intramuscularly at the base of the wing. The ulnar vein and brachial artery were then cannulated with polyethylene catheters.

The arterial catheter was connected to a Statham P-23-ID pressure transducer and a Grass 7D polygraph (Grass Instrument Co., Quincy, MA) for measurement of blood pressure and heart rate. The venous catheter was used for the administration of the disassociative anesthetic, ketamine hydrochloride (Fort Dodge laboratories, Fort Dodge, Iowa), and the anticoagulant, heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Once the hen was anesthetized, the ischiadic artery was exposed and cannulated both prograde and retrograde. The cannula were connected to a roller pump (Masterflex, Model 7520-25, Cole Parmer Inst. Co., Chicago, IL) and blood was shunted through the pump. A pressure transducer was connected to the pump outflow and pump flow was adjusted so that perfusion pressure matched systemic pressure. This flow rate was maintained throughout the experiment. Alterations in perfusion pressure indicated changes in vascular resistance. Hind limb vascular resistance was determined by dividing the arterial - venous pressure difference in mmHg by the hind limb blood flow in ml/min/100gm and is expressed as limb resistance units (LRU).

Dose-response curves of vascular resistance were generated for the cholinergic agonist, acetylcholine (ACh); the $\alpha_1$ adrenergic agonist, phenylephrine (PE); and the $ß_2$ adrenergic agonist, salbutamol (SAL). Concentrations of vasoactive agents used in this study were determined by preliminary investigation. The concentrations used ($10^{-8}$ to $10^{-4}$ moles/kg of body weight) produce a minimal response in vehicle-treated animals at the low

concentration and did not significantly alter systemic pressure or heart rate at the high concentration. The agents were injected into the influent pump tubing (100 µl/kg) and maximum response was recorded within 1 to 2 minutes of administration.

Hens were euthanized with an overdose of pentobarbital sodium at the completion of the experiment. The brain was removed from euthanized hens and frozen at -70° C for later spectrophotometric analysis of acetylcholinesterase and neurotoxic esterase activities.

**STATISTICAL ANALYSIS:** Parametric analysis of data included the use of the student's t-test and analysis of variance (ANOVA). If significant differences were noted using ANOVA, further analysis was accomplished using Fisher's least significant difference.

Data were considered significantly different from vehicle-treated hens when the probabilities were less than 0.05. Data are expressed as mean $\pm$ standard error of the mean (SEM).

**RESULTS:**

**CLINICAL DETERMINATION OF NEUROPATHY:** Alterations in walking behavior were observed in hens treated with PSP but not in those treated with PXN. Some hens given PSP only (4 of 10; clinical score : 0.4±0.2) displayed locomotor deficits as early as 7 days after treatment, however, most hens did not display clinical signs until day 9 (clinical score: 1.2±0.1, n=5). Signs of ataxia were notable in all hens by day 15 (clinical score: 2.6±0.2, n=5) and signs were maximal by day 18 (clinical score: 3.8±0.2, n=5). This increase in clinical scores is indicative of progressive distal axonopathy. Hens treated with PXN displayed no signs of locomotor deficiencies. However, they were lethargic the day after the PXN was administered.

Verapamil treatment delayed the onset and decreased the severity of clinical signs of neuropathy in hens treated with PSP although once initiated, the rate of progression of the ataxia was the same. No hen treated with PSP and verapamil displayed altered gait 7 days after treatment and only 1 hen displayed altered gait at 9 days after treatment. All hens treated with PSP and verapamil displayed altered walking behavior 11 days after PSP administration (clinical score: 1.0±0.3, n=5) and by day 15, these hens received an average clinical score of 2.6±0.6 (n=5). Maximal clinical scores were observed on day 21 for hens treated with verapamil and PSP was 3.2±0.2 (n=5).

**ESTERASE ACTIVITY:** Brain esterase activities in treated hens were differentially reduced by PSP and PXN (Table 1). PSP reduced

the activity of NTE while PXN hens lowered the activity of AChE in treated hens. PSP treated hens displayed an 87% decrease in the activity of brain NTE and one day after treatment a 17% reduction in brain AChE activity which was not statistically significant. NTE activity was significantly inhibited for 1 week in PSP treated hens and returned to control levels by day 21, which is when maximal clinical signs of OPIDN were observed. Hens treated with PXN displayed a nonsignificant 13% reduction in brain NTE activity and a significant 60% reduction in brain AChE activity 1 day after treatment. This level of AChE activity was significantly lower than the activity of both vehicle-treated and PSP treated hens 1 and 3 days after treatment and not different from other groups on day 21.

Verapamil treatment did not affect AChE or NTE activities in vehicle-treated hens and did not alter the esterase inhibiting effect of PSP or PXN in treated hens (data not shown).

**CARDIOVASCULAR RESPONSE TO TREATMENTS:** Several parameters, including mean systemic pressure, heart rate, limb blood flow and limb vascular resistance, were measured for each hen prior to the administration of vasoactive agents. Results of these measurements are displayed in Figure 1. In hens treated with PSP, blood pressure values were significantly elevated 1 and 3 days after treatment but were not different from values in vehicle-treated hens on days 7 and 21 (Figure 1B). In PXN treated hens there was no significant change in blood pressure throughout the study. Heart rate was significantly elevated in hens given PSP 1 day after the OP was

administered. PXN treatment caused no significant alteration in heart rate during the study (Figure 1A).

Limb blood flow was significantly decreased and limb resistance significantly increased 1 day after treatment with PSP (Figure 1C and 1D). Treatment with PSP reduced limb blood flow to 33% below values of vehicle-treated hens (Figure 1C) and limb vascular resistance was increased to 44% above vehicle-treated values at that time (Figure 1D). However, limb blood flow and resistance were not different from values of vehicle-treated hens during the remainder of the study. Limb blood flow and limb vascular resistance were not significantly altered by PXN on any of the days when measurements were taken.

Verapamil treatment had no significant effect on blood pressure, heart rate, and limb blood flow in vehicle-treated hens but limb resistance was significantly lowered (Figure 1). However, blood pressure was significantly elevated in hens given PSP and verapamil throughout the study when compared to that of vehicle-treated hens (Figure 1R). Blood pressure was also elevated in hens given PXN and verapamil 7 days after treatment (Figure 1R). Limb blood flow in hens treated with PSP and verapamil was significantly greater than that of hens given only verapamil 1 day after administration of the toxicant and limb resistance was significantly lower (Figure 1C and 1D). Hens treated with PXN and verapamil displayed no significant change in limb blood flow or resistance from that of vehicle treated hens throughout the study (Figure 1C and 1D).

**RESPONSE TO VASOACTIVE AGENTS:**

Acetylcholine: Perfusion pressure in response to acetylcholine (ACh) was different in hens treated with PSP than in hens treated with PXN (Figure 2). Hens treated with PSP displayed a biphasic response to ACh (Figure 2A). Perfusion pressure in response to ACh administration was increased 1 day (75.0±15.0 mmHg, @ $10^{-8}$ moles/kg, n=5) and 3 days (76.3±11.8 mmHg @ $10^{-8}$ moles/kg, n=5) following PSP treatment. In contrast, perfusion pressure was decreased after administration of ACh at higher concentrations 1 day (68.8±11.2 mmHg, @ $10^{-5}$ moles/kg, n=5) and 3 days (80.0±13.2 mmHg, @ $10^{-5}$ moles/kg, n=5) following PSP when responses were compared to the responses observed prior to the administration of ACh. The responses to ACh seen in PSP treated hens 1 and 3 days after administration of this OP were not seen in PXN treated hens (Figure 2B). The response of PXN treated hens to ACh administration was slightly elevated at concentrations of 10-7 moles/kg and 10-4 moles/kg one and 3 days after treatment and was not different from values of vehicle-treated hens on days 7 and 21. Vehicle-treated hens demonstrated a dose-dependent decrease in perfusion pressure to increasing concentrations of ACh with a threshold response at a concentration of $10^{-7}$ moles/kg (11.3±1.8 mmHg below values observed before administration of ACh, n=10) and a maximal response at a concentration of $10^{-4}$ (58.0±6.2 mmHg, n=10, above values obtained before administration of ACh).

The response to ACh in hens treated with PSP and verapamil (Figure 3A) was greatly reduced from the response observed in hens given PSP alone (Figure 2A), especially 1 and 3 days after OP administration. Verapamil had no measurable effect on the vascular response of PXN treated hens (Figure 2B, Figure 3B).

Phenylephrine: Hind limb perfusion pressure in response to phenylephrine (PE) was significantly different in OP treated hens from that in vehicle-treated hens (Figure 4). These differences were most marked 1 day after OP administration. The maximum response obtained after $10^{-4}$ moles/kg PE in PSP treated hens was 157.5$\pm$9.6 mmHg (n=5) over baseline values. This response was 37% greater than that obtained in vehicle-treated hens given $10^{-4}$ moles/kg PE. Furthermore, the initial response to PE in PSP treated hens occurred at 2 orders of magnitude lower concentrations of PE ($10^{-10}$ moles/kg) than it did in vehicle-treated hens ($10^{-8}$ moles/kg PE). On day 21, which was when hens had maximal clinical evidence of OPIDN, the response of PSP treated hens to PE was lower than values obtained from vehicle treated hens given this $\alpha_1$ agonist at concentrations of $10^{-7}$ and $10^{-6}$ moles/kg (Figure 4A). The response of PXN treated hens to a PE concentration of $10^{-4}$ moles/kg 1 day after treatment was 102.5$\pm$13.0 mmHg (n=5), a 23% increase over the response observed in vehicle-treated hens. The response of PXN treated hens to PE was slightly higher but not significantly different from the response of vehicle-treated hens on days 3, 7, and 21 (Figure 4B).

Verapamil treatment somewhat modified the vascular response of hens treated with PSP (Figure 5A). In addition, verapamil attenuated the effects of PE on vascular response in PXN treated hens (Figure 5B), with values obtained not different from those obtained in vehicle-treated hens throughout the 21 day study period. Vehicle-treated hens did display a dose-dependent increase in perfusion pressure to increasing concentrations of PE, with a maximum increase at a concentration of $10^{-4}$ moles/kg to $83\pm6.1$ mmHg (n=10), a 66% increase over baseline values: values in the figures represent increases above those obtained using vehicle treated hens.

Salbutamol: Perfusion pressure in response to SAL in PSP treated hens was also significantly different from that of vehicle-treated hens (Figure 6A). The response of vessels from PSP treated hens to this ß adrenergic agonist was unexpected in that a dose-dependent increase in perfusion pressure was observed 1 and 3 days following PSP treatment and a dose-dependent decrease was observed on day 7 (Figure 6A). On day 21, however, the response of PSP treated hens was near that of vehicle-treated hens. The response to SAL was not marked in hens treated with PXN as in PSP treated hens (Figure 6). Although a biphasic response to SAL was noted 1 and 3 days following treatment (Figure 6B). Concentrations of SAL at $10^{-8}$ and $10^{-7}$ moles/kg caused a significant increase in perfusion pressure to $22.5\pm5.4$, n=5 and $20.0\pm4.1$, n=5, respectively, over baseline perfusion pressure. Concentrations of $10^{-5}$ and $10^{-4}$ moles/kg resulted in a

significant decrease from baseline perfusion pressure in PXN treated hens to 43.8±8.9 mmHg, n=5 and 55±13.2 mmHg, n=5, respectively, 1 day after PXN treatment.

The response of hens treated with PSP and verapamil to SAL was modified considerably when compared to hens treated with PSP only (Figure 5A, Figure 6A). The response of PXN treated hens given verapamil to SAL was not different than that of vehicle treated controls (Figure 7B).

Dose-response curves for SAL generated in vehicle-treated hens indicated a dose-dependent decrease in perfusion pressure. The maximum decrease was 17.5±3.4 mmHg, (n=10) below values obtained before SAL was given and this decrease was seen at $10^{-4}$ moles/kg for this group (Figure 6).

## DISCUSSION

The data presented above indicate that treatment with OPs alters general cardiovascular parameters as well as vascular response as indicated by changes in perfusion pressure and by changes in response to cholinergic and adrenergic agonists. When compared to vehicle-treated hens: (1) the most prominent changes were observed 1 and 3 days after OP treatment; (2) heart rate, blood pressure and limb vascular resistance was increased and hind limb blood flow was decreased 1 day after PSP treatment but not significantly altered in PXN treated hens. (3) response to ACh was more marked in PSP treated hens; (4) the response to phenylephrine was increased in hens given both compounds but lasted for 3 days in PSP treated hens; (5) the response to salbutamol in PSP treated hens was greater than the response to this ß adrenergic agonist in PXN treated hens; (6) treatment with verapamil reduced the effects of PSP and PXN treatment on blood pressure, hind limb vascular resistance, hind limb blood flow, and vascular response to cholinergic and adrenergic agents. (7) treatment with verapamil reduced the effects of PXN treatment on vascular response to cholinergic and adrenergic agents.

Criteria for the determination of OPIDN include an early inhibition of brain NTE by at least 70% (Johnson and Lotti, 1980) and the onset of clinical signs 7 to 21 days after exposure to a neurotoxic OP. In this study, brain NTE of hens given PSP was reduced by 87%

from values obtained from vehicle-treated hens. Locomotor deficits were observed as early as day 7 and progressed throughout the study. This indicates that a sufficient dose of PSP was used to produce OPIDN in adult hens. This observation is consistent with that of other investigators using this compound (McCain,*et al*, 1993; El-Fawal *et al*, 1989; Jortner and Ehrich, 1987). Lack of NTE inhibition and clinical signs of OPIDN in PXN treated hens are also supported by earlier studies. These findings confirm that PXN is a suitable representative of an OP that significantly reduces the activity of AChE and does not produce OPIDN (McCain *et al*, 1993; DeNeef *et al*, 1982).

In this study, PXN did not produce any significant effects on blood pressure, heart rate or blood flow 1 day after treatment. This contrasts with observations made by other investigators who worked with PXN or other strong inhibitors of AChE (Maxwell *et al*, 1987; De Neef *et al*, 1982; Vetterlein and Haase, 1979; Kristic, 1978). Brezenoff *et al* (1984) indicated that a 70% inhibition of brain cholinesterase was necessary to produce a hypertensive response to soman in rats. This level of inhibition was reached in hens treated with PXN (72% inhibition) but no significant hypertension was observed. The hypertension observed by other investigators occurred early after treatment (up to 120 minutes) while the first observation in this study was made at 24 hours. It is possible that homeostatic mechanisms had reduced hypertension, if it occurred, by this time. In contrast to PXN, brain AChE activity was not reduced to

a great extent after administration of PSP (17% inhibition) yet systemic hypertension was observed in hens 1 and 3 days after PSP treatment. Since a 70% inhibition of brain AChE was not observed after administration of this compound, another mechanism must be involved in producing the observed hypertensive effect, possibly through local responses to peripheral esterase inhibition or a selective effect on central vasomotor centers. The increase in blood pressure observed in hens treated with PSP and verapamil, which was the same as that observed for PSP only, may indicate that the initial hypertensive response is not due to the increase of intracellular $Ca^{++}$.

In the present study, hind limb vascular resistance was increased and hind limb blood flow decreased in hens treated with PSP. There was also an increase in hind limb vascular resistance PSP treated hens (using doppler flow measurement) one day after treatment in a previous study (McCain *et al*, 1993). The increase in hind limb vascular resistance and decrease in hind limb blood flow of hens treated with PSP could contribute to the neuropathic condition through: (1) decreased oxygen supply (Lundborg, 1970); (2) increased cellular metabolites (Bjornberg *et al*, 1989); and (3) decreased detoxification (Maxwell *et al*, 1987). The PSP-induced effects on hind limb blood flow and hind limb vascular resistance were modified in hens given verapamil. This suggests a potential mechanism whereby this calcium channel blocker could serve to

ameliorate clinical and neuropathological effects of PSP that were noted in previous studies (El-Fawal *et al*, 1989).

Altered responses to ACh, PE, and SAL administration was observed in PSP treated hens. These changes were not the same as those produced in hens administered PXN. Especially notable was the difference in the response to ACh. It is possible that prolonged elevations of ACh leads to a decrease in responsiveness in PXN treated hens (Costa *et al*, 1982); an effect that would not be expected after administration of PSP, since it did not have a significant effect on AChE.

