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## INTRODUCTION

It has been proposed that organophosphates (OPs) and carbamates may have acted together in triggering low-grade neurological syndromes in a small cohort of Gulf War veterans (Jamal *et al.*, 1996; Haley and Kurt, 1997). There have been suggestions, for example, that prophylactic use of pyridostigmine bromide (PB) may have increased susceptibility to OP compounds (pesticides, nerve agents) present in the S.W. Asia theater of operations (Abou-Donia *et al.*, 1996). Some allied troops may have been exposed to low airborne concentrations of sarin in Coalition-occupied Iraq, and there is concern that such agents may be used offensively in future military and civilian conflicts. Recent experience with civilian exposures to sarin in Japan (Yokoyama *et al.*, 1998) have challenged the widely held belief that sarin is unable to trigger the peripheral neuropathy that follows OP-induced actions on neuropathy target esterase (NTE). High doses of sarin (30-60 times the LD<sub>50</sub>) are known to bring about organophosphate induced delayed neuropathy (OPIDN) in the chicken (Davies *et al.*, 1960; Gordon *et al.*, 1983). Additionally, the low-grade neurological syndromes reported in Gulf War veterans have been interpreted as mild forms of OPIDN (Haley and Kurt, 1997). This is a central and peripheral nervous system (CNS-PNS) distal axonopathy that is readily induced in hens treated with diisopropyl fluorophosphate (DFP) or tri-ortho-cresylphosphate (TOCP). TOCP has not been reported to induce the experimental myopathy associated with sarin, PB and other inhibitors of acetylcholinesterase (AChE; Dettbarn, 1984).

A goal of this research is to examine the hypothesis that PB may promote sarin's ability to induce muscle damage and axonal degeneration. The study is directed toward the following specific questions: (1) Will single and repeated low-level exposures to sarin and PB produce neurological and neuromuscular damage, respectively, in selected laboratory species chosen for their demonstrated sensitivity to OPIDN (hen) and ACh-induced myopathy (rodent)? (2) Even though motor endplates are vulnerable to damage after acute cholinesterase (ChE) inhibition and consequent excessive ACh receptor stimulation, will sensory nerve twigs and endings be more vulnerable than equivalent parts of motor fibers at the commencement of OPIDN? (3) Will PB exacerbate sub-clinical, sarin-induced CNS-PNS axonopathy when the carbamate is administered after the OP?

## BODY

### Materials

Strain Cd1 male mice were purchased from Charles River. White leghorn hens were purchased from Lakeview Farms as day-old chicks and raised in Animal Science Department facilities at UC Davis. Dilute sarin (XGB) was supplied by the US Army Medical Research Institute of Chemical Defense. Diisopropyl fluorophosphate (DFP), paraoxon (PO), atropine sulfate, and 2-pralidoxime (2-PAM) were purchased from Sigma Chemical Co. Tri-ortho-cresyl phosphate (TOCP) was purchased from Acros Organics.

### Methods

#### Animal Injections

Chickens were dosed by intramuscular (im) injection in the pectoral muscle. Chickens that received TOCP were dosed by subcutaneous (sc) injection at the inner thigh. Birds dosed with high levels of OP compounds were given 20 or 50 mg/kg atropine sulfate prior to the OP injection to prevent acute cholinergic effects. If necessary, 2-pralidoxime (2-PAM; 50 mg/kg) was administered intravenously (iv) to further alleviate acute effects (Wilson, *et al.*, 1988).

Mice were dosed by sc injection at the back of the neck. Atropine (1-2 mg/kg) and 2-PAM were administered as necessary to alleviate acute cholinergic effects of high OP doses. One of our objectives was to prevent convulsions and the consequent lack of oxygen in the brain and damage that might confound the histopathology.

#### Sacrifice

Mice and chickens were sacrificed by cervical dislocation for biochemical assessments. Animals were anesthetized and perfused with buffered fixative through the ascending aorta (as described below) for morphological assessments.

#### Tissue Sampling for Biochemistry

Chicken blood was drawn into heparinized syringes from the brachial (wing) or leg veins. The blood was centrifuged for 10 min at 1000 x g. The supernatant plasma was removed for ChE analysis (bird erythrocytes contain no cholinesterases). Brain and muscle were dissected after sacrifice. Tissues were kept on ice for immediate analysis, or frozen at -70°C for future study.

#### ChE Determinations

Samples were measured using the colorimetric method of Ellman, *et al.* (1961), modified for use with an automatic microplate reader.

#### Neuropathy Target Esterase Determinations

Brain neuropathy target esterase (NTE) activity was determined by the colorimetric method of Johnson (1977) as modified by Wilson's laboratory (Mackay, *et al.*, 1996) from that of Correll and Ehrich (1991).

#### Protein Determinations

Protein was measured by the method of Lowry (1951).

#### OPIDN Clinical Assessment

Chickens were observed in their cages for standing and walking behavior. Observations were also made while prompting stationary birds to move by approaching or reaching towards them. Birds were characterized as being in one of five clinical states. 1: normal. 2: loss of coordination; stumbles; rests on hocks, but is able to stand. 3: leg weakness; bird rests and moves on its hocks; is unable to stand. 4: paralysis; legs are held out straight; bird can move by back-pedaling; locomotion limited. 5: moribund; bird falls to its side; can't keep its body upright; bird can't move about.

