CARDIOVASCULAR COMPONENTS OF ORGANOPHOSPHORUS-INDUCED DELAYED NEUROPATHY

by

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Toxicology

(ABSTRACT)

The focus of this study was to assess the cardiovascular effects in hens of a single 2.5 mg/kg intramuscular injection of phenyl saligenin phosphate (PSP) into the breast muscle. Parameters were measured at 1, 3, 7, and 20 days post treatment. All hens developed clinical signs of delayed neuropathy by day 10 and these signs were maximal by day 20. Alterations of measured parameters were observed prior to the onset of clinical signs of organophosphorus-induced delayed neuropathy (OPIDN) (days 1, 3, and 7) as well as when maximal clinical signs were evident (days 15-21). Significant decreases in the activities of brain NTE and plasma cholinesterase as well as decreases in weight and the level of pCO2 and an increase in peripheral resistance were observed prior to evidence of clinical signs of OPIDN. When maximal signs of OPIDN were present, brain NTE and plasma cholinesterase were at control levels but brain cholinesterase was significantly increased. Significant decreases in body weight and arterial pCO2 and significant increases in limb venous flow, arterial blood pressure, and hematocrit were seen at this time.

I dedicate this thesis 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.

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ABBREVIATIONS

ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
ACTCh	-	Acetylthiocholine
ANOVA	-	Analysis of variance
BuTCh	-	Butyrylthiocholine
CPK	-	Creatinine phosphokinase
DMSO	-	Dimethyl sulfoxide
EDRF	-	Endothelium derived relaxing factor
NTE	-	Neurotoxic esterase
OP	-	Organophosphorus compound
OPIDN	-	Organophosphorus-induced delayed neuropathy
PSP	-	Phenyl saligenin phosphate
PV	-	Phenyl valerate
TOCP	-	Tri-o-cresyl phosphate

A. STATEMENT OF HYPOTHESIS AND JUSTIFICATION FOR THE STUDY:

The hypothesis tested is that organophosphorus induced delayed neuropathy (OPIDN) alters blood flow to the legs of the hen, which is the region most clinically altered in this type of toxicosis. Blood flow to skeletal muscles most severely affected by OPIDN has not been defined, although some cardiovascular parameters altered initially after the administration of cholinesterase-inhibiting organophosphorus esters (OPs), such as blood pressure and heart rate, have. Information on possible changes of cardiovascular parameters after administration of neuropathy-inducing OPs is important because such changes could contribute to the toxicosis. If it is found that the cardiovascular changes do contribute to OPIDN, modification of the cardiovascular parameters could be used exacerbate or ameliorate OPIDN.

Organophosphorus esters (OPs) have several properties that could contribute to changes in blood flow to the legs of hens. Many OPs elicit a pressor response when administered to animals, an effect abolished by spinal transection. This indicates that OPs alter a central component in the regulation of blood pressure. It has been suggested that the inhibition of cholinesterase in the hypothalamus and medulla resulting from OP administration causes an increase in sympathetic outflow resulting in peripheral vasoconstriction. This could contribute to decreases in blood flow to skeletal muscle when muscular activity is increased because cholinesterase-

inhibiting OPs have inhibited the degradation of the neurotransmitter, acetylcholine, at the motor end plate. It seems reasonable that damage could occur, then, when there is a high degree of metabolic activity in the skeletal muscle and a decrease in blood flow due to a decrease in the caliber of resistance vessels. A reduction in the flow of blood during a period of high oxygen demand could result in ischemic tissue damage. With high doses of some OPs, skeletal muscle damage has been demonstrated (Kibler, 1972; Linkheart and Wilson, 1978; Cisson and Wilson, 1982; Patterson et al, 1988). Although previous studies have demonstrated these effects with cholinesterase-inhibiting OPs, none have examined the possible contribution of this effect to OPIDN. The effects of a neuropathy-inducing OP on blood flow to the limbs and the relationship of blood flow to cholinesterase inhibition were included in the present study.

A change in blood flow caused by administration of OPs could contribute to the nerve damage characteristic of OPIDN. There is a high degree of activity within the nerves following cholinesterase inhibition. The vaso nervorum, which supplies nutrients to the nerve, could be compromised by an increase in vasomotor tone produced when OPs inhibit acetylcholinesterase. Distal portions of the nerve are not as richly supplied by blood vessels as are proximal portions and would be more severely affected by a reduction in flow. The sensitivity of distal portions of the nerves has been demonstrated by

histopathological studies of peripheral neural damage. These indicated that a Wallerian degeneration (distal to proximal axonopathy) occurs following exposure to OPIDN producing compounds (Drakontides and Baker, 1983; Jortner et al, 1988; Jortner and Ehrich, 1987).

If blood flow to the limbs of the hen is reduced by administration of neuropathy-inducing OPs, an accumulation of metabolic wastes could occur which would alter the environment of the muscle. Local tissue modulators such as CO2, adenosine, and prostaglandins would be released resulting in vasodilation and an increase in capillary permeability in response to metabolic accumulation. These changes could also contribute to the neuropathy and myopathy associated with OPIDN.

Cardiovascular effects of acute OP exposure have been well documented (Brezenoff et al 1984, De Neef, et al 1982, Kristc 1978, Brezenoff 1972). However, these studies deal only with the cholinergic effects of OPs and not with the delayed neuropathic effects. Much less is known about the cardiovascular effects which occur during OPIDN. Information does exist on alterations of blood flow and cardiac output distribution following nerve transection, a process somewhat similar to the chemical denervation caused by exposure to some OP compounds (Hudlicka and Renkin, 1968). Although somewhat similar, they are not, however, identical. Nerve transection ablates the action of all fibers distal to the lesion. OPs

damage only a portion of fibers in a nerve and, in addition, are selective in that they have been reported to affect primarily large diameter myelinated fibers (Bouldin and Cavanagh, 1979; Jortner and Ehrich, 1987; El-Fawal et al, 1988). For those OPs that inhibit AChE as well as NTE, stimulation of central cholinergic vasomotor pathways following cholinesterase inhibition may also cause a reduction of blood flow to the vaso-nervorum resulting in ischemia and could augment the progression of neuropathy. There are, however, OPs that induce OPIDN that do not have a significant effect on AChE. The cardiovascular effects of these compounds have not been described.

This study, then, is the first in an attempt to gain insight into the cardiovascular components of OPIDN and to determine to what extent cardiovascular changes can influence the initiation and progression of the neuropathy and its associated myopathy.

B. LITERATURE REVIEW:

1. HISTORICAL PERSPECTIVE.

Although the toxic effects of organophosphorus (OP) compounds in humans were known before 1930, the accidental poisoning of more than 10,000 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 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 preignition and plug fouling in gasolines and enhance the viscosity index of lubricating oils (Salem and Olajos, 1988; Abou-Donia, 1989).

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). 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 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 to the OPs. Signs may persist for one to three days in humans. Among these signs are stimulation of 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 an increase of acetylcholine in the heart. This effect is sometimes masked by central cholinergic stimulation of cardiac centers and by 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 fasiculations. 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 (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 appears to trigger 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 obviously evident in laboratory rodents (Abou-Donia, 1981).

The accepted treatment of cholinergic poisoning, which is characteristic of acute OP toxicosis, is the administration of atropine. This compound acts as a competitor for postsynaptic muscarinic receptors. This decreases the capability acetylcholine to bind with the receptor and decreases the effect of excess acetylcholine that is present when the enzyme responsible for the degradation is inhibited by the OP. 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 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 pralidoxime (2-PAM, pyridine-2-aldoxine methyl chloride) 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 nucleophilic attack on the phosphorous 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 pretreatment 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 defined. 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 necrosis. 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.

3. CHEMISTRY AND BIOCHEMISTRY OF ORGANOPHOSPHORUS COMPOUNDS:

The 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 phosphorous 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).

$$\text{ENZYME-OH} + \text{R-P(=O)-O-R'} \;\underset{}{\overset{}{<-\;->}}\; \text{ENZYME-O(R-P(=O)-O-R')} \qquad k1$$

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. 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 by competitively, but reversibly, inhibiting NTE (Gall, 1981; Deyi et al, 1981).