The response of the vasculature observed in PSP treated hens in this study could be caused by an increased intracellular calcium levels in vascular smooth muscle. This is a possibility because the response of the vasculature to cholinergic and adrenergic agonists was modified in hens given PSP and verapamil, a calcium channel blocker. Altered calcium metabolism by affected nerve cells has been suggested as a possible mechanism for OPIDN (Abou-Donia *et al*, 1984). His investigations suggest that an increase in intracellular calcium induces the phosphorylation of specific intracellular components through calcium-calmodulin modulated kinases. Previous work done in our laboratory which demonstrated that verapamil, a phenylalkamine calcium channel antagonist, could ameliorate the neuropathic condition associated with OPs (El-Fawal *et al*, 1989) also support the role of calcium in OPIDN. The calcium-calmodulin second messenger system is the major pathway for

neurogenic and humoral contraction in the vasculature (Mulvaney and Aalkjaer, 1990). This system is activated through $\alpha_1$ adrenergic receptors and results in phosphorylation of contractile elements by myosin light chain kinase (MLCK). This contraction is directly related to the amount of intracellular calcium (Kamm and Stull, 1985). If the level of intracellular calcium is increased in vascular smooth muscle as it is in nerve by OP treatment, it is possible to induce contraction without activation of a receptor. This could contribute to the decrease in hind limb blood flow and increase in hind limb vascular resistance seen in PSP treated hens in this study; an effect that did not occur in hens given PSP and verapamil.

This study has clearly indicated that verapamil altered the response of hind limb vasculature to PSP. The increased limb blood flow and decreased limb vascular resistance during a critical period may have, in part, been responsible for the decrease in clinical signs observed for PSP treated hens. A reduction in vascular resistance and an increase in blood flow have also been observed in in the lower leg of humans in response to verapamil treatment (Solti *et al*, 1978; Nissen and Alexander, 1975). Verapamil also reduces conduction at neuromuscular junctions which may also reduce hind limb vascular resistance by decreasing skeletal muscle tone. (Dretchen *et al*, 1986).

Responsiveness to adrenergic agents was included as a part of this study because skeletal muscle blood vessels are richly innervated by sympathetic nerves (Maspers *et al*, 1990; Rechtland *et*

*al*, 1986; Bevan and Bevan, 1984). The adrenergic receptors of these blood vessels are primarily $\beta_2$ and produce vasodilation when activated (Kamm and Stull, 1985), presumably through the hyperpolarization of the cell membrane and the reduction of cytosolic concentrations of free calcium. However, Celander's classic study (1954) indicated that high levels of norepinephrine, epinephrine, and ACh produce vasoconstriction. The present study has indicated that responses of the hind limb vasculature to PE and SAL ($\alpha_1$ and $\beta_2$, agonists, respectively) and ACh, a cholinergic agonist, were altered by PSP treatment. The effects were modified in hens given PSP and verapamil. Verapamil not only prevents calcium entry into the cell through voltage-operated channels, but it can also block receptor-operated channels (Katz *et al*, 1985), fast sodium channels (Opie, 1984), $\alpha_1$ receptors (Motulsky, *et al*, 1983) and $\alpha_2$ receptors (Opie, 1984). This complex blockade of channels and receptors would have an inhibitory effect on vasoconstriction; an effect observed in this study as PSP-induced decreases in hind limb blood flow and increases in hind limb vascular resistance were modified by PSP and verapamil. Although verapamil can block calcium entry, it has little or no effect on the efflux of calcium from the cell and may reduce intracellular calcium levels in hens treated with neuropathy inducing OPs. This action of verapamil may be responsible for the reduced response to vasoactive agents by the vasculature of the hind limb of the hen.

Alteration of vascular reactivity of treated hens to vasoactive agents may also be due to alterations of second messenger systems. Adrenergic receptors of the $\alpha 1$ type activate a G protein responsible for the stimulation of phospholipase C which ultimately leads to both an increase in intracellular Ca++ and activation of protein kinase C. This second messenger system is also used by muscarinic receptors ($M_1$, $M_3$, and $M_5$) (Berridge, 1988). Adrenergic receptors of the $\alpha 2$ type activate another type of G protein (inhibitory) which inhibits the activity of adenylate cyclase. This second messenger system is also used by other muscarinic receptors ($M_2$ and $M_4$) (Gilman, 1987). Beta-2 adrenergic receptors activate still another type of G protein (stimulatory) which enhances the activity of adenylate cyclase, which may influence the actions and disposition of intracellular calcium (England et al, 1984). Because adrenergic and cholinergic agents have common second messenger systems and because both adrenergic and cholinergic responses are altered by PSP treatment, it may be possible that PSP has an effect on either or both of these systems.

Blockade of calcium channels by verapamil may also have an indirect effect on the vasculature through the inhibition of neurotransmitter release by vasomotor fibers. In nerve cells, calcium facilitates the binding of vesicles to the presynaptic membrane and the subsequent release of vesicular contents into the synaptic cleft (Llinas, 1982). Furthermore, there is a direct relationship between intracellular levels and the amount of

transmitter released (Katz and Miledi, 1967). Verapamil, by reducing the calcium entry into the nerve terminal, reduces the amount of transmitter released and hence reduces the post-synaptic potential. It may be possible, therefore, that the increased limb blood flow observed in hens treated with verapamil and hens treated with OP and verapamil may have been due, in part, to a decrease in transmitter release from vasomotor fibers.

It is obvious that more work needs to be done in order to determine the role of the vasculature in OPIDN more precisely. Such work could include studies with isolated vascular preparations to determine whether the response to vasoactive agents is the result of altered vascular function or the response of the vessel to changes in its microenvironment. Studies with other OPs, including DEF (buitifos, S,-S,-S,-tri-n-butylphosphoro-thrithioate), an organophosphate cotton defoliant, which produces severe vascular effects that sometimes result in gangrene could be done to further elucidate the role of the cardiovascular system in OPIDN (B. Wilson, personal communication). Elucidation of the relationship between the vasculature and OPIDN could also include investigations with PMSF (phenyl-N-methyl-N-benzylcarbamate), a compound that is capable of significantly reducing NTE without producing a neuropathy. This may provide some answers to the role of NTE in vascular response (M.K. Johnson, personal communication). These suggestions will be taken into consideration and incorporated into

future research on the vascular contributions to OP-induced neuropathy.

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**Table 1:** Brain esterase activities in hens given 2.5mg/kg (im) phenyl saligenin phosphate (PSP) or 0.10 mg/kg (im) paraoxon (PXN). [1,2]

| Parameter Measured (units) | Days following OP administration | | | | |
|---|---|---|---|---|---|
| | 0 (Control) | 1 | 3 | 7 | 21 |
| Brain AChE (μM Hydrolyzed/min/ mg protein | | | | | |
| PSP | 21.33 ±1.42 | 17.76 ±1.85 | 17.45 ±1.01 | 21.08 ±1.53 | 24.15 ±1.67 |
| PXN | 21.33 ±1.42 | 6.03* ±1.17 | 10.32* ±0.90 | 18.72 ±1.32 | 22.18 ±1.28 |
| Brain NTE (nM Hydrolyzed/min/ mg protein) | | | | | |
| PSP | 27.84 ±1.85 | 3.85* ±0.58 | 7.67* ±0.36 | 15.46* ±1.49 | 27.43 ±0.66 |
| PXN | 27.84 ±1.85 | 26.97 ±0.88 | 27.20 ±1.16 | 27.44 ±0.74 | 26.88 ±1.15 |

[1] Control hens received vehicle (DMSO, 0.5 ml/kg) only (n=10). n=5 per day for PSP and PXN treated hens.

[2] Data are expressed as mean ± standard error of the mean.

* Significant at $p<0.05$ with Fisher's least significant difference following ANOVA.

**Figure 1:** Histograms of cardiovascular parameters from hens given OPs or OPs and verapamil. Measurements of blood pressure (Fig. 1A), heart rate (Fig. 1B), hind limb blood flow (Fig. 1C), and hind limb vascular resistance (Fig. 1D) were taken before the administration of general anesthesia. Values from control hens (DMSO, 0.5 ml/kg, n=10) were 125$\pm$4 mmHg for blood pressure, 308$\pm$14.8 beats/min for heart rate, 12.52$\pm$1.33 ml/min/100 gm for hind limb blood flow, and 9.99$\pm$1.24 limb resistance units (LRU, pressure difference / flow) for hind limb vascular resistance. OP treated groups received either PSP (2.5 mg/kg) or PXN (0.1 mg/kg). Groups of hens (n=5) received verapamil (7 mg/kg) the day before, the day of, and for two days after administration of PSP or PXN. Parameters were determined in verapamil treated hens after three days of treatment. Data were tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference; those values significantly different from controls are indicated on the figure by *. n=10 for control and n=5 for each treatment group.

**Figure 2:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) to consecutive log doses of acetylcholine chloride (ACh, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the vascular resistance in response to ACh displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from 125$\pm$2.56 mmHg @ $10^{-8}$ moles/kg to 66.2$\pm$6.2

mmHg @ $10^{-4}$ moles/kg. Data were tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. Values significantly different from controls are indicated by *. n=10 for control and n=5 for each treatment group.

**Figure 3:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) as well as verapamil (7 mg/kg given the day before, the day of, and for two days after OP treatment) to consecutive log doses of acetylcholine (ACh, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the response to ACh displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from 125$\pm$2.56 mmHg @ $10^{-8}$ moles/kg to 66.2$\pm$6.2 mmHg @ $10^{-4}$ moles/kg. Data were tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. Values significantly different from controls are indicated by *. n=10 for control and n=5 for each treatment group.

**Figure 4:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) to consecutive log doses of phenylephrine (PE, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the response to PE displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from 125$\pm$2.56 mmHg @ $10^{-8}$ moles/kg to 208.0$\pm$7.5 mmHg @ $10^{-4}$ moles/kg. Data were

tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. Values significantly different from controls are indicated by *. n=10 for control and n=5 for each treatment group.

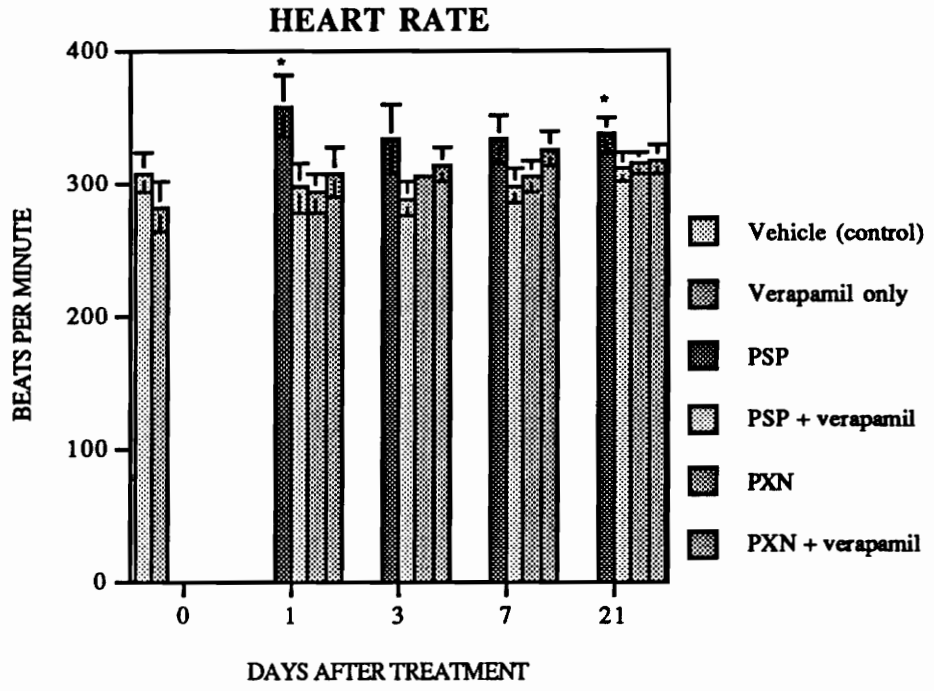
**Figure 5:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) as well as verapamil (7 mg/kg given the day before, the day of, and for two days after OP treatment) to consecutive log doses of phenylephrine (PE, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the response to PE displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from $125\pm2.56$ mmHg @ $10^{-8}$ moles/kg to $208.0\pm7.5$ mmHg @ $10^{-4}$ moles/kg. Data were tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. Values significantly different from controls are indicated by *. n=10 for control and n=5 for each treatment group.

**Figure 6:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) to consecutive log doses of salbutamol (SAL, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the response to SAL displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from $125\pm2.56$ mmHg @ $10^{-8}$ moles/kg to $107.5\pm2.2$ mmHg @ $10^{-4}$ moles/kg. Data were

tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. Values significantly different from controls are indicated by *. n=10 for control and n=5 for each treatment group.

**Figure 7:** Perfusion pressure of the hind limb vasculature of hens treated with either PSP (2.5 mg/kg) or PXN (0.1 mg/kg) as well as verapamil (7 mg/kg given the day before, the day of, and for two days after OP treatment) to consecutive log doses of salbutamol (SAL, $10^{-8}$ to $10^{-3}$ moles/kg). The data (mean $\pm$ SE) are displayed as a percent of the response to SAL displayed by vehicle treated hens serving as controls. Perfusion pressure in vehicle treated controls ranged from $125 \pm 2.56$ mmHg @ $10^{-8}$ moles/kg to $107.5 \pm 2.2$ mmHg @ $10^{-4}$ moles/kg. Data were tested for significance at the 0.05 level using ANOVA with Fisher's least significant difference. n=10 for control and n=5 for each treatment group.

A


HEART RATE
400
300
200
100
0
BEATS PER MINUTE
0
1
3
7
21
DAYS AFTER TREATMENT
Vehicle (control)
Verapamil only
PSP
PSP + verapamil
PXN
PXN + verapamil

B

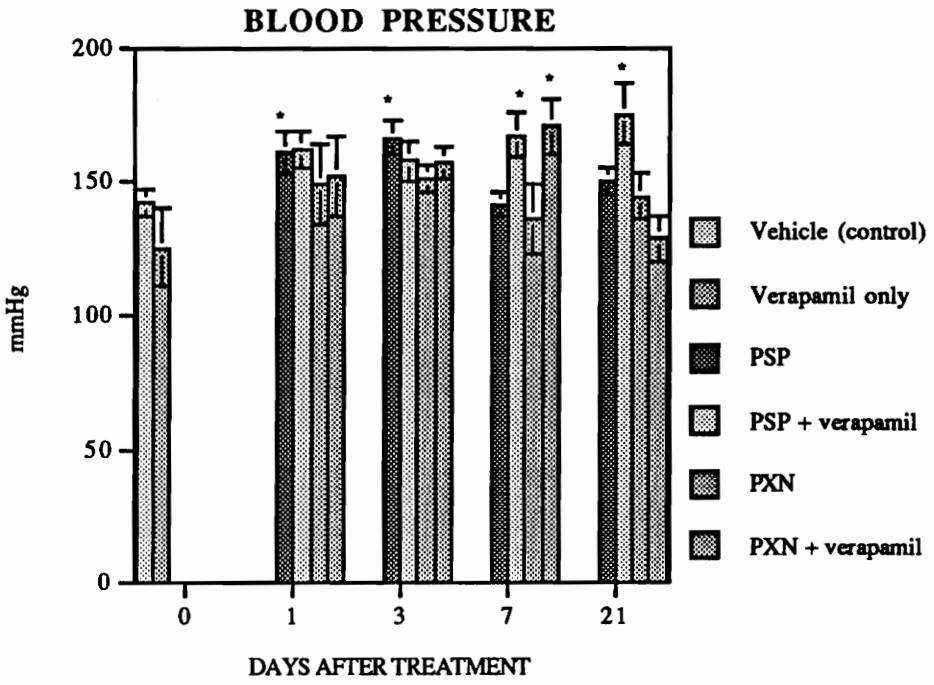
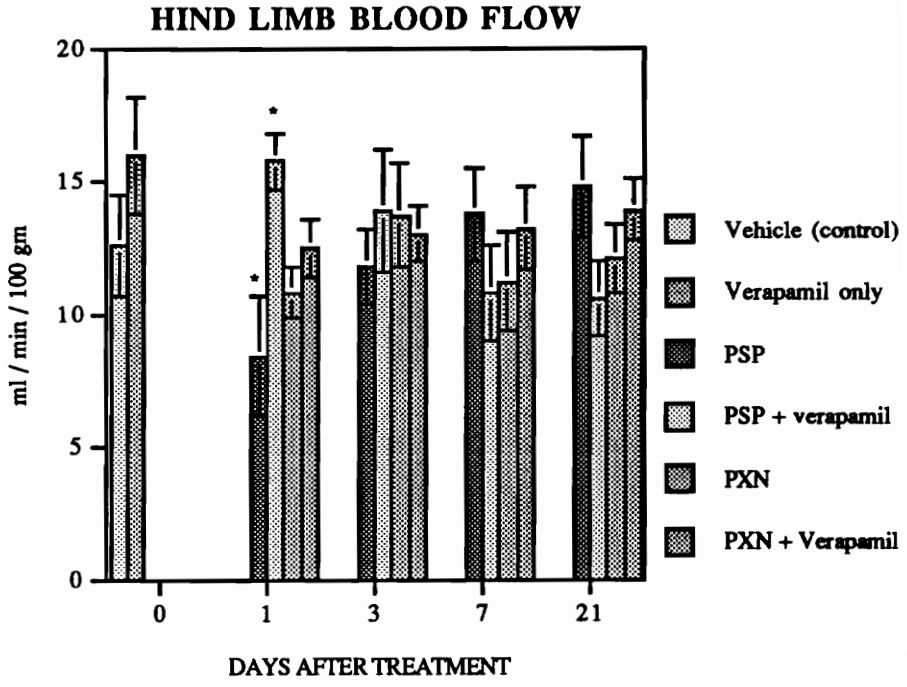