#### Morphological and Semi-quantitative Analyses

Animals were anesthetized, heparinized, the chest and heart opened, and perfused through the ascending aorta with 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 10 sec followed by 5% glutaraldehyde in the same buffer for 10 min. Perfused animals were sent from the University of California at Davis (UC Davis) to Oregon Health & Science University (OHSU) for further processing. Selected tissues were dissected (Table 1). Tissues were placed in 0.1M sodium phosphate (pH 7.4), postfixed with 2% osmium tetroxide in the same buffer, dehydrated in ascending concentrations of ethanol, and embedded in Spurr's epoxy resin. One-micrometer-thick sections were stained with 1% toluidine blue and examined by bright-field microscopy. Thin sections were prepared as needed from sensory and motor nerve terminals, treated with 2% uranyl acetate and 1% lead citrate, and examined with a JEOL 100CX transmission electron microscope. Semi-quantitative light-microscopic assessment of neuropathological changes was made by two independent observers working without knowledge of the treatment schedule. Observed changes were scored as follows: 0 = normal no observable pathology; 1 = normal

appearance or non-specific changes (technically-induced); 2 = scattered abnormal cells (1-4 per low-magnification field); 3 = moderate numbers (5-8) of abnormal cells per low-magnification field, mostly early pathology observed; 4 = abnormal cells common and advanced pathology observed; 5 = advanced alterations, cellular destruction and cell loss. The assessment focused on the distal regions of peripheral nerves because reproducible changes can be detected there early in the disease process. The other tissue samples prepared in blocks are available if further assessment is required.

Animal dosing, perfusion, biochemistry and toxicology were carried out at the Institute of Toxicology and Environmental Health (ITEH) and the Department of Animal Science, located at UC Davis under the direction of P.I. Barry Wilson. Morphological assessment of sample tissues was carried out under subcontract at the Center for Research on Occupational and Environmental Toxicology (CROET), located at OHSU under the direction of co-P.I. Peter Spencer.

**Task One:** This consisted of “scoping” trials to establish appropriate dose/response ranges for sarin and control chemicals (DFP, TOCP and paraoxon).

#### TOCP Dosing in Mice: Trial I

As reported previously, tissues were inadvertently frozen during their shipment from University of California Davis to Oregon Health and Science University, Portland. Frozen tissues are not usable for morphological examination.

#### TOCP Dosing in Mice: Trial II

The mouse trial was repeated using higher levels of TOCP since no clinical signs of OPIDN were observed in the first trial (as reported in the 2000 annual report). Pairs of male Cd1 mice were given a single s.c. injection at the base of the neck of 0, 800, 1600, or 3200 mg/kg TOCP in corn oil vehicle. One animal treated with 3200 mg/kg was found dead one week after treatment, apparently killed by the other mouse treated at the same level. The mice were observed for 25 days after treatment, and no limb weakness was found. Animals were perfused and shipped to OHSU in an inner container surrounded by foam packing material; they were received in good condition. Seven animals were received for processing. From each animal the indicated tissues (see Table I) were processed into Spurr's epoxy plastic for examination by light and, if needed, by electron microscopy. Focus was placed on vulnerable distal regions of the tibial, peroneal and sural nerves, all of which in all animals were within normal limits (Table 2).

#### TOCP Mouse Trial III

Pairs of mice received a single s.c. injection at the base of the neck of 0, 3.2 g/kg (the highest dose in the previous trial), or 6.4 g/kg undiluted TOCP. The agent leaked from the injection site in one high-dose animal. A higher dose was planned but not used as it appeared a single dose with a larger volume of TOCP could not be administered to the mice.

Animals were observed for 49 days for signs of OPIDN. No clinical signs were seen. Six mice were perfused and sent to OHSU for histological assessment. Hindlimb nerves were sectioned, mounted, stained, and scored. Focus was placed on vulnerable distal regions of the tibial, peroneal and sural nerves, all of which in all animals were within normal limits (Figure 1). One animal treated with 6.4g/kg TOCP showed minor abnormalities in the medial peroneal and distal tibial nerve (Table 3).

Taken together, the three TOCP Mouse Trials established that single doses of TOCP fail to induce OPIDN in the strain of the species examined.

### TOCP Dosing Trial in Chickens II

These trials sought to determine a threshold dose for TOCP-OPIDN for use in experiments seeking interactive (synergistic) effects of TOCP + PB in relation to OPIDN. The previous trial determined that a single s.c. dose (base of leg) of 50 mg/kg TOCP resulted in slight clinical signs of OPIDN (stage 1/2 by 23 days), while 100 mg/kg TOCP induced moderate signs (stage 3 by day 21-26). An intermediate dose was judged suitable for this work. Birds therefore received a single s.c. dose (base of leg) of 0 mg/kg (n = 1), 50 mg/kg (n = 2), 75 mg/kg (n = 3), or 100 mg/kg (n = 2) undiluted TOCP. Animals were observed for clinical signs of OPIDN for up to 28 days. No clinical signs were observed in animals treated with 0, 50 (n = 2) or 75 mg/kg (n = 1) TOCP. The remaining 75 mg/kg and 100 mg/kg birds all showed stage 2 signs at 19-20 days, and stage 3 by 27-28 days. One of the 75 mg/kg birds progressed to stage 3 at day 21 and advanced to a stage intermediate of 3 and 4 at the end of observation period.