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 Bunger (bands formed from the union of sheath cells during the regeneration of peripheral neurons). Ultrastructural examination revealed early signs to be 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 has 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).

5. PERIPHERAL INNERVATION AND NERVOUS CONTROL OF THE VASCULATURE:

It is the peripheral nerves that are most affected by OPIDN. These nerves are served by the vasculature and these nerves, themselves, play a role in vasomotor tone and blood flow, thus, in ways, affecting their own nutrient supply. The peripheral vasculature is controlled especially by the autonomic nervous system (ANS), primarily by 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 adventia of the vessel. Axons of these fibers come into contact with the external muscle layer of peripheral blood vessels and form a meshwork of unmyelinated processes approximately 1um in diameter with varicosities of 3um to 5um forming at intervals between 80um and 100um (Akester, 1971). These varicosities come into close contact with the smooth muscle at the adventital-medial boarder in arterioles (dia.=100um to 50um) and terminal arterioles (dia.=50um to

15um) and actually penetrate the media of veins and larger arteries (Fuxe and Sedvall, 1965). The meshwork is greater in resistance vessels (arterioles, metarterioles, terminal arterioles, precapillary sphincters, and, to some degree, postcapillary venules) than in capacitance vessels (the veins). Innervation of the larger muscle blood vessels (dia. > 100um) 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. < 100um) 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 are either adrenergic (Alquist, 1948) or cholinergic according to the type of transmitter released. The adrenergic fibers 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). 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 veins occurs at four to six hertz and flow is reduced by only 30% (Mellander, 1960). Stimulation of vasoconstrictor fibers not only increases resistance, small arterioles and precapillary sphincters may 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 at a frequency of 1hz and is maximal at 12hz. At frequencies above 15hz vasoconstriction occurs and masks the effect of vasodilation. A decrease in oxygen consumption and an increase in 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 was reported to be due to a reduced release of transmitter at the nerve terminals (Mauskopf et al, 1969; Amenta, 1988).

Isolated studies performed on vessels from skeletal muscle indicated that in the absence of sympathetic stimulation, low levels of acetylcholine (10^{-9} to 10^{-8}) caused relaxation of these vessels while higher concentrations (10^{-8} to 10^{-5}) caused 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).

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 (Kahyutin, 1966). These studies indicate that sensory stimulation composed of higher voltages and frequency and a longer pulse duration are 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 (Asmussen et al, 1965).

6. 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. Tonically active neurons in the medulla adjust their activity by the afferent input from peripheral cardiovascular receptors and 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 vasoconstrictor activity of the medullary pressor area is 1 to 2 hertz while vasodilation primarily occurs by inhibition of vasoconstrictor tone (Celander, 1954).

Neurons from both pressor and depressor centers have afferent and efferent connections in the hypothalamus and other supramedullary structures. With central inhibition of cholinesterase following proximal microinjections of echothiophate into the posterior hypothalamic nucleus (PHN), 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 also evoked a pressor response from the ventromedial hypothalmic nucleus and a depressor response from the dorsomedial and premammalary hypothalamic nuclei (Brezenoff, 1972).

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).

7. VASCULAR SUPPLY OF THE PERIPHERAL NERVES:

Nutrients and toxic agents are supplied to the peripheral nerves through the vasa nervorum. This network of blood vessels is formed from nutrient arteries 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 effects on 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).

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)

8. EFFECTS OF OPs ON CARDIOVASCULAR PARAMETERS:

Although investigations into the cardiovascular effects of exposure to OPs have been conducted (Maxwell et al, 1987; Vitterlein and Hasse, 1979; Vanhoutte, 1974; Batillard et al, 1990), the investigations conducted to date have examined the relatively short term effects of potent cholinesterase inhibitors. 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. No studies exist which follow the changes in cardiovascular parameters throughout the progression of OPIDN.

A. SPECIFIC OBJECTIVES:

The objective of this study was to describe the changes in blood flow to the hind limb of the hen which occured during the progression of OPIDN. Other cardiovascular parameters such as heart rate, blood pressure, P_{O2}, P_{CO2}, blood osmolarity and hematocrit were assessed as well. Particular emphasis was placed on changes which occurred early after exposure to a single dose of phenyl saligenin phosphate (PSP). This OP is known to produce the neuropathic condition without causing notable acute toxicosis as inhibition of neural acetylcholinesterase is not pronounced. This data on cardiovascular effects of PSP was correlated with the onset and progression of clinical signs and with inhibition of neurotoxic esterase (NTE) and other esterases. To meet the objective, hens were exposed to a neuropathy-inducing dose of PSP and the following parameters were examined:

1. Biochemical assay of NTE and cholinesterase.
2. Development of ataxia and effects of OPIDN on limb weight.
3. Blood pressure, heart rate, and hind limb blood flow.
4. PO_2, PCO_2, hematocrit and osmolarity.

B. INVESTIGATIVE PROTOCOL:

1. ANIMAL TREATMENT:

The experiment was conducted on 31 month old white leghorn hens obtained from the Department of Poultry Science at Virginia Polytechnical Institute and State University. Hens were placed in wire bottom cages (24 X 51 X 28 cm) with four birds per cage. They were housed in a temperature controlled building with a 12 hour day / night cycle. Water and commercial feed were administered ad libitum.

2. EXPERIMENTAL DESIGN

Birds in this study were weighed and randomly divided into two groups. One group was given phenyl saligenin phosphate (PSP, 2-phenoxy-4H-1-1-,3-2-benzodioxaphosphorin-2-oxide [Lark Enterprises, Webster, MA]) dissolved in dimethyl sulfoxide (DMSO) to yield a 5 mg/ml solution. The control group was given the vehicle, DMSO, only.

The dose of PSP given to the treated group was 2.5 mg/kg IM (5 mg/ml in DMSO) into the breast muscle. Control birds received an equivalent volume of intramuscular DMSO (0.5 ml/kg).

To gain information about the effects of PSP before as well as during OPIDN, data was collected during the first few days after exposure and when neuropathy was evident. Samples from at least 6 hens from each of the control and experimental groups were taken on days 1, 3, 7 and 20-24 after PSP administration. The last time point represents the pool of hens with notable clinical evidence of OPIDN. A total of 48

birds were used for this study. Several extra hens were dosed so that replacements were available for birds which died prior to the day they were to be examined for cardiovascular effects. A total of four birds needed to be replaced; two died the day after they were dosed and two died prior to day 20-24.

3. SAMPLE COLLECTION

Hens were brought to the laboratory on the day of examination, weighed, and restrained in lateral recumbancy with twine. A local anesthetic (0.5 to 0.7 cc of 2% lidocaine [Butler, Colombus, OH]) was injected intramuscularly at the wing root. Lidocaine was used as opposed to the general anesthetic, pentobarbital sodium, as cardiovascular effects are often seen with barbituates (Nightingale, 1977; Clement 1983; Cheney, 1984; Hartsfield and McGrath, 1986). The ulnar vein and brachial artery were cannulated with polyethylene catheters for measurement of cardiovascular parameters. A similar dose of lidocaine was given in the lateral portion of the hind limb at approximately the pelvic junction. Catheters were placed both prograde and retrograde in the sciatic vein and connected with a three-way stopcock. This allowed venous blood flow in the hind limb to be diverted into a graduated cylinder for a timed interval. Blood collected in this fashion was returned to the bird. A 2.5mm electromagnetic flow transducer was placed around the ischiadic artery and

grounded to the flesh of the upper thigh. The hen was then given 1000 IU of sodium heparin (iv) and allowed to equilibrate for 15 minutes. Measurements of heart rate, blood pressure, blood flow, and venous flow in the hind limb were taken 10, 20, and 30 minutes after the equilibration period.

Blood samples were collected 30 minutes after instrumentation was completed. These included a 2.0 cc heparinized sample for measurement of electrolytes, hematocrit, and osmolarity and a 2.0 cc sample for the assay of plasma cholinesterase activity was obtained. Venous blood was also measured for content of cholinesterase activity.

Just prior to euthanasia, the ischiadic artery distal to the flow transducer was occluded in order to verify a zero flow reading with the meter.

Hens were sacrificed with an intravenous overdose (2.5 to 3 ml) of sodium pentobarbital.

The brain was collected from all birds immediately after euthanasia and frozen at -70 degrees until time for assay of NTE.