BLOOD PRESSURE
200
150
100
50
0
mmHg
0
1
3
7
21
DAYS AFTER TREATMENT
Vehicle (control)
Verapamil only
PSP
PSP + verapamil
PXN
PXN + verapamil

**FIGURE 1**

C




D

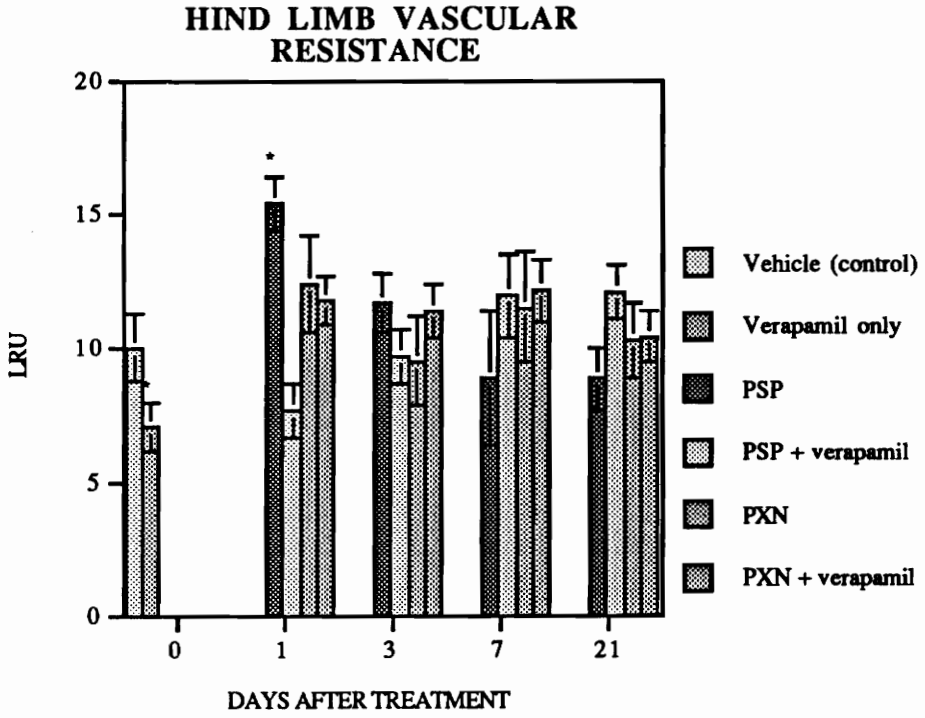
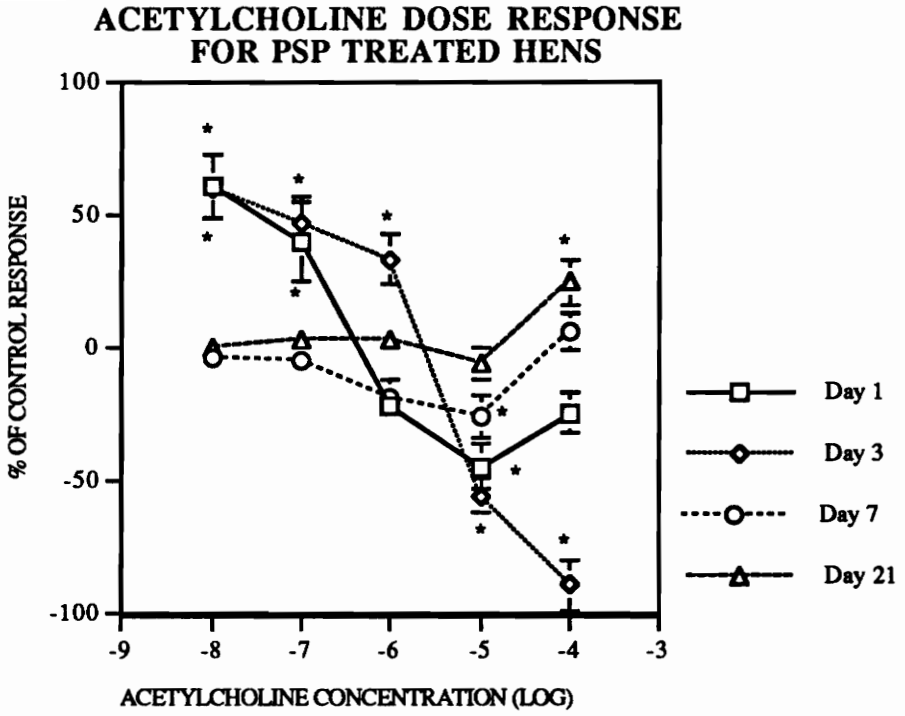



**FIGURE 1 (continued)**

A


ACETYLCHOLINE DOSE RESPONSE
FOR PSP TREATED HENS
% OF CONTROL RESPONSE
100
50
0
-50
-100
-9
-8
-7
-6
-5
-4
-3
ACETYLCHOLINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

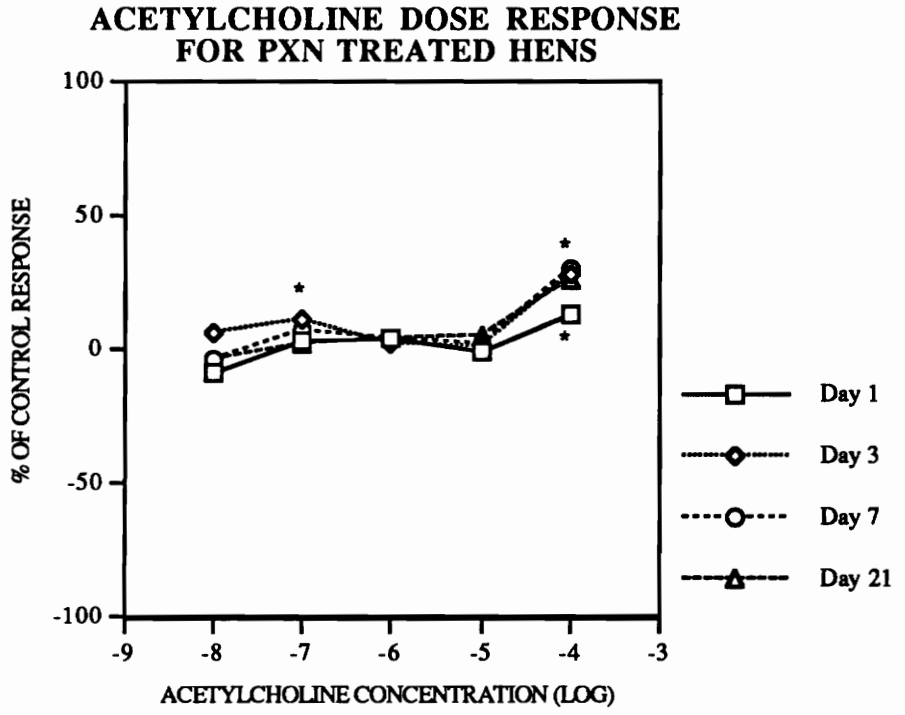
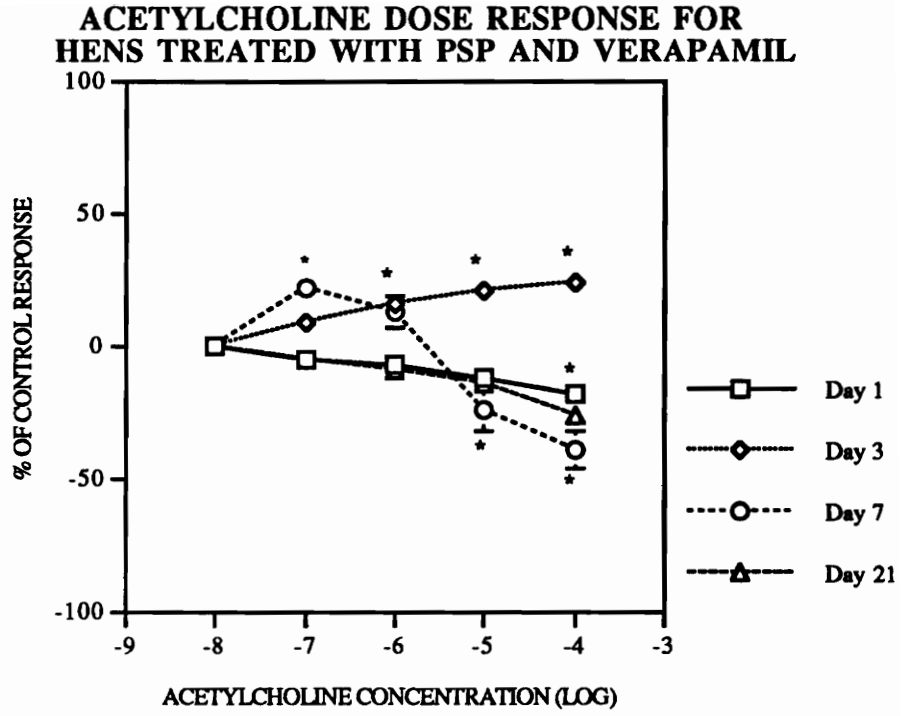

ACETYLCHOLINE DOSE RESPONSE
FOR PXN TREATED HENS
% OF CONTROL RESPONSE
100
50
0
-50
-100
-9
-8
-7
-6
-5
-4
-3
ACETYLCHOLINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21



FIGURE 2

A


ACETYLCHOLINE DOSE RESPONSE FOR
HENS TREATED WITH PSP AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
ACETYLCHOLINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

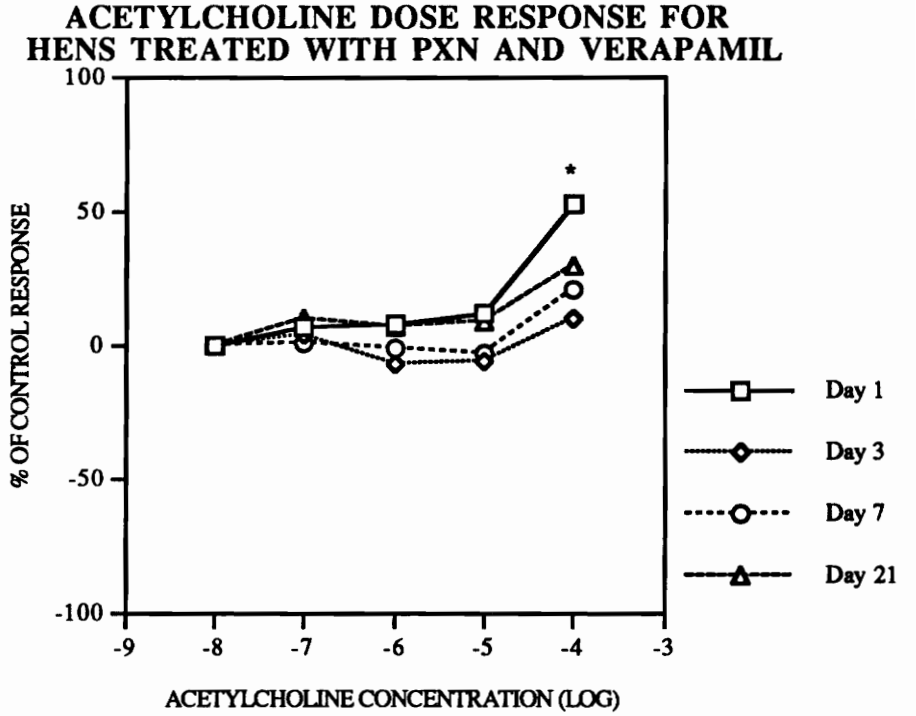
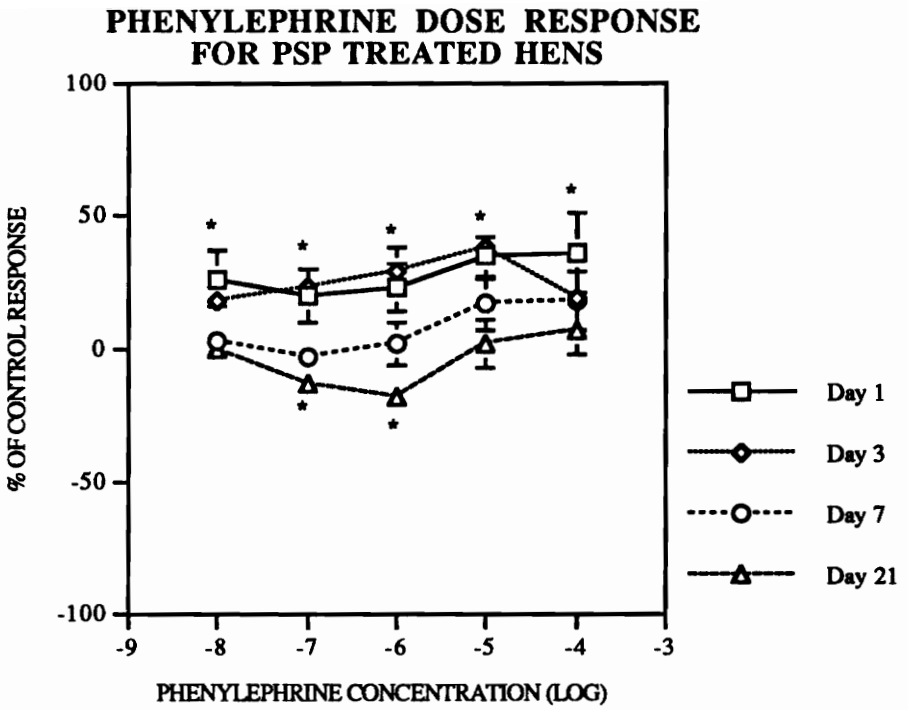

ACETYLCHOLINE DOSE RESPONSE FOR
HENS TREATED WITH PXN AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
ACETYLCHOLINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

**FIGURE 3**

A


PHENYLEPHRINE DOSE RESPONSE
FOR PSP TREATED HENS
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
PHENYLEPHRINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

