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THE EFFECT OF TRI-ORTHO-CRESYL PHOSPHATE ON THE DEVELOPMENT OF ORGANOPHOSPHATE-INDUCED DELAYED NEUROTOXICITY IN IMMATURE CHICKENS AND MODULATION BY SELECTED HORMONES AND HORMONE ANTAGONISTS
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Fowzy Abd Fathy

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Steven D. Burn
Major professor

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THE EFFECT OF TRI-ORTHO-CRESYL PHOSPHATE ON THE DEVELOPMENT OF ORGANOPHOSPHATE-INDUCED DELAYED NEUROTOXICITY IN IMMATURE CHICKENS AND MODULATION BY SELECTED HORMONES AND HORMONE ANTAGONISTS

by

Fowzy Abd Fathy

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ABSTRACT

THE EFFECT OF TRI-ORTHO-CRESYL PHOSPHATE
ON THE DEVELOPMENT OF ORGANOPHOSPHATE-INDUCED
DELAYED NEUROTOXICITY IN IMMATURE CHICKENS
AND MODULATION BY SELECTED HORMONES
AND HORMONE ANTAGONISTS

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The reason for the insusceptibility of young animals to organophosphorus-induced delayed neurotoxicity (OPIDN) remains to be elucidated. The present study was undertaken to determine the influence of age-related changes in hormone concentrations on the development of OPIDN in the domestic chicken. In the first experiment it was demonstrated that a relatively fast growing broiler-breed of chicken developed OPIDN when dosed with a single oral dose (500 mg/kg body weight) of tri-o-cresyl phosphate (TOCP) at 6 weeks of age while a slower growing layer-breed was not susceptible until 12 weeks of age. The serum growth hormone and testosterone profiles indicated the growth hormone concentrations began to decline in both breeds approximately 3 weeks before they became susceptible to OPIDN and that testosterone concentrations in the broiler-breed birds increased significantly from 1 to 9 weeks of age, while testosterone concentrations in the layer-breed birds remained relatively constant. In the second experiment, testosterone and estradiol were administered to broiler-breed cockerels from 6 through 10 weeks of age to interfere with sexual development and the organophosphorus delayed neurotoxin TOCP was administered in a single oral dose of 500 mg/kg body

weight at 7 weeks of age. One of 5 birds in the testosterone/TOCP group and 2 of 5 birds in the estradiol/TOCP group developed OPIDN as opposed to 5 of 5 birds in the TOCP group. This suggested that interference with sexual development via administration of testosterone or estradiol protected the bird against development of OPIDN.

The administration of the synthetic corticoid dexamethasone had no effect on the development of OPIDN in young broiler-breed chickens nor did thiouracil which has been demonstrated to cause a decrease in circulating growth hormone concentrations.

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INTRODUCTION

Organophosphorus (OP) compounds have been synthesized and utilized for the welfare of human beings in a variety of ways. They have been used as plasticizers, as stabilizers in lubricants and hydraulic fluids, and as flame retardants. They have also been widely used in agriculture as insecticides and as defoliants (Smith et al., 1959; Wilson et al., 1980; Metcalf, 1982; Francis et al., 1985).

The effects of organophosphorus compounds are of two types. The acute cholinergic effect involves the inhibition of acetylcholinesterase which hydrolyses the neurotransmitter acetylcholine. This inhibition leads to continuous stimulation of the post-synaptic cholinergic receptors of the affected cells resulting in the typical acute cholinergic clinical signs. A second toxic effect of some organophosphorus compounds unrelated to the inhibition of acetylcholinesterase is called organophosphorus-induced delayed neurotoxicity (OPIDN) (Johnson, 1975). The clinical signs of delayed neurotoxicity are not apparent until 8-14 days after exposure to organophosphorus compounds capable of inducing delayed neurotoxicity (Cavanagh, 1964, 1973; Johnson, 1980).

The manifestation of OPIDN in man has been the result of accidental exposure to organophosphorus delayed neurotoxins which first occurred in the 1930s (Metcalf, 1982). Clinical signs of OPIDN in man are similar to those

observed in sensitive animal species. Symptoms in man include painful muscle cramps, numbness of the feet, an unsteady stepping gait with subsequent weakness followed by a lower motor neuron type of paralysis (Bidstrup, 1953; Cavanagh, 1964). Most of the patients displayed symmetrical and bilateral flaccid paralysis of the distal muscles which was sometimes accompanied by spastic paralysis. Besides locomotor impairment, emotional and psychological signs have also been reported in man as a result of exposure to compounds causing organophosphorus-induced delayed neurotoxicity (Jajer et al., 1970).

In addition to man, some species of animals are also susceptible to the effects of compounds causing OPIDN. The clinical signs in the domestic chicken, which is very susceptible to OPIDN, are manifested as ataxia and subsequent limb paralysis. These signs develop concurrently with histopathological alterations appearing in the spinal cord and sciatic nerve (Cavanagh, 1973; Johnson, 1975; Abou-Donia, 1981; Metcalf, 1982). Other animal species for which clinical signs have been reported which were similar to those described for man and chickens include the cat (Taylor, 1967); pig (Cranmer and Hixon, 1984); cow (Beck et al., 1977); and slow loris (a primate) (Ahmed and Glees, 1977).

In general, the young of sensitive species are not susceptible to compounds causing OPIDN when such compounds are administered in a single oral dose capable of producing

signs in adults (Cavanagh, 1964; Johnson, 1975; Maydew et al., 1976). The reason for the insusceptibility of the young animal is still a question, as is the mechanism of OPIDN in adult animals. Baron (1981) concluded in his review that the relative insusceptibility of young animals to OPIDN compounds administered orally may be related to malabsorption of these compounds. Abou-Donia (1981) suggested that this insusceptibility may be due to enhanced metabolism and elimination of the compound in the young animal. Johnson (1982) stated that the insusceptibility of young animals to OPIDN may be associated with the dose threshold of the organophosphorus compounds which results in optimal inhibition and subsequent aging of the proposed target protein, neuropathy target esterase (NTE). He postulated that the effectiveness of NTE inhibition and the subsequent aging process is related to the anatomical development of the neuron.

Another possible explanation for the age-related susceptibility to OPIDN is that the hormonal environment of the developing animal is such that protection is afforded against compounds causing OPIDN. As the animal matures, the concentrations of numerous hormones change and it is possible that an increase or decrease of a specific hormone(s) may then trigger the sensitivity of the animal to the delayed effects of organophosphorus compounds. Examples of hormones whose concentrations vary inversely

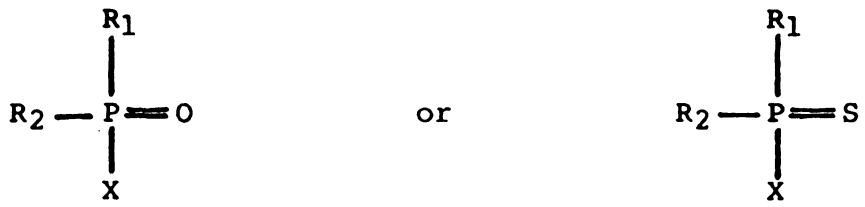
with age in the chicken include the glucocorticoids, growth hormone, and prolactin (Tanabe, 1982; Harvey et al., 1979). The concentrations of estradiol and testosterone have also been shown to vary according to age (Guichard et al., 1977; Tanabe et al., 1979; Tanabe, 1982).

The overall aim of this project was to examine the relationship between the age of the animal and its susceptibility to organophosphorus compounds causing delayed neurotoxicity in terms of a changing hormonal environment. The purpose of the first experiment was to determine the age of susceptibility to OPIDN in broiler-breed and layer-breed chickens which have different age-related circulating growth hormone profiles and as a result grow at different rates. The purpose of the second experiment was to determine if the administration of testosterone or estrogen to sexually immature broiler-breed chickens would influence the development of OPIDN. The purpose of the third experiment was to determine if the presumed alteration of endogenous corticoid concentrations by the administration of the synthetic corticoid dexamethasone would alleviate or enhance clinical signs characteristic of OPIDN in young broiler-breed birds. The purpose of the fourth experiment was to determine if a thiouracil-induced decrease in circulating growth hormone concentration would influence the development of organophosphorus-induced delayed neurotoxicity in young broiler-breed chickens.

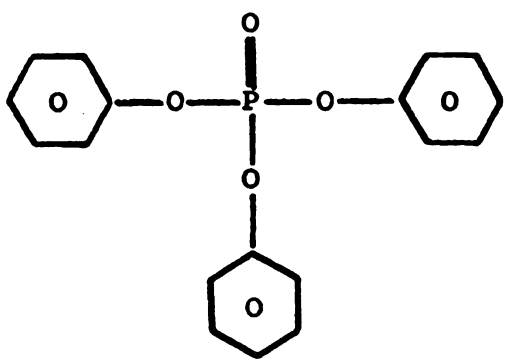
LITERATURE REVIEW

General Structure of Organophosphorus Compounds

Organophosphorus (OP) compounds can be defined as chemicals which contain carbon and phosphoric acid derivatives. The general formula for OPs can be represented as:



where R_1 and R_2 represent variable basic constituents such as alkoxy, alkyl, aryl, or amide groups while the X represents an acidic entity which may be a halide, cyanide, thiocyanate, phenoxy, phosphate, or carboxylate. Organophosphorus esters are named as derivatives of their corresponding parent phosphorus acid. Thus, a phosphate, $(RO)_3P=O$, is a derivative of phosphoric acid; phosphinate, $(R)_2(RO)P=O$, is a derivative of phosphinic acid, and phosphonate, $(R)(RO)_2P=O$, is a derivative of phosphonic acid. Organophosphorus compounds containing aryl or triaryl groups may be represented by the following general structural formula:



In Vivo Mechanism of OPs

In order to understand how OPs can affect a neuron, it is important to present a general overview of how a neuron functions physiologically. A neuron is composed of a cell body (perikaryon), numerous dendrites which function to receive signals, and a single axon which transmits signals away from the cell body. Information is transmitted in the form of electrical signals or nerve impulses along the entire length of the neuron (Caterall, 1984).

The positive ions of the extracellular fluid are principally sodium and potassium whereas the major negative ion is the chloride ion. In the resting state, the concentration of potassium ions is high on the inside of the neuron while the concentration of sodium ions is high on the outside of the neuron. At rest, the distribution of these ions results in the interior of the neuron being 60 to 90 mv more negative than the exterior of the neuron. This potential difference is called the resting membrane potential.

When any event happens which disrupts the resting membrane potential, an electrical impulse or action potential can be generated which occurs in two distinct phases; depolarization and repolarization. Depolarization of the neuron consists of two events which can occur concurrently. The first event consists of a rapid increase in membrane

permeability which allows sodium ions to enter the interior of the neuron. The second event is leakage of potassium ions from the interior of the neuron to the exterior. These ions have specific channels to pass through during depolarization. Repolarization is the stage during which the reverse of depolarization occurs. Immediately after depolarization takes place, the pores of the neuronal membrane again become almost impermeable to sodium ions and more permeable to potassium ions. Therefore, sodium ions stop moving to the inside of the fiber and are actively pumped to the exterior by a Na^+/K^+ pump. As sodium ions are pumped out of the neuron, potassium ions are pumped back into the neuron. As the excess positive charges inside the fiber are transferred back out of the fiber, the normal negative resting membrane is reestablished and the neuron is ready to fire again.

When the action potential reaches the nerve terminals at the end of the axon, a neurotransmitter is released from vesicles via a calcium-dependent process called exocytosis. The neurotransmitter diffuses across the synaptic cleft and binds to receptors on the post-synaptic cell causing a biological event typical of that cell. Once the event has occurred, the action of the neurotransmitter must be terminated. If the neurotransmitter is acetylcholine, it is hydrolysed into acetic acid and choline by the post-synaptic enzyme acetylcholinesterase (Goth, 1984; Caterall, 1984).

Acute Effect of Organophosphorus Compounds

The mechanism of action of OPs has been divided into two types. The acute cholinergic mechanism involves phosphorylation of the active site of acetylcholinesterase which causes permanent inhibition of the enzyme. The result of this inhibition is a continuous stimulation of the post-synaptic acetylcholine receptors of the affected cells (neurons, muscles, and glands) which produces the typical acute cholinergic clinical signs. These signs are characterized by mild to profuse salivation, dyspnea, signs of abdominal pain, diarrhea, sweating, muscular fasciculations, tremors or severe spasmodic events, ataxia, and occasional convulsions (Archibald et al., 1979).

Clinically, the acute intoxication can be treated by atropine and pralidoxine (pyridine-2-aldoxime-methiodide or 2-PAM) (Archibald et al., 1979; Goth, 1984). Atropine binds to cholinergic receptors thereby competively inhibiting the activation of post-synaptic receptors by acetylcholine. Early administration of 2-PAM results in regeneration of acetylcholinesterase because it has a greater affinity for the phosphate moiety than does the enzyme. However, 2-PAM must be administered before aging of the enzyme occurs. Aging is the process whereby one of the residual groups on the phosphate moiety is cleaved off, leaving a negatively charged oxygen. Once aging occurs, the enzyme is permanently phosphorylated and restoration of cholinesterase activity occurs only as a result of de novo synthesis of the enzyme.

The Delayed Neuropathy Effect of Certain Organophosphorus Compounds

The second type of toxic effect of certain organophosphorus compounds that is not related to the acute inhibition of acetylcholinesterase is called organophosphorus-induced delayed neurotoxicity (OPIDN) (Johnson, 1975). It is characterized by lower leg weakness, ataxia, and paralysis which is not apparent until 8-14 days after exposure to the organophosphorus compound. That is why it is called delayed neurotoxicity (Bidstrup et al., 1953; Cavanagh, 1964, 1973; Johnson, 1975).

History of Organophosphorus-Induced Delayed Neurotoxicity

Paralysis due to OP compounds was first reported in the 19th century when phosphocreosote was used as a treatment for patients with pulmonary tuberculosis (Cavanagh, 1964). The first major incident of OPIDN occurred in the United States in the early 1930s (Cavanagh, 1964; Baron, 1981; Metcalf, 1982). During the prohibition era, there was a wide-spread use of ginger extract as a beverage because of its 60-80% alcohol content. The ginger extract was diluted with an oil called Lindol which consisted primarily of cresyl phosphate esters. Consumption of the ginger-based drink resulted in an epidemic of paralysis known as "Ginger Jake" paralysis which subsequently affected some 10,000 people. It was eventually determined that the neurotoxic agent causing "Ginger Jake" paralysis was tri-o-cresyl phosphate (TOCP) (Cavanagh, 1964; Baron, 1981; Metcalf, 1982).

In various European countries, an abortifacient drug known as Apiol was widely used. Apiol, which contained 28-50% TOCP, caused OPIDN in 40 women in Holland and in at least 50 women in Germany, Yugoslavia, and Switzerland during 1930 (Cavanagh, 1964; Metcalf, 1982).

In 1937, an outbreak of paralysis occurred in Durban, South Africa due to the use of cooking oil contaminated with lubricating oil containing TOCP. Another accident happened in Durban in 1955 when drums used for shipping TOCP were subsequently used for drinking water (Metcalf, 1982). Separate incidents of OPIDN involving German, Swiss, and British soldiers occurred between 1939 and 1945 which resulted from the use of cooking oil contaminated with TOCP (Cavanagh, 1964; Metcalf, 1982). The most recent incident of TOCP-induced delayed neurotoxicity occurred in 1959 in Menkes, Morroco where 10,000 people were paralyzed as a result of using cooking oil contaminated with TOCP (Smith and Spalding, 1959).

Organophosphorus compounds other than TOCP have also been reported to induce delayed paralysis in man. Mipafox (bis-monoisopropyl aminophosphorofluoramidate), an insecticide under development in the 1950s, induced ataxia and eventual paralysis of the lower limbs in three laboratory personnel working on the compound (Bidstrup et al., 1953). Leptophos, an organophosphate hailed as a replacement for DDT, caused paralysis in hundreds of water buffalo in Egypt

in 1974 (Abou-Donia et al., 1974). Leptophos was also implicated in the development of clinical signs characteristic of OPIDN observed in several employees of a chemical plant in Bayport, Texas which manufactured the pesticide (Committee on Judiciary, 1977).

Because of these reported accidents, the United States Environmental Protection Agency (EPA), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO) focused attention on the regulatory aspects of organophosphorus-induced delayed neurotoxicity in the U.S. as well as in other countries (EPA, 1976). The Environmental Protection Agency now requires that all organophosphorus compounds must be tested for their delayed neurotoxicity potential before they can be registered for use as pesticides in the United States (EPA, 1976).

In 1975, EPA established tolerance requirements for organophosphorus delayed neurotoxins in the environment. Similarly, WHO and FAO jointly established residue limits ranging from 0.05 to 20 ppm for Leptophos on 20 different crops. The actions of EPA and WHO/FAO reflected the concern for the effects of organophosphorus delayed neurotoxins in man and in animals (Casida and Baron, 1976).

Species Susceptibility to OPIDN

The susceptibility of animal species other than man to OPIDN is quite variable. Cavanagh (1973) reported that man and the adult chicken are probably equally susceptible

to compounds causing OPIDN. This is one of the reasons why the adult hen has become the standard test animal in delayed neurotoxicity trials. The extensive work of Smith and his associates in the 1930s (Cavanagh, 1964) demonstrated the differences in susceptibility to compounds causing OPIDN in such species as the chicken, cat, dog, monkey, rat, and guinea pig. The chicken responded in a consistent manner. In the cat and guinea pig, signs typical of OPIDN were clearly developed and were dose-, route-, and vehicle-dependent. However, the dog, monkey, and rat were resistant to the delayed effects of TOCP at different dose levels (Cavanagh, 1964; Johnson, 1975; Baron, 1981). Subsequent studies by Smith (Cavanagh, 1964) suggested that the insusceptibility of the dog and monkey to TOCP-induced delayed neurotoxicity was due to inadequate absorption of the compound through the gastrointestinal tract. It was later shown that repeated dosing with aryl phosphate compounds resulted in degenerative changes in the peripheral nerves typical of OPIDN (Barnes and Denz, 1953).