After euthanasia, the contralateral hind limb, which had not been catheterized, was removed, skinned, rinsed in saline, blotted dry, and weighed. The gastrocnemius muscle of this leg was removed from all birds, rinsed in saline, blotted, and weighed.

Samples of nerve, muscle, and blood were also taken at this time and properly preserved for future investigations of

PSP effects on catecholamine levels and on nerve and muscle histology.

C. SPECIFIC PROCEDURES:

Neurotoxic esterase activity was determined spectrophometrically using the method of Sprague et al (1981), in which capability to hydrolyze phenylvalerate is assessed. Cholinesterase activity was assayed spectrophometrically as described by Ellman et al (1961) by measuring the yellow anion produced by hydrolysis of acetylthiocholine.

Clinical signs were assessed every other day following treatment according to the scale developed by Sprague et al (1980). Using this system, normal hens are given a score of 0. Hens with altered gait were given scores of 1, with scores of 2, hens had difficulty walking, a score of 3 was given when hens displayed moderate ataxia, and a score of 4 was assigned for severe ataxia and paralysis of the hind limbs. Hens received a score of 5 when wing droop, a sign of upper limb involvement, was observed.

Heart rate and blood pressure were measured with a Statham P-23-ID pressure transducer coupled to a Grass 7D polygraph.

Venous flow in the hind limb was determined by a timed collection of blood from the sciatic vein. Limb arterial blood flow was measured using a blood flow meter (Model BL-

613 [Biotronix Laboratory, Inc; Kensington, MD]) with a 2.5 mm flow transducer. Cabling for this instrument was restricted to 10 ohms of resistance.

Arterial and venous blood samples were measured on a BMS-3 MK II blood gas analyzer for PO_2 and PCO_2. Hematocrit was measured using microcapillary tubes centrifuged at 3000 rpm for three minutes. Blood osmolarity was measured with a microosmometer (Precision Systems, Inc.) based on freezing point determination.

D. STATISTICAL ANALYSIS:

Parametric analysis of data included the following: 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 (Fisher's LSD). Regression analysis was performed as necessary in order to determine changes in measured parameters over time (Zar, 1984).

Data was considered significantly different from controls if the probability was less than 0.05.

RESULTS

Neuropathy-inducing phenyl saligenin phosphate (PSP, 2.5 mg/kg im) caused statistically significant changes in several parameters determined in hens following its administration. These included decreased activities of plasma cholinesterase and NTE. Body weight and partial pressure of carbon dioxide in arterial blood were also decreased after PSP administration. Heart rate, mean blood pressure, limb vascular resistance, venous flow in the hind limb, and hematocrit were increased in hens given PSP when compared to control hens at one or more time points after PSP administration. Parameters were examined 1, 3, 7, and 20-24 days after PSP was given. The 20-24 day pool of hens represents the group in which clinical effects of OPIDN were maximal, as the hens were unable to walk without great difficulty.

Changes observed after PSP administration were compared to controls examined over the time course of the study. Analysis of variance indicated that no differences among parameters examined on different days occurred within the control group. Data from the control birds were, therefore, pooled and used to provide baseline values for each parameter.

The effect of exposure to PSP was most dramatic when examining esterase activities (Table 1). There were significant reductions in plasma cholinesterase and neurotoxic esterase activities ($p<.001$) 24 hours following administration. Brain cholinesterase activity was reduced at

this time, but not significantly (Figure 1). There was no statistical difference between arterial and venous cholinesterase activity, although arterial values were slightly lower on days one and three and higher on days seven and when maximal clinical effects of OPIDN were evident (hens examined 20-24 days after PSP)(Table 1). The activity of NTE remained statistically lower than control values ($p<.001$) when determined three and seven days after PSP, but was at near control level in birds sacrificed on days 20-24. Brain cholinesterase, although initially lower than control levels on days 1 and 3, was at normal levels by day seven and was statistically higher on days 20-24.(Figure 1)

Clinical signs of OPIDN were first observed on day eight in three of the 26 hens given PSP. Initial signs of OPIDN were not seen until day ten in others given this OP. An average clinical score of $2.4 \pm .1$ (mean $\pm$ SEM, n=6) on a scale of five was given to birds by day 12. All birds displayed the severe ataxia associated with OPIDN by the time they were used to provide data on blood flow (days 20-24). They average clinical score was $4.1 \pm .1$ (mean $\pm$ SEM) by this time. (Figure 2). Upper limb involvement was observed in 6 of 8 birds by day 18 and they were given a score of 5. Two of the hens with OPIDN died before the end of the observation period and were replaced.

TABLE 1

The effects on brain cholinesterase, brain neurotoxic esterase, plasma cholinesterase, body weight, and limb weight of hens given 2.5mg/kg (im) phenyl saligenin phosphate (PSP) compared with levels in control hens receiving vehicle (DMSO) only.[1,2]

Parameter Measured (units)	Days following PSP administration				
	Control	1	3	7	20-24
Brain Cholinesterase (uM Hydrolyzed/gm/min)	20.57 ±1.31	16.75 ±1.61	16.41 ±0.51	21.21 ±1.53	27.06* ±1.67
Brain NTE (nM Hydrolyzed/gm/min)	29.87 ±1.85	4.50** ±0.58	6.88** ±0.36	12.66** ±1.49	26.66 ±0.66
Plasma Cholinesterase (uM Hydrolyzed/gm/min)	5.01 ±0.23	0.67** ±0.27	0.69* ±0.28	4.88 ±0.57	4.72 ±0.40
Body Weight (grams)	1767 ±76	1650 ±57	1616 ±101	1516* ±54	1300** ±51
Limb Weight (grams)	59.04 ±1.24	56.10 ±1.87	55.31 ±4.02	55.03 ±3.13	54.38 ±2.14

[1] Control n=22; PSP treated n=6 per day.

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

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

** Significant at $p<0.005$ with Fisher's least significant difference following ANOVA.

Figure 1.

The effects of PSP treatment on esterase activity in hens. Data points are mean ± standard error of the mean. n=22 control; n=6 for each treatment day. (ACHE=arterial acetylcholinesterase, BRCHE=brain acetylcholinesterase, NTE=neurotoxic esterase, C=control, and P=PSP treated).