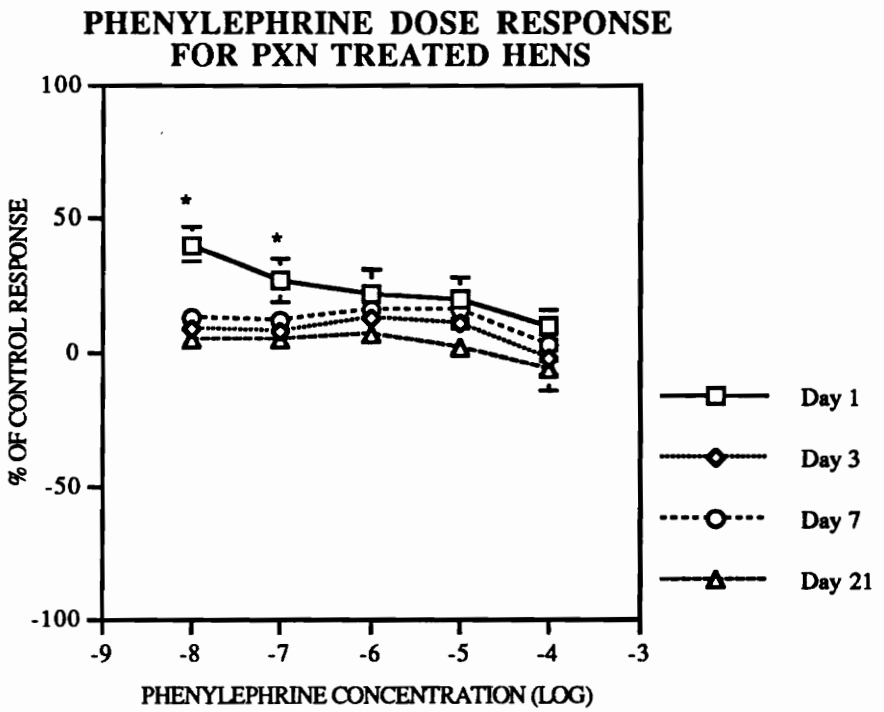
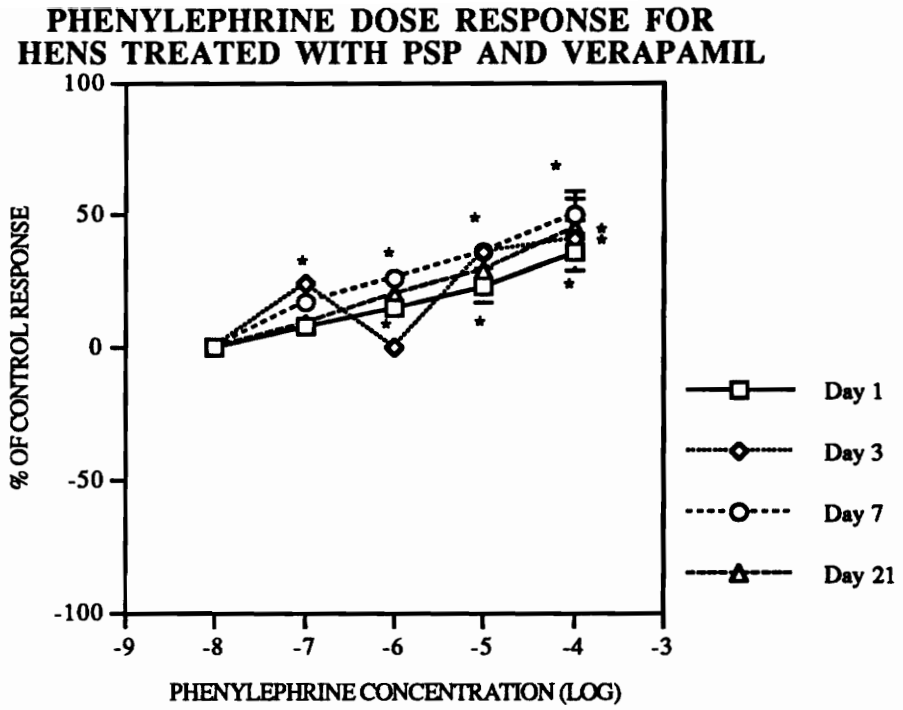

PHENYLEPHRINE DOSE RESPONSE
FOR PXN TREATED HENS
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
PHENYLEPHRINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

**FIGURE 4**

A


PHENYLEPHRINE DOSE RESPONSE FOR
HENS TREATED WITH PSP AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
PHENYLEPHRINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

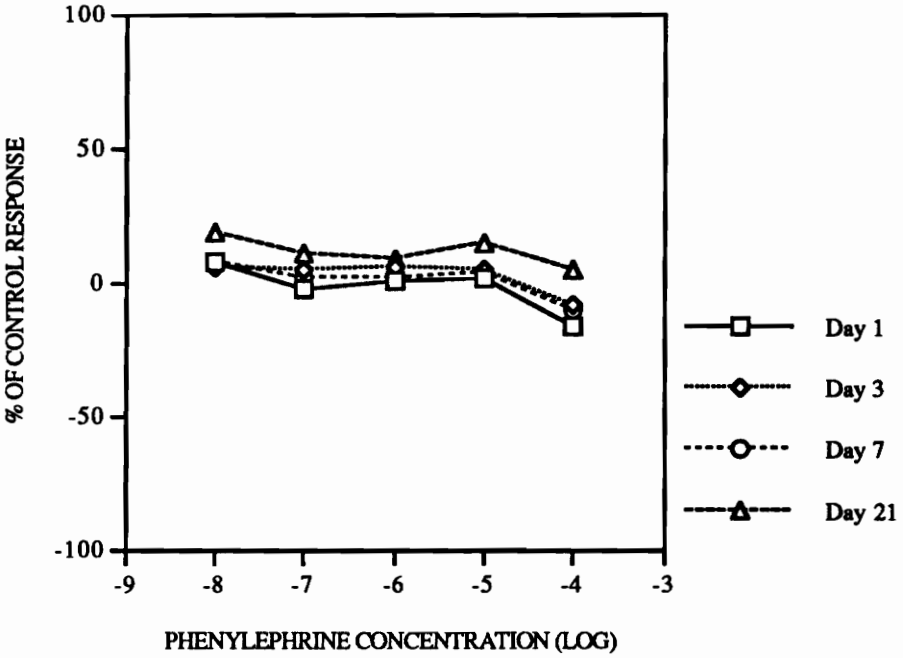
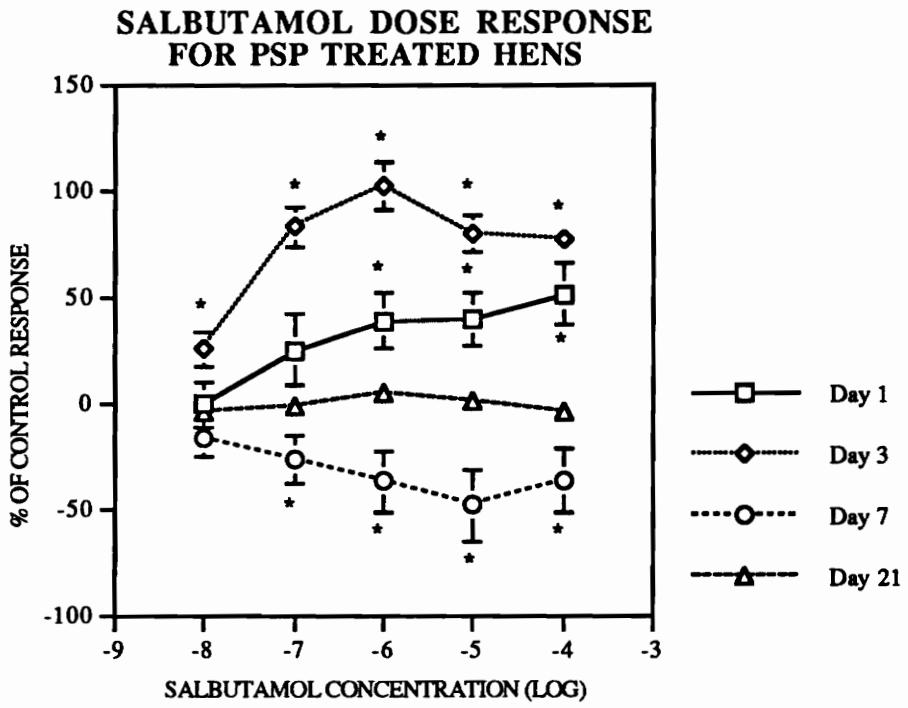

PHENYLEPHRINE DOSE RESPONSE FOR
HENS TREATED WITH PXN AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
PHENYLEPHRINE CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21



FIGURE 5

A


SALBUTAMOL DOSE RESPONSE
FOR PSP TREATED HENS
150
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
SALBUTAMOL CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

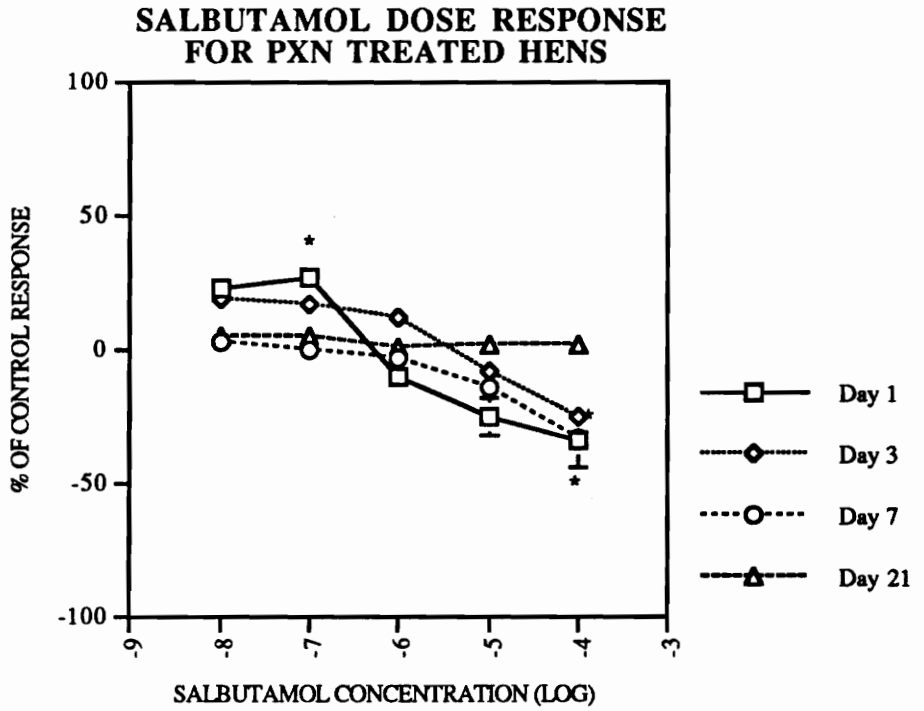
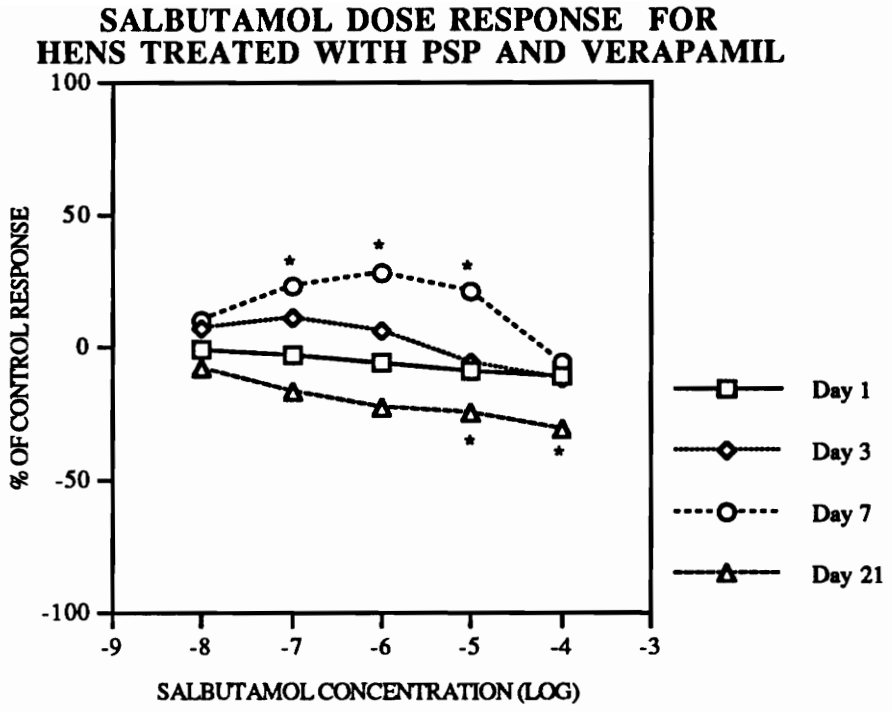

SALBUTAMOL DOSE RESPONSE
FOR PXN TREATED HENS
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
SALBUTAMOL CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21



FIGURE 6

A


SALBUTAMOL DOSE RESPONSE FOR
HENS TREATED WITH PSP AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
SALBUTAMOL CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

B

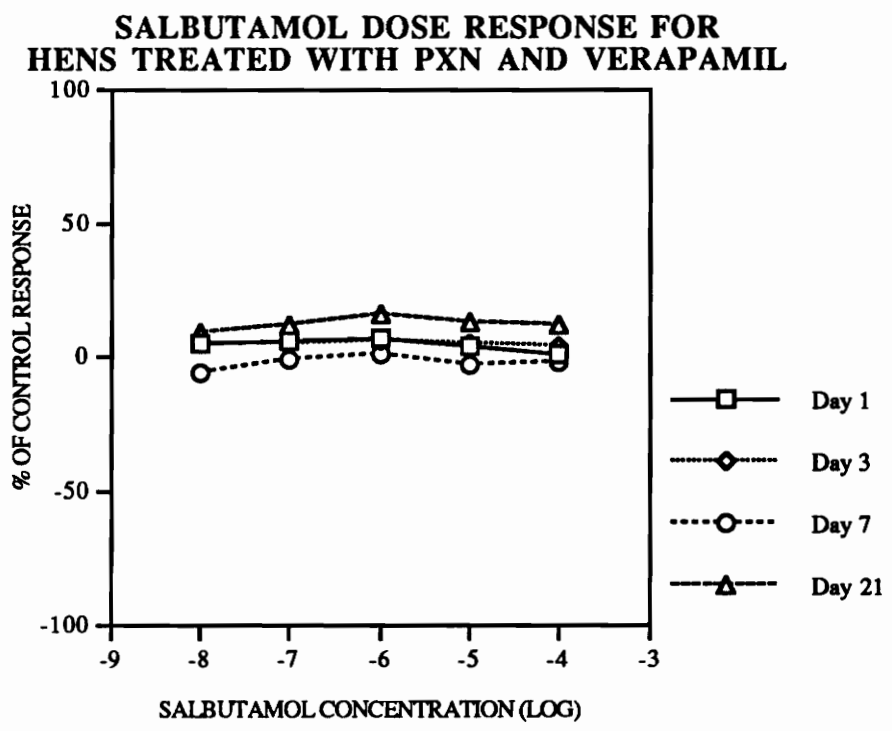

SALBUTAMOL DOSE RESPONSE FOR
HENS TREATED WITH PXN AND VERAPAMIL
100
50
0
-50
-100
% OF CONTROL RESPONSE
-9
-8
-7
-6
-5
-4
-3
SALBUTAMOL CONCENTRATION (LOG)
Day 1
Day 3
Day 7
Day 21

FIGURE 7

# CHAPTER 7

# CATECHOLAMINES AND THE CONTRACTILE RESPONSE OF ISOLATED VESSELS FROM HENS TREATED WITH CYCLIC PHENYL SALIGENIN PHOSPHATE OR PARAOXON IN THE PRESENCE OR ABSENCE OF VERAPAMIL

Wilfred C. McCain, Diane M. Flaherty, Linda Correll,
Bernard Jortner, and Marion Ehrich

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# CATECHOLAMINES AND THE CONTRACTILE RESPONSE OF ISOLATED VESSELS FROM HENS TREATED WITH CYCLIC PHENYL SALIGENIN PHOSPHATE OR PARAOXON IN THE PRESENCE OR ABSENCE OF VERAPAMIL

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Running title: OP effects on blood vessels and catecholamines.

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**ABSTRACT:**

**CATECHOLAMINES AND THE CONTRACTILE RESPONSE OF ISOLATED VESSELS FROM HENS TREATED WITH CYCLIC PHENYL SALIGENIN PHOSPHATE OR PARAOXONIN THE PRESENCE OR ABSENCE OF VERAPAMIL** W.C. McCain, D.M. Flaherty, L. Correll, B. Jortner, and M.F. Ehrich. VA-MD Regional College of Veterinary Medicine, Virginia Polytechnic Inst. and State Univ., Blacksburg, VA.

Blood samples and vascular segments from the ischiadic artery of hens treated with either cyclic phenyl saligenin phosphate (PSP; 2.5 mg/kg, i.m.) or paraoxon (PXN; 0.1 mg/Kg, im) in the presence or absence of verapamil, a calcium channel antagonist, (7 mg/kg, i.m. given 4 consecutive days beginning the day before OP administration) were examined 1, 3, 7, and 21 days after OP administration in order to determine the contribution of catecholamines and peripheral blood vessel physiology and morphology to organophosphorus-induced delayed neuropathy (OPIDN). The levels of plasma catecholamines were measured by HPLC and indicated a different effect with PSP, which causes OPIDN, and PXN, which does not. PSP treatment elevated the levels of norepinephrine and epinephrine throughout the study while PXN treatment depressed these levels. Verapamil treatment attenuated the OP response by approximately 50% for both compounds.