Clinical Signs Characteristic of OPIDN

The clinical signs resulting from exposure to organophosphorus compounds causing delayed neurotoxicity are similar in man and susceptible animals. During the "Ginger Jake paralysis" era in the late 1920s and early 1930s, the symptomatology for man was clearly defined

(Cavanagh, 1964). These symptoms included gastrointestinal disturbances such as vomiting and diarrhea which occurred soon after consumption of the contaminated drink or food. The neurological symptoms were characterized by painful cramps in the muscles of the legs, numbness and tingling of the feet, an unsteady stepping gait followed by foot weakness and foot drop, and weakness of hand muscles (Bidstrup et al., 1953; Cavanagh 1964). These symptoms were also followed or accompanied by fasciculations, tremors, and wasting of the muscles. Ultimately, a lower motor neuron type of paralysis occurred (Bidstrup, 1953; Cavanagh 1964). Most of the "Ginger Jake" patients had symmetrical and bilateral flaccid paralysis of the distal muscles, while a smaller percentage of patients suffered spastic (hypertonic) paralysis, which is typical of cerebral palsy.

OPIDN can also affect higher centers of the human brain. The resulting effects may be manifested as insomnia, excessive dreaming, increased libido, paraesthesia, visual hallucinations, and tremors (Jajer et al., 1970). Another characteristic of OPIDN associated with the central nervous system can be demonstrated by electromyography, electroneurography, or electroencephalography which are considered to be sensitive and objective methods for detecting nerve damage (Jajer, 1976). These electrophysiological techniques were initially designed to evaluate the normal conduction velocity of action potentials in nerves and at the

neuromuscular junction. These tools are being used in industrial medicine to monitor the occupational exposure of workers to OPs (Jajer, 1976). The main changes reported in exposed workers were a decrease in the voltage of muscle action potentials (4 mv compared to 11-12 mv in a normal individual) and a decrease in the conduction velocity of nerve fibers when compared to non-exposed humans (Jajer, 1976).

When patients were tested for sensory responses to such stimuli as pin pricks or changes in temperature, they showed a state of anesthesia or hypoesthesia. This implied that the sensory tracts were also involved in the OP neuropathy (Bidstrup et al., 1953; Cavanagh, 1964, 1973; Herns, 1971; Bradley, 1976).

Cavanagh (1954) reported that the first clinical sign he observed in chickens orally dosed with TOCP was a steady drop in body weight. At 8 to 14 days after dosing, birds had overt signs of weakness and fatigue and preferred to squat when forced to walk. Cavanagh (1954) also observed that following a short period of exercise, treated chickens displayed an unsteady and clumsy gait. Additionally, affected chickens gave a negative response in an ankle jerk clinical test designed to assess leg muscle hypertonicity (Cavanagh, 1954). Permanent leg paralysis eventually developed and in severe cases, the bird's wings became paralyzed (Cavanagh, 1954, 1961, 1973; Johnson,

1975). At this stage of paralysis, the chicken had difficulty in swallowing which suggested involvement of cranial or spinal nerves. In mild cases of OPIDN, recovery may be almost or entirely complete (Cavanagh, 1964, 1973).

The cat has also been used as a test animal to study OPIDN because of its sensitivity to organophosphorus compounds causing delayed neurotoxicity (Smith et al., 1932; Cavanagh, 1964; Taylor, 1967; Bouldin et al., 1979a,b; Baron, 1981; Drakontides et al., 1982). Smith et al. (1932) reported that TOCP and related phenyl esters caused a specific neurological deficit in the cat which appeared several days post-treatment. The clinical signs observed in the cat were similar to those observed in the chicken with the exception of respiratory involvement which was observed only in the cat. In the cat, the signs characteristic of OPIDN were initially manifested as weakness of the hind limbs. The animals would squat on their metatarsal joints instead of thrusting on their toes in response to stroking. The flaccid paresis was later associated with extensor muscle rigidity of the hind limbs. Also, the end of the tail was curled in a semiflaccid shape accompanied by frequent twitching.

Julian et al. (1975) reported the accidental poisoning of a herd of cattle as the result of dermal treatment for ringworm with organophosphorus-contaminated oil. One week after exposure, the first signs observed were posterior

limb weakness and stiffness. The affected cattle staggered and had difficulty standing. A few days later, ataxia as well as sensory and motor reflex deficiencies in the hind quarters were apparent. This incoordination and weakness then progressed to the front legs. Beck et al. (1977) reported similar delayed effects in cattle naturally and experimentally exposed to TOCP.

The pig, like the chicken, is highly susceptible to OPIDN. This susceptibility is route-, age-, and dose-dependent according to Cranmer and Hixon (1984), Kruckenberg et al. (1973), and Wilson et al. (1982). Kruckenberg et al. (1973) induced posterior paralysis in the pig after dermal application of TOCP. Cases of hind leg paralysis were reported in swine after dosing with the organophosphate anthelmintic, Haloxon (Wilson et al., 1982). Yorkshire gilts, 6-9 months of age, developed variable degrees of posterior paralysis two weeks after TOCP treatment at dose levels ranging from 100 to 1600 mg/kgm body weight. The occurrence and severity of clinical signs were age- and dose-related (Maydew et al., 1976).

Larson et al. (1986) reported that adult turkeys dosed with TOCP orally or diisopropyl fluorophosphate (DFP) subcutaneously developed OPIDN. The clinical signs were mild ataxia, followed by complete paralysis of the legs. They also observed that the rate of clinical progression was related to the dose. Male turkeys were consistently more severely affected than females.

Typical laboratory animals such as the rat, rabbit, and mouse develop acute cholinergic clinical symptoms after treatment with organophosphorus compounds causing OPIDN, but do not develop a delayed permanent paralysis. However, neuropathological lesions of the spinal cord and peripheral nerves resulted after repeated dosing with an organophosphorus compound causing OPIDN (Barnes and Denz, 1953; Baron, 1981; Veronesi, 1984; Veronesi et al., 1986).

Insusceptibility of Immature Animals to OPIDN

It has been reported that immature animals are not susceptible to OPIDN while adult animals are (Johnson and Barnes, 1970; Cavanagh, 1964; Beck et al., 1977; Baron, 1981). Susser and Stein (1957) (cited by Cavanagh, 1964) reported that during the second occurrence of OPIDN in Durban, South Africa, children younger than seven years of age did not show any clinical symptoms.

Similarly, layer-breed chickens less than 55 days of age were not susceptible to the delayed effects of organophosphorus compounds (Baron and Denz, 1953). Johnson and Barnes (1970) were unable to produce clinical signs typical of OPIDN in chickens 7-49 days old which were orally dosed with DFP at 2-5 mg/kgm body weight. However, during the period of 60-100 days of age, birds showed a striking increase in their susceptibility to the delayed neurotoxic effects of DFP. When chickens were dosed at 100-130 days of age, the effects were both more severe and longer lasting.

In contrast to this, Baron (1981) reported that the administration of TOCP by intraperitoneal injection resulted in OPIDN in four-week old chicks. He attributed the apparent insensitivity of the young bird after oral dosing to its inability to absorb the compound through the gastrointestinal tract. However, data from this laboratory indicated that four-week old chickens were not susceptible to TOCP-induced delayed neurotoxicity regardless of the route of administration (Olson and Bursian, in press).

In an accidental outbreak of OPIDN in cattle (Beck et al., 1977), calves were not affected by the organophosphorus compound while adult animals were moderately or severely disabled. In a second incident, a herd of cattle was dermally exposed to an organophosphorus compound causing delayed neurotoxicity and none of the two-year old steers or heifers were susceptible to the delayed effects of the compound.

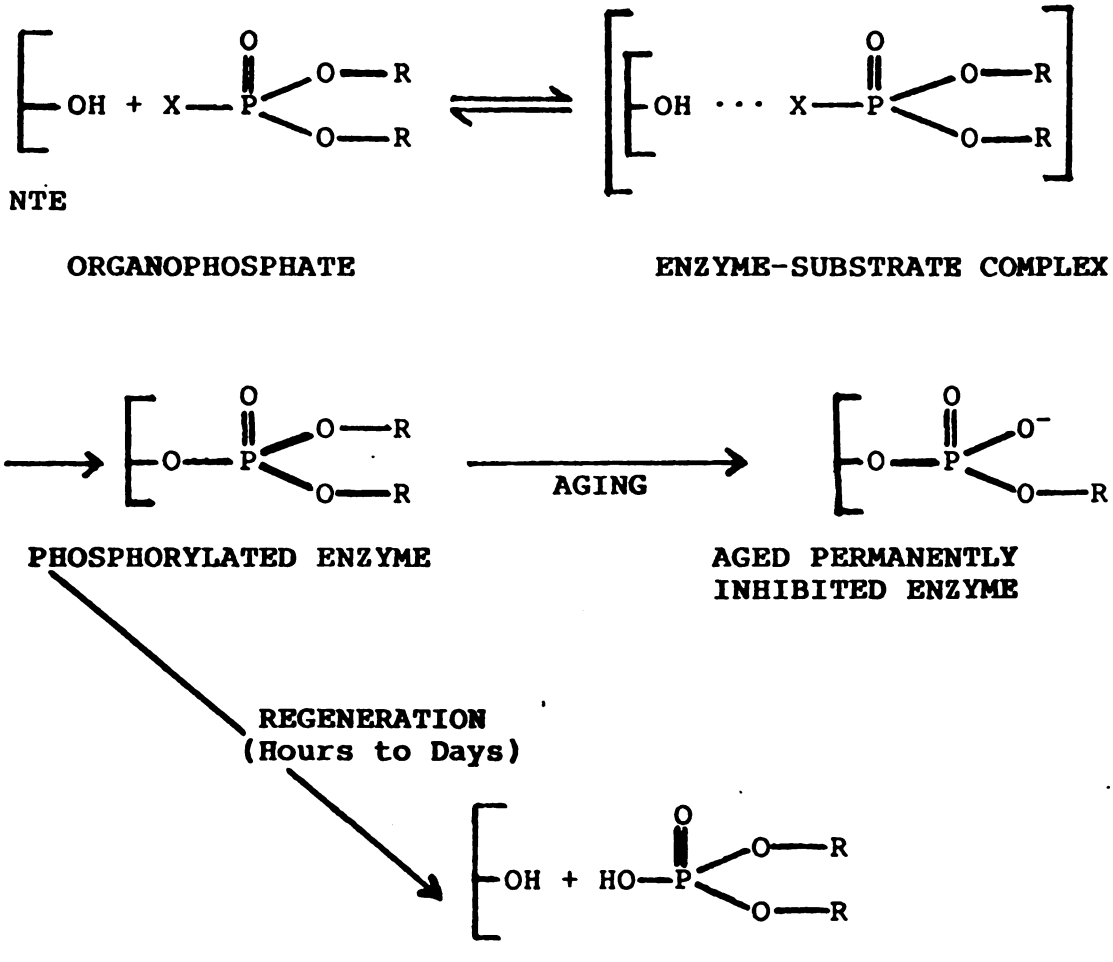
A number of hypotheses have been proposed concerning the resistance or insusceptibility of young animals to organophosphorus compounds causing delayed neurotoxicity. Baron (1981) attributed the insusceptibility of 12-week old chickens to orally administered TOCP to incomplete anatomical and physiological maturation of the digestive tract. Abou-Donia (1981) suggested that species sensitivity to delayed neurotoxicity compounds may be related to differences between young and older animals in the metabolism

and elimination of the compound. Johnson (1982) suggested that this age-related insusceptibility may be associated with the threshold dose of the OPIDN compound required to maximally inhibit the proposed target enzyme, neuropathy target esterase (NTE). Perhaps NTE must be inhibited to a greater extent in young birds than older birds for the condition to develop which in turn could be related to the age-dependent anatomical development of the neuron.

Biochemical Basis of Organophosphorus-Induced Delayed Neurotoxicity

While the exact mechanism of OPIDN is not known, Johnson (1970, 1979) postulated that the initial biochemical event involves inhibition of a nervous system protein initially called "neurotoxic esterase" and now known as neuropathy target esterase (NTE). The process includes two molecular steps which occur within a few hours after exposure to the organophosphorus compound causing delayed neurotoxicity (Figure 1). The first step involves covalent binding of the parent organophosphorus compound or its metabolically activated metabolite to the active site of the target protein which results in the loss of its catalytic esteratic activity (Johnson, 1982). The second essential step for manifestation of OPIDN is the "aging process" by which a constituent group is cleaved from the phosphorus after the enzyme has been phosphorylated. This results in a negatively charged residue on the phosphorylated enzyme thereby rendering the enzyme permanently inhibited (Johnson,

FIGURE 1. Inhibition of Neuropathy Target Esterase (NTE) by an Organophosphate Compound.



1982). However, the exact sequence of subsequent events that leads to the clinical signs and histopathological lesions 7 to 21 days later is not known (Johnson, 1975, 1982; Lotti and Johnson, 1978).

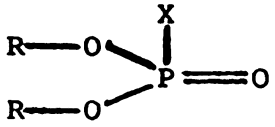
If the organophosphorus compound cannot undergo aging, it will not cause OPIDN. Johnson (1975, 1982) has classified inhibitors of NTE into two groups of compounds, A and B (Figure 2). Compounds in both of these classes can bind to the active site of NTE and thus inhibit the enzyme. Group A compounds include phosphates, phosphonates, and phosphoramidates. These compounds are capable of phosphorylating NTE and undergoing the aging process, thereby causing OPIDN. Group B compounds, which include phosphinates, sulfonates, and carbamates, inhibit the enzyme but can't undergo aging. For this reason, Class B compounds do not cause OPIDN. Johnson (1975) has demonstrated that if a Class B compound is administered to an adult chicken prior to dosing with a Class A compound, the bird is protected against OPIDN because the active site of NTE is occupied by a constituent which cannot undergo aging.

Distribution of Neuropathy Target Esterase

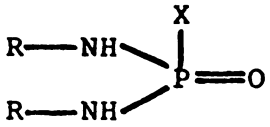
Neuropathy target esterase (NTE) activity in the human brain is higher than activity in the spinal cord. Within the brain, high NTE activity has been reported for the cortex, thalamus, hippocampus, and nucleus caudatus. The lowest NTE activity in the human is reported to be

FIGURE 2. Structures of neuropathy target esterase inhibitors. Group A compounds phosphorylate neuropathy target esterase and undergo the aging process while Group B compounds can inhibit the enzyme but can not undergo aging.

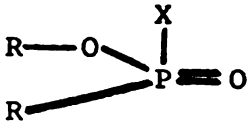
Group A Compounds



PHOSPHATE

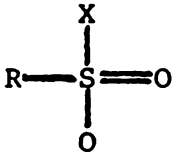


PHOSPHORAMIDATE



PHOSPHONATE

Group B Compounds



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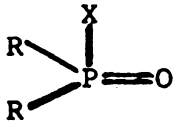

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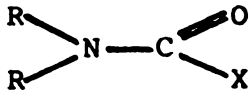


CARBAMATE

in peripheral nerves (Lotti and Johnson, 1980). Similarly, Olajos and Rosenblum (1979) and Johnson (1969) reported that NTE activity in chicken brain homogenate was higher than activity in the spinal cord. Olajos et al. (1978) indicated that substrate hydrolysis in sciatic nerve extracts from the chicken were approximately 10 times less than for whole-brain homogenates. Accordingly, they concluded that a definite quantitative difference in the amount and activity of neuropathy target esterase exists between the peripheral and central nervous systems. Non-neuronal tissues such as heart, spleen, and lymphocytes possess variable NTE activity. Other tissues, such as skeletal muscle, kidney, erythrocytes, and plasma do not have any NTE activity (Dudek and Richardson, 1982).

In terms of subcellular localization of NTE, Richardson et al. (1979) reported that the highest activity of chicken brain and sciatic nerve NTE was recovered in the microsomal fraction. Brain cytosolic fraction contained lower NTE activity than the cytosolic fraction of the sciatic nerve. The myelin sheath of the axon contained no NTE activity (Olajos et al., 1978; Richardson et al., 1979; Olajos and Rosenblum, 1979).

Relationship Between NTE Inhibition and Development of OPIDN

The inhibition of NTE is regarded as an early event of OPIDN. NTE is the site at which the biochemical lesion of delayed neurotoxicity is thought to occur (Johnson,

1969). Johnson's contention is that if this enzyme is inhibited by at least 70-80%, then clinical signs characteristic of delayed neurotoxicity will subsequently develop. If inhibition of NTE is less than 70%, then these symptoms do not occur in susceptible species (Johnson, 1975, 1982).

In the rat, a species which does not develop the typical delayed clinical signs, Veronesi et al. (1986) reported a correlation between neuropathic changes and NTE inhibition after repeated dosing with an organophosphorus compound which causes OPIDN. The inhibition of NTE activity was greater than 73% and 67% in the spinal cord and brain, respectively, with 85% of the rats experiencing degeneration of the spinal cord.

It should be pointed out, however, that inhibition of NTE in excess of 70% does not guarantee development of OPIDN. Bursian et al. (1983) reported that in both the Japanese quail and bobwhite, TOCP caused inhibition of whole-brain NTE in excess of 70%, but did not cause clinical signs typical of OPIDN. In the pheasant, TOCP resulted in more than 70% inhibition of whole-brain NTE activity at all dose levels, but clinical symptoms developed only in those birds receiving the highest dose of TOCP. Johnson and Barnes (1970) reported that single doses of DFP caused greater than 70% inhibition of whole-brain NTE in young birds, but clinical signs typical of OPIDN did not develop.

Histological Lesions of Organophosphorus-Induced Delayed Neurotoxicity

Cavanagh (1954, 1961, 1964) has characterized the lesions of OPIDN in the central and peripheral nervous systems as a "dying-back" or Wallerian degeneration of the axons with subsequent disruption of the myelin sheath. As the term "dying-back" suggests, degeneration is thought to occur at the distal end of long fibers and extend back toward the cell body. More recently, Bouldin and Cavanagh (1979a,b) have proposed that instead of a dying-back process, the degeneration of the nerve fiber may be focal and not an event necessarily originating at the nerve terminal. In any event, animal studies suggest that the development of histopathological lesions occurs concomitantly with the appearance of clinical signs typical of OPIDN (Cavanagh, 1964).

In general, lesions typical of OPIDN are more severe in the spinal cord than in peripheral nerves (Bradley, 1976; Abou-Donia et al., 1980). Cavanagh (1954, 1964, 1973) reported that in TOCP- and DFP-treated chickens, the ascending sensory (spinocerebellar) dorsolateral tract of the spinal cord was severely damaged at the cervical level but not at the thoracolumbar level. The posterior gracilus and cuneatus tracts were also severely degenerated at the cervical level of the cord. The only descending tracts to be consistently affected in the chicken were the ventral horn tracts.