Hens were perfused and sent to OHSU for histological assessment. Vulnerable regions of the peripheral and central nervous system were sectioned, mounted, stained, and scored (Table 4). Relative to controls, animals treated with 75 or 100 mg/kg TOCP which displayed clinical signs of OPIDN, showed pathological changes in peripheral nerves (distal regions > proximal regions), spinal cord and medulla oblongata (Figure 2). The pattern and distribution of neuropathology matched that expected for OPIDN.

**Task Two:** To determine thresholds and relative dose-effect levels for biochemical and morphological end-points using multiple sarin exposures. This will be the basis to estimate the "highest no-effect dosage" (HNED) of sarin.

And

**Task Three:** Similar to Task Two, but the goal is to estimate the "highest no-effect dosage" (HNED) of PB.

### Multiple Dosing Trial in Chickens

The purpose of this trial was to assess the effect of multiple doses and levels of XGB (sarin) or PB on ChE levels and clinical status. DFP was chosen as the positive OPIDN control because of prior experience in using multiple low dosages of this agent to induce OPIDN (TOCP is an effective control only when single doses of agents are assessed).

Hens were dosed 20 times (5 days/week for 4 weeks) via i.m. injection in the pectoral muscle. Animals were treated in pairs with the following agents administered daily: atropine control (see below); 100 µg/kg DFP (positive OPIDN control); 25, 50 or 100 µg/kg XGB; 100, 250 or 500 µg/kg PB. The XGB treated birds received 20 mg/kg atropine 15-30 minutes prior to the sarin treatment. The atropine dose was raised to 50 mg/kg at the start of the second week. The atropine controls received the same dosage. The 100 µg/kg XGB birds also received 50 mg/kg 2-PAM i.v. (leg vein) immediately after the XGB dose; one of the 25 µg/kg XGB birds required 2-PAM on one occasion (at the start of the second week). The 2-PAM was given to birds that displayed marked acute toxic signs, including laying prone with the head down, often with eyes closed. One of the 100 µg/kg XGB birds was found dead the morning following the first dose (though it showed only mild signs such as sitting 2 hours after treatment). The other 100 µg/kg XGB bird died following the 5th dose. It had a mild convulsion while the 2-PAM was being administered.

Effects of dosing on body weight and plasma ChE levels were reported in the 2000 annual report. In summary, PB and atropine had minor effects on body weight. The XGB-treated birds had a

rapid weight loss of 15-25%, with recovery after dosing was stopped. The DFP controls showed a steady loss of weight over the first 37 days, even after dosing stopped. The DFP bird showing signs of OPIDN had lost close to 40% of its body weight by day 37. As expected, OP and carbamate treatment caused inhibition of plasma ChE. The atropine controls had no ChE inhibition, the 100 µg/kg PB-treated birds were inhibited 50% of initial ChE activity, and the other PB, DFP and XGB treatments resulted in ChE inhibitions  $\geq 80\%$ . ChE inhibition did not advance with repeated dosing.

Only one of the DFP positive controls showed clinical signs of OPIDN. It first showed stage 2 symptoms on day 23 (during the last week of dosing; it received the last 4 doses after showing signs). The OPIDN progressed to stage 3 on day 30, and to borderline stage 3/4 on 35. The bird remained at this stage until day 38, when it was perfused (along with one of the atropine controls) and shipped to OHSU for histological assessment. The remaining birds showed no clinical signs of OPIDN through day 50 (24 days after the last dose). These birds were perfused over days 50-52 and shipped to OHSU for histological assessment. From each of fourteen animals received, the following tissues (Table I) were sampled and are embedded in plastic. Samples were taken from a group of fourteen animals. The most distal nerves were sectioned, mounted, stained, and scored (Table 5).

Sampled nerves of animals treated with sarin or atropine were within normal limits. One of the six animals treated with PB (Hen #9, 100 µg/kg) had an atypical distal peroneal nerve but the tibial and sural nerves were within normal limits. Of the two animals treated with DFP, one was within normal limits and the other (Hen #16, 100µg/kg DFP) showed pathology in all three distal nerves. In sum, there was a variable individual animal response to 100 µg/kg DFP.

**Task Four:** Examine whether PB induces responses to sub-threshold doses of sarin or DFP when the carbamate is given before or after OP administration.

#### DFP/PB Multiple Dosing Trial

Groups of chickens ( $n = 3$ ) were dosed with atropine (50 mg/kg; control group), 100 µg/kg PB, 100 µg/kg DFP, 200 µg/kg DFP, 100 µg/kg DFP + PB, or 200 µg/kg DFP + PB. All birds received the stated dose of atropine, and PB treatment was given at the same time as the OP. Injections were given i.m. in the pectoral muscle; each agent was injected in a different area. Dosing was 5 days/week for up to 25 injections. Dosing ended when birds exhibited clinical signs of OPIDN.

Clinical signs of OPIDN were observed in the treatment groups as follows: no signs were seen in the atropine controls; no signs were seen in animals treated with 100 µg/kg PB; birds in the 100 µg/kg DFP group first exhibited stage 2 at day 22 and day 28, and the third bird progressed only to borderline stage 1/2 at day 34; the 200 µg/kg DFP birds reached stage 2 at day 17/18 and 2 birds reached stage 3 at day 21/22 (then perfused); only 1 of the 100 µg/kg DFP + PB birds reached stage 2, at day 37; the 200 µg/kg DFP + PB birds reached stage 2 at day 14/16 and stage 3 at day 17/21.