Ischiadic vessel segments were isolated from OP treated hens, and perfused at a constant flow rate of 12 ml/min, then examined for their response to potassium chloride (KCl, 3 x $10^{-3}$ M), acetylcholine, phenylephrine (PE), an $\alpha_1$ agonist, and salbutamol (SAL), a $\beta_2$ agonist. Agents were delivered in concentrations of $10^{-8}$ to $10^{-3}$ M. Vascular segments did not respond to ACh or SAL at any concentration used. Vessels displayed a significant reduction in contractile response to both KCl (3 x $10^{-3}$ M) and PE ($10^{-5}$ to $10^{-3}$) three and 21 days after exposure to either PSP or PXN. This reduced response was not altered by the presence of verapamil. Innervation of the peripheral vasculature was unchanged after OP treatment. This study indicates that plasma catecholamine levels are differentially altered by OP treatment and that the alterations involve intracellular calcium. In contrast, vascular response of the ischiadic artery was altered following OP treatment but the effect was not specific for the neuropathy-inducing OP, PSP, and response was not mediated by $Ca^{++}$ or the result of autonomic nerve deterioration.

## INTRODUCTION:

The general population is frequently at risk from exposure to organophosphorus compounds (OPs). OPs are used as plasticizers, additives in petroleum and lubricants, agricultural and household insecticides, and medicines. Toxic exposure to these OPs may result in the significant inhibition of several esterases including acetylcholinesterase (AChE). The inhibition of AChE prolongs the effect of acetylcholine (ACh) at neural and neuromuscular junctions. Some OPs also have the ability to inhibit the activity of neurotoxic esterase (NTE, neuropathy target esterase). A 70% reduction in the activity of NTE by OP esters early after exposure (24 hours) has been associated with the production of a distal axonopathy 7 to 21 days after exposure to a single toxic dose in man and other susceptible species. Organophosphorus-induced delayed neuropathy (OPIDN) has been diagnosed in a number of accidental poisonings around the world. In the 1930's, more than 50,000 people were accidently poisoned by drinking ginger beer that had been contaminated with tri-o-cresyl phosphate (TOCP). Since that time, more neurotoxic OPs have been identified and more than 60,000 people have been exposed to the neurotoxic effects of these compounds (Ecobichon, 1991).

Although most studies have focused on the neurotoxic effects of OPs, the cardiovascular system is also affected. The cardiovascular system has long been known to be susceptible to the effects of AChE inhibition as a result of exposure to OPs. Proximal injection of the OP,

DFP (diisopropyl phosphorofluoridate), in the medulla and midbrain of dogs produces a pressor response (Brezenoff, 1982; Kristic 1978). This pressor response is due to an increase in sympathetic outflow caused by central inhibition of AChE at vasomotor centers in the hypothalamus and medulla (Dowman, 1972). A reduction in blood flow to skeletal muscle, kidney and spleen following OP-induced AChE inhibition was reported in a study conducted by Vetterlein and Haase (1979). Sympathetic stimulation generally increases blood flow to skeletal muscles through the action of norepinephrine on $\beta_2$ adrenergic receptors as part of the "fight or flight" response (Parkinson, 1990).

Although the majority of studies have been conducted using OPs that cause significant inhibition of AChE, cardiovascular changes may follow administration of neuropathy-inducing OPs as well. Experimentation in our laboratory demonstrated that there were significant cardiovascular changes which occurred in the hen following a single exposure to PSP (cyclic phenyl saligenin phosphate), a neuropathy-inducing OP which was not an inhibitor of acetylcholinesterase (AChE). Cardiovascular changes occurred both before and after the onset of clinical signs of OPIDN. Administration of PSP was shown to decrease blood flow and increase vascular resistance of skeletal muscles of the hind limb of hens, an effect not expected if there had been ganglionic stimulation by AChE inhibition (McCain, *et al*, 1993a). This decrease in blood flow and increase in vascular resistance was also seen in another experiment which used

the perfused hind limb of the hen as a model. This model indicated that vessels in hens treated with PSP responded differently *in vivo* to selective $\alpha_1$, $ß_2$, and cholinergic agonists than did those of control hens and hens treated with paraoxon, an AChE inhibitor (McCain *et al*, 1993b). This study, however, used the whole hind limb and did not separate events which occurred within the vessels from events which occurred in the vessel's environment (endogenous vasoactive compounds or skeletal muscle activity).

The present study was therefore designed to examine the direct effects of OPs on the peripheral vasculature. OP-induced changes that directly affect vascular function could contribute to decreased blood flow and tissue damage seen after administration of these toxicants (Patterson, *et al*, 1988; Cisson and Wilson, 1982). This study included examination of the temporal effects of the neuropathy-inducing OP, PSP, on *ex vivo* vascular reactivity of isolated large-bore arteries to adrenergic and cholinergic agents. The effects of PXN, which does not cause OPIDN, were also examined. Because the level of circulating catecholamines can alter the density of receptors, levels of epinephrine and norepinephrine were examined in this study. As with our previous study, verapamil, a calcium channel blocker known to ameliorate the neuropathic effects of PSP (El-Fawal *et al*, 1989), was given to half of the hens in each group to determine whether its effect on vascular response could be a mechanism for its amelioration of OPIDN. In addition, because morphological changes could contribute to changes in vascular

function, nerves and vessels in the area in which these samples were taken were subjected to histopathological examination. Also, because changes in the levels of serum calcium and circulating catecholamines could contribute to changes in vascular function, these were measured as well.

## MATERIALS AND METHODS:

Animals:

Experiments were conducted on adult white leghorn hens obtained from the Department of Poultry Science at Virginia Polytechnic Institute and State University. The hens were between 18 and 24 months of age and weighed between 1.4 and 2.2 Kg. Hens were placed in wire bottom cages (52.8 x 42.4 x 42.4 cm.) with four birds per cage and were housed in a temperature controlled building with a 12 hour day / night cycle. Water and commercial chicken feed were provided ad libitum.

Treatment:

Hens used in this study were weighed and randomly divided into three groups with 40 hens in each group. One group of 40 hens was given 2.5 mg/kg phenyl saligenin cyclic phosphate (PSP, 2-phenoxy-4H-1-1-,3-2-benzodioxaphosphorin-2-oxide [Lark Enterprises, Webster, MA]) by intramuscular injection. PSP was dissolved in dimethyl sulfoxide (DMSO) to yield a 5 mg/ml solution. A second group of 40 hens was given paraoxon (PXN, O-O-diethyl,O-p-nitrophenyl phosphate), 0.10 mg/kg by intramuscular injection. A stock solution of paraoxon (13.76 mg/ml in acetone) was diluted with saline to yield a 0.30 mg/ml solution immediately prior to injection. The control group of 40 hens was given an equivalent volume (0.5 ml/kg) of the vehicle, DMSO. One half of the hens in each group (20) were given the calcium channel antagonist, verapamil (7 mg/kg, i.m.)

the day before, the day of, and daily for two days after OP treatment. Tissues were collected and examined 1, 3, 7, and 21 days after OP treatment. Days 1 and 3 represent time points prior to histological evidence of PSP-induced neuropathy and day 7 is a time point prior to clinical signs of neuropathy but after histological signs are evident (El-Fawal *et al*, 1990). Day 21 represents a time point when clinical signs of OPIDN are maximal.

Assessment of Clinical score:

The walking behavior of hens was evaluated every other day after administration of OPs (or vehicle). Clinical scores were given using the method of Sprague *et al* (1980) where 0 = normal, 1 = altered gait, 3 = difficulty in walking or standing, 4 = leg paralysis, and 5 = paralysis with both leg and wing involvement.

Enzyme assays:

Enzyme assays were conducted to determine the activity of neurotoxic esterase (NTE) and acetylcholinesterase (AChE). NTE activity was measured spectrophotometrically in frozen hen brains using the method of Sprague *et al* (1981) in which the ability to hydrolyze phenyl valerate is determined in the presence of paraoxon and/or mipafox. AChE activities were determined spectrophotometrically in brain samples by measuring the yellow anion produced by hydrolysis of acetylthiocholine (Ellman *et al*,1961).

Biochemical assays:

The level of plasma catecholamines was examined in samples obtained sequentially from a group of 20 hens (5 per treatment group). Blood was obtained from the brachial vein and separated into two 3 ml sample tubes. One tube contained 0.1 ml EGTA-glutathione solution for catecholamine analysis and the other contained 0.1 ml sequestersol. Samples were placed on ice, returned to the laboratory and centrifuged. The plasma was removed and frozen at -70°C until analysis. Samples collected prior to treatment were used to determine baseline levels of epinephrine and norepinephrine for each hen and subsequent samples were compared to this baseline value. Samples were measured using an electrochemical detector (LCEC, Bioanalytical Systems Inc., West Lafayette, IN) with dihydroxy benzyl amine (DHBA) as an internal standard.

Physiological and pharmacological assay:

For these studies, daily measurements included arteries from control and experimental subjects. The 2 cm segment of the ischiadic artery was removed from hens euthanized by cervical dislocation and placed in a petri dish containing warm (37°C), aerated (95% $O_2$ - 5% $CO_2$) Krebs-Henseleit (K-H) buffer solution (composition in g/l: NaCl 6.9; $MgSO_4 \cdot 7H_2O$, 0.29; $KH_2PO_4$, 0.15; glucose, 2.0; $NaHCO_3$, 2.1; KCl, 0.35; $CaCl_2$, 0.28). The vessel was then cleared of extraneous

tissue, muscular arterioles were ligated with silk suture (5-0 or 70, Ethicon, Somerville, NY), and trimmed to 1.5 cm. The vessel was suspended between two glass cannulae and tied in place. The tips of the cannulae, which were fitted to the luminal diameter of the vessel (1.5 - 2.0 mm), were separated by 1 cm. The preparation was transferred to an organ bath (Radnoti Glass Technologies Inc., Monrovia, CA) containing K-H buffer. A pressure transducer (Cobe Laboratories Inc, Lakewood, CO) was placed in the inflow cannula and connected to a physiographic recorder (TA-240, Gould Instrument Co., Valley View, OH). The cannula were connected to a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Chicago, IL) via silicone tubing (size 14, Masterflex, Cole-Parmer Instrument Co., Chicago, IL) and the lumen of the vessel was perfused with oxygenated K-H buffer at a flow rate of 6.0 ml/min. The volume of the luminal circuit was 10 ml. Bath solution and luminal solutions were changed every 15 minutes. The vessel preparation was allowed to recover for 30 minutes prior to treatment with vasoactive agents.

Log concentration-response curves in response to phenylephrine (PE), salbutamol (SAL) and acetylcholine chloride (ACh) ($10^{-8}$ to $10^{-3}$ moles/l) (Sigma Chemical Co., St. Louis, MO) were generated by plotting cumulative increments of agent concentration against pressure change (expressed as a % of potassium contraction) within the luminal circuit. The vessels were precontracted with potassium chloride ($3 \times 10^{-3}$ M) prior to administration of

salbutamol and acetylcholine, the vasodilating agents. Agents were injected (0.1 ml aliquot) into the luminal tubing prior to its entry into the pump. After the final measurements were taken for each agent, fresh K-H buffer was perfused through the luminal circuit for 5 minutes. The vessel preparation was allowed to recover for 15 minutes, the luminal circuit and bath were refilled with fresh K-H buffer, and the next agent was administered. When the final agent had been tested, the vessel was removed and measured. Vessels from the contralateral limb of several hens of each group were removed and fixed in phosphate buffered formalin and later processed for histological examination.

Vessels from hens of several groups were examined on each day in order to prevent errors which could occur from slight alterations in buffer composition, temperature, and other extraneous factors. At least one vessel from a vehicle-treated hen was examined each day that vessels from OP-treated or OP and verapamil treated hens were examined

Histology:

Tissues selected for examination in this study included the ischiadic artery, vein, and nerve as well as a section of the gastrocnemius muscle. The artery, vein and nerve were taken from the mid-femoral region and the section of gastrocnemius muscle examined was taken from the medial head at a point 1-2 mm distal to the point of entry of the sural nerve, which has displayed

evidence of pathological changes following PSP treatment (Jortner and Ehrich, 1987). Cross sections of the muscle would, therefore, include a section of the sural nerve and its associated artery and vein. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded and 10 μm sections were cut and stained. Tissues were stained for general histological purposes with luxol fast blue, periodic acid-Schiff, and hematoxylin. This staining process allowed the examination of muscle fiber diameter and vessel to fiber ratio (number and area) (Goto, 1988). Nerve cross sections were examined for the presence and structure of endoneurial blood vessels and to assess nerve fiber damage associated with OPIDN.

Statistics:

Data were analyzed using analysis of variance with the Newman-Keuls method of multiple comparisons for determination of statistical differences among control and experimental groups, with $p<0.05$ considered significant. All data are expressed as mean $\pm$ SEM, except where otherwise indicated.

**RESULTS:**

Clinical Scores:

Clinical scores in hens treated with PSP were similar to those observed in other studies conducted in our laboratory (El-Fawal *et al*, 1989; McCain *et al*, 1993). Hens developed a decrease in locomotor activity starting on day 6 if given PSP only and not until day 8 if given PSP and verapamil. All hens given only PSP were affected 8 days after PSP administration, whereas 10 days were needed before all hens given PSP and verapamil were affected. Signs progressed in all hens through day 21 when the study was terminated. Hens had manifested maximal clinical signs of OPIDN on day 20. The clinical scores were $4.2\pm0.3$ in hens treated with PSP only which included 2 (of 15) hens with "wing droop", an indication of upper limb involvement and $3.6\pm0.2$ in hens given PSP and verapamil.

Hens treated with PXN or PXN with verapamil did not develop locomotor deficits.

Enzyme assays:

The activity level of neurotoxic esterase (NTE) in brain homogenates of hens treated with PSP was significantly inhibited 1, 3 and 7 days after exposure and the activity of brain acetylcholinesterase (AChE) was not greatly affected. The activity level of brain NTE in hens treated with PSP was 63% inhibited 1 day after treatment when compared to the activity of brain NTE in vehicle-treated hens (from $26.2\pm1.6$ nmol/min/mg protein to $9.9\pm1.8$

nmol/min/mg protein; n=10 and n=5, respectively). The activity level of brain AChE was reduced by 11%, (from 18.6±1.2 μmol/min/mg protein to 16.6±1.6 μmol/min/mg protein; n=10 and n=5, respectively) 1 day after PSP treatment. Verapamil treatment had no significant effect on the inhibitory action of PSP on these enzymes in treated hens.

PXN treated hens exhibited no decrease in the activity level of NTE in this study but the activity level of AChE was significantly inhibited 1 day after exposure. Brain AChE activity in PXN treated hens was reduced to 25% of the levels observed in vehicle-treated hens (from 18.6±1.2 μmol/min/mg protein to 4.6±1.3 μmol/min/mg protein). The activity of brain NTE reduced by less than 5% one day after exposure to PXN (from 26.2±1.6 nmol/min/mg protein to 24.9±1.1 nmol/min/mg protein). The activity of brain AChE in PXN treated hens was still slightly lower than that of vehicle-treated hens 3 and 7 days after treatment and slightly elevated 21 days after treatment.

The inhibition of brain AChE activity in hens treated with PXN was not altered by treatment with verapamil.

Biochemistry:

The level of epinepherine (EPI) observed in the plasma of hens treated with PSP was elevated throughout the 21 day study period (range = 9.09±4.55 ng/ml on day 1 to 17.41±8.71 ng/ml on day 7, n=5 each, compared to 6.02±2.63 ng/ml base values in these hens).

A 54% increase in EPI over control values was observed 1 day after PSP treatment (Figure 1A). EPI increased by 76% three days after treatment and reached a maximal level (192% of control) at 7 days. The level of EPI was 98% greater than control values 21 days after treatment with PSP. The level of EPI observed in hens treated with PSP and verapamil was approximately 50% lower than that observed in hens treated with PSP only on all sampling days (Figure 1A).

Hens treated with PXN displayed a decrease in the level of circulating EPI on all sampling days (range = 3.65±1.57 ng/ml on day 1 to 5.57±2.93 ng/ml on day 3, n=5 each) (Figure 1A). The greatest reduction occurred 3 days after PXN treatment when the level of EPI was reduced by 54%, although circulating EPI was reduced by more than 30% seven and 21 days after treatment. The levels of EPI observed in PXN treated hens were significantly lower than those observed in hens treated with PSP on all examination days (Figure 1A). The inhibitory effect on EPI levels seen in hens given PXN only was also reduced by approximately 50% when hens were administered verapamil as well as PXN.