The earliest observations of peripheral neuropathy caused by organophosphorus compounds resulting in OPIDN were reported by Lillie and Smith (1932). They observed in the cat a mild axonopathy in a few fibers which was characterized by swelling and other degenerative changes at the nodes of Ranvier. Cavanagh (1964) reported that lesions typical of OPIDN in the cat tended to be more extensive in the distal portion than in the proximal portion of the nerve. Degenerative changes in the intramuscular nerve bundles were more severe than in nerves located outside the muscle. The most extensively affected part of the neuromuscular junction was the annulospinal formation of the muscle spindle which is innervated by large diameter fibers (Cavanagh, 1964; Drakontides et al., 1982).

Bischoff (1970) and Bradley (1976) reported severe axonal damage of the larger fibers comprising the sciatic nerve of chickens exposed to TOCP. This damage was characterized by axonal swelling, distintegration of the myelin sheath, and formation of intraaxonal vacuoles. Abou-Donia et al. (1980) reported axonal degeneration in the sciatic, tibial, and peroneal nerves in chickens exposed to Leptophos. Higher doses of the compound resulted in myelin disintegration. Lesions of the sciatic nerve and its branches (the tibial and peroneal nerves) have also been described for the slow loris (Ahmed and Glees, 1971), the cat (Bouldin and Cavanagh, 1979a,b), the cow (Julian

et al., 1975), and the turkey (Larsen et al., 1986). Lesions typical of OPIDN have also been reported in the rat (Veronesi, 1984; Veronesi et al., 1986), a species which does not display clinical signs typical of OPIDN.

Organophosphorus-Induced Ultrastructural Changes

Peripheral Nerve

At the electron microscopic (EM) level, chicken and cat peripheral nerves had lesions similar to those reported for spinal nerve tracts (Bischoff, 1970; Bouldin and Cavanagh, 1979b). In the latter study, teased fiber preparations of the left recurrent laryngeal nerve from a cat treated with a single intraperitoneal dose of DFP had axonopathic changes located primarily in the distal part of the nerve. These axonopathic alterations were characterized by a granular transformation of the axoplasm or its total disappearance. Changes in organelles included loss of microfilaments as well as swelling and degeneration of mitochondria. Other changes included formation of large vacuoles within the axon as well as within the myelin sheath (Bouldin and Cavanagh, 1979a,b). They also demonstrated multimembranous or ellipsoid formation of degenerated myelin. Similar lesions have been reported for the rat as well (Veronesi, 1984; Veronesi et al., 1986). The mechanism of the intramyelinic and intraaxonal vacuolation may be related to the impairment of the energy-dependent mechanism which regulates the ionic gradient across the cell membrane (Veronesi, 1984).

Spinal Cord

Ultrastructural changes of the spinal cord have been described in different susceptible species exposed to various compounds causing OPIDN (Bischoff, 1970; Bouldin and Cavanagh, 1979a,b; Sterman et al., 1984; Brown et al., 1984). Bischoff (1970) demonstrated that the ultrastructural changes of the chicken spinal cord induced by TOCP were similar to those observed in the axons of peripheral nerves. These changes were manifested as giant axonal swelling, intraaxonoplasmic and intramyelinic vacuole formation associated with a thin myelin sheath. Also observed was an accumulation of rough endoplasmic reticulum debris forming a lattice of branched tubules. Similar changes were also reported by Veronesi (1984) and Veronesi et al. (1986) in the rat.

Since the susceptibility to OPIDN is age-dependent, there are obviously physiological changes occurring in the maturing animal which trigger the sensitivity to organophosphorus delayed neurotoxins. Among the numerous physiological parameters which change with age are endogenous hormone levels. The possibility exists that the susceptibility of the domestic chicken to compounds causing OPIDN is in some way related to an age-related increase or decrease in the concentration of a particular hormone(s).

One such hormone whose concentration changes with age is growth hormone (GH). In layer-type birds, plasma

GH concentrations are highest in birds from 3 to 9 weeks of age with a subsequent decline in adult birds which begins at 10 to 11 weeks of age (Harvey et al., 1979). The age at which GH concentrations begin to decline in layer-type birds roughly corresponds to the age at which this breed of chicken becomes susceptible to organophosphorus compounds causing delayed neurotoxicity. In broiler-type birds, which grow at a faster rate than do layer-type birds, a similar trend of high plasma GH levels in young animals with a subsequent decline to adult levels was observed (Harvey et al., 1979). However, in comparing the two breeds, mean relative growth rate and plasma GH concentrations were higher in broiler-breed birds during the first four weeks, while after four weeks of age, both the mean relative growth rate as well as plasma GH concentrations were significantly higher in the layer-breed (Harvey et al., 1979; Scanes et al., 1980). If the susceptibility to organophosphorus compounds causing delayed neurotoxicity is related to body size, as has been suggested by Johnson (1987), or growth rate, then it is possible that growth hormone may play a role in age susceptibility. It is also possible that two breeds of chickens possessing different age-related GH profiles would respond differently to compounds causing OPIDN.

Testosterone and estrogen also vary with age in the domestic chicken. In male layer-breed chickens, plasma

concentrations of testosterone increase approximately two-fold during the last days prior to hatching and then decrease slightly within the first three to seven days after hatching. Plasma testosterone concentrations stay relatively constant until about 16 weeks of age and then increase, reaching a peak at 24 weeks of age (Tanabe et al., 1979). In the female, plasma estradiol increases from less than 100 pg/ml 6 weeks before lay (14-16 weeks of age) to a peak of 350 pg/ml 2 to 3 weeks before lay and then decreases to basal concentrations (100-150 pg/ml) at time of first lay (Senior, 1974). Differences in estrogen and testosterone profiles between breeds have not been reported.

A third class of hormones which may have a relationship to the development of OPIDN is comprised of the corticosteroids. These hormones are also characterized by age-related changes in their concentrations. Tanabe (1982) reported that the concentrations of corticosterone and cortisol in the plasma of the domestic chicken were low in embryos, reached peaks at one day after hatching which were 20 times higher than adult concentrations, and then slowly declined with age. No breed differences in corticosteroid concentrations have been reported.

Numerous studies have been conducted which examined the effects of corticosteroids on the development of OPIDN in adult chickens. Drakontides et al. (1982) reported

that glucocorticoid pre-treatment of cats subsequently dosed with DFP caused a marked reduction in peripheral nerve morphological abnormalities characteristic of OPIDN. In studies conducted with chickens, mixed results have been reported. Ehrich et al. (1986) stated that corticosteroid treatment at a level of 50 ppm in the feed offered a degree of protection against the development of OPIDN in adult chickens treated with TOCP based on a clinical assessment as well as histological examination of the sciatic nerve and spinal cord. Conversely, if the corticosteroid was administered at a higher level (in excess of 200 ppm), chickens dosed with TOCP, were more severely affected than birds treated with TOCP alone.

EXPERIMENT 1

THE EFFECT OF AGE ON THE SUSCEPTIBILITY TO TOCP-INDUCED DELAYED NEUROTOXICITY IN TWO DIFFERENT BREEDS OF CHICKENS

INTRODUCTION

One of the areas relating to OPIDN which continues to receive attention is the apparent insensitivity of the young of susceptible species to organophosphorus compounds causing delayed neurotoxicity based on clinical and histological observations. Numerous reports suggest that the chicken must be at least 55 to 70 days old at time of exposure in order for the development of OPIDN to occur (Barnes and Denz, 1953; Bondy et al., 1960; Johnson and Barnes, 1970).

Johnson (1987) made the observation that larger species seem to be more susceptible to OPIDN than smaller species. Data reported by Bursian et al. (1983) lend support to this observation for avian species in that the small Japanese quail and bobwhite were not susceptible to TOCP-induced delayed neurotoxicity while the larger chicken and pheasant were. If body size and/or rate of growth influence the susceptibility to OPIDN, then it is possible that two different breeds of chickens which have different rates of growth will have different ages of susceptibility to compounds causing OPIDN.

It is known that growth hormone influences the rate of growth of avian species. Studies by Harvey et al. (1979) have demonstrated that plasma GH levels are high

in the young bird and then decline to adult levels at a breed-specific age. In layer-breed birds, the decline in circulating GH begins at 10 to 11 weeks of age while in the faster growing broiler-breed bird, GH concentrations begin to drop at four weeks of age. Since the age at which circulating GH concentrations begin to decline in layer-breed birds corresponds to the age at which this breed is susceptible to OPIDN, it was of interest to test the hypothesis that broiler-breed chickens, which grow at a relatively fast rate and have an early drop in circulating GH concentrations will develop OPIDN at an earlier age than a slower growing layer-breed chicken which has a relatively late decline in circulating GH concentrations.

MATERIALS AND METHODS

Day-old male White Mountain Hubbard (broiler-breed) chicks were purchased from a commercial hatchery and day-old male White Leghorn (layer-breed) chicks were obtained from the Department of Animal Science, Michigan State University. Birds were raised in brooder batteries until four weeks of age. There were 25 birds per compartment which measured 100 x 75 x 63 cm (L x W x H). At four weeks of age, birds were transferred to growing batteries until termination of the experiment. There were 5 broiler-breed birds or 10 layer-breed birds per compartment which measured 98 x 79 x 38 cm (L x W x H). Birds were supplied with feed (Purina chick starter) and water ad libitum and exposed to a 16 hour light:8 hour dark photoperiod.

Beginning at 1 week of age, 10 birds of each breed were administered either a single oral dose of the delayed neurotoxin tri-o-cresyl phosphate (TOCP) at 500 mg/kg body weight or the corn oil vehicle. At 2 weeks of age, 10 more birds from each breed received a single oral dose of TOCP or corn oil. This dosing regime continued weekly through 10 weeks of age for the broiler-breed birds and through 12 weeks of age for the layer-breed chickens.

Half of the birds in each group were bled via cardiac puncture 48 hours after dosing between noon and 3 p.m. for subsequent hormone analysis. The remaining 5 birds per group were maintained for an additional 19 days. These birds were observed daily from 8 days post-TOCP exposure through 21 days post-TOCP exposure for the development of clinical signs characteristic of OPIDN. Birds were assessed utilizing an 8-point scale which was modified from Cavanagh (1964).

Serum for hormone analyses was obtained from the blood collected by cardiac puncture. Two aliquots of serum from each bird were prepared when possible and immediately frozen for subsequent hormone analysis. One aliquot was used for the determination of growth hormone by personnel in the laboratory of Dr. Colin Scanes (Rutgers University, New Brunswick, NJ) and the other aliquot was analyzed for testosterone using a radioimmunoassay kit purchased from Sigma Chemical Company.

Broiler-breed birds dosed with TOTP or corn oil at 6 weeks of age were utilized for histological examination at the end of their respective 21-day test periods. Chickens were anesthetized with ether and perfused intracardially with physiological saline followed by a phosphate-buffered 1% formalin solution containing 1% glutaraldehyde using the procedure of Gary et al. (1979). The spinal cord, sciatic nerves, adrenal glands, and testes were removed and prepared for examination by light or electron microscopy. The tissue sample preparation techniques for scanning and transmission electron microscopic examination are described in Appendex 1. Tissues utilized for light microscopic examination were embedded in paraffin and sectioned at 9 to 15 μm. Hemotoxylin and eosin (H & E), Luxol fast blue (LFB), and toluidine were utilized as the stains (Luna, 1968).

Hormone data were analyzed using analysis of variance with statements of significance based on $P < 0.05$. When the F test was significant, Bonferroni's t-test was used to test for differences between treatment means (Gill, 1978).

RESULTS

The severity of clinical signs typical of OPIDN in broiler- and layer-breed chickens administered a single oral dose of 500 mg TOCP/kg body weight at different ages is summarized in Table 1. Broiler-breed birds were initially

Table 1. Average degree of ataxia in broiler and layer breeds of chickens administered a single oral dose of 500 mg TOCP/kgm body weight at different ages.

Age (wks)	n[b]	Days post-TOCP administration[a]													
		8	9	10	11	12	13	14	15	16	17	18	19	20	21
Broiler															
5	5/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	3/5	0	0	0	0.3	0.7	0.7	0.7	0.7	0.7	0.7	1.0	2.3	2.7	2.7
7	5/5	0	0	0	0.6	0.6	1.3	1.8	1.8	2.6	2.6	3.8	3.8	3.8	3.8
9	4/5	1.8	2.8	3.3	3.5	3.5	4.5	4.8	5.5	5.5	5.5	6.0	6.3	6.3	6.3
10	5/5	0	0.8	1.8	1.8	6.0	7.6	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Layer															
11	5/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	5/5	0	0	0	0	0	0	1.0	1.6	2.4	2.4	2.6	3.0	3.6	3.6

[a] Mean degree of ataxia based on the number of birds which were ataxic during the 21 day observation period. Birds were scored on an 8-point scale which was modified from Cavanagh (1964) where 0 = normal; 1-2 = slight but definite ataxia; 3-4 = ataxia but without serious incapacitation; 5-6 = marked ataxia with an inability to maintain an upright stance for any length of time; 7-8 = total inability to rise or walk.

[b] Number of ataxic birds/number of birds dosed.

susceptible to OPIDN at 6 weeks of age. Three of the 5 birds dosed at this age developed ataxia which was first apparent at 11 days post-TOCP and progressed to mild ataxia by the end of the 21-day test period. All 5 birds dosed at 7 weeks of age developed OPIDN. Signs were first apparent at 11 days after dosing and by the end of the 21-day test period, the average degree of ataxia was moderate. Birds dosed at 9 and 10 weeks of age were similarly affected with clinical signs beginning at 8 and 9 days post-TOCP, respectively. At the end of the 21-day test period, the broiler-breed birds dosed at 9 weeks of age were severely ataxic and the birds dosed at 10 weeks of age were paralyzed. In contrast to the broiler-breed chickens which were susceptible to OPIDN beginning at 6 weeks of age, layer-breed birds were susceptible at 12 weeks of age. In this group of chickens, clinical signs were first apparent at 14 days post-TOCP and by 21 days post-TOCP, birds were moderately ataxic.

Serum GH concentrations in 1- through 10-week old broiler- and layer-breed birds are presented in Table 2. Serum samples from 11- and 12-week old layer-breed birds were inadvertently discarded before the assay was completed. In both breeds, serum GH concentrations declined significantly with age as determined in the corn oil-treated birds. In broiler-breed birds, a sharp decline in GH concentration occurred at 3 weeks of age while in layer-

Table 2. Serum growth hormone (GH) concentrations (ng/ml) in broiler- and layer-breed chickens 48 hours after administration of 500 mg TOCP/kgm body weight at different ages.

Breed	Age of dosing with TOCP (wks)	Treatment	
		Control	TOCP
Broiler	1	398 ± 79.6(5)[a]	76 ± 79.6(4)[b]
	2	391 ± 79.6(5)	230 ± 79.6(5)
	3	108 ± 79.6(5)	234 ± 89.0(4)
	4	143 ± 79.6(5)	139 ± 102.7(3)
	5	114 ± 79.6(5)	285 ± 79.6(5)
	6	76 ± 79.6(5)	102 ± 102.7(3)
	7	84 ± 79.6(5)	59 ± 79.6(5)
	8	41 ± 79.6(5)	54 ± 79.6(5)
	9	58 ± 89.0(4)	58 ± 79.6(5)
	10	104 ± 89.0(4)	205 ± 102.7(3)
Layer	1	550 ± 89.0(4)	172 ± 102.7(3)
	2	546 ± 79.6(5)	728 ± 79.6(5)
	3	542 ± 89.0(4)	361 ± 79.6(5)
	4	418 ± 89.0(4)	731 ± 102.7(3)
	5	76 ± 79.6(5)	312 ± 89.0(4)
	6	338 ± 79.6(5)	304 ± 79.6(5)
	7	118 ± 79.6(5)	239 ± 79.6(5)
	8	214 ± 79.6(5)	368 ± 79.6(5)
	9	243 ± 79.6(5)	351 ± 79.6(5)
	10	210 ± 102.7(3)	210 ± 79.6(5)

[a] Mean ± standard error. Numbers in parentheses refer to sample size.

[b] Significantly different from control value at same age.

breed birds, the decline was more gradual over the 10-week period. The administration of TOCP had no significant effect on serum GH concentrations except in 1-week old broiler-breed birds where the organophosphate-treated group had a significantly lower mean concentration than the control group.

Serum testosterone concentrations in broiler- and layer-breed chickens from 1 to 9 weeks of age are presented in Table 3. Serum samples from 10-week old broiler-breed birds and 11- and 12-week old layer-breed birds were inadvertently discarded before the assay was completed. In the broiler-breed birds, there was an age-related increase in testosterone concentrations over the 9 week period represented while testosterone concentrations in the layer-breed birds did not significantly vary with age. Eight- and 9-week old broilers treated with 500 mg TOCP/kg body weight had significantly higher serum testosterone concentrations when compared to their respective controls. Serum testosterone concentrations were not significantly affected by TOCP administration in the layer-breed birds.

Figure 3 presents light micrographs of sciatic nerve longitudinal sections stained with H & E or LFB from broiler-breed birds dosed with TOCP or corn oil at 6 weeks of age and killed 21 days later. Sciatic nerve fibers from the TOCP-treated bird (Figure 3B and 3D) appear as long twisted threads surrounded by degenerated myelin.

Table 3. Serum testosterone concentrations (µg/100 ml) in broiler- and layer-breed chickens 48 hours after administration of 500 mg TOCP/kgm body weight at different ages.

Breed	Age of dosing with TOCP (wks)	Treatment	
		Control	TOCP
Broiler	1	0.1 ± 0.22(5)[a]	0.1 ± 0.22(5)
	2	0.1 ± 0.22(3)	0.2 ± 0.22(4)
	3	0.6 ± 0.20(4)	0.4 ± 0.24(4)
	4	0.4 ± 0.20(5)	0.2 ± 0.25(5)
	5	0.4 ± 0.22(5)	0.5 ± 0.20(5)
	6	0.6 ± 0.24(4)	0.3 ± 0.20(5)
	7	0.9 ± 0.25(5)	0.8 ± 0.20(3)
	8	1.2 ± 0.20(4)	1.9 ± 0.22(5)[b]
	9	0.8 ± 0.23(5)	1.5 ± 0.22(5)[b]
Layer	1	0.3 ± 0.22(5)	0.3 ± 0.22(4)
	2	0.4 ± 0.23(4)	0.3 ± 0.21(6)
	3	0.5 ± 0.22(5)	0.6 ± 0.23(5)
	4	0.3 ± 0.24(4)	0.5 ± 0.25(3)
	5	0.9 ± 0.25(5)	0.5 ± 0.24(4)
	6	0.4 ± 0.24(3)	0.7 ± 0.20(4)
	7	0.3 ± 0.22(5)	0.4 ± 0.20(5)
	8	0.5 ± 0.24(4)	0.3 ± 0.21(5)
	9	0.3 ± 0.25(4)	0.4 ± 0.23(4)

[a] Mean ± standard error. Numbers in parentheses refer to sample size.