DFP-treated birds showing signs of OPIDN (stage 2 or 3) were perfused, along with Atropine and PB controls. The 200 µg/kg DFP birds were perfused at 21/22 days, and the 100 µg/kg DFP birds were perfused at either 29 or 42 days. The perfused chickens were sent to OHSU for histological assessment (see Table 1). There is a general correlation between the OPIDN clinical



stage observed (1 = normal to 5 = moribund) and the level of histological damage (0/1 = normal to 5 = advanced alterations, cellular destruction and cell loss).

The plasma ChE was strongly inhibited in the DFP-dosed birds (with or without PB); by approximately 90% in birds receiving 100 µg/kg DFP and 98% in birds dosed with 200 µg/kg DFP (Graph 1). The ChE was depressed to this level after the initial dose and the depression level was maintained throughout the period of treatment. Plasma ChE of birds receiving PB alone was initially inhibited 30%, with inhibition increasing to ~70% with subsequent carbamate dosing.

Microscopical examination of sampled hind limb nerves of all animals treated with PB was within normal limits (Figure 3). DFP showed distal nerve fiber damage in the absence (Figure 4A, 4C) and presence (Figure 4B, 4D) of concurrent PB treatment. In the absence of PB treatment, one animal (#30) showed little change, a second (#31) showed distal nerve changes, and a third (#43) showed changes distally and proximally (Table 6). Animals treated with 200 µg/kg DFP showed distal and proximal neuropathologic changes. However, one animal (#32) showed sural and peroneal damage in the absence of pathology in the tibial nerve, while others (#'s 30 & 32) showed advanced pathology at all sampled sites.

Given that pathological changes were reliably detected in sural and peroneal nerves, it is possible to use these sites to compare nerve fiber damage between DFP- and DFP/PB-treated animals. Graph 2 provides data that are consistent with a dose response for DFP alone and an enhanced dose response for DFP/PB-treated animals. However, one animal in each of the two groups of three did not fit this pattern. Thus, it would be inappropriate to conclude the presence of a dose-response for both DFP and DFP/PB because of the very small numbers of animals (n=2 per group) that showed suggestions of this pattern.

#### XGB/PB Multiple Dosing Trial

An experiment studying the effects of sarin in the presence and absence of PB is in progress. Groups of chickens (n = 3) are dosed with atropine (50 mg/kg; control group), 100 µg/kg PB, 100 µg/kg DFP, 100 µg/kg DFP + PB, 100 µg/kg XGB, or 100 µg/kg XGB + PB. All birds receive atropine. Injections were given i.m. (pectoral muscle); each agent is injected in a different area. Dosing is 5 days/week for 6 weeks. Blood samples are being taken for plasma ChE measurements. At the end of the dosing period, the birds will be observed for 3 weeks for clinical signs of OPIDN, and then perfused and shipped to OHSU for histological assessment. Treatment of animals with DFP (OPIDN positive control) was stopped after they developed neurological signs.

#### KEY RESEARCH ACCOMPLISHMENTS

- ◆ Mice treated with single large doses of TOCP developed no clinical or pathological signs of OPIDN.
- ◆ A single dose of TOCP near the no-effect level for OPIDN induction was determined in hens. For the birds that exhibited clinical signs, the pattern and distribution of neuropathology match that expected for OPIDN.
- ◆ Multiple doses (20 over 4 weeks) of sarin did not induce clinical or neuropathological signs of OPIDN in hens.

- ◆ Concurrent treatment of hens with multiple doses of DFP + PB appeared to result in a greater degree of nerve damage than in animals treated with DFP alone. There was variability amongst birds; additional study is needed to confirm this result.

## REPORTABLE OUTCOMES

Wilson BW, Henderson JD, Coatney EM, Nieberg PS and Spencer PS. 2001. Actions of pyridostigmine and organophosphate agents on chick cells, mice and chickens. Drug and Chemical Toxicology. In press.

Wilson BW, Henderson JD, Ramirez A, Kayton R and Spencer PS. 2001. Low level effects of pyridostigmine bromide and delayed neuropathy organophosphates in experimental animals. Proceedings of the Conference on Illnesses among Gulf War Veterans: A Decade of Scientific Research. Alexandria, Virginia. January 24-26, 2001.

Spencer PS, Henderson JD, Coatney EM, Nieberg PS and Wilson BW. 2001. Pyridostigmine and organophosphate agents actions on chick cells, mice and chickens. Presented at Society of Toxicology 40<sup>th</sup> Annual Meeting. San Francisco, California. March 25-29, 2001.

We thank reviewers of past reports for their comments and note the lack of research papers produced to date. The experiments are time-intensive, involving subchronic dosing of animals, the observation period for the delayed neuropathy, and the preparation and assessment of numerous histological specimens. These experiments are now coming to fruition.

## CONCLUSIONS

A low-level of TOCP in hens that induces OPIDN was determined. Mice treated with 85x this level of TOCP did not develop signs of OPIDN. Nevertheless, because others (Husain, *et al.*, 1993) have reported OP-induced nerve damage, multiple exposure experiments with TOCP, DFP, sarin and PB will be undertaken.

Multiple dose levels of DFP that induce OPIDN in hens were established. The effect of PB exposures alone and in conjunction with DFP was studied. Preliminary evidence has been obtained to suggest that repeated treatment with DFP + PB damages hen nerves to a greater degree than DFP alone.