The level of norepinephrine (NE) was slightly increased in hens treated with PSP 1, 3 and 21 days after treatment and significantly elevated 7 days after PSP treatment (70%; from a base value of 21.74±5.86 ng/ml to 33.02+6.34 ng/ml, n=5) (Figure 1A). Treatment with verapamil reduced the effect of PSP on serum NE levels by more than 50% one day after treatment and actually reduced the

level of of NE to below pretreatment levels on days 3, 7, and 21 (Figure 1A).

PXN treatment reduced the level of serum NE significantly 1, 3, and 7 days after treatment (Figure 1B), with greatest reduction 7 days after PXN treatment. At that time, NE was reduced by 67% (from base values of 23.96±7.82 ng/ml to 7.78±6.37 ng/ml, n=5). The level of NE was lower on day 21 but not significantly so. Verapamil treatment significantly reduced the effect of PXN on serum NE levels at all times when samples were evaluated. There was no difference between pretreatment values and those obtained from hens treated with PXN and verapamil (Figure 1B).

Vascular response to vasoactive agents:

The ischiadic artery responded to the vasoconstrictor effects of potassium chloride (KCl) and phenylephrine (PE) (Figure 2) but not to the vasodilator effects of acetylcholine (ACh) or salbutamol (SAL) (data not shown). Vascular segments from all treatment groups responded in the presence of 3 x $10^{-3}$M potassium chloride (KCl) producing increases in pressure when perfusion rate was kept constant. The response of vessels to PE administration occurred only at the higher concentrations ($10^{-5}$M to $10^{-3}$M). The responses were dose-dependent with a maximal response occurring at PE concentrations of $10^{-3}$M.

Vehicle-treated hens demonstrated an increase in resistance of 19.86±1.76 mmHg (n=10) in the presence of 3 x $10^{-3}$M KCl (Figure

2A). The response of vessels from vehicle-treated hens to PE was maximal at a concentration $10^{-3}$M (7.88±0.76 mmHg, 39.8% of the KCl response, n=10) (Figure 2B). The mean maximal response of vessels from vehicle-treated hens to ACh was less than 1 mmHg and no vessel responded to the $ß_2$ agonist, SAL.

A significant decrease in response to KCl or to PE, when compared to response in arteries from vehicle-treated hens was observed 3 and 21 days after treatment of hens with either PSP or PXN. The vascular responses observed in hens treated with OP and verapamil were not different from those response observed in hens treated with OP only. Vessels from hens given only verapamil responded to KCl and PE like vessels from vehicle-treated hens (Figure 2).

Histology:

Muscle tissue obtained from hens treated with PSP displayed a significant reduction in fiber diameter 21 days after exposure when compared to muscles obtained from vehicle-treated hens. The average fiber diameter determined in the medial head of the gastrocnemius was 51.0±1.0 µm (n=10 hens, 25 fibers/hen) in vehicle-treated hens. The diameter of fibers in PSP treated hens 21 days after exposure was 47.3±1.3 µm (n=5 hens, 25 fibers/hen) Verapamil treatment reduced the effect of PSP on fiber diameter in gastrocnemius muscle of hens examined 21 days after treatment

(49.9±1.4 μm; n=5 hens, 25 fibers/hen), an effect that was not significantly different from that of vehicle-treated hens.

The diameter of fibers examined 21 days after treatment with PXN were not different from those examined from vehicle-treated hens (50.6±1.1 μm; n=5 hens, 25 fibers/hen) and verapamil had no effect on fiber diameter in either vehicle-treated or PXN treated hens when examined 21 days after administration of the toxicant.

The muscle fiber to vessel ratio of OP-treated hens was not different from that of vehicle-treated hens. Nerve fibers examined at the adventitial / medial border of the sural artery of treated hens were not different from those examined in vehicle-treated hens and there was also no differnce in the vascularization of the sciatic nerve of OP-treated hens when compared to vehicle-treated hens (data not shown).

## DISCUSSION:

The major findings of this study were the following: (1) the responses to KCl and PE of large-bore vessels *ex vivo* were altered 3 and 21 days after treatment with either PSP or PXN. (2) Verapamil treatment did not alter the response of large-bore vessels *ex vivo* to vasoactive agents. (3) PSP treatment increased and PXN treatment decreased the levels of circulating catecholamines. (4) Verapamil treatment reduced the effect of OPs on circulating catecholamine levels by approximately 50 percent.

In this study, the inhibition of brain NTE approached 70%, a criterion set by Johnson and Lotti (1980), and hens in this study did develop clinical signs of OPIDN which progressed in a manner similar to other studies in which PSP was used at the same concentration (McCain *et al*, 1993; El-Fawal *et al*, 1989, 1990; Jortner and Ehrich, 1987; Baron *et al*, 1962). In the present study, the activity of brain NTE was inhibited by 68% and deficits in walking behavior were observed as early as day 7 and progressed throughout the study. A previous study from our laboratory indicated that a specific threshold for NTE was not necessary and that both NTE inhibition and OPIDN were dose related (Ehrich *et al*, 1993) The degree of NTE activity inhibition by PSP was unaffected by treatment with verapamil, an indication that the reduction in activity of this enzyme is probably not calcium dependent.

The activity of NTE was not significantly inhibited in hens treated with PXN and clinical signs of OPIDN were not noted in these

animals. This data is consistent with that of other investigators using this compound (McCain *et al*, 1993a; 1993b; DeNeef *et al*, 1982).

The response of isolated vessels to vasoactive agents was altered by treatment with OPs. The responses observed were not limited to the neuropathic OP, PSP, and were unaltered by treatment with verapamil. The similarity of responses in PSP and PXN treated hens in the presence or absence of verapamil indicate that OPs can reduce the response of large caliber arteries to vasoactive agents and that this change is probably not due to an increase in intracellular calcium through verapamil sensitive membrane channels. This data contrasts with data obtained in a previous study in which the response of hind limb vasculature to the same vasoactive agents was examined *in vivo* (McCain *et al*, 1993b). In the previous study, vascular response differed between hens treated with PSP and hens treated with PXN. Furthermore, in the previous study, verapamil treatment significantly reduced the response to vasoactive agents in hens treated with either PSP or PXN. Since vessels are highly sensitive to metabolic alterations such as those observed during graded exercise (Bjornberg *et al*, 1989), the differences in response may be accounted for by the absence of endogenous mediators in the present study. The increase in the level of catecholamines in response to PSP treatment and their reduction following PXN treatment could account for some of the differences observed between the *in vivo* and this *ex vivo* study. The temporal difference in the response of vessels from treated hens may indicate a

difference in response to two phases in the development of OPIDN. The response observed at three days may represent the vascular response to esterase inhibition while the response observed at 21 days may be the result of neuropathic effects.

Another factor to consider when comparing the present *ex vivo* study with previous *in vivo* studies is that large caliber vessels are different from resistance arteries and arterioles in several ways. The present study used large caliber arteries; vessels of all sizes contributed to results obtained *in vivo*. Large caliber arteries differ from resistance arteries in their receptor population and density (Bevan and Bevan, 1984). Large arteries also have a greater smooth muscle content (Mulvaney and Aalkjaer 1990), have increased synaptic cleft space, are less dependent on myogenic tone, and are less dependent on extracellular calcium for NE induced contraction (Bevan, 1985) than small arteries. These differences indicate that a differential response could exist within the arterial tree. The response to changes in catecholamine levels and myogenic activity could, therefore, differ between these sets of vessels. Large caliber vessels were used in this study to both validate an *ex vivo* perfusion system and examine one segment, the largest, of the vascular tree of the hind limb. More traditional studies are planned using vascular ring segments to measure alterations in generated force in response to vasoactive agents. These studies will examine the response of smaller resistance arteries and arterioles of the hind limb vascular tree of OP treated hens.

Another factor to consider is that perfusion pressure at a constant flow rate of 12.0 ml/min (the flow rate determined for vehicle-treated hens *in vivo*) ranged between 60 and 80 mmHg which was 40 to 60 mmHg lower than that observed for these hens *in vivo*. This decrease in pressure is at a similar flow rate was probably due to the combination of 3 factors: 1) The reduction in vascular tone supplied by autonomic innervation 2) the reduction of pressure from surrounding skeletal muscle tissue and 3) the absence of resistance vessels distal to the arterial segment used. The decrease in wall tension of the vessel probably blunted the response to all vasoactive agents used in this study. However, when flow was increased to produce a pressure of 125 mmHg, vessels often produced "leaks". It would be interesting to determine the effects of OP treatment on hind limb vasculature using the perfused hind limb preparation (Ploucha *et al*, 1981; McCain *et al*, 1993b) and an osmotically balanced perfusion solution of a known composition and volume. This technique could isolate the vasoactive effects of tissue factors from those blood factors.

The levels of circulating catecholamines were elevated by treatment with PSP. This information is supported by *ex vivo* work using chromaffin cells which displayed an increase in the level of catecholamine secretion following administration of the type 1 OPIDN producing compound, DFP (Knoth-Anderson and Abou Donia, 1993). The enhancement of catecholamine secretion by chromaffin cells and the resulting increase in the levels of circulating catecholamines

could have a profound effect on the response of the vasculature. There is evidence that a down regulation of receptors occurs in response to sustained elevations of catecholamines (Guyton, 1991), which may reduce the response of vessels, both *in vivo* and *ex vivo*, to administration of these agents.

PXN treatment reduced the levels of circulating catecholamines in treated hens. This information contrasts with data obtained from that of Knoth-Anderson and Abou-Donia (1993) in which no decrease in catecholamine secretion was observed in response to PXN treatment. Catecholamine secretion in chromaffin cells was, however, inhibited by the type II neuropathic OP, triphenyl phosphite (TPP) (Knoth-Anderson and Abou Donia, 1993) and indicates a difference in the response of the adrenal medulla to different types of OPs as well as a difference between *in vivo* and *ex vivo* responses.

Measurements of skeletal muscle fiber diameter observed in this study was consistent with that obtained by Cisson and Wilson (1982) in which a reduction in fiber diameter was observed 20 days after exposure to tri-ortho-cresyl phosphate (TOCP). In this study, it was demonstrated that the reduction of fiber diameter was attenuated by treatment with verapamil. The attenuation, however, may also be a result of a less severe neuropathy with a greater ability to obtain water. PSP treated hens examined on day 21, when the greatest reduction in fiber diameter was observed, were also ataxic and may not have been able to obtain an adequate supply of

water from the automatic watering system located in the cages. These hens appeared dehydrated (not quantitatively examined). Dehydration has been associated with a decrease in skeletal muscle fiber diameter and an increase in specific electrical resistance of lean body mass (De-Boer *et al*, 1992). Future studies in this area might include measurements of bioimpedence and water intake in order to determine their contribution to decreased fiber diameter associated with OPIDN.

The fiber to vessel ratio and the capillary tortuosity pattern were not altered by treatment with OPs. Changes in vascular architecture may not become evident early after the neuropathy manifests itself clinically. Since hens were sacrificed less than two weeks after the first clinical signs were evident, these long term effects may not have been evident.

No alterations of nerve fibers innervating the vasculature were evident in hens treated with OPs. This information is consistent with data presented by Cavanaugh (1961) in which he examined ganglia of TOCP treated hens and found no pathological lesions, an indication that autonomic nerve damage did not occur in this type of toxicity.

It has been demonstrated in our laboratory that verapamil, a phenylalkamine calcium channel antagonist, can ameliorate the neuropathic condition (El-Fawal *et al*, 1989; 1990; McCain *et al*, 1993b). Furthermore, verapamil treatment attenuates the response of the vasculature to vasoactive agents *in vivo* (McCain *et al*, 1993b). These studies, as well as those of others investigating the role of

calcium in OPIDN (Abou-Donia *et al*, 1984; 1988; Patton *et al*, 1985; 1986; Suwita *et al*, 1986), indicate that increases in intracellular calcium can alter the clinical and pathological outcome of delayed neuropathy associated with OPs and that effects can be reduced by calcium channel blockade.

There are still unanswered questions about the role of the cardiovascular system in OPIDN. The difference in effects between PSP and PXN on catecholamine levels engenders as many questions as does the lack of difference in effects of these two compounds on isolated vascular response to vasoactive agents. Several suggestions, however, have been made which will further elucidate the role of the vasculature in OPIDN and include an examination of endothelial response to OPs, the response of adrenalectomized hens to OPs, and the response of the isolated perfused limb from OP-treated hens to vasoactive agents. These suggestions will be taken into consideration for future investigations.

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**Figure 1:** Serum levels of A) epinephrine (EPI) and B) norepinephrine (NE) in hens treated with PSP, PSP and verapamil, PXN, and PXN and verapamil compared to values obtained from each hen before treatment. Data are presented as a percent of the pretreatment values. Analysis of variance followed by Newman-Keuls post test was used to determine significant differences ($p<0.05$).

a. indicates a significant difference from pretreatment values.

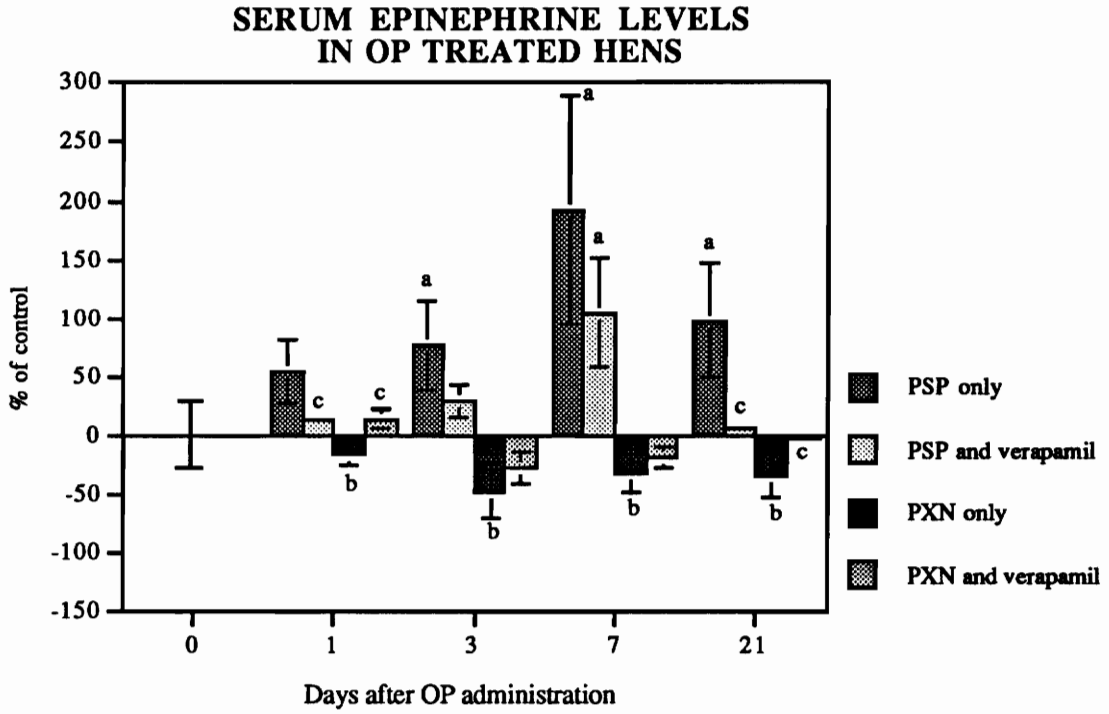
b. Indicates that PXN treatment gave results significantly different from PSP treatment.

c. indicates that verapamil significantly reduced the OP effect.