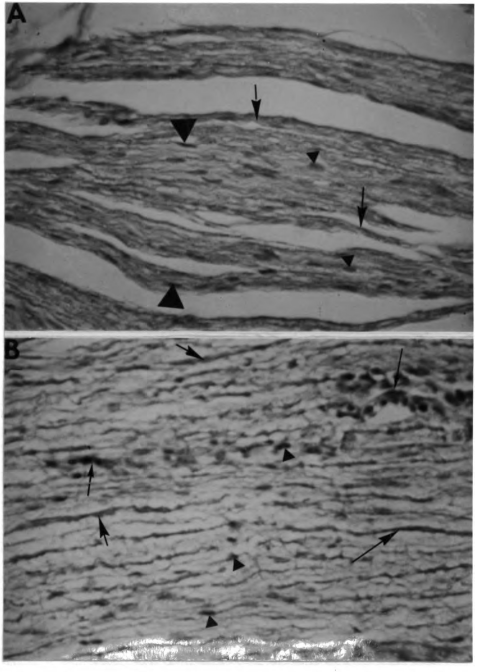
[b] Significantly different from control value at same age.

FIGURE 3. A. Longitudinal section of a sciatic nerve from a broiler-breed chicken treated with corn oil at six weeks of age. Intact fibers (large arrow) with normal Schwann cells (large arrow head). H & E x 400.

B. Longitudinal section of sciatic nerve from a broiler-breed chicken treated with TOCP at six weeks of age. Long, thick, irregular fibers (large arrow); aggregations of mononuclear cells (small arrow); Schwann cells (arrow head). H & E x 400.

C. Longitudinal section of sciatic nerve from a broiler-breed chicken treated with corn oil at six weeks of age. An intact axon (large arrow) and myelin sheath (small arrow). LFB x 100.

D. Longitudinal section of sciatic nerve from a broiler-breed chicken treated with TOCP at six weeks of age. Fragmented myelin debris (large arrow); degenerated axonoplasmic materials (small arrow). LFB x 400.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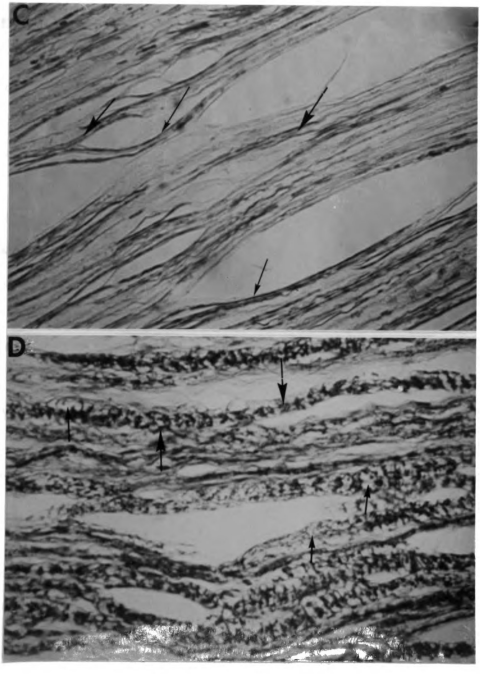
B


C
D

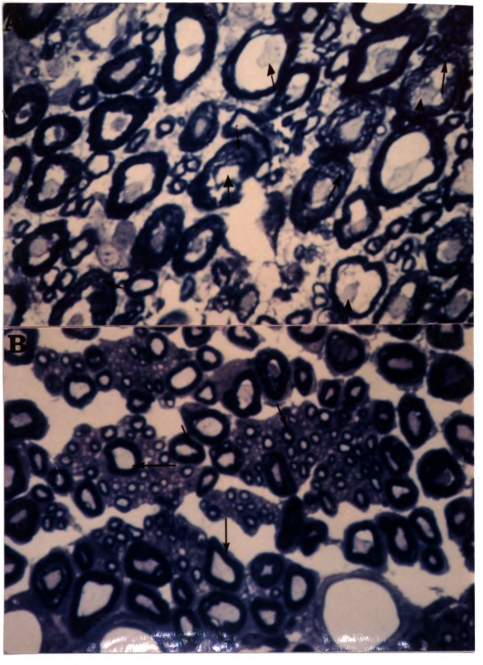
The acidophilic irregular rods within the axon represent disintegrated axonoplasmic material. Acidophilic mononuclear cells are located in close proximity to the degenerating axons. The vacuolation and fragmentation of myelin is well illustrated with the LFB stain (Figure 3D). The sciatic nerve fibers from the control bird (Figure 3A and 3C) are intact, without the foamy appearance of disintegrated myelin surrounding the acidophilic axoplasm. Infiltration of mononuclear cells is also absent in the control section.

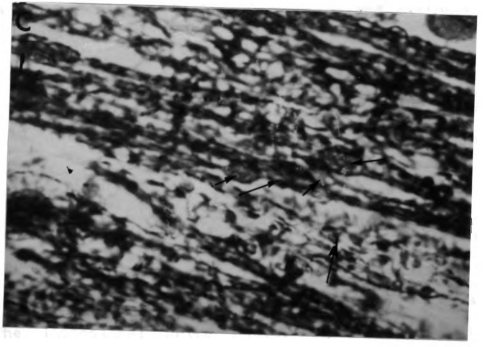
Figure 4 presents cross sections of the lumbosacral region of the spinal cord from a broiler-breed bird dosed with TOCP at 6 weeks of age (A) and a broiler-breed bird dosed with corn oil at 6 weeks of age (B) as well as a longitudinal section of the lumbosacral spinal cord from a TOCP-treated bird (C). The spinal cord lesions of TOCP-treated birds were usually restricted to the axons rather than the cell bodies. When lesions of the perikaryon do occur, they are characterized by chromatolysis and a peripheral dislocation of the nuclei. Multiple intramyelinic vacuoles are present in several affected axons. The axoplasm is compressed and is not filling the whole axon as is evident in the control section. Numerous focal swellings of the axoplasm in addition to small intraaxonoplasmic vacuoles are also apparent in the sections from the TOCP-treated birds.

FIGURE 4. A. Cross section of lumbosacral spinal cord white matter from a broiler-breed chicken dosed with TOCP at six weeks of age. Numerous small intramyelinic vacuoles (small arrow) associated with degenerated axonoplasmic materials (large arrow). Toluidine blue stain x 1000.

B. Cross section of lumbosacral spinal cord white matter from a broiler-breed chicken dosed with corn oil at six weeks of age. Smooth, uniformly intact axons devoid of intramyelinic vacuoles (small arrow). Toluidine blue stain x 1000.

C. Longitudinal section of lumbosacral spinal cord from broiler-breed chicken treated with TOCP at six weeks of age. Multiple, axonoplasmic focal swellings (small arrow) associated with small intra-axonoplasmic vacuoles; debris of fragmented axons and disintegrated myelin (large arrows). LFB x 1000.


A
B



Lesions of the dorsomedial tract (DMT), dorsolateral tract (DLT), ventromedial tract (VMT), and ventrolateral tract (VLT) in the lumbosacral region of the spinal cord were variable in severity (Figure 5). The VMT was affected the least and lesions in this tract were usually bilateral. The axons of the DMT from a TOCP-treated bird (Figure 5A) stained with H & E show remnants of myelin debris. Most of the debris has been engulfed by macrophages which are shown as clear spaces with foamy cytoplasm and peripherally located nuclei. Axonal degeneration is indicated by vacuolated swellings or as eosinophilic bodies of variable size. There are also fewer axons present in the cord from the TOCP-treated bird when compared to the spinal cord from the control animal (Figure 5B). The lesions in the DLT are similar to those present in the DMT.

Figure 6 presents transmission electron micrographs of the spinal cord (A and B) and sciatic nerve (C) from a broiler-breed chicken treated with TOCP at 6 weeks of age. The lumbosacral white matter is characterized by axonoplasmic and myelinic alterations. Figure 6A shows a swollen axon surrounded by a thin layer of myelin. Multiple intraaxonoplasmic vacuoles may indicate depletion of neurofilaments and/or microfilaments. Figure 6B illustrates multiple intramyelinic vacuoles. In Figure 6C, changes observed in the sciatic nerve are shown. The most prominent lesion is the fragmentation of axonoplasmic components into small pieces associated with formation

FIGURE 5. A. Cross section of the lumbosacral region of the spinal cord from a broiler-breed chicken dosed with TOCP at six weeks of age. Diminished number of axons which are infiltrated with macrophages (large arrow) engulfing myelin and axonoplasmic debris. The large eosinophilic sphere (upper middle) may represent a swollen axon. H & E x 100.

B. Cross section of the lumbosacral region of the spinal cord from a control broiler-breed chicken. Eosinophilic dots represent intact axons (small arrow). H & E x 100.

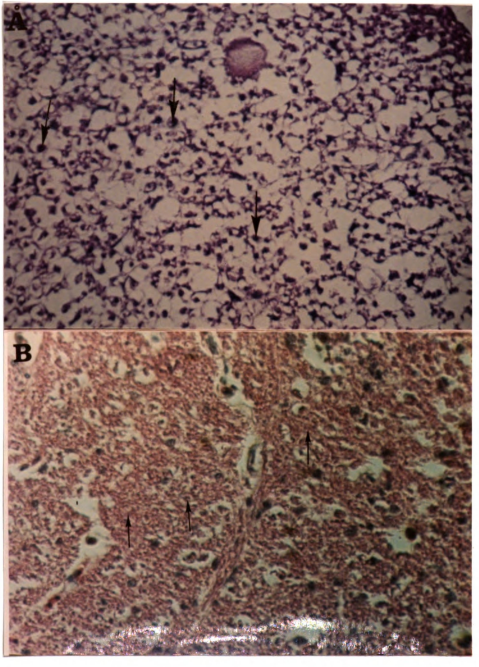
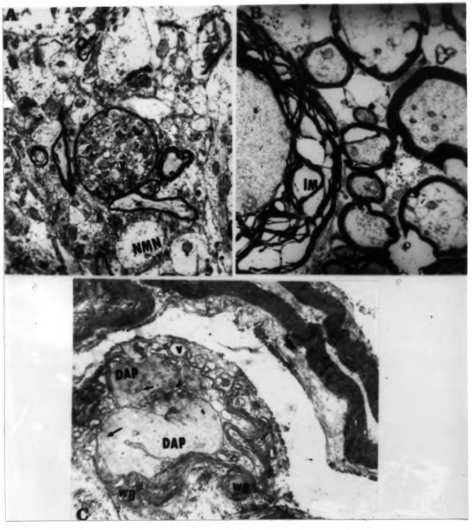

A
B

FIGURE 6. A. Transmission electron micrograph of lumbosacral region of the spinal cord from a broiler-breed chicken treated with TOCP at six weeks of age. Intra-axonoplasmic vacuoles (V); non-myelinated axons (NMN) show a loss of axonoplasmic organelles. Uranyl acetate/lead citrate x 4500.

B. Transmission electron micrograph of lumbosacral spinal cord from a TOCP-treated broiler-breed chicken illustrating numerous intramyelinic vacuoles (IM). Uranyl acetate/lead citrate x 10,000.

C. Transmission electron micrograph of sciatic nerve from a broiler-breed chicken dosed with TOCP at six weeks of age. Myelin whorl bodies (WB); disintegrated axonoplasmic products (DAP); degenerated mitochondria (arrow); vacuoles (V). Uranyl acetate/lead citrate x 4500.


HMN
IM
DAP
DAP
MP
MP
C

of intraaxonoplasmic vacuoles of variable sizes. The myelin changes involve distingration of the myelin and formation of dense osmiophilic whorls. For comparison, sections taken from the lumbosacral region of the spinal cord and from the sciatic nerve of a control broiler-breed chicken are shown in Figure 7. Normal axoplasm and smooth, intact myelin sheaths are illustrated in the micrograph of the spinal cord (A). The micrograph of the sciatic nerve (B) also shows normal axoplasm and axonoplasmic components such as neurofilaments and mitochondria.

Scanning electron micrographs of the lumbosacral region of the spinal cord and the sciatic nerve from a broiler-breed bird dosed with TOCP at 6 weeks of age are shown in Figures 8 and 9. Micrographs of the lumbosacral region of the spinal cord from a control bird are presented in Figure 10. The predominant features in the sections from the treated bird include twisted axons of varying diameters and distingrated myelin.

Histological examination of testes from broiler-breed chickens dosed with TOCP at 6 weeks of age indicated numerous alterations (Figure 11). These changes were characterized by little or no maturation of germinal cells. There was also a lack of spermatogenic activity as indicated by abnormalities in Leydig cells and Sertoli cells as well as shrinkage of seminiferous tubules. Ultrastructural

FIGURE 7. A. Transmission electron micrograph of lumbosacral spinal cord from a broiler-breed chicken treated with corn oil at six weeks of age. Intact osmiophilic myelin sheath (1); oligodendrogliocyte nucleus (2); intact mitochondria within the axoplasm (arrow). Uranyl acetate/lead citrate x 4500.

B. Transmission electron micrograph of sciatic nerve from a broiler-breed chicken treated with corn oil at six weeks of age. Intact, dense osmiophilic myelin sheath (1); neurofilament within the axoplasm (arrow); normal Schwann cell mitochondria (2). Uranyl acetate/lead citrate x 10,000.

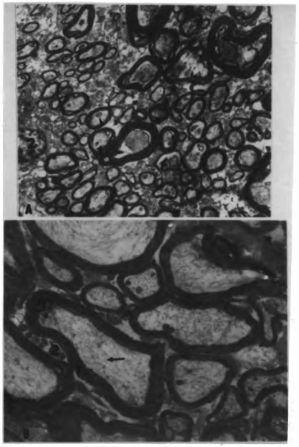


FIGURE 8. A. Scanning electron micrograph of lumbosacral spinal cord from a broiler-breed chicken dosed with TOCP at six weeks of age. Disintegrated myelin or collogen (large arrow); fiber of varying diameter (small arrow); glial or mononuclear blood cells (GC). Glutaraldehyde/osmium tetraoxide x 400.

B. Scanning electron micrograph of sciatic nerve from a broiler-breed chicken dosed with TOCP at six weeks of age. Damaged fascicle with myelin debris (large arrow); intact fascicle (IF). Glutaraldehyde/osmium tetraoxide x 400.

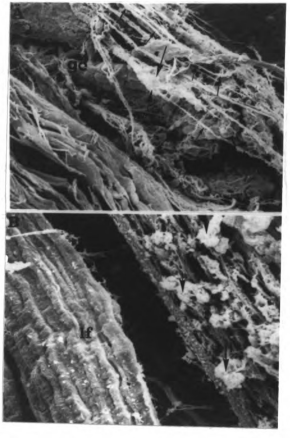


FIGURE 9. A. Scanning electron micrograph of lumbosacral spinal cord from a broiler-breed chicken dosed with TOCP at six weeks of age. Debris of disintegrated myelin (large arrow); glial cell, mononuclear blood cell or connective cell (small arrow). Glutaraldehyde/osmium tetraoxide x 600.

B. Higher power (x 800) of A. Arrows refer to the same features described in A.

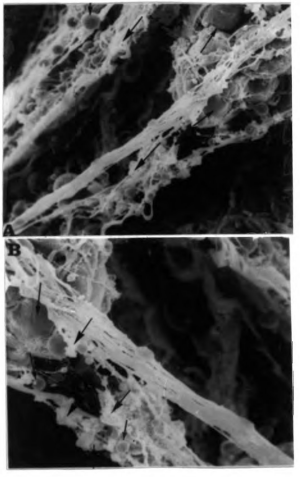

A
B

FIGURE 10. A. Scanning electron micrograph of lumbosacral spinal cord from a broiler-breed chicken treated with corn oil at six weeks of age. Smooth, intact fiber fascicles (IF). Glutaraldehyde/osmium tetraoxide x 1700.

B. Higher power (x 3000) of A.


A
lf
lf
B

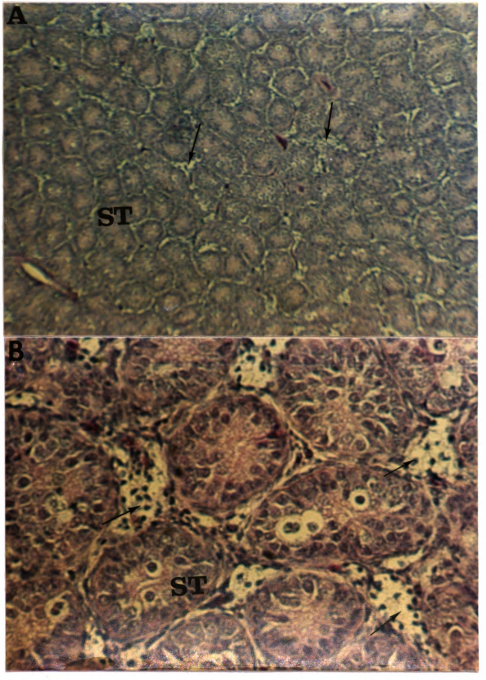
FIGURE 11. A. Cross section of a testis from a broiler-breed chicken dosed with TOCP at six weeks of age. The seminiferous tubules (ST) are atrophied; Leydig cells containing lipid-like foamy materials at the intertubular area (arrow). H & E x 100.

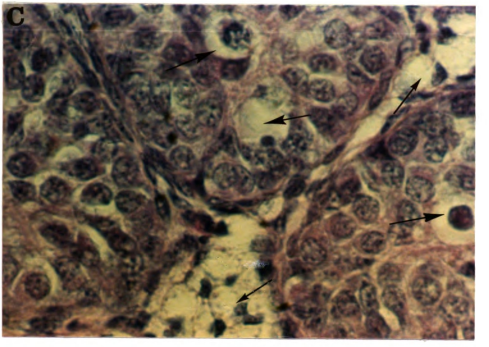
B. Cross section of a testis from a broiler-breed chicken dosed with TOCP at six weeks of age. Seminiferous tubules (ST) are undergoing atrophic changes and are without spermatogenic activity; Leydig cells appear as clear spaces (small arrow) and are within the intertubular area. H & E x 400.