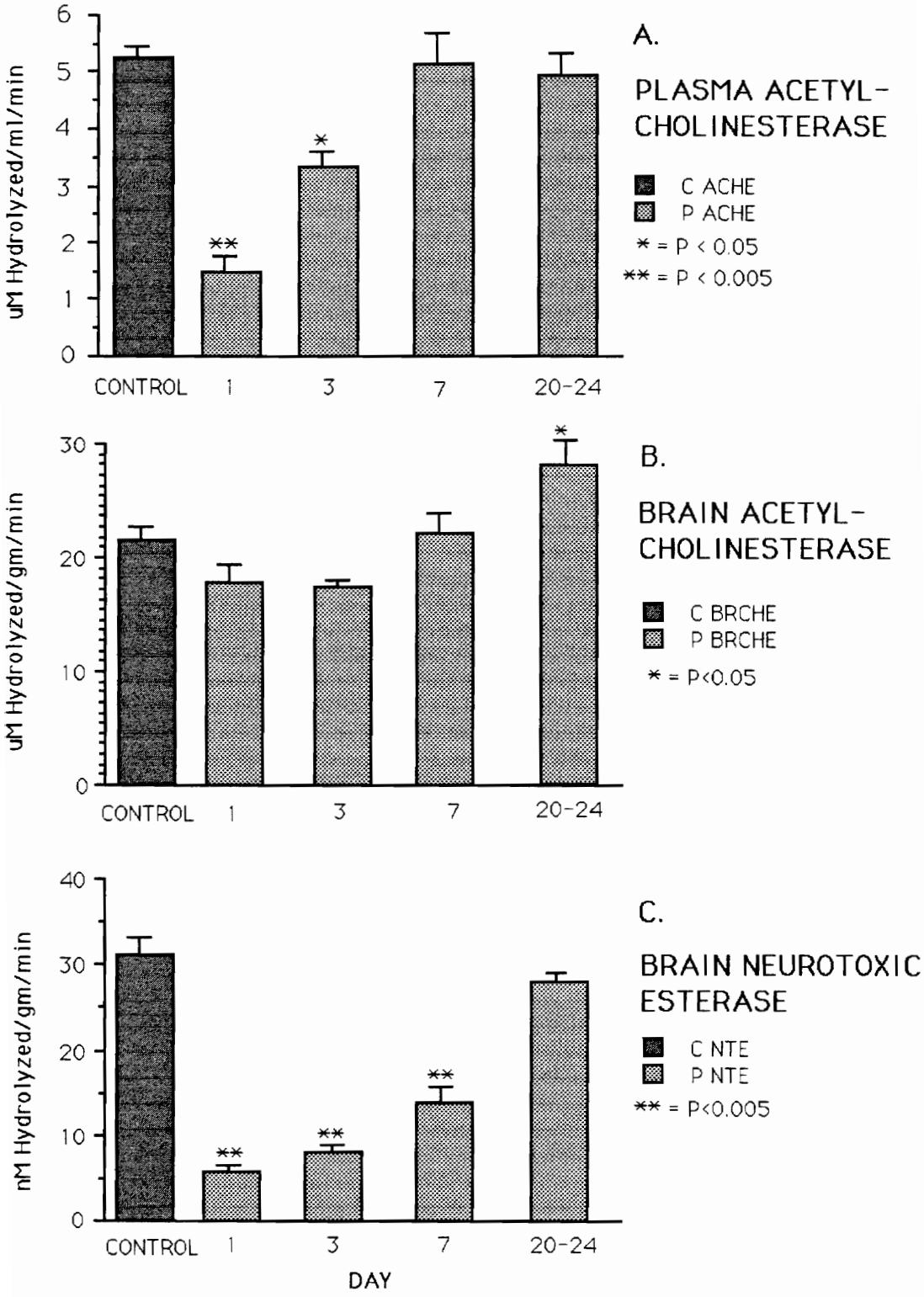
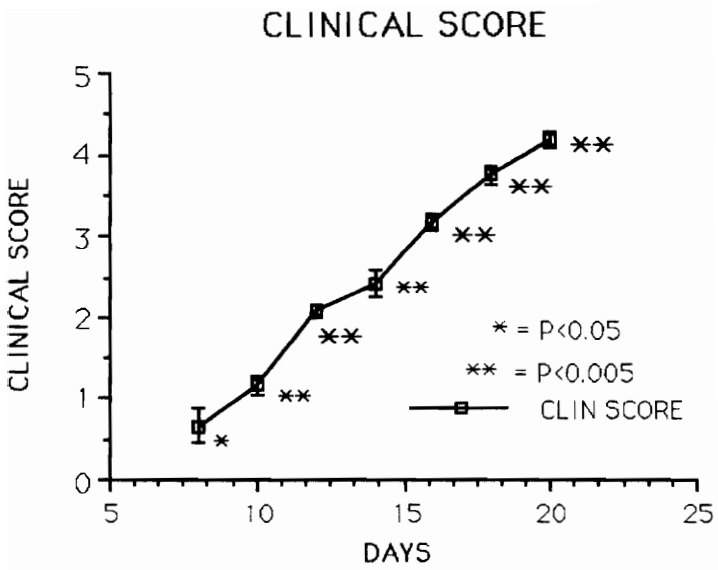

A.
PLASMA ACETYL-CHOLINESTERASE
C ACHE
P ACHE
* = P < 0.05
** = P < 0.005
uM Hydrolyzed/ml/min
0
1
2
3
4
5
6
CONTROL
1
3
7
20-24
B.
BRAIN ACETYL-CHOLINESTERASE
C BRCHE
P BRCHE
* = P<0.05
uM Hydrolyzed/gm/min
0
10
20
30
CONTROL
1
3
7
20-24
C.
BRAIN NEUROTOXIC ESTERASE
C NTE
P NTE
** = P<0.005
nM Hydrolyzed/gm/min
0
10
20
30
40
CONTROL
1
3
7
20-24
DAY

Figure 2.

Clinical scores of hens treated with PSP. Data points are mean ± standard error of the mean. (n=6). Clinical scores were significantly different in groups of hens given PSP compared to control hens from day 8 until the end of the study.


CLINICAL SCORE
CLINICAL SCORE
5
4
3
2
1
0
5
10
15
20
25
DAYS
* = P<0.05
** = P<0.005
CLIN SCORE

The loss of body weight associated with exposure to PSP averaged approximately 23 gm/day (Figure 3). This loss became statistically significant by days 7 ($p=.030$). The hind limb of hens given PSP displayed a logarithmic decrease in weight over the study period (Figure 4). The proportion of weight of the gastrocnemius muscle with respect to the hind limb remained at 13% in both treated and untreated hens.

Alterations in arterial blood flow to the hind limb after PSP administration were not determined to be statistically significant (Table 2). A 13% reduction in flow was observed one day following a single dose of PSP compared with a control value of 28.98 $\pm$ 2.13 ml/100gm/min (mean $\pm$ SEM, n=22). This was accompanied by a 10% increase in limb venous flow and a significant increase in vascular resistance ($p=.030$) (Table 2, Figure 5). Control levels for these parameters were 13.78 $\pm$ 1.44 ml/100gm/min (mean $\pm$ SEM, n=22)and 7.23 $\pm$ 1.27 PRU (mean $\pm$ SEM, n=22) respectively. Three days after PSP administration, arterial blood flow, limb venous flow, and limb resistance were all slightly elevated. The greatest reduction (18%) in arterial blood flow to the hind limb ($p=.132$) was seen at seven days. Limb vascular resistance was increased by 28% seven days after PSP, a statistically significant ($p=.034$) change from control values (Figure 6). Limb venous flow was not altered at this time. Arterial blood flow was increased by 14% when OPIDN was clinically evident ($p=.194$) and venous flow was nearly doubled ($p<.001$) at this

Figure 3.

Body weight in hens following PSP administration compared to weights of vehicle-treated hens. Data points are mean $\pm$ standard error of the mean. (n=22 control; n=6 per treatment day). (WT=weight; C=control; P=PSP treated).

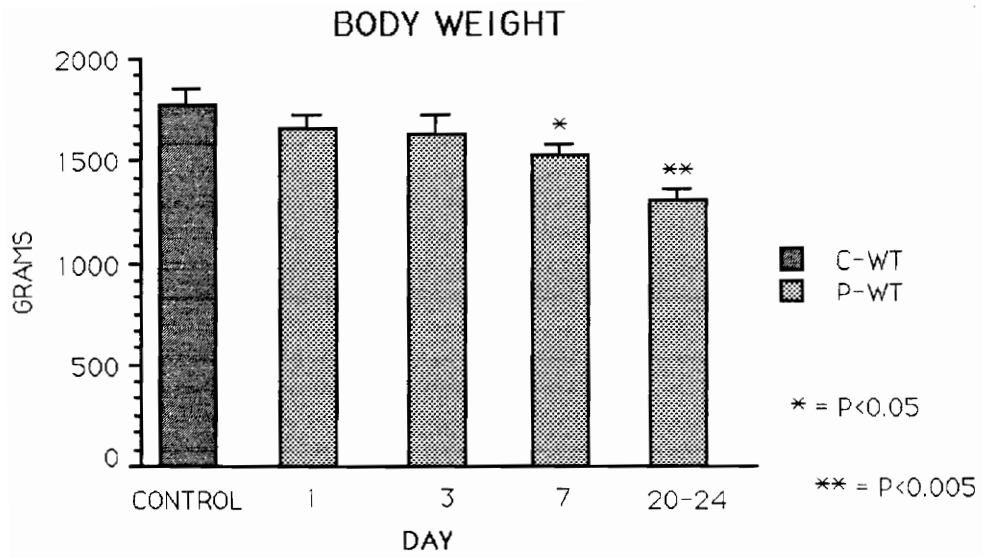

BODY WEIGHT
2000
1500
1000
500
0
GRAMS
*
**
CONTROL
1
3
7
20-24
DAY
C-WT
P-WT
* = P<0.05
** = P<0.005

Figure 4.

Limb weights of hens treated with PSP. Curve fitting indicated a logarithmic decrease in weight. Data points are mean ± standard error of the mean. (n=6 for each point).