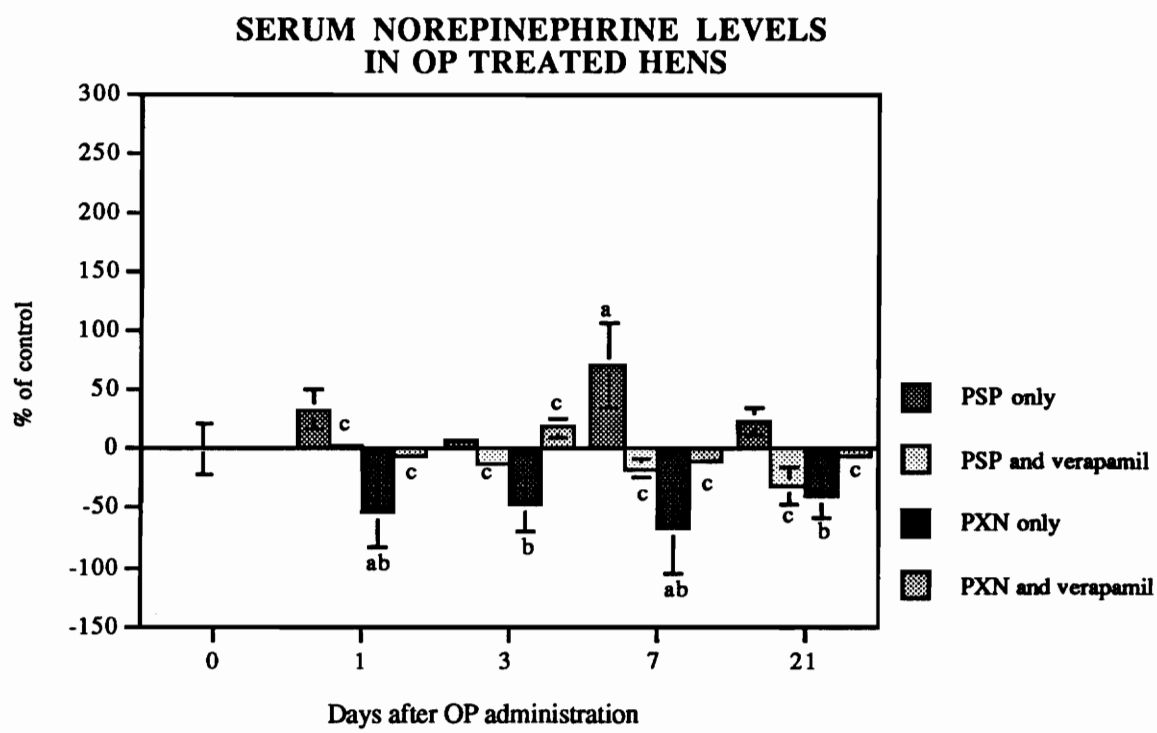
**Figure 2:** The response of isolated vessels from hens treated with PSP, PSP and verapamil, PXN, and PXN and verapamil to A) potassium chloride (KCl, $3x10^{-3}$ M) and B) phenylephrine (PE, $10^{-3}$ M) compared to the responses in hens given the vehicle, dimethyl sulfoxide (DMSO), or DMSO and verapamil (displayed as day 0).

* indicates a significant difference from the response in vehicle-treated hens using analysis of variance and Newman-Keuls post test. ($p<0.05$, n=10 for day 0 and n=5 for treatment groups).

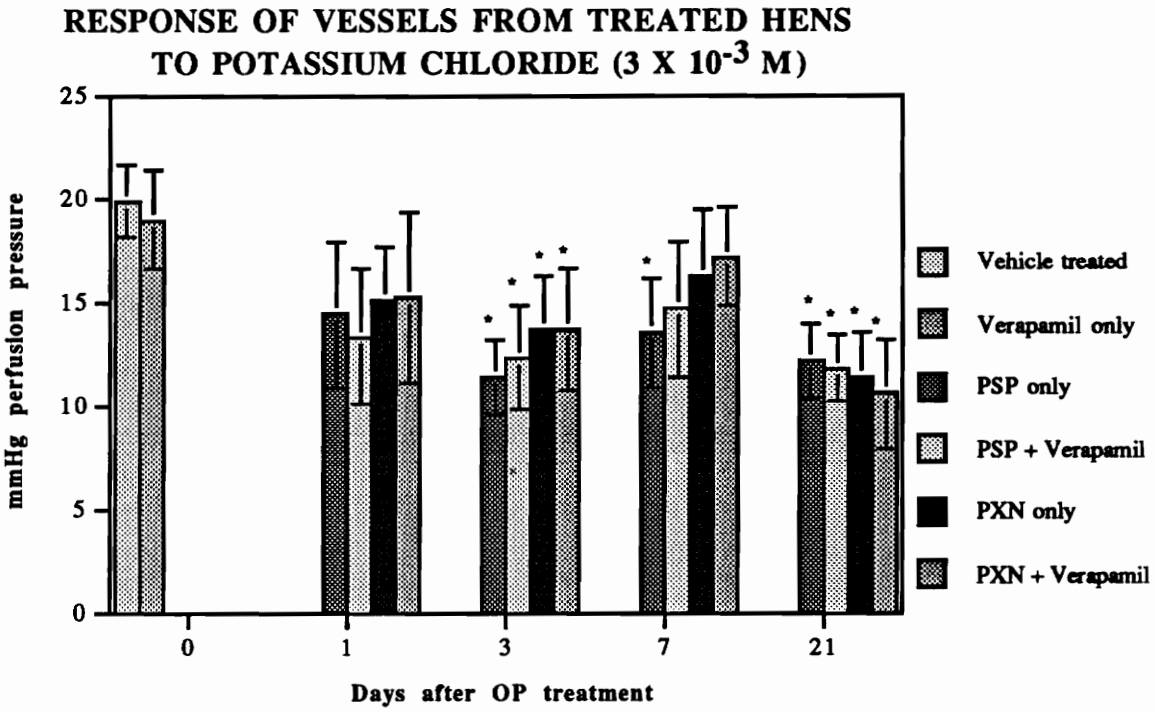
A


SERUM EPINEPHRINE LEVELS
IN OP TREATED HENS
% of control
300
250
200
150
100
50
0
-50
-100
-150
0
1
3
7
21
Days after OP administration
PSP only
PSP and verapamil
PXN only
PXN and verapamil

B


SERUM NOREPINEPHRINE LEVELS
IN OP TREATED HENS
% of control
300
250
200
150
100
50
0
-50
-100
-150
0
1
3
7
21
Days after OP administration
PSP only
PSP and verapamil
PXN only
PXN and verapamil

A


RESPONSE OF VESSELS FROM TREATED HENS
TO POTASSIUM CHLORIDE (3 X 10-3 M)
mmHg perfusion pressure
25
20
15
10
5
0
0
1
3
7
21
Days after OP treatment
Vehicle treated
Verapamil only
PSP only
PSP + Verapamil
PXN only
PXN + Verapamil

B

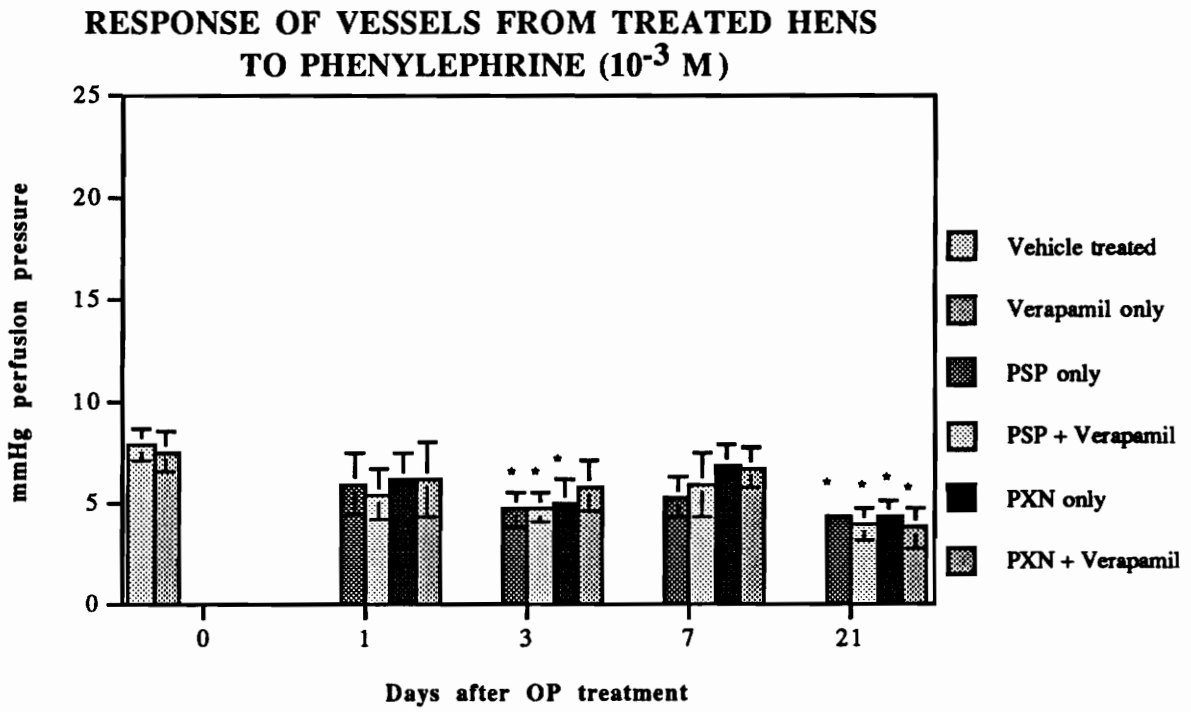

RESPONSE OF VESSELS FROM TREATED HENS
TO PHENYLEPHRINE (10-3 M)
mmHg perfusion pressure
25
20
15
10
5
0
0
1
3
7
21
Days after OP treatment
Vehicle treated
Verapamil only
PSP only
PSP + Verapamil
PXN only
PXN + Verapamil



Figure 2

# CHAPTER 8

# ADDITIONAL OBSERVATIONS

## ADDITIONAL OBSERVATIONS

### 8.1 Effects of nerve crush on limb vascular resistance *in vivo* and *ex vivo*

One group of hens in this study were subjected to unilateral nerve crush, with and without verapamil, as a method of inducing nerve damage without the use of OPs. Hens in this group displayed no alteration in either brain NTE or AChE at any time point following treatment. Walking behavior was maximally impaired immediately after recovery from the surgical procedure. Hens subjected to unilateral nerve crush were unable to walk and moved very little during the 21 day observation period and were given a clinical score of 4. Verapamil treatment did not affect the clinical scores.

Assessment of cardiovascular parameters indicated an increase in blood flow and a decrease in vascular resistance 1 and 3 days after treatment. Seven and 21 days after nerve crush, blood flow was not different from that of vehicle-treated hens. Heart rate was slightly elevated 1 day after treatment and blood pressure was slightly reduced but not significantly so. On days 3, 7, and 21, heart rate and blood pressure were similar to or slightly below that of vehicle treated hens. The response was not different in hens given verapamil prior to nerve crush. These observations are consistent with those of other investigators (Hudlicka, 1973).

The response of the hind limb vasculature to vasoactive agents was different from that of OP treated hens. Little response was noted to administration of ACh, PE, or SAL 1 and 3 days after treatment, however, the response to ACh and PE was significantly enhanced 7 days after treatment. The response to ACh and PE produced a dose dependent increase in vascular resistance on day 7 and a slight increase on day 21 only at the higher concentrations of these agents. The response of the hind limb to SAL was slightly less than that of vehicle-treated hens 7 and 21 days after treatment.

The response of isolated vessels from hens with unilateral nerve crush to vasoactive agents was also different from that of OP treated hens. There was a significant reduction in the response of these vessels to KCl 7 and 21 days after treatment. Furthermore, hens given verapamil displayed a significant reduction in response on day 1 as well. The response of vessels from hens with nerve crush was also different for PE. A reduction in the response to PE was noted 1 and 21 days after treatment. The response of vessels from hens treated with verapamil as well as nerve crush was reduced on days 7 and 21 but not on day 1.

# PART V

# DISCUSSION AND CONCLUSIONS

# CHAPTER 9

# DISCUSSION

## DISCUSSION

The results obtained from these studies present new information on the relationship of the vasculature to OP intoxication and OPIDN in the following ways: 1) the studies demonstrated that the response of vessels to vasoactive agents is altered both *in vivo* and *ex vivo* prior to and after the manifestation of clinical signs and pathological lesions associated with OPIDN. 2) the level of circulating catecholamines, which can produce vascular responses, was differentially altered in hens treated with neuropathy-inducing OP, PSP, and the nonneuropathy-inducing OP, PXN. 3) treatment with the calcium channel antagonist, verapamil, reduced the effects of OP administration on *in vivo* but not on *ex vivo* vascular response to vasoactive agents. 4) Verapamil treatment reduced the effect of OPs on the levels of circulating catecholamines. 5) There were no morphological alterations of the vasculature of the hind limb associated with OPIDN.

Criteria for the development of OPIDN include an early and significant inhibition of brain NTE, with onset assured if inhibition and binding is at least 70% (Johnson and Lotti, 1980; Ehrich *et al*, 1993). The onset of clinical signs after a single exposure to a neurotoxic OP follows a 7 to 21 day latent period. In these studies, brain NTE of hens given PSP was reduced sufficiently to produce locomotor deficits which were observed as early as day 7 and progressed throughout the studies in a manner similar to other

studies in which PSP was used at the same dosage (McCain *et al*, 1993; El-Fawal *et al*, 1989, 1990; Jortner and Ehrich, 1987). The degree of NTE activity inhibition by PSP was unaffected by treatment with verapamil in this and previous studies (El-Fawal *et al*, 1989), an indication that the reduction in activity of this enzyme is probably not calcium dependent. Verapamil did, however, attenuate other effects that followed PSP administration, indicating that other processes, such as vascular response to OPs seen in these studies, are likely to be calcium dependent. NTE inhibition, then, may act as a marker for OPIDN induction but is probably not associated with the alterations in vascular response or catecholamine release.

The activity of NTE was not significantly inhibited in hens treated with PXN and clinical signs of OPIDN were not noted in these animals. This data is consistent with that of other investigators using this compound (McCain et al, 1993a; 1993b; DeNeef et al, 1982).

The development of OPIDN is triggered by an event (or events) which occurs early after the administration of a neuropathic OP. This event occurs prior to the development of pathological lesions or the onset of clinical signs of neuropathy. Neuropathologic lesions have been observed in the biventer nerve of hens as early as 4 days after treatment with PSP (El Fawal *et al*, 1989). Alterations observed in hens 1 and 3 days after PSP are, therefore, associated with the neurophysiologic phase of OPIDN. During this phase, observed effects are probably due to the action of the OP on physiologic systems. More emphasis was placed on the examination of events which

occurred during this phase as it is likely that they may contribute to the onset of the neuropathic condition. Events which occurred at 7 and 21 days after PSP administration were most likely responsive changes due to the neuropathic condition. Seven and 21 days after PSP treatment there is no longer a significant level of OP remaining in the hens (Davis and Richardson, 1985) and the protective effects of verapamil are also reduced (Gilman *et al*, 1991).

PSP treatment altered several general cardiovascular parameters during the neurophysiologic phase (days 1 and 3) and was significantly different from the responses observed after treatment with PXN during this time period. The elevation in blood pressure and heart rate 1 day after PSP treatment may have been caused by the increase in circulating epinephrine and norepinephrine observed at this time. No increase in heart rate or blood pressure was observed in PXN-treated hens and the levels of circulating catecholamine were reduced. Heart rate and blood pressure, which were near control levels three days after PSP treatment, may have been reduced by a down-regulation of adrenergic receptors. It has been demonstrated that such a down-regulation occurs in response to sustained elevations of catecholamine levels similar to those observed in this study (Guyton, 1991). Verapamil treatment prevented the increase in heart rate and blood pressure observed in hens given PSP. This decrease may have been due to the direct effect of verapamil on cardiac and vascular calcium channels which

would increase blood flow and decrease heart rate (Fleckenstein, 1985).

The increase in hind limb vascular resistance and associated decrease in hind limb blood flow observed 1 day after PSP treatment is supported by a previous study in which blood flow was measured with a doppler flowmeter and resistance was calculated (McCain *et al*, 1993). The increase in hind limb vascular resistance and decrease in hind limb blood flow of hens treated with PSP could contribute to the neuropathic condition through: (1) decreased oxygen supply (Lundborg, 1970); (2) increased cellular metabolites (Bjornberg *et al*, 1989); and (3) decreased detoxification (Maxwell *et al*, 1987). PXN-treated hens, which did not display a decrease in blood flow during the same time period, would not be subjected to these phenomena and may contribute to the lack of neuropathic effects associated with this compound. The PSP-induced effects on hind limb blood flow and hind limb vascular resistance were modified in hens given verapamil as well as PSP. This provides a potential mechanism whereby this calcium channel blocker could serve to ameliorate clinical and neuropathological effects of PSP that were noted in previous studies (El-Fawal *et al*, 1989).