An experiment with multiple exposures of GB in the presence or absence of PB is in progress. Included in this study are animals treated with DFP in the presence or absence of PB

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## APPENDICES

Table 1. Tissues Sampled for Morphological Assessment

Table 2. Morphological Assessment of Mice Treated with TOCP

Table 3. Morphological Assessment of TOCP-Treated Mice

Table 4. Morphological Assessment of Hens Treated with TOCP

Table 5. Morphological Assessment of Hens Treated with Multiple Doses of Sarin

Table 6. Morphological Assessment of Hens Treated with DFP +/- PB

Graph 1. ChE Inhibition in Hens Treated with DFP +/- PB

Graph 2. Average Morphological Scores of Nerve Damage in Hens Treated with DFP +/- PB

- Figure 1. Distal Sural Nerves Taken from Mice Treated with TOCP  
Figure 2. Medulla Oblongata Taken from Hens Treated with TOCP  
Figure 3. Distal Peroneal Taken from a Hen Treated with PB  
Figure 4. Proximal Sural Nerves Taken from Hens Treated with DFP +/- PB

**Table 1. Tissues Sampled for Morphological Assessment**

Frontal Cortex	Soleus Muscle
Basal Ganglia	Sural Nerve Proximal
Hippocampus	Sciatic Nerve Distal
Cerebellar Vermis	Diaphragm Muscle
Medulla Oblongata	Interosseous Muscle
Spinal Cord Cervical	Lumbrical Muscle
Spinal Cord Thoracic	Tibial Nerve Mid
Spinal Cord Lumbar	Tibial Nerve Distal
Lumbar Dorsal Root Ganglia	Sciatic Nerve Mid
Sciatic Nerve Proximal	Sural Nerve Mid
Sciatic Nerve Mid-thigh	Tibial Nerve Proximal
Gastrocnemius	Tibial Nerve at Ankle
Sural Nerve Distal	Peroneal Nerve Proximal
Peroneal Nerve Mid	Peroneal Nerve Distal

**Table 2. Morphological Assessment of Mice Treated with TOCP**

<b>Animal</b>	<b>TOCP Dose</b>	<b>Histological Score</b>	<b>Examined Tissue</b>
5A	0 mg/kg	1	Peroneal Nerve Distal
		1	Sciatic Nerve Distal
		0	Sural Nerve Distal
		1	Tibial Nerve Distal
5B	0 mg/kg	0	Peroneal Nerve Distal
		1	Sural Nerve Distal
		2	Tibial Nerve Distal
6A	0.8mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
6B	0.8mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		0	Tibial Nerve Distal
7A	1.6 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
7B	1.6 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
8B	3.2 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal

**Table 3. Morphological Assessment of TOCP-Treated Mice**

<b>Animal</b>	<b>TOCP Dose</b>	<b>Histological Score</b>	<b>Examined Tissue</b>
15	0 mg/kg	0	Sural Nerve Distal
		1	Tibial Nerve Distal
16	0 mg/kg	0	Peroneal Nerve Distal
		0	Spinal Cord Cervial
		1	Tibial Nerve Distal
13	3.2 g/kg	0	Peroneal Nerve Distal
		0	Sural Nerve Distal
		1	Tibial Nerve Distal
14	3.2 g/kg	1	Peroneal Nerve Distal
		0	Sural Nerve Distal
		0	Tibial Nerve Distal
11	6.4 g/kg	2	Peroneal Nerve Medial
		0	Sural Nerve Distal
		2	Tibial Nerve Distal
12	6.4 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
		1	Medulla Oblongata

**Table 4. Morphological Assessment of Hens Treated with TOCP**

Animal	TOCP Dose	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
19	0 mg/kg	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Sural Nerve Distal
			1	Sural Nerve Proximal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			1	Spinal Cord Lumbar
			1	Medulla Oblongata
22	50 mg/kg	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
25	50 mg/kg	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
			1	Spinal Cord Cervical
2	Medulla Oblongata			
17	75 mg/kg	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			0	Tibial Nerve Distal
18	75 mg/kg	3/4	3	Peroneal Nerve Distal
			3	Peroneal Nerve Proximal
			3	Sural Nerve Distal
			3	Sural Nerve Proximal
			3	Tibial Nerve Distal
			3	Tibial Nerve Proximal
			3	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			2	Medulla Oblongata



**Table 4. Morphological Assessment of Hens Treated with TOCP (Cont.)**

<b>Animal</b>	<b>TOCP Dose</b>	<b>OPIDN Sign at Perfusion</b>	<b>Histological Score</b>	<b>Examined Tissue</b>
20	75 mg/kg	3	3	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			2	Medulla Oblongata
23	100 mg/kg	3	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			4	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			4	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			1	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
24	100 mg/kg	3	2	Medulla Oblongata
			3	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			5	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			3	Medulla Oblongata
3	Tibial Nerve Proximal			

**Table 5. Morphological Assessment of Hens Treated with Multiple Doses of Sarin**

<b>Animal</b>	<b>Treatment</b>	<b>Score</b>	<b>Tissue</b>
1	Sarin 25 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		0	Tibial Nerve Distal
2	Sarin 25 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
3	Sarin 50 ug/kg	0	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
4	Sarin 50 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1.5	Tibial Nerve Distal
7	Atropine 20 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
8	Atropine 20 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
9	PB 0.1 mg/kg	5	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
10	PB 0.1 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
11	PB 0.25 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
12	PB 0.25 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
13	PB 0.5 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
14	PB 0.5 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
15	DFP 100 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
16	DFP 100 ug/kg	2	Peroneal Nerve Distal
		2	Sural Nerve Distal
		4	Tibial Nerve Distal