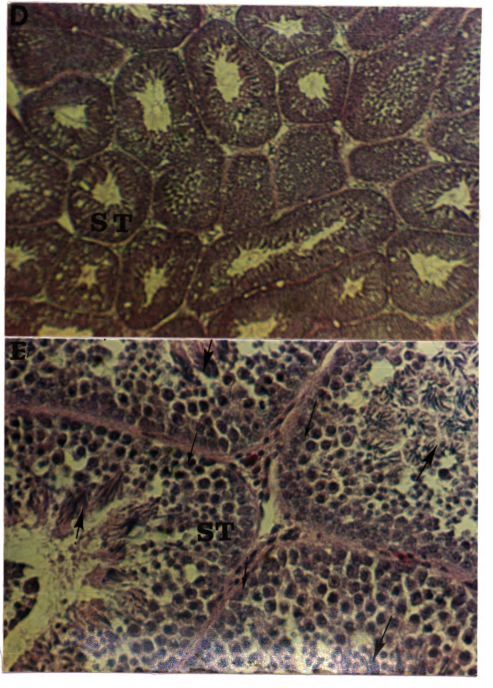
C. Higher power of A. Intratubular Sertoli cells filled with lipid-like foamy material (large arrow); intertubular Leydig cells containing pyknotic degenerative nuclei (small arrow). H & E x 1000.

D. Cross section of testis from a broiler-breed chicken dosed with corn oil at six weeks of age. H & E x 100.

E. Higher power of D. H & E x 400.


A
ST
B
ST


C


D
ST
E
ST

changes in a testis from a broiler-breed chicken dosed with TOCP at 6 weeks of age are illustrated in Figure 12. Germ cells with nuclei of variable sizes are spherical and hypoplastic. The mitochondria of these cells are reduced in size and number. Sertoli cells appear atrophied and contain nuclei of variable sizes.

Light micrographs of adrenal gland cross sections from broiler-breed chickens dosed with TOCP or corn oil at 6 weeks of age are presented in Figure 13. The columnar arrangement of cortical cells from the TOCP-treated bird is disrupted and the cell outlines are ill-defined. The cytoplasm contains irregularly scattered vacuoles and the nuclei are small, dark, and occasionally spindle-shaped. Subcellular details of adrenal cortical cells from TOCP-treated chickens were not visible by transmission electron microscopy because of the presence of large vacuoles of lipid filling the cytoplasm. However, within the medullary cells from broiler-breed chickens dosed with TOCP or corn oil at 6 weeks of age, catecholamine-containing granules are visible (Figures 14 and 15, respectively). In the control bird, the granules appear as osmiophilic bodies of variable shapes and sizes with smooth outlines free of vacuoles. Smooth and intact membranes encompassing the core of the granules can also be observed. However, the granules from the TOCP-dosed bird contain numerous vacuoles.

FIGURE 12. A. Transmission electron micrograph of a testis from a broiler-breed chicken dosed with corn oil at six weeks of age. Spermatogonia with normal appearing nuclei (star); mitochondria (MC); artifact (AF). Uranyl acetate/lead citrate x 4500.

B. Transmission electron micrograph of a testis from a broiler-breed chicken dosed with TOCP at six weeks of age. Germ cells (clear star) and Sertoli cells (dark star) are atrophied and contain shrunken nuclei; subcellular organelles are reduced in size or absent. Uranyl acetate/lead citrate x 4500.

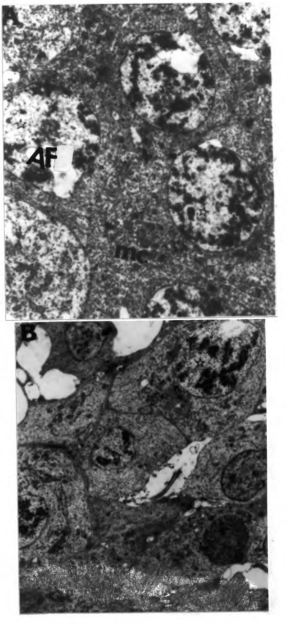

AF
mc

FIGURE 13. A. Cross section of an adrenal gland from a broiler-breed chicken dosed with TOCP at six weeks of age. The normal architecture of the columnar cell is disrupted; the cytoplasm is deeply stained and small cytoplasmic vacuoles are present (large arrow). H & E x 400.

B. Cross section of an adrenal gland from a broiler-breed chicken dosed with corn oil at six weeks of age. Cortical cells have normal columnar arrangement; cytoplasm is well defined and contains small vacuoles. H & E x 400.

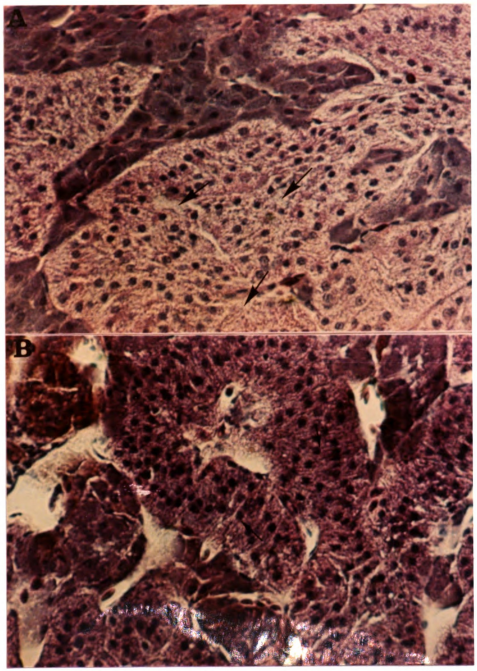

A
B

FIGURE 14. A. Transmission electron micrograph of adrenal medullary cells from a broiler-breed chicken dosed with TOCP at six weeks of age. Osmiophilic granules having irregular membranes (arrow) encompassing a core; vacuoles (V). Uranyl acetate/lead citrate x 20,000.

B. Higher power (x 30,000) of A.

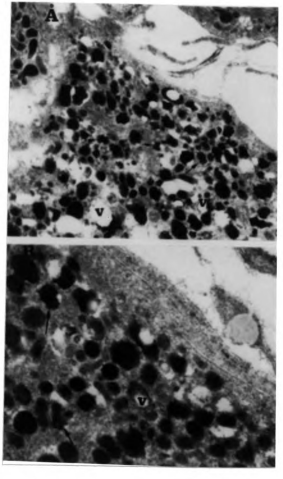
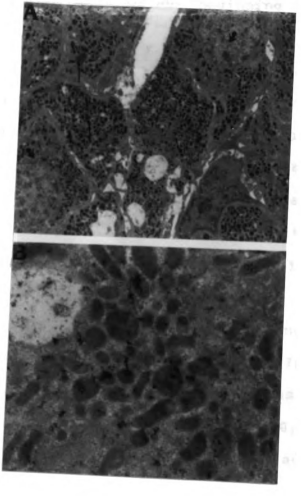

A
V
V
V

FIGURE 15. A. Transmission electron micrograph of adrenal medullary cells from a broiler-breed chicken treated with corn oil at six weeks of age. Osmiophilic catecholamine-containing granules (arrow). Uranyl acetate/lead citrate x 7000.

B. Higher power (x 30,000) of A.



DISCUSSION

Previous studies have indicated that the immature chicken is not susceptible to the delayed effects of certain organophosphorus compounds. For example, Barnes and Denz (1953) reported that 4 chicks (Rhode Island Red x Light Sussex) which received 10 successive weekly subcutaneous doses of 1 mg DFP/kg body weight beginning at 2 weeks of age did not develop paralysis which typically would have been observed in adult birds administered a single dose of 1 mg DFP/kg body weight. In a similar experiment, Johnson and Barnes (1970) injected chicks (various unspecified breeds) subcutaneously with a single dose of 2 to 5 mg DFP/kg body weight from 7 to 49 days of age and observed no delayed effects. However, the susceptibility of the chicks to the delayed effects of DFP progressively increased when the compound was injected between 60 and 100 days of age. Bondy et al. (1960) administered single oral doses of 1000 mg TOCP/kg body weight to chicks (Incross strain) of varying ages. They reported that TOCP was not effective in producing OPIDN when administered at 10, 20, 30, 40, or 50 days of age but was effective if administered at 72 and 100 days of age.

Baron (1981) has suggested that the resistance of the young bird to the delayed effects of orally administered TOCP is due to poor absorption of the compound through the gastrointestinal tract. He cited unpublished data

indicating that if TOCP were administered to 4-week old chicks (unspecified breed) by intraperitoneal injection, then clinical signs characteristic of OPIDN could be observed while if TOCP were administered orally, clinical signs were evident only in birds 12 weeks of age or older. In contrast to Baron's study, Olson and Bursian (1988) demonstrated that 4-week old White Leghorn chicks were not susceptible to TOCP or its neuroactive metabolite, o-tolyl saligenin phosphate, regardless of the route of administration. This suggested that the resistance of the young chicken to the delayed effects of organophosphorus compounds is due to factors other than poor absorption of the compound through the gastrointestinal tract.

The present study indicates that the breed of chicken influences the age of susceptibility to the delayed effects of TOCP (Table 1). Broiler-breed birds began having clinical signs characteristic of OPIDN when dosed with TOCP at 6 weeks of age. As successively older chickens were dosed with TOCP, clinical signs were apparent earlier and were more severe at the end of the 21-day test period. In contrast, the layer-breed birds did not begin to show clinical signs when dosed with TOCP until 12 weeks of age which is consistent with earlier reports. The influence of breed on the development of OPIDN has not been systematically studied. Indeed, in many studies the breed of chicken is not specified, leaving one to assume that

the typical layer-breed bird (White Leghorn) is the experimental animal.

Given the fact that breed does influence the age of susceptibility to the delayed effects of TOCP, there must be physiological differences between the layer-breed bird and broiler-breed bird which account for the difference in age susceptibility. One obvious difference between the 2 breeds is rate of growth. For example, at 6 weeks of age, the average body weight of a broiler-breed male is 1300 gms while the average weight of a White Leghorn cockerel (a laying-breed bird) is 600 gms. Harvey et al. (1979) reported that the difference in growth rate between broiler-breed and layer-breed birds was related to the difference in circulating growth hormone concentrations. Results of the present study (Table 2) confirm those reported by Harvey et al. (1979) and Scanes et al. (1980) in that there was a negative relationship between age and GH concentrations. They reported that GH concentrations in layer-breed birds were highest from 3 to 9 weeks of age with a subsequent decline to adult concentrations at about 12 weeks of age. In broiler-breed birds, the same phenomenon occurred, but the major drop in GH concentration was around three weeks of age. In both breeds, the decline in GH concentrations began about 3 weeks before the bird was susceptible to OPIDN. It is possible that the decline in GH concentration is related in some way to the age of susceptibility to OPIDN.

The other endocrine parameter which was examined in relation to the age at which OPIDN first developed was serum testosterone concentration. In the broiler-breed birds, serum testosterone increased over the 9-week period it was analyzed, while in the layer-breed birds, testosterone concentrations remained relatively constant over the 9-week period (Table 3). As with growth hormone, it is possible that increasing concentrations of testosterone in the broiler-breed birds are contributing to their susceptibility to OPIDN at an earlier age when compared to the layer-breed chickens.

The histopathological changes in the sciatic nerve reported in the present study are similar to those reported for adult chickens and cats (Barnes and Denz, 1953; Cavanagh, 1954; Fenton, 1955; Bouldin and Cavanagh, 1979; Abou-Donia et al., 1979, 1980; Bickford and Sprague, 1982). Lesions resulting from exposure to TOCP included swollen and fragmented axons associated with disintegrated myelin. Axonal and myelin debris of injured fibers stimulates infiltration of mononuclear cells to damaged areas (Abou-Donia et al., 1979, 1980; Bickford and Sprague, 1982). Lesions of the spinal cord were also evident in the young birds having clinical signs typical of OPIDN which were similar to those reported for adult chickens (Fenton, 1955; Abou-Donia and Graham, 1979; Bickford and Sprague, 1982). The ultrastructural changes of the spinal cord and sciatic

nerve reported in the present study agree with those reported for adult hens treated with TOCP (Bischoff, 1970), for cats treated with DFP (Bouldin and Cavanagh, 1979) as well as for rats receiving multiple doses of TOCP (Veronesi, 1984) and mipafox (Veronesi et al., 1986).

While the effect of TOCP on testes morphology reported in the present study is probably not related to age susceptibility to OPIDN, the data do support recent studies in which the effects of TOCP on the reproductive tracts of male rats and roosters were examined (Somkuti et al., 1987a,b,c). These authors reported that TOCP administered from 21 to 63 days to rats and for 18 days to White Leghorn roosters caused a decrease in sperm motility and density as well as vacuolation of and disorganization in the seminiferous epithelium.

In conclusion, the results of the present study indicate that broiler-breed birds were susceptible to the delayed effects of TOCP at 6 weeks of age as opposed to layer-breed birds which were susceptible beginning at 12 weeks of age. The differences in growth rate and the profiles of serum growth hormone and testosterone between the 2 breeds suggest that these endocrine parameters warrant further investigation in relation to OPIDN.

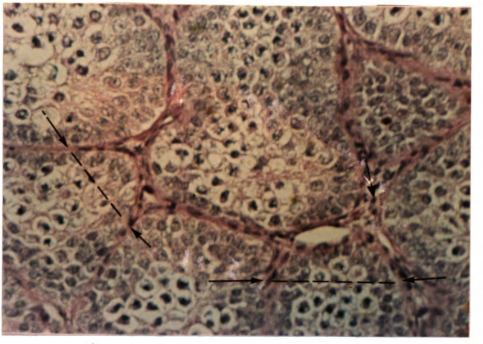
EXPERIMENT 2

THE EFFECT OF TESTOSTERONE OR ESTRADIOL ON THE DEVELOPMENT OF TOCP-INDUCED DELAYED NEUROTOXICITY IN BROILER-BREED COCKERELS

INTRODUCTION

In Experiment 1, broiler-breed cockerels were susceptible to OPIDN when dosed at 6 weeks of age, while the slower growing layer-breed birds were not susceptible until 12 weeks of age. One of the physiological differences noted between the 2 breeds was the serum testosterone profile. In broiler-breed males, there was an age-related increase in serum testosterone concentrations, while in layer-breed birds, testosterone concentrations remained steady from 1 to 9 weeks of age. It is possible that the age-dependent development of OPIDN is related to the process of sexual maturation and that modification of this process will in turn alter the development of OPIDN. Early studies demonstrated that treatment of male chickens with androgen decreased testes size and depressed spermatogenesis somewhat by suppressing the output of pituitary FSH (Kumaran and Turner, 1949b). The same authors also reported that estrogen administration to male chickens depressed the output of pituitary gonadotrophins (Kumaran and Turner, 1949a). The purpose of the present experiment was to test the hypothesis that administration of testosterone or estradiol to broiler-breed cockerels dosed with TOCP at 7 weeks of age will interfere with the development of OPIDN.

FIGURE 16. Cross section of a testis from a broiler-breed cockerel dosed with TOCP at seven weeks of age. No spermatogenic activity is evident; the seminiferous tubules (between arrows) are reduced in size; a small group of intertubular cells (large arrow). H & E x 400.



MATERIALS AND METHODS

White Mountain Hubbard (broiler-breed) cockerels were purchased from a commercial hatchery at 1 day of age and raised as described in Experiment 1. Beginning at 6 weeks of age, 10 birds each received 28 daily intramuscular injections of either 50 µg estradiol/bird, 100 µg testosterone/bird, or 0.1 ml of the vehicle which consisted of a 15.75:8.75:10.5 ratio of propylene glycol, distilled water, and 95% ethanol (Davies et al., 1976; Lax et al., 1983). At 7 weeks of age, 5 birds in each of the 3 groups were administered a single oral dose of 500 mg TOCP/kg body weight while the remaining 5 birds per group were given corn oil. Chickens were observed daily for 14 days beginning on day 8 post-TOCP exposure for the development of clinical signs characteristic of organophosphorus-induced delayed neurotoxicity. Clinical signs were evaluated following the 8-point scale of Cavanagh (1964). At 21 days post-TOCP, birds were killed and the adrenal glands and testes prepared for histopathological examination by light microscopy as described in Experiment 1.

RESULTS

The effects of exogenous testosterone and estradiol on the development of clinical signs characteristic of OPIDN in broiler-breed cockerels are presented in Table 4. All 5 birds dosed with TOCP had clinical signs which were first apparent at 11 days post-TOCP. At the end of

Table 4. The effect of testosterone or estradiol on the development of TOCP-induced delayed neurotoxicity in young broiler-breed cockerels based on the average degree of ataxia.

Treatment[b]	n[c]	Days post-TOCP administration[a]													
		8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control	5/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testosterone	5/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Estradiol	5/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOCP	5/5	0	0	0	1.6	1.8	2.2	3.2	3.4	3.4	3.0	2.8	2.8	2.8	2.8
TOCP/ Testosterone	1/5	0	0	2.0	3.0	4.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	6.0	7.0
TOCP/Estradiol	2/5	0.5	1.0	1.0	1.5	2.0	2.0	2.5	3.0	3.0	3.5	3.5	3.5	3.5	3.5

[a] Mean degree of ataxia based on the number of birds which were ataxic during the 21-day observation period. Birds were scored on an 8-point scale which was modified from Cavanagh (1964) where 0 = normal; 1-2 = slight but definite ataxia; 3-4 = ataxia but without serious incapacitation; 5-6 = marked ataxia with an inability to maintain an upright stance for any length of time; 7-8 = total inability to rise or walk.

[b] Testosterone was administered by intramuscular injection at a dose of 100 µgm/bird/day for 28 days beginning at 5 weeks of age. Estradiol was administered by intramuscular injection at a dose of 50 µgm/bird/day for 28 days beginning at 5 weeks of age. TOCP was administered in a single oral dose of 500 mg/kg body weight at seven weeks of age.

[c] Number of ataxic birds/number of birds dosed with TOCP.

the 21-day observation period, birds in this group were moderately ataxic (average score of 2.8). In the group which was injected with testosterone for 7 days before and 21 days after administration of TOCP, 1 bird of 5 had clinical signs typical of OPIDN. Ataxia was first observed on day 10 post-TOCP and by day 21 the bird was unable to rise or walk (score of 7). Two birds of the 5 which were administered estradiol for 28 days were ataxic as a result of TOCP. Signs were initially observed on day 8 post-TOCP and by day 21 the ataxia had progressed to a moderate degree (average score of 3.5).