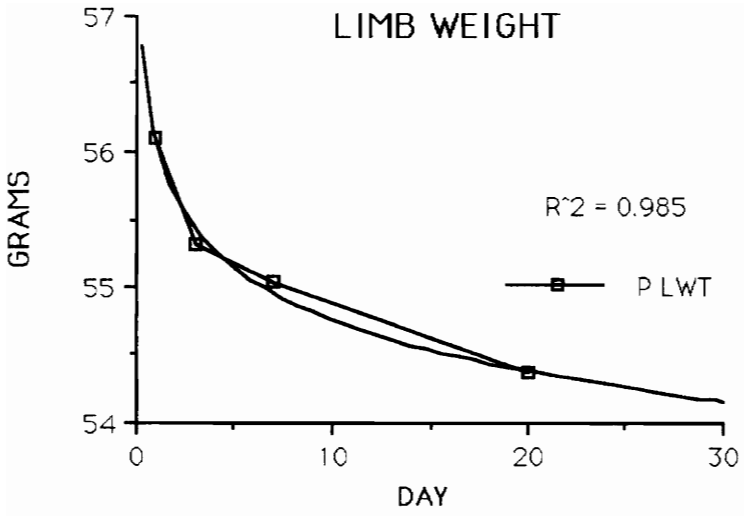

LIMB WEIGHT
57
56
55
54
GRAMS
R^2 = 0.985
P LWT
0
10
20
30
DAY

TABLE 2

The effects on blood pressure, heart rate, flow rates and resistance of hens given 2.5mg/kg (im) phenyl saligenin phosphate (PSP) compared with levels in control hens receiving vehicle (DMSO) only. [1,2]

Parameter Measured (units)	Days following PSP administration				
	Control	1	3	7	20-24
Arterial Blood Pressure (mmHg)	126 ±2.99	129 ±2.75	132 ±2.38	122 ±4.70	139* ±5.22
Heart Rate (Beats/min)	352 ±7.82	336 ±5.82	369 ±10.41	386* ±10.83	360 ±5.57
Limb Arterial Blood Flow (ml/100gm/min)	28.98 ±2.13	25.33 ±2.55	31.64 ±2.65	23.93 ±1.89	35.97 ±1.87
Limb Venous Flow (ml/100gm/min)	13.78 ±1.44	16.08 ±1.56	15.78 ±1.94	13.85 ±1.25	25.16** ±1.43
Limb Resistance (LRU)[3]	7.23 ±0.39	9.08* ±1.30	7.54 ±0.60	9.26* ±1.09	7.10 ±0.17

[1] Control n=22; PSP treated n=6 per day.
[2] Data is expressed as mean ± standard error of the mean.
[3] LRU = Arterial-venous pressure / Limb arterial blood flow (ml/min).
* Significant at $p<0.05$ with Fisher's least significant difference following ANOVA.
** Significant at $p<0.005$ with Fisher's least significant difference following ANOVA.

Figure 5.

Values for (a) arterial blood flow, (b) venous flow, and (c) vascular resistance of the hind limb of hens treated with PSP compared with control levels. Data points are mean ± standard error of the mean (n=22 control; n=6 each treatment day). (FLO=arterial blood flow, RET=limb venous flow, RES=vascular resistance, C=control, P=PSP treated).

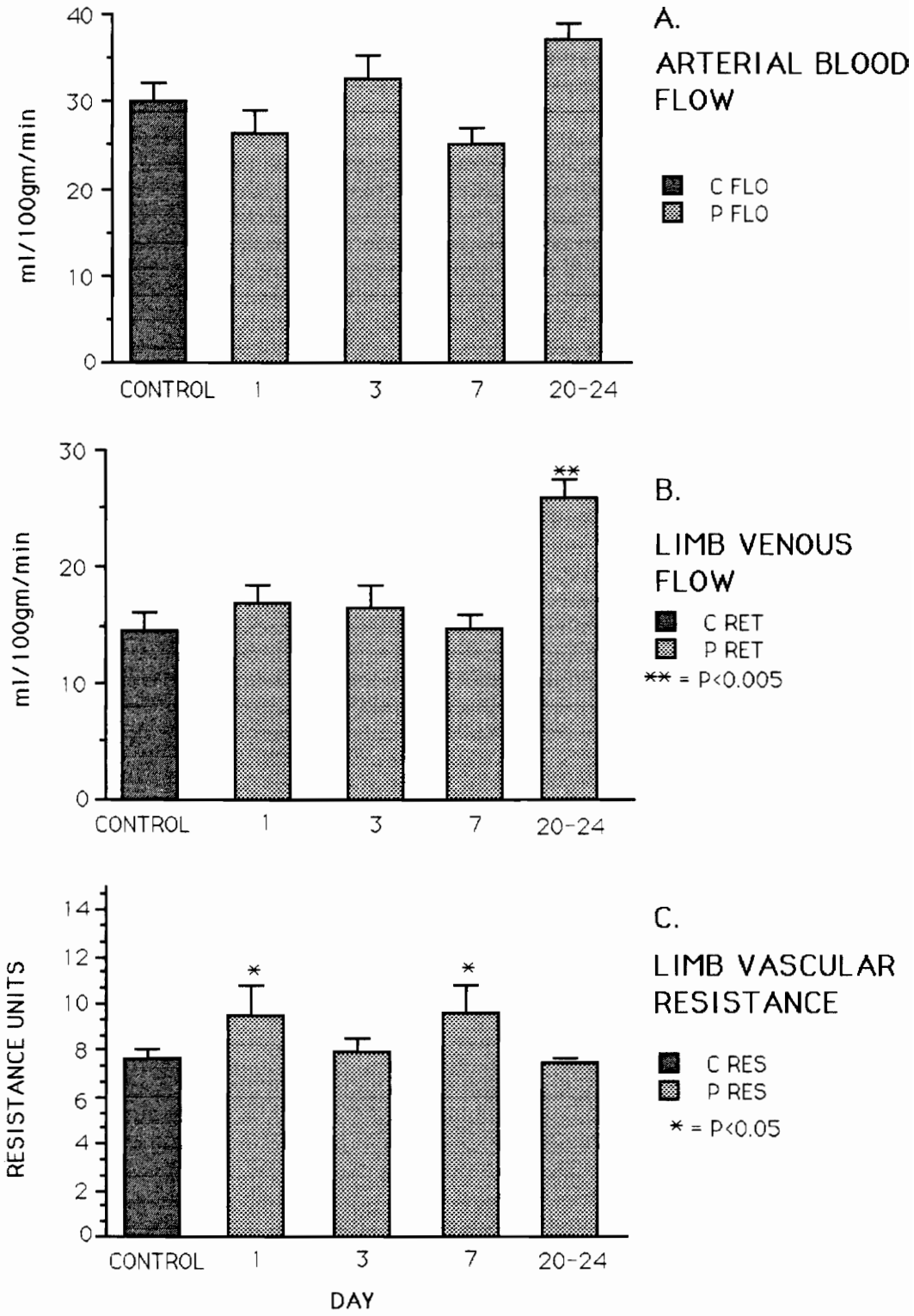
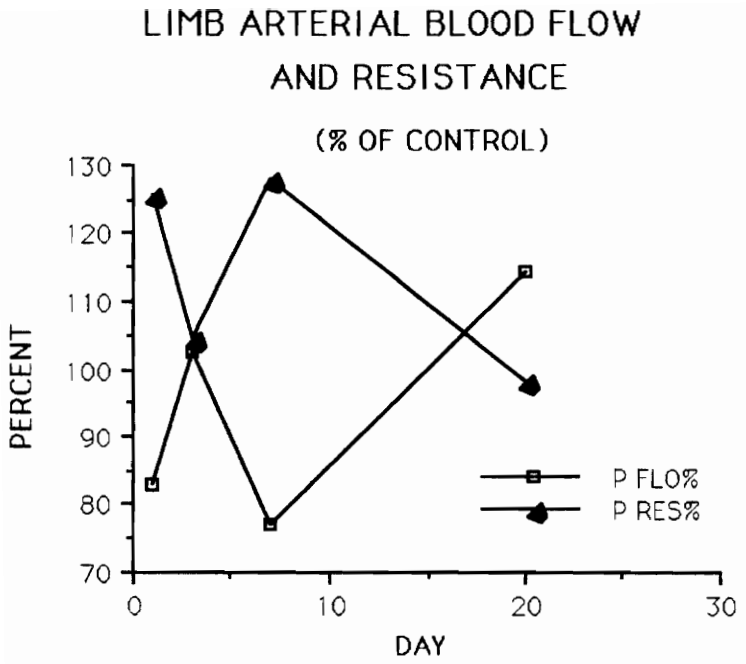

A.
ARTERIAL BLOOD FLOW
C FLO
P FLO
ml/100gm/min
40
30
20
10
0
CONTROL
1
3
7
20-24
B.
LIMB VENOUS FLOW
C RET
P RET
** = P<0.005
ml/100gm/min
30
20
10
0
**
CONTROL
1
3
7
20-24
C.
LIMB VASCULAR RESISTANCE
C RES
P RES
* = P<0.05
RESISTANCE UNITS
14
12
10
8
6
4
2
0
*
*
CONTROL
1
3
7
20-24
DAY

Figure 6.