**Table 6. Morphological Assessment of Hens Treated with DFP +/- PB**

Animal	Dose Group	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
26	Atropine	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Tibial Nerve Distal
27	Atropine	1	0	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
39	Atropine	1	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
28	PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
29	PB	1	0	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
42	PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
30	DFP (100)	2	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Lumbar Dorsal Root Ganglia
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
31	DFP (100)	1.5	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Lumbar Dorsal Root Ganglia
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			2	Tibial Nerve Distal
			1	Tibial Nerve Proximal
43	DFP (100)	2	2	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			2	Sural Nerve Proximal
			2.5	Tibial Nerve Distal
			3	Tibial Nerve Proximal

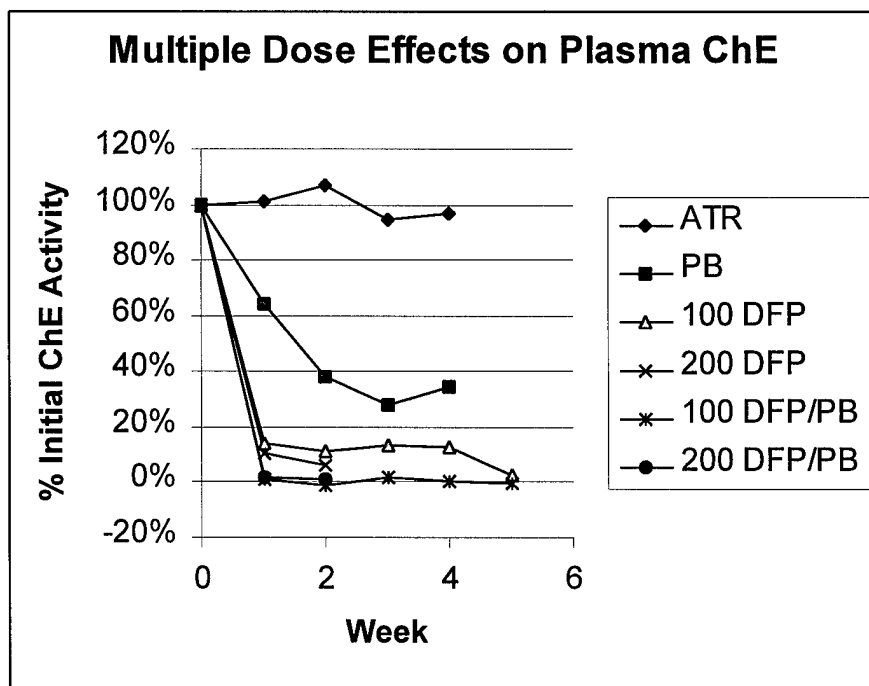
Table 6. Morphological Assessment of Hens Treated with DFP +/- PB (Cont.)

Animal	Dose Group	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
32	DFP (200)	2	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1.5	Sural Nerve Distal
			4	Sural Nerve Proximal
			1	Tibial Nerve Distal
			2	Tibial Nerve Proximal
			1	Sciatic Nerve Proximal
33	DFP (200)	3	3	Peroneal Nerve Distal
			3	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			2	Sural Nerve Proximal
			2.5	Tibial Nerve Distal
			4	Tibial Nerve Proximal
44	DFP (200)	3	2	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Tibial Nerve Proximal
34	DFP (100)/PB	2.5	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			3	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			2	Tibial Nerve Proximal
35	DFP (100)/PB	1	2	Peroneal Nerve Distal
			1.5	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			2	Tibial Nerve Distal
			1.5	Tibial Nerve Proximal
45	DFP (100)/PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
36	DFP (200)/PB	3	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			4	Tibial Nerve Distal
			4	Tibial Nerve Proximal

**Table 6. Morphological Assessment of Hens Treated with DFP +/- PB (Cont.)**

<b>Animal</b>	<b>Dose Group</b>	<b>OPIDN Sign at Perfusion</b>	<b>Histological Score</b>	<b>Examined Tissue</b>
38	DFP (200)/PB	3	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			3	Tibial Nerve Distal
			4	Tibial Nerve Proximal
46	DFP (200)/PB	3	1	Peroneal Nerve Distal
			2	Sural Nerve Distal
			1	Tibial Nerve Distal

PB dose is 100 ug/kg; DFP dose is number in parentheses, in ug/kg.