The cross section of the testis from a broiler-breed cockerel treated with TOCP at 7 weeks of age is shown in Figure 16. The seminiferous tubles are reduced in size and there is no evidence of spermatogenic activity. Cross sections from TOCP/testosterone-treated cockerels are shown in Figure 17. In Figure 17A, which is the testis cross section from a bird which was not ataxic, the seminiferous tubules appear normal and there is evidence of spermatogenic activity. However, there are a large number of lipid-like cells and clear spaces in the lumen of tubules. Distended Sertoli cells with lipid-filled cytoplasm and Leydig cells with foamy cytoplasm are also apparent. In Figure 17B, which is a testis cross section from the bird which was severely ataxic, there is a reduction in the size of seminiferous tubules, a lack of spermatogenic activity and a presence of foamy intertubular cells.

FIGURE 17. A. Cross section of a testis from a TOCP/ testosterone-treated broiler-breed cockerel which did not display clinical signs typical of OPIDN. Seminiferous tubules are normal in size with spermatogenic activity (large arrow); Sertoli cells with active nuclei lipid-filled cytoplasm (SC) and large lipid-like materials in the lumen (LM). H & E x 400.

B. Cross section of a testis from a TOCP/ testosterone-treated broiler-breed cockerel which displayed clinical signs typical of OPIDN. Seminiferous tubules are atrophied (between thick arrows) with an absence of spermatogenic activity; intertubular foamy cells (arrow). H & E x 400.

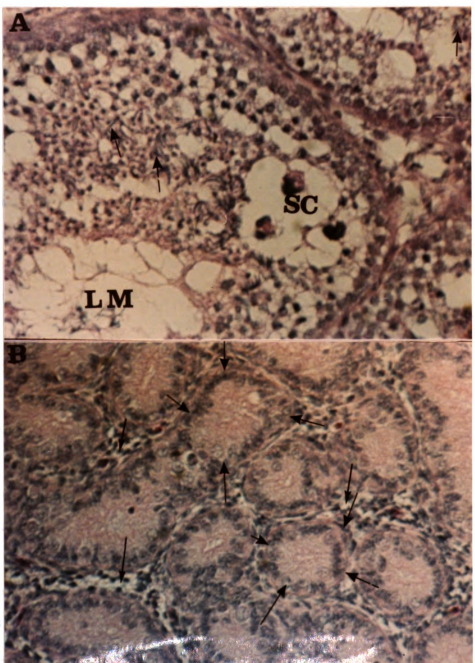

A
SC
LM
B

Figure 18 shows cross sections of testes from a broiler-breed cockerel treated with estradiol only and from a bird treated with estradiol and dosed with TOCP. In the section from the estradiol-treated bird (Figure 18A), seminiferous tubules are of normal size but there are alterations of germinal and Sertoli cells. Pyknotic nuclei of cells and accumulation of lipid can also be observed. In the section from the TOCP/estradiol-treated bird (Figure 18B), the seminiferous tubules are reduced in size with a concomitant absence of spermatogenic activity. Germinal cells are reduced in number as is the size of their nuclei. The Sertoli cells are reduced in size and have pyknotic nuclei.

Figures 19 through 24 show cross sections of adrenal glands from control, TOCP-, testosterone-, TOCP testosterone-, estradiol-, and TOCP/estradiol-treated broiler breed cockerels, respectfully. General features of the control section (Figure 19) include columnar arrangement of the cortical cells. Nuclei are identical in shape and location. Small vacuoles are uniformly distributed throughout the cytoplasm. In the adrenal gland cross section from the TOCP-treated bird (Figure 20), the cytoplasmic vacuoles are larger in size and less uniformly distributed. Adrenal cross sections from the testosterone and estradiol-treated birds (Figures 21 and 23, respectively) show essentially the same features as the control section.

FIGURE 18. A. Cross section of a testis from a broiler-breed cockerel treated with estradiol for 28 days. Seminiferous tubules are of normal size (between arrows); degenerative changes in Sertoli cells and germinal cells (arrow). H & E x 400.

B. Cross section of a testis from a broiler-breed bird treated with estradiol for 28 days beginning at six weeks of age and dosed with TOCP at seven weeks of age. Seminiferous tubules are reduced in size (between large arrows); degenerative changes of Sertoli cells (small arrow) are characterized by pyknotic nuclei; intertubular foamy cells (arrow head). H & E x 400.

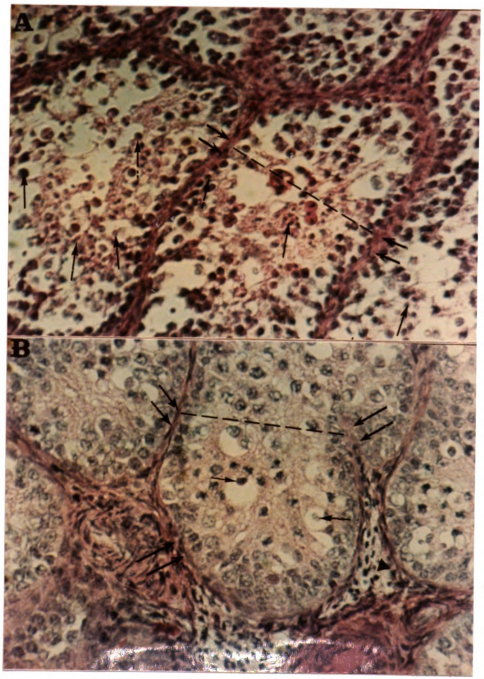

A
B

FIGURE 19. A. Cross section of an adrenal gland from a control broiler-breed cockerel. Columnar arrangement of cortical cells (small arrow) with nuclei of similar size. H & E x 400.

B. Higher power (x 1000) of A. Small cytoplasmic vacuoles (small arrow); nucleated blood cells within blood vessels (arrow head).

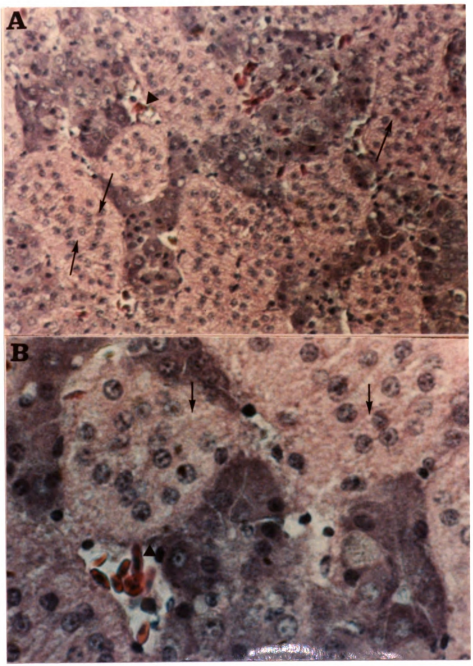

A
B

FIGURE 20. Cross section of an adrenal gland from a broiler-breed cockerel dosed with TOCP at seven weeks of age. A reduction in cortical cell nuclei associated with predominant cytoplasmic vacuolation (small arrow); blood vessel (arrow head). H & E x 1000.

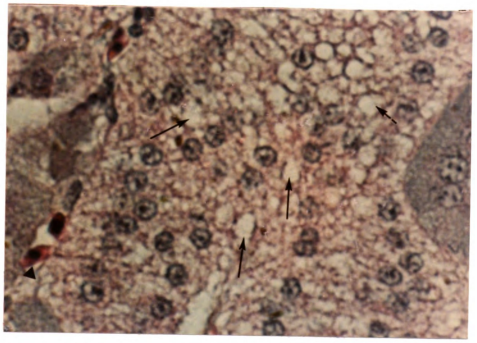


FIGURE 21. A. Cross section of an adrenal gland from a broiler-breed cockerel administered testosterone from six through nine weeks of age. Histologically similar to control section. H & E x 400.

B. Higher power of A (x 1000).

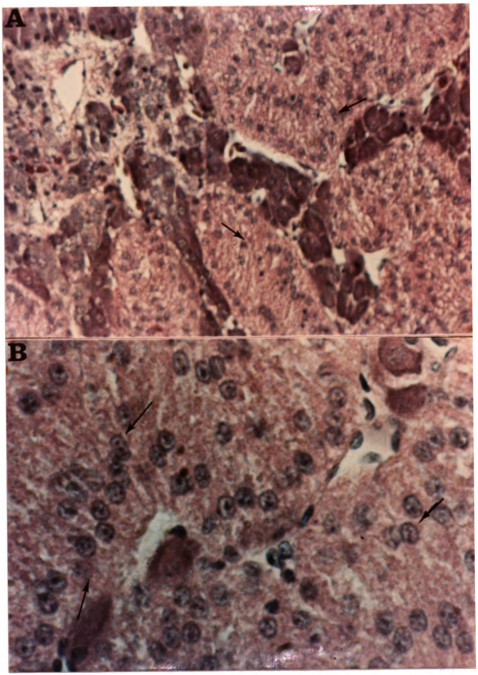

A
B

FIGURE 22. A. Cross section of an adrenal gland from a broiler-breed cockerel administered testosterone from six through nine weeks of age and dosed with TOCP at seven weeks of age; disturbance in columnar dominance (small arrow); nucleated red blood cells (arrow head). H & E x 400.

B. Higher power of A (x 1000). A reduced number of cortical cells with variable size vacuoles (large arrow head); hypoplastic (small arrow head) and pyknotic (arrow) nuclei.

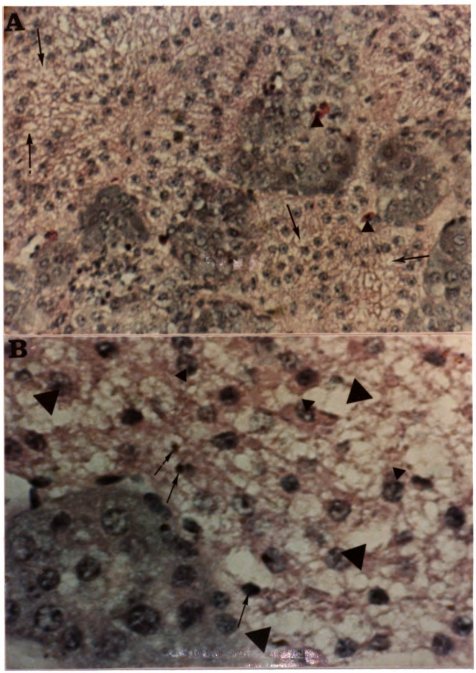

A
B

FIGURE 23. A. Cross-section of an adrenal gland from a broiler-breed cockerel administered estradiol from six through nine weeks of age. Slight change in the columnar arrangement of cells and their nuclei (arrow). H & E x 400.

B. Higher power of A (x 1000). Moderate size vacuoles with darker basophilic nuclei (arrow).

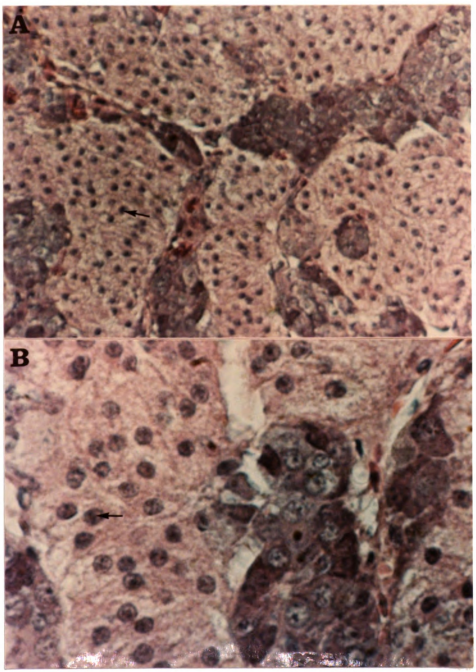
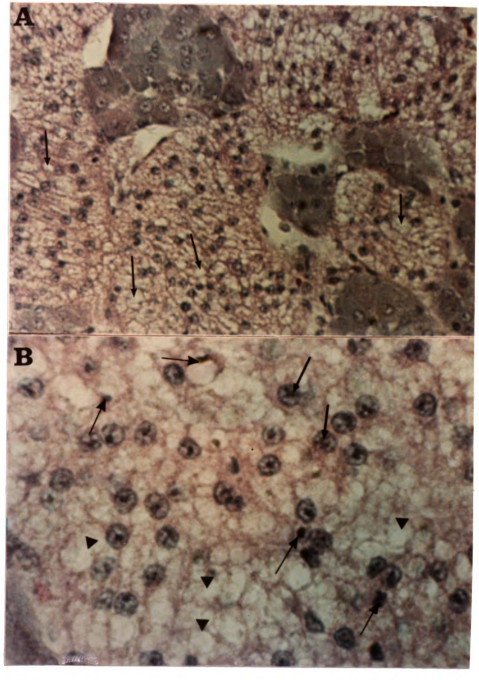

A
B

FIGURE 24. A. Cross section of an adrenal gland from a broiler-breed cockerel administered estradiol from six through nine weeks of age and dosed with TOCP at seven weeks of age. A reduction in the number of nuclei of cortical cells associated with variable size vacuoles (small arrow). H & E x 400,

B. Higher power of A (x 1000). Large vacuoles (arrow heads); hypoplastic nuclei (arrows).


A
B

In the adrenal cross sections from the testosterone/TOCP- and estradiol/TOCP-treated birds, there are reductions in the number of nuclei of cortical cells as well as an increase in the number of cytoplasmic vacuoles.

DISCUSSION

Broiler-breed cockerels administered testosterone or estradiol from 6 through 9 weeks of age and dosed with TOCP at 7 weeks of age were generally not susceptible to OPIDN when compared to birds not receiving testosterone or estradiol. Four of 5 birds in the TOCP/testosterone group did not develop ataxia while 3 of 5 birds in the TOCP/estradiol group were resistant (Table 4). The hypothesis was that prolonged administration of testosterone or estradiol to sexually immature cockerels would interfere with the maturation process and as a result prolong the insensitivity to OPIDN typical of young animals.

The testes of chickens undergo marked changes during the development of spermatogenesis. During the first 5 weeks of age, the seminiferous tubules are organized and multiplication of the basal layer of cells (spermatogonia) occurs. The primary spermatocytes begin to appear at about the 6th week. During the next 2 to 3 weeks, growth of the primary spermatocytes is the primary activity. The secondary spermatocytes begin to appear at 10 weeks of age. Spermatids appear in the seminiferous tubules at 12 weeks of age and by the 20th week are usually in all

the tubules. The seminiferous tubules of prepuberal males are small and are lined with a single layer of cells while tubules of mature testes have a multilayered epithelium containing spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, Sertoli cells, and spermatozoa (Sturkie, 1976).

In the present study, administration of estradiol or testosterone to immature cockerels from week 6 through week 9 of age generally resulted in a decrease in the size of germinal cells and Sertoli cells which suggested a delay in the maturation process. There was, however, no change in the size of the seminiferous tubules.

In those birds which received a single oral dose of TOCP at 7 weeks of age, there was a decrease in seminiferous tubule size and no evidence of spermatogenic activity. Somkuti et al. (1987) reported that adult White Leghorn (layer-breed) roosters dosed with 100 mg TOCP/kg body weight for 18 days had decreased sperm motility and that testes from these birds were characterized by vacuolation and disorganization of the seminiferous epithelium. However, adult Leghorn roosters administered a single oral dose of 750 mg TOCP/kg body weight were not similarly affected. The reason for an effect on the testes due to a single dose in the present study and the lack of an effect in the study by Somkuti et al. (1987) may be related to the age and/or breed of chicken.

The histology of the testes from birds exposed to both steroid and TOCP seemed to differ between birds which showed clinical signs typical of OPIDN and those which did not. The 1 bird in the TOCP/testosterone group which was ataxic had testes characterized by a decrease in the size and number of seminiferous tubules as well as a decrease in spermatogenic activity. A non-ataxic bird in the same group had testes characterized by normal appearing seminiferous tubules and normal spermatogenic activity. It is possible that there is a relationship between the apparent sparing effect of testosterone or estradiol on TOCP-induced alterations of seminiferous tubule morphology and spermatogenic activity and the development of OPIDN. Perhaps estradiol or testosterone treatment delayed the maturation process enough that the animals were refractory to the effects of TOCP.

Estradiol and testosterone alone had no effect on histological features of the adrenal gland. The administration of TOCP alone or in conjunction with steroid administration caused an increase in cytoplasmic vacuoles in cortical cells. It seems unlikely that the protective effect offered by estradiol or testosterone against the development of OPIDN is due to an effect on the adrenal gland.

EXPERIMENT 3

THE EFFECT OF DEXAMETHASONE ON THE DEVELOPMENT OF TOCP-INDUCED DELAYED NUEROTOXICITY IN YOUNG BROILER-BREED CHICKENS

INTRODUCTION

Corticosteroids are hormones whose endogenous levels are age-dependent. Tanabe (1982) reported that plasma concentrations of corticosterone and cortisol in the domestic chicken peaked at 1 day post-hatching and then slowly declined with age. If the age-related susceptibility to OPIDN is associated with the decline in adrenal cortical hormones in the maturing bird, then alteration of these levels could influence the development of OPIDN. Dexamethasone (DX) is a synthetic analogue of corticosterone (Goth, 1984) which can inhibit the release of andrenocorticotropic hormone (ACTH) via a negative feedback mechanism (Farner et al., 1973; Mountcastle, 1974). Inhibition of ACTH release from the pituitary will in turn cause a decrease in plasma concentrations of adrenal cortical hormones. The purpose of the present experiment was to test the hypothesis that administration of the synthetic corticoid dexamethasone to young broiler-breed chickens would increase the severity of OPIDN as a result of lowering concentrations of circulating adrenal cortical hormones.

MATERIALS AND METHODS

White Mountain Hubbard chicks of mixed sex were obtained at 1 day of age and raised in brooder batteries as previously described. At 4 weeks of age, birds were

transferred to the Michigan State University Poultry Science Research and Teaching Center and raised on the floor in pens measuring 3 x 1.5 x 3 meters (L x W x H). Each pen contained between 10 to 20 birds. Birds were provided with feed and water ad libitum. The feed used was Purina Chick Starter and the water was fortified with a vitamin and mineral supplement (Appendix 2). Birds were maintained on continuous light.