Limb arterial blood flow and vascular resistance expressed as a percent of control levels in PSP treated hens. (FLO%=arterial blood flow, RES%=limb vascular resistance).


LIMB ARTERIAL BLOOD FLOW
AND RESISTANCE
(% OF CONTROL)
PERCENT
130
120
110
100
90
80
70
0
10
20
30
DAY
P FLO%
P RES%

time yet resistance remained at control levels (Table 2).

Blood pressure was altered when PSP-induced neuropathy was evident (Table 2, Figure 7). Until OPIDN was clinically evident, blood pressure varied within a few percent of baseline (Figure 6), but 20-24 days after PSP, diastolic and mean pressures were increased by 10%, a statistically significant ($p=0.043$ and $p=0.055$, respectively) rise (Table 2, Figure 7). Although heart rate was slightly decreased (5%) on the first day after PSP administration, rates were slightly elevated at other time points and significantly elevated on day 7 (Table 2, Figure 7). This significant increase in heart rate ($p=.039$) was a 9% rise over the baseline value of 352 ± 7.82 bpm (mean $\pm$ SEM, $n=22$).

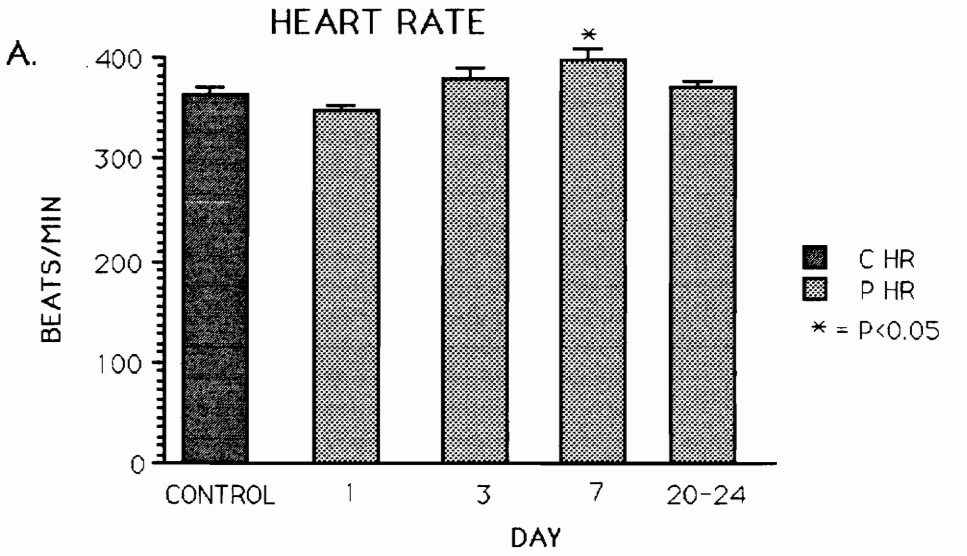
The effect of PSP administration in blood gases, respiratory rate, and hematocrit are given in table 3. The evaluation of dissolved gases in the blood indicated that the level of CO2 in treated hens was at least 9% lower than the 24.62 ± 0.71 mmHg (mean $\pm$ SEM, $n=22$) of control birds on all examination days (Figure 8). Statistically significant decreases were noted on day 3 ($p=.009$) and day 20-24 ($p=.001$). Respiration rate was reduced from 41 ± 1 (mean $\pm$ SEM, $n=22$) to 36 ± 2 (mean $\pm$ SEM, $n=6$) breaths per minute on day 3 and days 20-24. The content of dissolved oxygen in the blood never varied more than 10% during the first week following PSP

exposure. On day 20-24, PO2 was 12% higher than control values (p=.167) (Figure 8).

Hematocrit values remained near control levels of 27.9 $\pm$ 0.7 % during the first week after PSP administration (Table 3). A statistically significant increase (p=.028) was observed during OPIDN (days 20-24), when the observed value was 31.2 $\pm$ 1.2 %. Blood osmolarity was not altered during the study.

Figure 7.

(a) Heart rate and (b) blood pressure of hens following administration of PSP. Data points are mean ± standard error of the mean. (n=22 control; n=6 for each treatment day). (HR=heart rate, MP=mean arterial blood pressure, C=control, P=PSP treated).