The response of the vasculature in PSP-treated hens to cholinergic and adrenergic agonists was greatly altered during the neurophysiologic phase. The hypertensive response to PE observed in PSP treated hens may be a result of an increase in the level of circulating catecholamines. This may explain why the threshold

response to PE occurred at two orders of magnitude lower than that of control hens. The response to SAL, however, cannot be explained so easily. There is some evidence that β-adrenergic receptors can convert to α-adrenergic receptors which may explain the hypertensive response to SAL observed 1 and 3 days after PSP treatment. Other evidence indicates that $\beta_2$ receptors may respond to humoral stimuli (circulating epinephrine and norepinephrine) while $\beta_1$ receptors respond only to neuronal stimuli (Bryan et al, 1981). Another possible mechanism for this effect could be an increase in intracellular phosphorylation following treatment with a neuropathic OP. An increase in the phosphorylation of structural proteins of the brain and spinal cord has been demonstrated after administration of TOCP (Abou Donia *et al*, 1988; Patton *et al*, 1985). This increase was detectable in the 50, 60, and 80 kd bands at 24 hours after TOCP administration, reached a maximal level 7 days after treatment, was sustained until 21 days after treatment, then activity declined. Furthermore, phosphorylation in the 80 kd band was maintained by the addition of calcium and calmodulin. If an increase in intracellular phosphorylation occurs in vascular smooth muscle as it does in nerve, either through direct phosphorylation by OPs or through the increase in intracellular $Ca^{++}$ which mediates phosphorylation, this could be a potential mechanism for the alteration of responses to adrenergic and cholinergic agonists.

The response of the vasculature to cholinergic and adrenergic agonists was modified in hens given PSP and verapamil, a calcium

channel blocker. Therefore the response of the vasculature could be caused by an increase in intracellular calcium levels of vascular smooth muscle. Previous work done in our laboratory demonstrated that verapamil, a phenylalkamine calcium channel antagonist, could ameliorate the neuropathic condition associated with OPs (El-Fawal *et al*, 1989). The calcium-calmodulin second messenger system is the major pathway for neurogenic and humoral contraction in the vasculature (Mulvaney and Aalkjaer, 1990). This system is activated through $\alpha_1$ adrenergic receptors, with contraction directly related to the amount of intracellular calcium (Kamm and Stull, 1985). If the level of intracellular calcium was increased in vascular smooth muscle by PSP treatment, it is possible to induce contraction without activation of a receptor. The decrease in hind limb blood flow and increase in hind limb vascular resistance seen in PSP treated hens in this study would follow, and, as noted, these effects would not occur in hens given PSP and verapamil. The study clearly indicates that verapamil had an effect on the hind limb vasculature that modified the effect of PSP.

The modification of vascular effects in hens given PSP and verapamil may be due to a number of factors. Verapamil not only prevents calcium entry into the cell through voltage-operated channels, but it can also block receptor-operated channels (Katz *et al*, 1985), fast sodium channels (Opie, 1984), $\alpha_1$ receptors (Motulsky, *et al*, 1983) and $\alpha_2$ receptors (Opie, 1984). This blockade of channels and receptors would have an inhibitory effect on vasoconstriction, an

effect observed in this study as PSP-induced decreases in hind limb blood flow and increases in hind limb vascular resistance were modified in hens given PSP and verapamil. Although verapamil can block calcium entry, it has little or no effect on the efflux of calcium from the cell and may reduce intracellular calcium levels in hens treated with neuropathy inducing OPs. This action of verapamil may be responsible for the reduced response to vasoactive agents by the vasculature of the hind limb of the hen.

Blockade of calcium channels by verapamil may also have an indirect effect on the vasculature through the inhibition of neurotransmitter release by vasomotor fibers. In nerve cells, calcium facilitates the binding of vesicles to the presynaptic membrane and the subsequent release of vesicular contents into the synaptic cleft (Llinas, 1982). Furthermore, there is a direct relationship between intracellular levels and the amount of transmitter released (Katz and Miledi, 1967). Verapamil, by reducing the calcium entry into the nerve terminal, reduces the amount of transmitter release and post-synaptic potential. It may be possible, therefore, that the increased limb blood flow observed in hens treated with verapamil and hens treated with OP and verapamil may have been due, in part, to a decrease in transmitter release from vasomotor fibers. This reduction in post ganglionic transmission may also be responsible for the decrease in the levels of circulation catecholamines observed in hens treated with PSP and verapamil.

The levels of circulating catecholamines were elevated by treatment with PSP during the neurophysiologic phase and were sustained throughout the 21 day study period while the levels of circulating catecholamines were decreased in PXN-treated hens. The ability of PSP to increase the levels of circulating catecholamines is supported by *ex vivo* work using bovine chromaffin cells which displayed an increase in the level of catecholamine secretion following administration of the type I OPIDN producing compound, DFP (Knoth-Anderson and Abou Donia, 1993). The enhancement of catecholamine secretion by chromaffin cells and the resulting increase in the levels of circulating catecholamines could have a profound effect on the response of the vasculature. There is evidence that a down-regulation of receptors occurs in response to sustained elevations of catecholamines (Guyton, 1991) which may reduce the response of vessels, both *in vivo* and *ex vivo*, to administration of these agents. Vascular adrenergic receptors in hens treated with PXN may have either been up-regulated or been unaltered by the decrease in catecholamine levels. The differential effect on circulating catecholamine levels between hens treated with PSP only and hens treated with PSP and verapamil indicates a possible alteration in the calcium metabolism of chromaffin cells exists in hens treated with PSP.

The percent of increase in the levels of epinephrine was greater than the percent increase in the levels of norepinephrine in hens treated with PSP. This difference could produce different

effects in the response of the cardiovascular system. Epinephrine has a greater effect on cardiac stimulation through its action on the $\beta_1$ cardiac receptors than does norepinephrine. Epinephrine also causes only a weak constriction of the vessels in skeletal muscles when compared to the constriction caused by norepinephrine. Epinephrine also has a greater effect on the metabolic rate of the body than does norepinephrine. Indeed, the metabolic rate of the body can be increased by as much as 100% above normal (Guyton, 1991). The increase in epinepherine levels and associated increase in $\beta_1$ stimulation and metabolic increase may have been responsible for the increased heart rate and core temperature observed in rats 3 and 7 days after treatment with DFP (Gordon, 1993). Our investigation indicated the greatest increase in catecholamine levels during this time period.

Morphological alterations of the peripheral vasculature or its innervation did not occur in hens that developed OPIDN after treatment with PSP. Skeletal muscle fiber diameter did, however, decrease in hens with OPIDN. Measurements of skeletal muscle fiber diameter observed in this study was consistent with that obtained by Cisson and Wilson (1982) in which a reduction in fiber diameter was observed 20 days after exposure to tri-ortho-cresyl phosphate (TOCP). In this study, it was demonstrated that the reduction of fiber diameter was attenuated by treatment with verapamil. The attenuation could, however, be a result either of a less severe neuropathy or of a greater ability to obtain water, as hens given PSP

only were more ataxic and could have been somewhat dehydrated compared to hens given PSP and verapamil. Dehydration has been associated with a decrease in skeletal muscle fiber diameter and an increase in specific electrical resistance of lean body mass (De-Boer *et al*, 1992).

The fiber to vessel ratio and capillary tortuosity pattern was not altered by treatment with OPs. Changes in vascular architecture may not become evident early after the neuropathy manifests itself clinically. Since hens were sacrificed only two weeks after the first clinical signs were evident, these long term effects may not have been evident.

No alterations of autonomic nerve fibers were evident in hens treated with OPs. This information is consistent with data presented by Cavanaugh (1961) in which he examined ganglia of TOCP treated hens and found no pathological lesions, an indication that autonomic nerve damage did not occur in this type of toxicity.

There are still unanswered questions about the role of the cardiovascular system in OPIDN. The difference in effects between PSP and PXN on catecholamine levels engenders as many questions as does the lack of difference in effects of these two compounds on isolated vascular response to vasoactive agents. Several suggestions, however, have been made which will further elucidate the role of the vasculature in OPIDN and include an examination of endothelial response to OPs, the response of adrenalectomized hens to OPs, and the response of the isolated perfused limb from OP treated hens to

vasoactive agents. These suggestions will be taken into consideration prior to planning the next series of investigations.

# CHAPTER 10

# CONCLUSIONS

## CONCLUSIONS

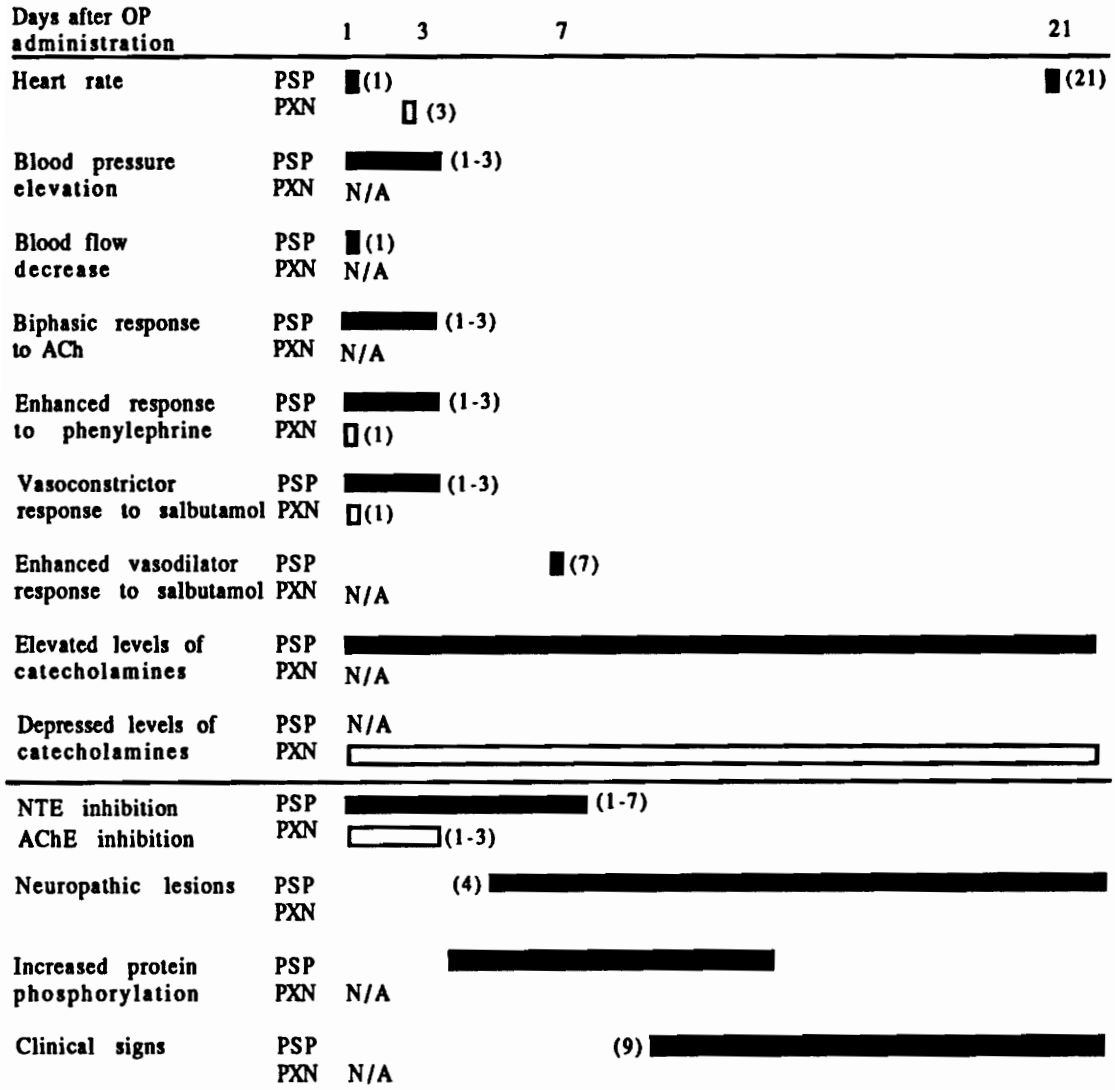
The data presented above indicate that treatment with OPs alters vascular response as indicated by changes in perfusion pressure and by changes in response to cholinergic and adrenergic agonists. When compared to vehicle-treated hens: (1) the most prominent changes *in vivo* were measured 1 and 3 days after OP treatment; (2) response to ACh was considerably more notable in PSP treated hens; (3) the response to phenylephrine was increased in hens given both compounds but lasted for 3 days in PSP treated hens; (4) the response to salbutamol in PSP treated hens was more notable than response to this ß adrenergic agonist in PXN treated hens; (5) Treatment with verapamil reduced the effects of PSP and PXN treatment on vascular response to cholinergic and adrenergic agents, (6) The response of large diameter vessels *ex vivo* were altered 3 and 21 days after treatment with either PSP or PXN. (7) Verapamil treatment did not alter the response of vessels to vasoactive agents. (8) PSP treatment increased and PXN treatment decreased the levels of circulating catecholamines. (9) Verapamil treatment reduced the effect of OPs on circulating catecholamine levels by approximately 50 percent.

The data obtained from these studies supports some portions of the hypothesis while other portions are either not supported or are still in question. For instance, the hypothesis stated that OPs that cause OPIDN would cause alterations within the vascular system. Alterations in the function of vessels *in vivo* were, indeed, observed

in hens treated with PSP and these changes were different from those of hens treated with PXN. The hypothesis suggested that cardiovascular alterations could be due to structural changes, but structural alterations of the vessels or their innervation were not observed in OP-treated hens. Although the hypothesis suggested that OPIDN producing compounds may cause direct functional changes to blood vessels, *ex vivo* studies with isolated vessels did not distinguish between an OP causing OPIDN and an OP that did not. The increase in the levels of circulating catecholamines in PSP treated hens and their decrease in PXN treated hens was a serendipitous observation which may provide a potential mechanism for the observed cardiovascular effects. It can not be determined if this alone was responsible for the effects, however, the calcium channel blocker, verapamil, which ameliorated clinical signs of OPIDN and reduced the extent of neuropathic lesions in this and previous studies, attenuated both general cardiovascular responses associated with PSP treatment and responses of vessels to vasoactive agents. This supported the hypothesis, which suggested that the beneficial effects of verapamil in OPIDN could be related to its effects on the cardiovascular system. Verapamil treatment also attenuated the effects of OPs on alterations of plasma catecholamine levels. These observations indicates that an alteration in calcium homeostasis may be responsible to some degree for the initiation and development of OPIDN.

It is obvious that more work needs to be done in order to precisely determine the role of the vasculature in OPIDN. Such work could include in depth pharmacological studies with both perfused limb and isolated vascular preparations to precisely determine the relationship of receptors to vascular function. Other studies may include the measurment of intracellular calcium levels in blood vessels and ligand binding to determine if changes observed in these studies are due to ionic alterations within the cell or changes in receptor type or density. Studies with other OPs, including DEF (buitifos, S,-S,-S,-tri-n-butylphosphoro-thrithioate), an organophosphate cotton defoliant, which produces severe vascular effects that sometimes result in gangrene could be done to further elucidate the role of cardiovascular effects in OPIDN (B. Wilson, personal communication). Elucidation of the relationship between the vasculature and OPIDN could also include investigations with PMSF (phenyl-N-methyl-N-benzylcarbamate), a compound that is capable of significantly reducing NTE without producing a neuropathy. This may provide some answers to the role of NTE in vascular response (M.K. Johnson, personal communication).

## CARDIOVASCULAR RESPONSES TO OP TREATMENT




The time sequence for cardiovascular events in hens treated with the neuropathic OP, cyclic phenyl saligenin phosphate (PSP), are compared to those same parameters in hens treated with the non-neuropathy inducing OP, paraoxon (PXN). The cardiovascular changes seen in hens treated with PSP are significantly different from those observed in vehicle treated hens or those treated with PXN. The time of other observed phenomena associated with OPIDN are displayed at the bottom of the table. The observed cardiovascular events preceed both the development of neuropathic lesions and the onset of clinical signs.

# PART VI

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# VITA

Wilfred Carl McCain was born in Stuttgart, Germany on September 2, 1950 and moved to the United States with his parents in 1954, eventually settling in New Bern, North Carolina. He graduated from New Bern High School in 1968 and entered the Army. After serving tours in Europe and Viet Nam, he was honorably discharged in 1971. He earned an Associate degree in general studies from New River Community College in 1978 and a Bachelor of Science degree from Radford University in 1980. He subsequently taught biology at New River Community College, managed the quality control program at Dominion Laboratories, and, in 1985, was hired as a research technician at the VA-MD Regional College of Veterinary Medicine. Wilfred started graduate studies under the supervision of Dr. Marion Ehrich in 1986 while maintaining his position as a technician in the physiology research laboratory. He earned his Masters degree in Veterinary Medical Science in 1991, and entered the Doctoral program as a full-time student at that time.

Wilfred married Carolyn Cox in 1976. They have two daughters, Kathy, born in 1982 and Jennifer, born in 1985.