Beginning at 6 weeks of age, 45 broiler-breed birds (males and females) were administered daily doses of 2 mg dexamethasone/bird via intramuscular injection for a total of 23 days. Forty broiler-breed birds (males and females) were administered the saline vehicle (1 ml/bird) over the same time period. At 7 weeks of age, 20 birds in the DX group and 19 birds in the saline group received a single oral dose of 500 mg TOCP/kg body weight. The remaining 25 birds in the DX group and 21 birds in the saline group received a single oral dose (1 ml/kg) of the corn oil vehicle. Thus, the 4 treatment groups in this experiment were control (saline injections for 23 days beginning at 6 weeks of age and a single oral dose of corn oil at 7 weeks of age), DX (dexamethasone injections for 23 days beginning at 6 weeks of age), TOCP (a single oral dose of TOCP at 7 weeks of age), and DX/TOCP (dexamethasone for 23 days beginning at 6 weeks of age and a single dose of TOCP at 7 weeks of age). The treatment groups contained

unequal numbers of males and females because birds were assigned to treatment groups before the gender could be determined. It has been reported that the sex of the bird does not influence the development of OPIDN (Abou-Donia, 1981).

Forty-eight hours after administration of TOCP, 5 birds from each of the 4 groups were killed by cervical dislocation. Their brains were removed, weighed, and immediately frozen for subsequent analysis of whole-brain neuropathy target esterase activity as described by Johnson (1969). The remaining birds were maintained for an additional 19 days. These birds were observed daily from 8 days post-TOCP exposure through 21 days post-TOCP exposure for development of clinical signs characteristic of OPIDN. Birds were assessed utilizing the 8-point scale of Cavanagh (1964).

RESULTS

The effect of dexamethasone on the development of TOCP-induced delayed neurotoxicity in young broiler birds is presented in Table 5. Birds dosed with TOCP only began to be ataxic on day 10 post-TOCP exposure and by 21 days post-TOCP had progressed to moderate ataxia. Treatment of birds with dexamethasone had no effect on the development of OPIDN in that birds in the DX/TOCP group were also ataxic beginning at 10 days and had progressed to moderate ataxia by 21 days post-TOCP.

Table 5. The effect of dexamethasone on the development of TOCP-induced delayed neurotoxicity in young broiler-breed chickens based on the average degree of ataxia.

Treatment[b]	n[c]	Days post-TOCP administration[a]													
		8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control	16/16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dexamethasone	20/20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOCP	12/14	0	0	0.2	0.5	1.1	1.1	1.5	1.8	1.8	2.5	2.5	2.5	2.5	2.5
TOCP/ Dexamethasone	10/15	0	0	0.3	0.8	0.9	1.4	1.7	1.7	1.7	1.7	2.0	2.0	2.4	2.6

[a] Mean degree of ataxia based on the number of birds which were ataxic during the 21-day observation period. Birds were scored on an 8-point scale which was modified from Cavanagh (1964) where 0 = normal; 1-2 = slight but definite ataxia; 3-4 = ataxia but without serious incapacitation; 5-6 = marked ataxia with an inability to maintain an upright stance for any length of time; 7-8 = total inability to rise or walk.

[b] Dexamethasone (DX) was administered by intramuscular injection at a dose of 2 mg/bird/day for 23 days beginning at 6 weeks of age. TOCP was administered in a single oral dose of 500 mg/kg body weight at 7 weeks of age.

[c] Number of ataxic birds/number of birds dosed with TOCP.

Dexamethasone did not have a significant effect on the inhibition of whole-brain neuropathy target esterase activity by TOCP (Table 6). NTE activity was inhibited by 95% in those birds receiving TOCP only, while it was inhibited by 94% in those birds which had received dexamethasone for 9 days (7 days prior to TOCP and 2 days post-TOCP).

DISCUSSION

In the present experiment, the administration of the synthetic corticosterone analogue dexamethasone at a dose of 2 mg/bird/day for 23 days beginning at 6 weeks of age had no effect on the development or severity of OPIDN in broiler-breed birds dosed with TOCP at 7 weeks of age. The original hypothesis was that administration of dexamethasone would cause a decrease in circulating corticosteroid concentrations as a result of inhibition of ACTH release from the pituitary via a negative feedback mechanism. Since adrenal cortical hormone levels decrease with age, it was thought that artificially depressing the levels in young birds would enhance the severity of OPIDN if such hormones were in fact involved in the development of OPIDN. The lack of an effect by dexamethasone on the development of OPIDN could be due to the lack of an effect of dexamethasone on circulating corticosteroid concentrations in the present experiment or it is possible that corticosteroids have no relationship to the age-dependent development of OPIDN.

Table 6. The effect of dexamethasone on TOCP-induced inhibition of whole-brain neuropathy target esterase activity in young broiler-breed chickens.

Treatment[a]	Whole-brain neuropathy target esterase activity (nmole phenyl valerate hydrolyzed/min/gm brain)
Control	2461 ± 232[b]
Dexamethasone	2476 ± 609
TOCP	121 ± 135 (95%)
TOCP/Dexamethasone	140 ± 105 (94%)

[a] Dexamethasone was administered by intramuscular injection at a dose of 2 mg/bird/day for 9 days beginning at 6 weeks of age. TOCP was administered in a single oral dose of 500 mg/kg body weight at 7 weeks of age.

[b] Mean ± standard error. Sample size was 5. Numbers in parentheses refer to percent inhibition when compared to its appropriate control value.

It has been shown that corticosteroids do influence OPIDN in mature birds. Cats administered a single intravenous dose of 90 mg methyl prednisolone/kg body weight, followed by a single injection of the delayed neurotoxin DFP (2 mg/kg body weight) and then subsequently administered seven intramuscular injections of triamcinalone (9 mg/kg body weight) over the next 20 days did not develop lesions typical of the DFP-dosed animals. Ehrich et al. (1986) reported that adult chickens fed corticosterone at a concentration of 50 ppm or less during a typical 21-day delayed neurotoxicity test were protected against the effects of TOCP administered in a single oral dose of 360 mg/kg body weight. However, TOCP-induced delayed neurotoxicity was more severe in birds fed corticosterone at concentrations equal to or exceeding 200 ppm. They suggested that the protective effect offered by the low concentrations of corticosterone was due to enhanced reactivation of NTE and that the deleterious effect caused by the higher concentrations of corticosterone was due to an inhibition of hepatic enzymes responsible for the inactivation of the organophosphorus compound. Since the synthetic corticoid utilized in the present experiment had no effect on the development or severity of OPIDN, it is not known if the dose of dexamethasone used was inadequate or if dexamethasone itself is not an effective modulator of OPIDN.

EXPERIMENT 4

THE EFFECT OF THIOURACIL ON THE DEVELOPMENT OF TOCP-INDUCED DELAYED NEUROTOXICITY IN YOUNG BROILER-BREED CHICKENS

INTRODUCTION

Experiment 1 demonstrated that broiler-breed chickens were susceptible to OPIDN at 6 weeks of age while layer-breed birds were not susceptible until 12 weeks of age. One difference between the two breeds was the serum growth hormone (GH) profile. Serum growth hormone concentrations in the broiler-breed birds declined rapidly beginning at 3 weeks of age while the decline was more gradual in the layer-breed birds. Thiouracil causes a decrease in growth hormone concentrations through stimulation of thyroid stimulating hormone (TSH) release (Franklyn et al., 1986). If the age-related development of OPIDN is dependent upon the drop in GH concentrations, then depression of GH through administration of thiouracil should increase the severity of OPIDN in birds which are near the susceptible age. The purpose of the present experiment was to test this hypothesis.

MATERIALS AND METHODS

Thirty-six broiler-breed chickens (males and females) were fed a starter ration containing 0.2% thiouracil while 36 broiler-breed birds were fed the starter ration without thiouracil beginning at 8 weeks of age for 28 days. At 9 weeks of age, 16 birds from the thiouracil group and 18 birds from the group fed non-treated feed were

administered a single oral dose of 500 mg TOCP/kg body weight. The remaining 20 birds in the thiouracil group and 18 birds in the non-treated feed group were administered the corn oil vehicle (1 ml/kg body weight). This resulted in 4 different treatment groups: control, thiouracil (0.2% in the diet for 28 days beginning at 8 weeks of age), TOCP (500 mg/kg body weight at 9 weeks of age), and thiouracil/TOCP.

Forty-eight hours after administration of TOCP, 5 birds in each of the 4 groups were killed by cervical dislocation. Brains were removed, weighed, and immediately frozen for subsequent analysis of whole-brain neuropathy target esterase activity (Johnson, 1969). The remaining birds were observed for the subsequent 19 days to assess development of clinical signs typical of organophosphorus-induced delayed neurotoxicity using the 8-point scale of Cavanagh (1964).

RESULTS

All broilers in the thiouracil/TOCP group developed clinical signs characteristic of OPIDN which were similar with respect to timing and severity when compared to birds dosed with TOCP only (Table 7). In both groups, ataxia was first apparent at 8 days post-TOCP and was severe by the end of the 21 day observation period. Whole-brain neuropathy target esterase was inhibited by TOCP to the same extent in birds fed the non-treated diet as in birds fed the diet containing 0.2% thiouracil (Table 8).

Table 7. The effect of thiouracil on the development of TOCP-induced delayed neurotoxicity in young broiler-breed chickens based on the average degree of ataxia.

Treatment[b]	n[c]	Days post-TOCP administration[a]													
		8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control	13/13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiouracil	12/12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOCP	12/12	0.2	1.2	1.1	3.2	3.6	4.9	3.9	4.4	4.7	4.2	4.9	4.9	5.1	5.0
TOCP/ Thiouracil	11/11	0.5	0.9	1.2	1.9	2.6	4.3	5.2	5.8	5.7	5.6	6.0	5.9	6.0	6.6

[a] Mean degree of ataxia based on the number of birds which were ataxic during the 21-day observation period. Birds were scored on an 8-point scale which was modified from Cavanagh (1964) where 0 = normal; 1-2 = slight but definite ataxia; 3-4 = ataxia but without serious incapacitation; 5-6 = marked ataxia with an inability to maintain an upright stance for any length of time; 7-8 = total inability to rise or walk.

[b] Thiouracil was administered in the diet at 0.2% for 28 days beginning at 8 weeks of age. TOCP was administered in a single oral dose of 500 mg/kg body weight at 9 weeks of age.

[c] Number of ataxic birds/number of birds dosed with TOCP.

Table 8. The effect of thiouracil on TOCP-induced inhibition of whole-brain neuropathy target esterase activity in young broiler-breed chickens.

Treatment[a]	Whole-brain neuropathy target esterase activity (nmole phenyl valerate hydrolyzed/min/gm brain)
Control	1984 ± 72[b]
Thiouracil	1727 ± 347
TOCP	72 ± 67 (96%)
TOCP/Thiouracil	33 ± 74 (98%)

[a] Thiouracil was administered at a dietary level of 0.2% for 9 days beginning at 8 weeks of age. TOCP was administered in a single oral dose of 500 mg/kg body weight at 9 weeks of age.

[b] Mean ± standard error. Sample size was 5. Numbers in parentheses refer to percent inhibition when compared to its appropriate control value.

DISCUSSION

It was hypothesized that administration of thiouracil to young birds would increase the severity of clinical signs typical of OPIDN through a decrease in circulating growth hormone concentrations. The results of this study indicated that thiouracil had no significant effect on the development of OPIDN when birds were dosed with TOCP at 9 weeks of age. One possible explanation for this is that TOCP was administered after birds had reached the susceptible age and that a further decrease in growth hormone concentration would not influence the development of OPIDN. Another explanation is that the age-related development of OPIDN has nothing to do with circulating growth hormone concentrations. To more adequately test the hypothesis, thiouracil could be administered to broiler-breed birds prior to the drop in GH which occurs at 3 weeks of age or it could be administered to layer-breed birds before the susceptible age of 12 weeks. If OPIDN occurred at an earlier age in birds given thiouracil, this would suggest that GH might play a role in the age-dependent development of OPIDN.

SUMMARY AND CONCLUSIONS

Broiler-breed cockerels were susceptible to tri-o-cresyl phosphate-induced delayed neurotoxicity when dosed (a single oral dose of 500 mg/kg body weight) at 6 weeks of age while layer-breed cockerels had clinical signs characteristic of OPIDN when dosed at 12 weeks of age. Serum growth hormone concentrations in both breeds declined during the period of 1 to 10 weeks of age. In broiler-breed birds, a large drop in GH occurred at 3 weeks of age while in layer-breed cockerels, the decline was more gradual. Serum testosterone concentrations increased over a period of 1 to 9 weeks of age in the broiler-breed birds, but remained relatively constant in the layer-breed birds. The administration of TOCP had no consistent effect on the serum concentrations of growth hormone or testosterone. Lesions characteristic of OPIDN were observed in sections of the sciatic nerve and spinal cord from birds having clinical signs of OPIDN. Additionally, TOCP had a disruptive effect on the morphology of the testes and adrenal glands.

Broiler-breed cockerels administered estradiol or testosterone for a 28 day period from 6 weeks of age through 10 weeks of age and administered a single oral dose of 500 mg TOCP/kg body weight at 7 weeks of age were generally protected against OPIDN. Histological examination of testes from these birds indicated a decrease in the size of the germinal cells and Sertoli cells which suggested a delay

in the maturation process. It is possible that susceptibility to OPIDN is related to the degree of sexual development. It is not known if this could explain the difference in the ages of susceptibility between the broiler- and layer-breed birds.

Dexamethasone, a synthetic analogue of corticosterone, had no significant effect when administered for 23 days beginning at 6 weeks of age on the development of OPIDN in broiler breed birds dosed with 500 mg TOCP/kg body weight at 7 weeks of age. These data suggested that changes in circulating cortocisteroid levels are probably not related to the age-susceptibility to OPIDN.

Thiouracil administered to broiler-breed chickens via the feed for 28 days beginning at 8 weeks of age had no significant effect on the development of OPIDN in birds dosed with TOCP (500 mg/kg body weight) at 9 weeks of age. Since thiouracil has shown to decrease circulating GH concentrations, the lack of an effect of thiouracil on OPIDN would suggest that GH is not involved in the development of OPIDN. However, it is possible that modulating GH levels in birds which are already at a sensitive age would have no additional effect.

APPENDIX 1

TRANSMISSION AND SCANNING ELECTRION MICROSCOPE SAMPLE PREPARATION

After appropriate perfusion, the sciatic nerve and the lumbosacral and thoracic regions of the spinal cord were removed. Small pieces of spinal cord from each region as well as segments of both sciatic nerves were minced into pieces one mm in length. These pieces were then placed into vials containing ice-cold 1% glutaraldehyde in a phosphate buffer solution according to Gary et al. (1979). After 2 to 4 hours in the buffered glutaraldehyde solution, the samples were washed with phosphate buffer for 15 minutes. The samples were then transferred to a 2% osmium tetraoxide solution for 2 to 3 hours. Samples were again washed with a 2N phosphate buffer solution for 15 minutes. Samples were then dehydrated in successively stronger solutions of ethanol (15 minutes each in 25, 50, 75, 95, and 100% ethanol). Dehydration in 100% ethanol was repeated 2 more times for 1 hour and then overnight.

Samples for transmission electron microscopy were then transferred to a 2:1 ethanol:acetone solution for 15 minutes, a 1:2 ethanol:acetone solution for 15 minutes, and 100% acetone solution twice for 30 minutes each. The samples were infiltrated at room temperature. Samples were then transferred to a 3:1 acetone:epon solution for 2 hours, a 1:1 acetone:epon solution for 2 hours, and a 100% epon solution overnight on a shaker. Samples were

then embedded in freshly prepared 100% epon in electron microscopic molds and placed in a desiccator for 2 hours. Samples were then heated at 65°C for 48 hours to cause polymerization. A Sorvall MT-2 ultramicrotome equipped with glass knives was used to make ultrathin sections. Thin silver or gold sections (90-60 nm) were picked up with 300 mesh grids for insertion into the electron microscope. Sections were stained with uranyl acetate and lead citrate according to Gary et al. (1979). A Philips 201 electron microscope was utilized to obtain images of the tissue sections.

For scanning electron microscopy, samples, after dehydration in alcohol as described above, were processed by the critical point dry technique to remove excess moisture (Gary et al., 1979). Gold was then sprayed on the samples by a sputter coater machine. A Super II scanning electron microscope was used to obtain the image.

APPENDIX 2

VITAMIN AND MINERAL SUPPLEMENT ADDED TO THE WATER IN THE DEXAMETHASONE EXPERIMENT[a]

Vitamin A	2,500,000 i.u.[b]
Vitamin D_3	1,000,000 i.u.
Vitamin E	1,000 i.u.
Riboflavin	750 mg
d-Pantothenic acid	1,250 mg
Niacin	2,500 mg
Vitamin B_{12}	2.5 mg
Menadione sodium bisulfide	1,000 mg
Folic acid	65 mg
Thiamine mononitrate	250 mg
Pyridoxin hydrochloride	250 mg
Ascorbic acid	3,750 mg

[a] Manufactured by Agriculture and Nutrition Company, Inc., Kansas 66110.

[b] Per 8 oz.

APPENDIX 3

VITAMIN AND MINERAL SUPPLEMENT ADDED TO THE WATER IN THE THIOURACIL EXPERIMENT[a]

Vitamin A	5,000,000 i.u.[b]
Vitamin D_3	750,000 i.u.
Vitamin E	2,500 i.u.
Riboflavin	500 mg
d-Pantothenic acid	4,000 mg
Calcium d-pantothenate	4,384 mg
Potassium &	1,000 mg

[a] Manufactured by Salsbury Laboratories, Inc., Charles, Iowa, 50616-9989.

[b] Per 8 oz.

REFERENCES

Abou-Donia, M.B., D.G. Graham, R.T. Philip, and B.L. Reichart, 1979. Delayed neurotoxic and late acute effects of S,S,S-tributyl phosphorotrithioate on the hen: Effect of route of administration. Neurotoxicol. 1:425-447.

Abou-Donia, M.B., 1981. Organophosphorus ester-induced delayed neurotoxicity. Ann. Rev. Pharmacol. Toxicol. 21:511-548.

Abou-Donia, M.B. and D.G. Graham, 1979. Delayed neurotoxicology of a single oral dose of 0-ethyl 0-4-cyanophenyl phenylphosphono-thioate in the hen. Neurotoxicol. 1:449-466.

Abou-Donia, M.B., M.A. Othman, G. Tantawy, A.Z. Khalil, and M.F. Shawer, 1974. Neurotoxicity effect of leptophos. Experientia 30:63-64.