A.
HEART RATE
BEATS/MIN
400
300
200
100
0
*
CONTROL
1
3
7
20-24
DAY
C HR
P HR
* = P<0.05

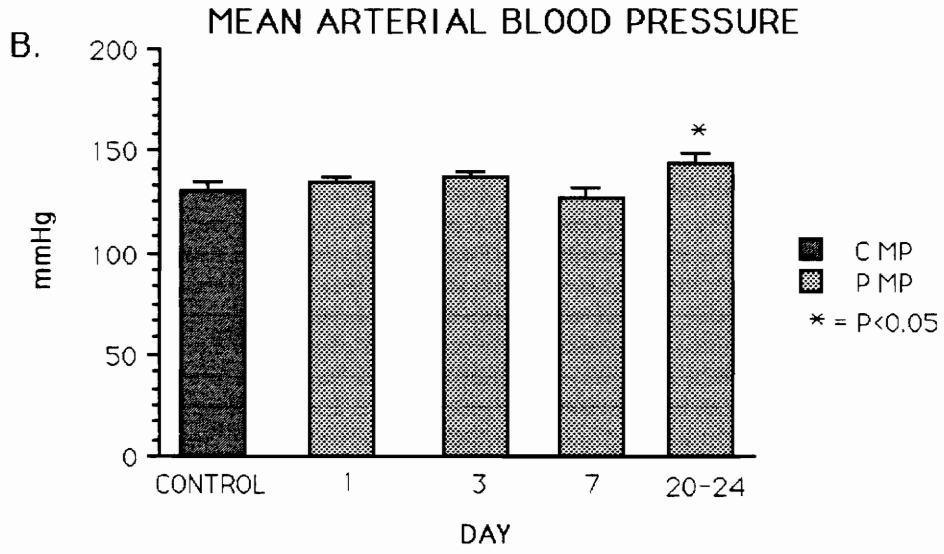

B.
MEAN ARTERIAL BLOOD PRESSURE
mmHg
200
150
100
50
0
*
CONTROL
1
3
7
20-24
DAY
C MP
P MP
* = P<0.05

TABLE 3

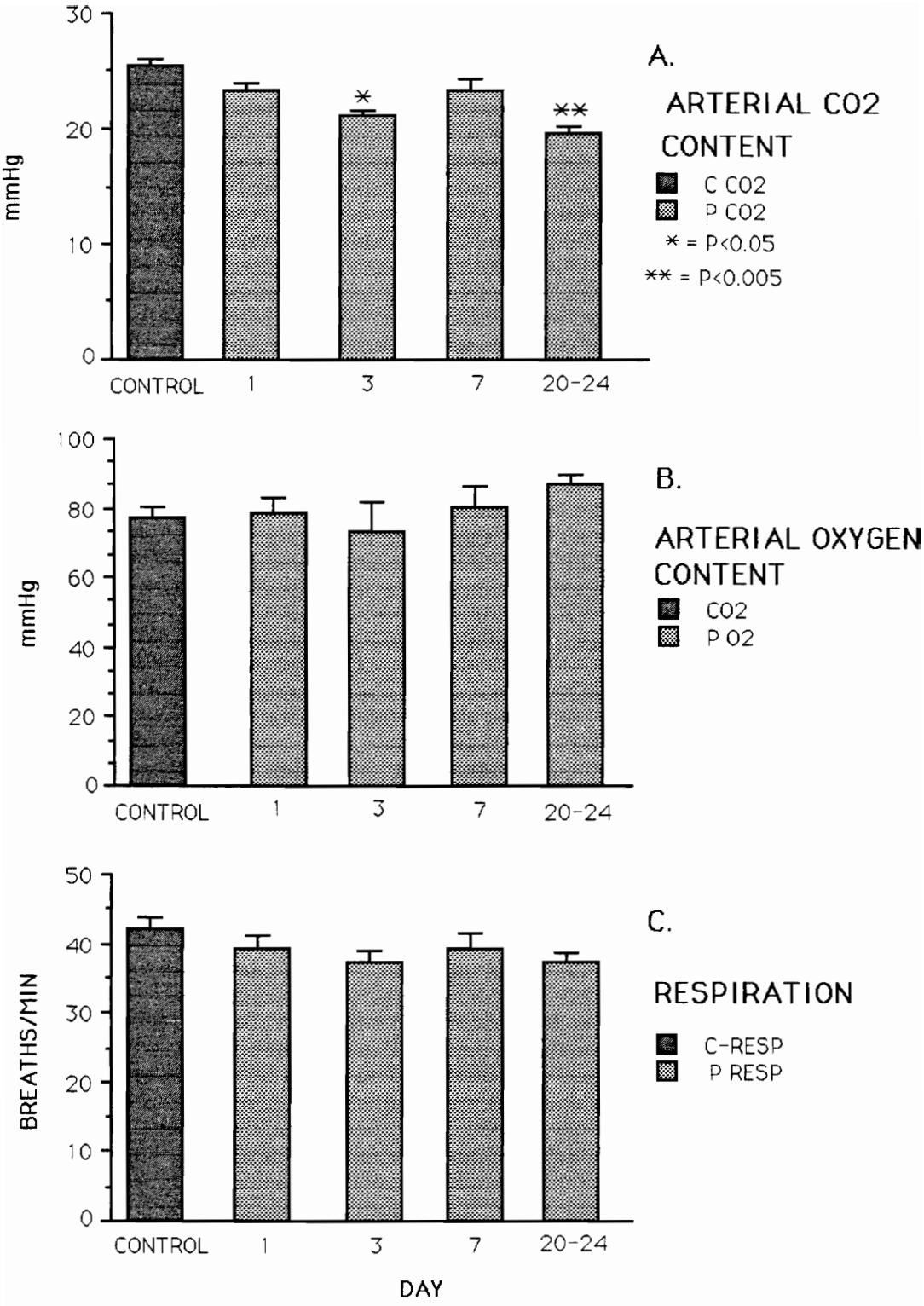
The effects on dissolved blood gases, respiratory rate, and hematocrit in hens given 2.5mg/kg (im) phenyl saligenin phosphate (PSP) compared with levels in control hens receiving vehicle (DMSO) only.[1,2]

Parameter Measured (units)	Days following PSP administration				
	Control	1	3	7	20-24
Arterial PCO_2 (mmHg)	24.62 ±0.71	22.52 ±0.65	20.46* ±0.24	22.48 ±1.01	18.18** ±0.52
Arterial PO_2 (mmHg)	74.78 ±2.78	75.80 ±4.51	70.85 ±8.32	78.14 ±5.74	84.23 ±2.53
Respiratory Rate (Breaths/min)	41 ±1.33	38 ±1.89	36 ±1.51	38 ±2.16	36 ±1.20
Hematocrit (%)	28 ±0.79	29 ±1.37	28 ±1.25	26 ±0.80	31* ±1.22

[1] Control n=22; PSP treated n=6 per day.
[2] Data is expressed as mean ± standard error of the mean.
* Significant at $p<0.05$ with Fisher's least significant difference following ANOVA.
** Significant at $p<0.005$ with Fisher's least significant difference following ANOVA.

Figure 8.

The effects of PSP administration on (a) PCO_2, (b) PO_2, and (c) respiration in hens. Data points are mean $\pm$ standard error of the mean (n=22 control; n=6 for each treatment day). (CO_2=arterial PCO_2, O2=arterial PO_2, C=control hens, P=PSP treated hens).


30
20
10
0
mmHg
*
**
CONTROL
1
3
7
20-24
A.
ARTERIAL CO2
CONTENT
C CO2
P CO2
* = P<0.05
** = P<0.005
100
80
60
40
20
0
mmHg
CONTROL
1
3
7
20-24
B.
ARTERIAL OXYGEN
CONTENT
CO2
P O2
50
40
30
20
10
0
BREATHS/MIN
CONTROL
1
3
7
20-24
DAY
C.
RESPIRATION
C-RESP
P RESP

DISCUSSION

The data indicate that neuropathy producing PSP, when given to hens, could produce alterations in cardiovascular parameters. Organophosphorus-induced effects were biphasic and are usually identified as being either neurophysiologic or neuropathic responses depending on the time of their appearance following exposure. The neurophysiologic responses would be those observed when esterase activities are inhibited (i.e., on days 1 and 3), while neuropathic effects would be seen later (i.e., on days 7 and 20-24) in this study.

In this study PSP inhibited cholinesterase activities in hen plasma to a much greater extent than it inhibited cholinesterase activity in the brain. Esterase activities were similar in arterial and venous plasma, indicating that tissues were not sufficiently damaged to increase cholinesterase activities determined on the venous side (Cisson and Wilson, 1982). The data on esterase inhibition following PSP administration corresponds well with that of previous studies with this compound (Jortner and Ehrich, 1987; Ehrich et al, 1988; El-Fawal et al, 1990a; 1990b). The difference in activity levels may reflect the increased susceptibility of pseudocholinesterase to inhibition following exposure to this particular OP, as this is the primary type of cholinesterase in hen plasma (Pickering and Pickering, 1976; Chambers and Chambers, 1990). It is the true

acetylcholinesterase of neural tissue that is responsible for cholinergic effects seen following OP administration (Murphy, 1986; Thompson,1991). Brain cholinesterase activities were not significantly affected within 1 to 7 days of PSP administration. During the later time points at which brain cholinesterase was measured, there appeared to be an increase in activity of this esterase. Increases in enzyme activities following tissue damage in hens have been reported (Cisson and Wilson, 1982).

The temporal distribution of clinical scores were also similar to those reported in previous studies using PSP (El-Fawal et al, 1988, 1990a, 1990b). The scores obtained by hens on day 20-24 were indicative of severe distal axonopathy (Jortner and Ehrich, 1987). As in these previous studies with PSP, significant inhibition of brain NTE activities preceded onset of clinical signs. Neurotoxic esterase activities had returned to control levels when OPIDN was clinically evident, which also supports previous observations (Jortner and Ehrich, 1987).

The experiments reported here provide some new data about the effects of PSP in hens. For example, decreases in limb weight were observed in hens with OPIDN. This loss in limb weight was proportionally greater than the loss in body weight. This loss was not, however, due to specific loss of gastrocnemius muscle because the ratio of weight of this muscle and the hind limb did not change with PSP treatment.

The logarithmic decrease in limb weight associated with a linear decrease in body weight could have been due, in part, to shifts in blood volume. The distribution of blood within body tissues following exposure to OPs has been described (Maxwell et al, 1987). This shift of blood volume favors the brain, heart, and lungs at the expense of muscle, skin, and kidneys. Increased sympathetic stimulation could cause the closure of some precapillary sphincters, decreasing the area through which blood could flow, thereby decreasing the volume of blood in the muscle. In this study, the average limb arterial blood flow was slightly decreased and the average limb venous flow was slightly increased on the first day after PSP administration, which would indicate a decrease in blood volume to the hind limb. A decrease in blood volume to the hind limb would be reflected by a decrease in limb weight. If the redistribution of blood did not alter the blood volume in the hen, the change in total body weight loss would not be as dramatic as the weight loss in the limb. Total body weight loss during this study is similar to that observed in other studies of OPIDN (Baron et al, 1962; Cisson and Wilson, 1982).