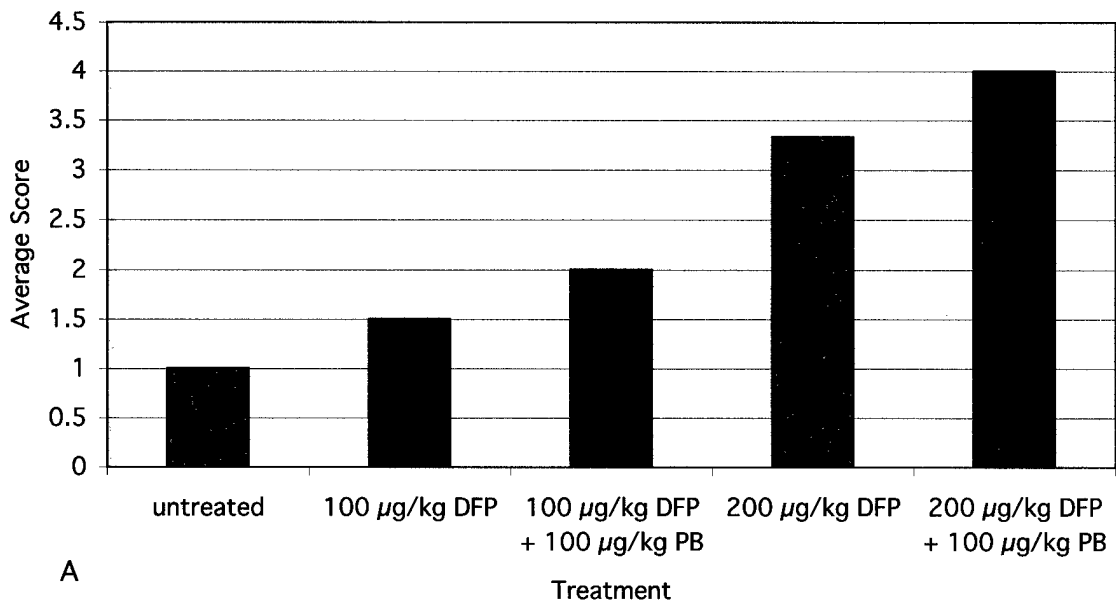


**Graph 1. ChE Inhibition in Hens Treated with DFP +/- PB**

Graph 2. Graphic representations of averaged scores for the proximal sural (A) and proximal peroneal (B) nerves.

- A. Demonstrates the semi quantitative trend for greater damage when DFP and PB are in combination. This trend is seen for both concentrations of DFP.
- B. This graph demonstrates the same trend for the proximal peroneal nerve.

### Sural Proximal



### Peroneal Proximal

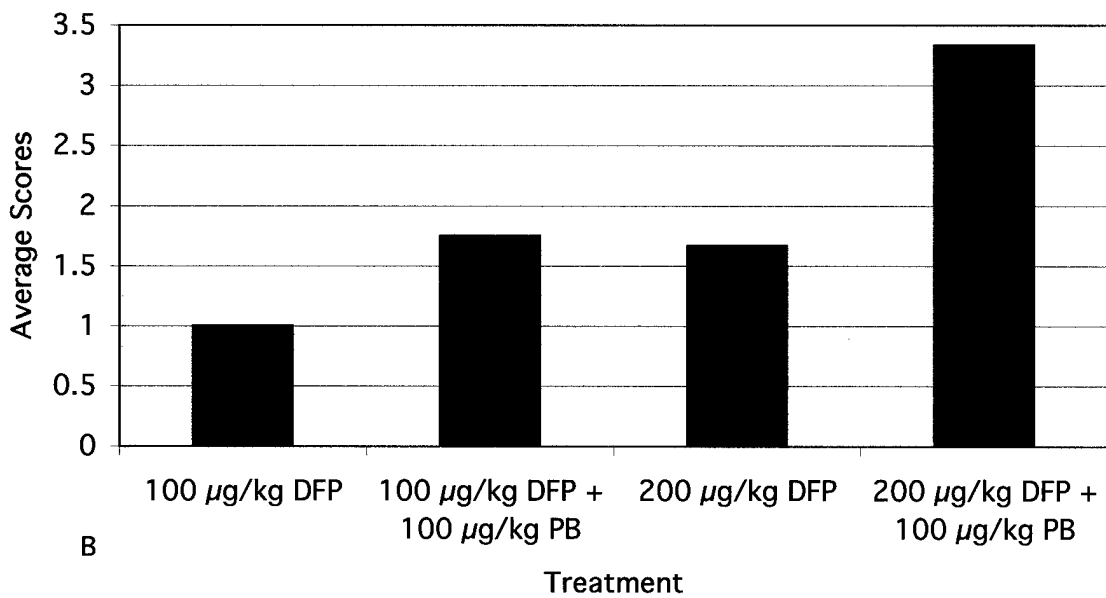
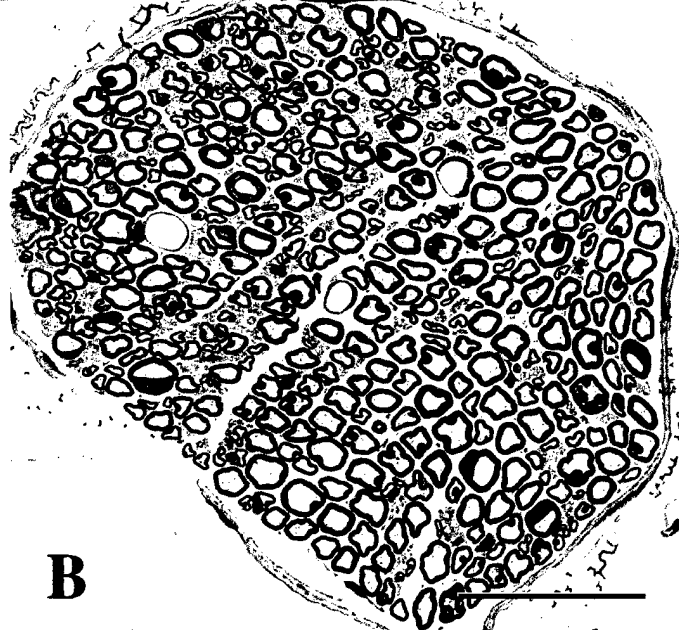
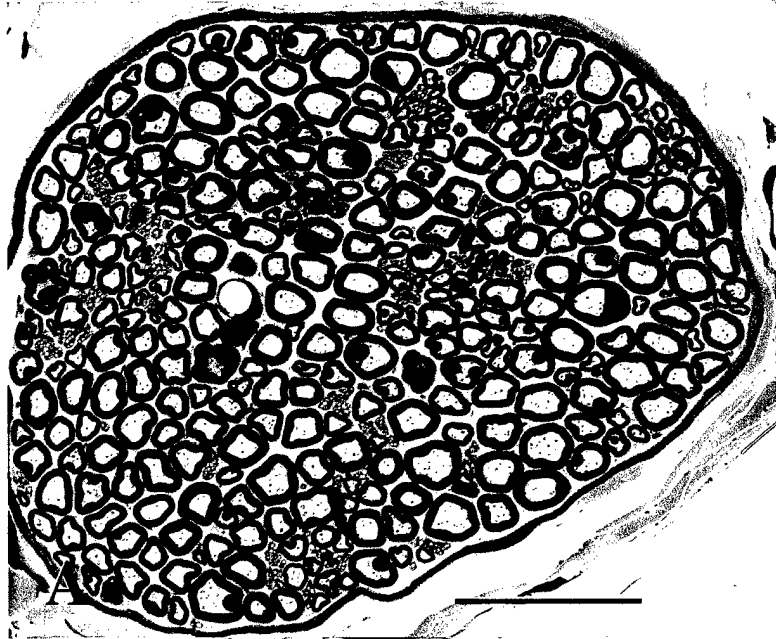