Abou-Donia, M.B., D.G. Graham, M.A. Ashry, and P.R. Timmons, 1980. Delayed neurotoxicity of leptophos and related compounds: Differential effects of subchronic oral administration of pure, technical grade, and degradation products on the hen. Toxicol. Appl. Pharmacol. 53:150-163.

Abou-Donia, M.B., D.G. Graham, K.M. Abdo, and A.A. Komeil, 1979. Delayed neurotoxic, late acute, and cholinergic effects of S,S,S-tributyl phosphorotrithioate (DEF): Subchronic (90 days) administration in hens. Toxicol. 14:229-243.

Abou-Donia, M.B., D.G. Graham, P.R. Timmons, and B.L. Reicherrt, 1980. Late acute, delayed neurotoxic, and cholinergic effects of S,S,S-tributyl phosphorotrithioate (Merphos) in hens. Toxicol. Appl. Pharmacol. 53:439-557.

Ahmad, M.M. and P. Glees, 1971. Neurotoxicity of tricresyl phosphate (TCP) in slow loris (Nycticebus coucang coucang). Acta Neuropath. (Berl.) 19:94-98.

Archibald, J., D.C. Blood, J.A. Henderson, P.M. Newberne, G.H. Snoeyenbos, and W.L. Weipers, 1979. **The Merck Veterinary Manual, A Handbook of Diagnosis and Therapy for the Veterinarian.** Fifth Edition, Merck and Co., Inc.

Barnes, J.M. and F.A. Denz, 1953. Experimental demyelination with organophosphorus compounds. J. Path. Bacteriol. 65:597-605.

Baron, R.L., 1981. Delayed neurotoxicity and other consequences of organophosphate esters. Ann. Rev. Entomol. 26:29-48.

Beck, B.E., C.D. Wood, and G.R. Whenham, 1977. Triaryl phosphate poisoning in cattle. Vet. Pathol. 14:128-137.

Bickford, A.A., 1982. Sources of hens for laboratory use and background neuropathology. Neurotox. 3(4):315-320.

Bickford, A.A. and G.L. Sprague, 1982. Critical neurohistopathologic evaluation of adult specific-pathogen-free (SPF) Single Comb White Leghorn hens. 3(4):321-332.

Bidstrup, P.L., J.A. Bonnell, and A.G. Beckett, 1953. Paralysis following poisoning by a new organic phosphorus insecticide (Mipafox) report on two cases. Brit. Med. J. May 16, pp. 1068-1072.

Bischoff, A., 1970. Ultrastructure of tri-ortho-cresyl-phosphate poisoning in the chicken. II. Studies on spinal cord alteration. Acta Neuropath. (Berl.) 15:142-155.

Bondy, H.F., E.J. Field, A.N. Worden, and J.P.W. Hughes, 1960. A study on the acute toxicity of the triaryl phosphates used as plasticizers. Brit. J. Ind. Med. 17:190-200.

Bondy, D., S.J. Bursian, and F.W. Edens, 1980. Neurochemical alterations induced by manganese chloride in neonatal rats. Neurotoxicol. 2:65-73.

Bouldin, T.W. and J.B. Cavanagh, 1979. Organophosphorus neuropathy. I. A teased-fiber study of the spatio-temporal spread of axonal degeneration. Am. J. Pathol. 94:241-525.

Bouldin, T.W. and J.B. Cavanagh, 1979. Organophosphorus neuropathy. II. A fine-structural study of the early stages of axonal degeneration. Am. J. Pathol. 94:253-270.

Brown, A.W., J.B. Cavanagh, R.D. Verschoyle, H.B. Gysberbs, H.B. Jones, and W.N. Alderidge, 1984. Evolution of the intercellular changes in neurons caused by trimethyltin. Neuropathol. Appl. Neurobiol. 10:267-283.

Bursian, S.J., J.S. Brewster, and R.K. Ringer, 1983. Differential sensitivity to the delayed neurotoxin tri-o-tolyl phosphate in several avian species. J. Toxicol. Environ. Health 11:907-916.

Bradley, W.A., 1976. The pathology of delayed neurotoxicity due to organophosphates. Environmental Health Effects Research Series. Pesticide Induced Delayed Neurotoxicity. Proceedings of a Conference, EPA-60011-76-025. pp. 84-101.

Catterall, A.W., 1984. The molecular basics of neuronal excitability. Sci. 223(4637):653-661.

Casida, J.E. and R.L. Baron, 1976. Recognition and overview of the organophosphorus induced delayed neurotoxicity problem. Environmental Health Effects Research Series. Pesticide Induced Delayed Neurotoxicity. Proceedings of a Conference. EPA 600/1-76-025. pp. 7-23.

Casida, J.E. and R.L. Baron, 1979. Recognition and overview of the organophosphorus induced delayed neurotoxicity problem. EPA.

Cavanagh, J.B., 1964a. The significance of the "Dying Back" process in experimental and human neurological disease. Int. Rev. Expt. Path. 3:219-267.

Cavanagh, J.B., 1964b. Peripheral nerve changes in ortho-cresyl-phosphate poisoning in the cat. J. Path. Bact. 87:365-383.

Cavanagh, J.B., 1973. Peripheral neuropathy caused by chemical agents. Crit. Rev. Toxicol. 2:365-417.

Cavanagh, J.B., 1954. The toxic effects of tri-ortho-cresyl phosphate on the nervous system. J. Neurol. Neurosurg. Psy. 17:163-170.

Cavanagh, J.B., D.R. Davies, D. Halland, and M. Lancaster, 1961. Comparison of functional effects of dyflos, tri-o-cresyl phosphate, and tri-p-ethylphenyl phosphate in chickens. Brit. J. Pharmacol. 17:21-27.

Committee on Judiciary, United States Senate, 1977. **The Environmental Protection Agency and the Regulation of Pesticides.** Appendix to Staff Report to the Subcommittee on Administrative Practice and Procedure, U.S. Government Printing Office, pp. 762.

Cranmer, J.M. and E.L. Hixson, 1984. **Delayed Neurotoxicity.** Intox Press.

Davies, D.T., L.P. Goulden, B.K. Follett, and N.L. Brown, 1976. Testosterone feedback on luteinizing hormone (LH) secretion during a photoperiodically induced breeding cycle in Japanese quail. Gen. Comp. Endocrin. 30:477-486.

Drakontides, A.B., T. Baker, and W.F. Riker Jr., 1982. A morphological study of the effect of glucocorticoid

treatment on delayed organophosphorus neuropathy. Neurotoxicol. 3(4):165-178.

Dudek, B.R. and R.J. Richardson, 1982. Evidence for the existence of neurotoxic esterase in neural and lymphatic tissues of the adult hen. Biochem. Pharmacol. 31(6):1117-1121.

Ehrich, M., R.W. Briles, W.E. Briles, E.A. Dunnington, A. Martin, P.B. Siegel, and W.B. Gross, 1986. Neurotoxicity of tri-ortho-tolyl phosphate in chickens of different genotypes in the presence and absence of deoxycorticosterone. Poultry Sci. 65:375-379.

Environmental Protection Agency, 1976. U.S. Environmental Health Effect Series. EPA-600/1-76-025.

Farner, D.S. and J.R. King, 1973. **The peripheral endocrine glands, Avian Biology, Vol. II.** Academic Press, New York. pp. 183-236.

Fenton, J.C.B., 1955. The nature of the paralysis in chickens following organophosphorus poisonings. J. Path. Bact. Vol. IXIX.

Francis, C.G and G.S. Hoskin, 1985. Inhibition of A-soman and diisopropyl-phosphofluoridate (DFP)-detoxifying enyzyme by Mipafox. Biochem. Pharmacol. 34(12):2069-2072.

Francis, B.M., R.L. Metcalf, and L.G. Hansen, 1985. Toxicity of organophosphorus esters to laying hens after oral and dermal administration. J. Environ. Sci. Health B20(1):73-95.

Franklyn, J.A., T. Lyham, K. Docherty, D.B. Ramsden, and M.C. Sheppard, 1986. Effect of hypothyroidism on pituitary cytoplasmic concentrations of messenger RNA encoding thyrotrophin B and L subunits, prolactin, and growth hormone. J. Endocrin. 108:43-49.

Gary, R.H., K. Karen, and S.L. Flegler, 1979. Exercises in electron microscopy. A Laboratory Manual for Biological and Medical Sciences. Michigan State University.

Gill, J.L., 1978. **Design and Analysis of Experiments in the Animal and Medical Sciences.** Vol. 1. The Iowa State University Press.

Goth, A., 1984. **Medical Pharmacology.** The C.V. Mosby Co., 11th edition.

Harvey, S., C.G. Scanes, D.K. Chadwick, and N.J. Bolton, 1979. Growth hormone and prolactin secretion in growing domestic fowl. Influence of sex and breed. Brit. Poultry Sci. 20:9-17.

Hern, J.E.C., 1971. Some effects of experimental organophosphorus intoxication in primates. D.M. Thesis, University of Oxford.

Jajer, K.W., D.V. Roberts, and A. Wilson, 1970. Neuromuscular function insecticide workers. Brit. J. Ind. Med. 27:273-278.

Jajer, K.W., 1976. Organophosphate exposure from industrual usage electraneuromyography in occupational medical

supervision of exposed workers. Environmental Health Effects Research Series, Pesticide Induced Delayed Neurotoxicity Proceedings of a Conference. EPA-600/1-76-025. pp. 152-160.

Johnson, M.K., 1970. Organophosphorus and other inhibitors of neurotoxic esterase and the development of delayed neurotoxicity in hens. Biochem. J. 120:523-531.

Johnson, M.K. and R.R. Lauwerys, 1969. Protection by some carbamates against the delayed neurotoxic effect of diisopropyl phosphorofluoridate. Nature (London) 222:1066-1067.

Johnson, M.K., 1974. The primary biochemical lesion to the delayed neurotoxic effects of some organophosphorus compound. J. Neurochem. 23:785-789.

Johnson, M.K., 1975. Organophosphorus esters causing delayed neurotoxic effects. Mechanism of action and structure-activity studies. Arch. Toxicol. 31:259-288.

Johnson, M.K., 1969. The delayed neurotoxic effect of some organophosphorus compounds. Identification of the phosphorylation site as an esterase. Biochem. J. 114:711-717.

Johnson, M.K. and J.M. Barnes, 1970. Age and the sensitivity of chicks to the delayed neurotoxic effects on some organophosphorus compounds. Biochem. Pharmacol. 19:3045-3047.

Johnson, M.K., 1975. Organophosphorus esters causing delayed neurotoxic effects. Mechanism of action and structure-activity studies. Arch. Toxicol. 31: 259-288.

Johnson, M.K., 1975. Structure-activity relationships for substrates and inhibitors of hen brain neurotoxic esterase. Biochem. Pharmacol. 24:797-805.

Johnson, M.K., 1977. Improved essay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. Arch. Toxicol. 37:113-115.

Johnson, M.K., 1978. Anomalous behavior of dimethyl phosphates in the biochemical test or delayed neurotoxicity. Arch. Toxicol. 41:107-110.

Johnson, M.K., 1980. The mechanism of delayed neuropathy caused by some organophosphorus esters: Using the understanding to improve safety. J. Environ. Sci. Health BIS(6):823-841.

Johnson, M.K., 1982. Initiation of organophosphate-induced delayed neuropathy. Neurobehav. Toxicol. Teratol. 4:759-765.

Johnson, M.K., 1987. Receptor of enzyme: The puzzle of NTE and organophosphate-induced delayed poly-neuropathy. T.I.P.S. 8:174-197.

Julian, R.J., D.E. Galt, and D. Butler, 1975. Diagnosis of tri-ortho-cresyl phosphate poisoning. Proceeding 7, 18th Annual Meeting of the American Association of Vet. Lab. Diagnosticians. 7:407-418.

Kumaran, J.D.S. and C.W. Turner, 1949a. The endocrinology of spermatogenesis in birds. I. Effect of estrogen and androgen. Poultry Sci. 28:593-602.

Kumaran, J.D.S. and C.W. Turner, 1949b. The endocrinology of spermatogenesis in birds. II. The effect of androgens. Poultry Sci. 28:739-746.

Kruckenberg, S.M., A.C. Strafuss, W.P. Marsland, B.S. Blauch, R.T. March, J.G.E. Vestweber, and W.D. Winter, 1973. Posterior paresis in swine due to dermal absorption of neurotoxic organophosphate tri-ortho-tolyl phosphate. Am. J. Vet. Res. 34:403-404.

Larsen, C., B.S. Jortner, and M. Ehrich, 1986. Effect of neurotoxic organophosphorus compounds in turkeys. J. Toxicol. Environ. Health 17:365-374.

Lax, E.R., F. Rumstadt, H.P. Plasczyk, and H. Schriefers, 1983. Antagonistic action of estrogens, flutamide, and human growth hormone on androgen-induced changes in the activities of some enzymes of hepatic steroid metabolism in the rat. Endocrin. 113:1043-1055.

Lillie, R.D. and M.I. Smith, 1932. The histopathology of some neurotoxic phenol esters. NIH Bull. 160:54-62.

Lotti, M. and M.K. Johnson, 1978. Neurotoxicity of organophosphorus pesticides: Predictions can be based on vitro studies with hen and human enzymes. Arch. Toxicol. 41:215-221.

Lotti, M. and M.K. Johnson, 1980. Neurotoxic esterase in human nervous tissue. J. Neurochem. 34(3):747-749.

Luna, L.G., 1968. **Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology.** Third edition. McGraw-Hill Book Co.

Maydew, M.A., S.M. Krukenberg, D.A. Schoneweis, J.E. Cook, and S.M. Dennis, 1984. Clinical signs and histopathologic changes of the spinal cord in pigs treated with tri-ortho-cresyl phosphate. **Delayed Neurotoxicity.** Granmer, J.M. and J.E. Xixon (eds.), Intox., Little Rock, AR.

Metcalf, R.L., 1982. Historical perspective of organophosphorus ester-induced delayed neurotoxicity. Neurotoxicol. 3(4):269-284.

Mountcastle, B.V., 1974. **Medical Physiology.** 13th edition, Vol. 2, Mosby.

Olajos, E.J., A.P. DeCaprio, and I. Rosenblum, 1978. Central and peripheral neurotoxic esterase activity and dose response relationship in adult hens after acute and chronic oral administration of diisopropyl fluorophosphate. Ecotoxicol. Environ. Safety 2:383-399.

Olajos, E.J. and I. Rosenblum, 1979. Measurement of neurotoxic esterase activity in various subcellular fractions of hen brain and sciatic nerve homogenates and the effect of diisopropyl fluorophosphate (DFP) administration. Ecotoxicol. Environ. Safety 3:18-28.

Olson, B.A. and S.J. Bursian. The effect of route of administration on the development of organophosphate-induced delayed neurotoxicity in four-week old chicks. J. Toxicol. Environ. Health (in press).

Richardson, R.J., C.S. Davis, and M.K. Johnson, 1979. Subcellular distribution of marker enzymes and of neurotoxic esterase in adult hen brain. J. Neurochem. 32:607-615.

Scanes, C.G., J.H. van Middelkoop, P.J. Sharp, and S. Harvey, 1980. Strain differences in the blood concentrations of luteinizing hormone, prolactin, and growth hormone in female chickens. Poultry Sci. 59:159-163.

Smith, M.I., E.W. Engel, and F.F. Stohlman, 1932. Further studies on the pharmacology of certain phenol esters with special references ot the relation of chemical constitution and physiologic action. Nat. Inst. Health Bull. 160:1-53.

Smith, H.V. and J.M.K. Spalding, 1959. Outbreak of paralysis in Morocco due to ortho-cresyl-phosphate poisoning. Lancet. 11:1019-1021.

Somkuti, S.G., D.M. Lapadula, R.E. Chapin, J.C. Lamb, and M.B. Abou-Donia, 1986. The morphogenesis and ultrastructural changes of the tri-o-cresyl phosphate (TOCP) induced testicular lesion in F344 rats. The Toxicologist. Abstracts of the 25 Anniv. Mtg., Vol. 6(1).

Somkuti, S.G., D.M. Lapadula, R.E. Chapin, J.C. Lamb, and M.B. Abou-Donia, 1987a. Reproductive tract lesions resulting from subchronic administration (63 days) of tri-o-cresyl phosphate in male rats. Toxicol. Appl. Pharmacol. 98:49-63.

Somkuti, S.G., D.M. Lapadula, R.E. Chapin, J.C. Lamb, and M.B. Abou-Donia, 1987b. The course of the tri-o-cresyl phosphate-induced testicular lesion in F-344 rats. Enzymatic, hormonal, and sperm parameter studies. Toxicol. Appl. Pharmacol. 89:64-72.

Sterman, A.B. and N. Sposito, 1984. Motorneuron axosomatic synapses are altered in axonopathy. J. Neuropath. Exptl. Neurol. 43(2):201-209.

Sturkie, P.D., 1976. **Avian Physiology**. Third edition, Springer-Verlag.

Tanabe, Y., T. Nakamura, K. Fujioka, and O. Doi, 1979. Production and secretion of sex steroid hormones by the testes, ovary, and adrenal glands of embryonic and young chickens (Gallus domesticus). Gen. Comp. Endocrin. 39:26-33.

Tanabe, Y., 1982. Onto-genetic aspects of steroid genesis with special reference to corticosteroidogenesis of the chicken (Gallus domesticus). **Aspect of Avian Endocrinology Practical and Theoretical Implications.** C.G. Scanes (ed.), Grad. Studies, Texas Tech. University 26:1-411.

Taylor, J.D., 1967. A neurotoxic syndrome produced in cats by a cyclic phosphate metabolism of tri-o-cresyl phosphate. Toxicol. Appl. Pharmacol. 11(3):197-205.

Veronesi, B., 1984. A rodent model of organophosphorus induced delayed neuropathy distribution of central (spinal cord) and peripheral nerve damage. Neuropath. Appl. Neurobiol. 10:357-368.

Veronesi, B., S. Padilla, and D. Lyerly, 1986. The correlation between neurotoxic esterase inhibition and mipafox induced neuropathic damage in rats. Neurotoxicol. 7:207:216.

Wilson, B.W., C.M. Cisson, W.R. Randall, J.E. Woodrow, J.N. Seiber, and J.B. Knaak, 1980. Organophosphate risk assessment. Field testing of DEF with scaleless chickens. Bull. Environ. Contam. Toxicol. 24:921-928.