Blood pressure was significantly altered as the hens developed OPIDN, but no significant changes were observed shortly after administration of PSP. The fact that PSP did not have a significant effect on neural acetylcholinesterase activity would make the lack of early effects on blood pressure expected. According to Brezenoff and Giuliano

(1982), a 70% inhibition of brain cholinesterase is necessary to trigger the hypertensive response. This reduction was not obtained and no hypertension was observed. A preliminary experiment that preceded the study done for this thesis investigation indicated that a 2.5 mg/kg (im) dose of PSP to 10 month old hens produced significant increase in blood pressure 5 days following exposure. Hens used in this study were 31 months old, which suggests that the age of the hens could have contributed to the lack of hypertensive response. Hens examined at 38 months, approximately the same age as hens used in this study, have been reported to have blood pressures 15-24% higher than hens only 10 months old (Sturkie et al, 1953; Muller and Caroll, 1966). This level of increase in baseline blood pressure may have masked any pressor effects produced by PSP administration. It is also possible that the blood brain barrier is less permeable in older hens. Several studies have demonstrated that substances injected into chicks enter the brain more rapidly than in adult birds (Spooner and Winters, 1966; Bulat and Supek, 1968). Blood pressure is also affected by restraint, and hens in this study were restrained while measurements were taken and blood samples collected. Following 120 minutes of restraint, which was similar to the time for restraint of all hens used in these experiments, blood pressure and vascular resistance decreased while heart rate and cardiac output were shown to increase (Whittow et al, 1965). Blood pressure was, however, significantly increased

in hens with OPIDN. The increased hematocrit and increased viscosity associated with this could have contributed to this increase. Also unknown at present is the possible relationship of this increase in blood pressure with the increase in central nervous system cholinesterase activity noted at this time period.

In this study, vascular resistance was increased within the first week after PSP was administered. Mechanisms that could have contributed to this effect include cholinergic stimulation and release of local modulators, such as CO2, adenosine, inorganic phosphate, and prostaglandins (Polucha et al, 1981; Mulvaney and Aalkjaer, 1990). Although whole brain cholinesterase activities were not notably inhibited when determined 1 to 7 days or more after administration of PSP, it may be possible that cholinesterase inhibition could have been more pronounced in specific regions of the central nervous system. If this occurred in the sympathetic vasomotor centers in the hypothalamus and medulla, for example, cholinergic stimulation could be responsible for increase in vascular resistance observed during the early days of this study. The cholinergic stimulation would increase the sympathetic outflow (Buccafusco and Brezenoff, 1978; Brezenoff et al, 1984). Another possible mechanism to explain why brain cholinesterase remained at control levels on days 1 to 7 while resistance was altered may be that local modulators may have responded to the decreased flow and induced vasodilation. Several mediators

exist which could produce this effect including CO_2, lactic acid, H+, potassium, inorganic phosphate, adenosine and ischemia. These mediators act primarily on precapillary segments of the resistance vessels (Hudlicka, 1973; Mulvaney and Aalkjae, 1990). Further studies will be necessary to determine if OP administration could affect these modulators.

Among the cardiovascular parameters altered during OPIDN induced by PSP was limb venous flow. As OPIDN has been described as a "chemical transection" of peripheral nerves (Davis and Richardson, 1980), a mechanism similar to that described following nerve transection may have contributed to the effects noted, such as the increases in both flow and return. In the studies of Hudlicka (1967), for example, the somatic motor nerves were sectioned while leaving the vasomotor nerves intact. After this manipulation, blood flow to the muscle remained the same even though there was a 50% reduction in muscle weight. When the flow was calculated as a proportion of the weight, as was done for the results obtained for this study, then flow nearly doubled. Hudlicka (1967) suggested, therefore, that skeletal muscle tone played a role in blood flow in and out of the limbs. In the present study, we noted an increase in the arterial blood flow to the limb of treated hens 20-24 days following PSP administration, but, due to variation among the experimental hens, this did not turn out to be statistically significant ($p=0.158$). Flow through the nutritive vascular bed is augmented and the

diffusion of substances from the vascular bed is increased following section of both vasoconstrictor fibers and somatic motor fibers. If such fibers were affected during OPIDN, flow through the vascular bed could be augmented in a similar manner. An increase in blood flow has also been associated with an increase in axonal regeneration following nerve injury (de la Torre and Goldsmith, 1988). Since venous flow was increased in the limb when clinical signs of OPIDN are present, it is possible that some sympathetic vasomotor damage may have occurred along with damage to motor nerves that is known to occur in OPIDN (Davis and Richardson, 1980; Jortner and Ehrich, 1985).

Hens given PSP in the present study also had decreases in PCO2. This was noted both 3 and 20-24 days after its administration. Cholinesterase activities on muscle were not measured in this study, so we do not know if inhibition of this enzyme, early after OP administration, caused an increase in muscle cholinergic activity. If it had, the associated increase in muscle activity would lead to increased levels of CO2 in the interstitial fluid which would diffuse into capillary circulation, opposite of what was seen in this study. There is also evidence that a 100 to 1000 fold increase in sensitivity to acetylcholine of skeletal muscle occurs due to the de novo synthesis of receptors on the sarcomere rather than just at the motor end plate (Axelsson and Thesleff, 1957; Miledi, 1960; El-Fawal et al, 1990b) .

This would also tend to increase the activity of skeletal muscles. Other investigators have noted that denervated muscles, such as those that exist during OPIDN, undergo fibrillation (Lindsley and Holmes, 1984). This, too, could have contributed to increases in the CO_2 levels of treated birds. Maxwell (1987), however, observed that flow to the lung was increased by 225% over control levels after the administration of the OP soman. Therefore it is possible that increased lung blood flow could contribute to decreased levels of CO2 at 3 days after PSP administration. The decrease in pCO_2 during OPIDN (days 20-24) could also be caused by a decrease in muscle activity and a reduced flow of blood through the nutritive capillary bed of the muscle (Hudlicka, 1973). Hens with OPIDN were also generally depressed, and this, too, could have contributed to the alterations in PCO_2.

The significant increase in hematocrit observed in blood samples taken from hens 20-24 days after PSP administration was probably the result of dehydration. When hens received a clinical score of four on a scale of five, they were severely ataxic and had difficulty standing, so their ability to obtain adequate water from the automatic watering system was inhibited. This dehydration may also have contributed to weight loss. Further studies would have to be undertaken in order to determine the role of tissue hydration in OPIDN, as its importance in the manifestations of neuropathy has not been described.

CONCLUSIONS

The experiments performed for this research have indicated that administration of an OP that induces neuropathy can affect cardiovascular parameters in the hen. Significant alterations of blood pressure, limb venous flow, limb vascular resistance, arterial PCO_2, and hematocrit were noted, especially after OPIDN was clinically evident. Further studies are needed to determine the mechanisms associated with these changes and the contributions they make to the manifestations of OPIDN.

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VITA

Wilfred Carl McCain was born in Stuttgart, Germany on September 2, 1950 and moved with his parents, Kenneth and Annalisa McCain, to New Bern, North Carolina in 1954. He graduated from New Bern High School in 1968. He was inducted into the Army where he was trained as a petroleum products analyst and served tours in Germany and Viet Nam. He supervised laboratories during both tours. He entered New River Community College in 1977 and graduated with an Associate in Science degree in general studies in 1978. He entered Radford University in 1978 and earned a Bachelor of Science degree in Biology in 1980 and was given a position as instructor of biology at New River Community College where he taught biology, anatomy and physiology, microbiology, and microtechnique for the following four years. He advanced his education by taking classes in Science Education Department at Radford University. He became manager of quality control and chief biologist at Dominion Laboratories in 1984. In 1986, he took a position as a research technician in the physiology laboratory at the College of Veterinary Medicine, Virginia Polytechnic Institute and State University. He started taking classes in Veterinary Medical Science in 1987.

Willie married Carolyn Cox in 1976. Their first daughter, Kathy, was born in 1982 and Jennifer was born in 1985.

Willie is a member of the National Speleological Society, American Association of Biological Laboratory Educators, and the American Association for the Advancement of Science. He is currently a student member of the American Society of Toxicology.