Figure 1. Cross sections of mouse distal sural nerves from the same level (ankle) of mice treated with TOCP. Mice were given single s.c. injections (base of neck). Animals were perfused 25 days after treatment. All of these nerves appear normal, with only preparation artifacts evident.

- A. Control mouse (#15) distal sural nerve (Bar = 38  $\mu\text{m}$ ).
- B. Distal sural nerve from a mouse (#13) dosed with 3.2 g/kg TOCP (Bar = 47  $\mu\text{m}$ ).
- C. Distal sural nerve from mouse (#12) dosed with 6.4 g/kg TOCP (Bar = 38  $\mu\text{m}$ ).



**B**

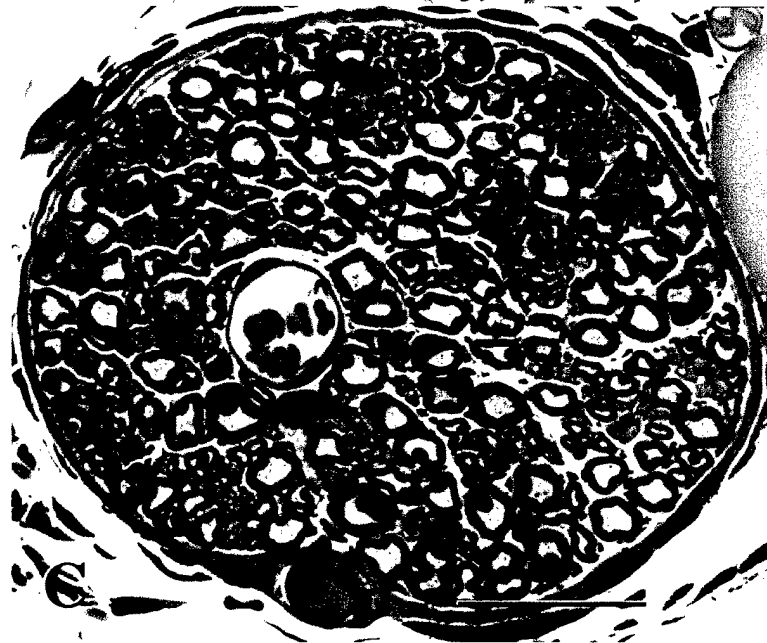


Figure 2. Cross sections of hen medulla oblongata showing areas of myelinated nerve fibers in tracts corresponding to the distal ends of ascending spinal pathways. Hens were given a single s.c. injection (base of leg). Animals were perfused 27-28 days after treatment.

- A. #19 (control), showing normal morphology (stage 0-1) (bar = 40  $\mu\text{m}$ ).
- B. #18 (75 mg/kg TOCP) showing extensive myelinated nerve fiber degeneration (arrows). Arrows denote stages in axonal degeneration and asterisks are intact fibers (stage 2) (bar = 44  $\mu\text{m}$ ).
- C. #24 (100 mg/kg TOCP) showing similar stages of degeneration as B (arrows) (stage 3) (bar = 56  $\mu\text{m}$ ).
- D. #23 (100 mg/kg TOCP) demonstrating primary axonal degeneration (arrows) . Note myelinated nerve fibers that are normal in appearance (asterisks) (stage 2) (bar = 20  $\mu\text{m}$ ).

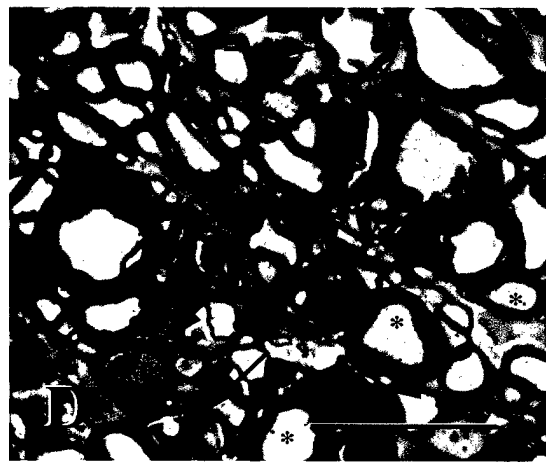
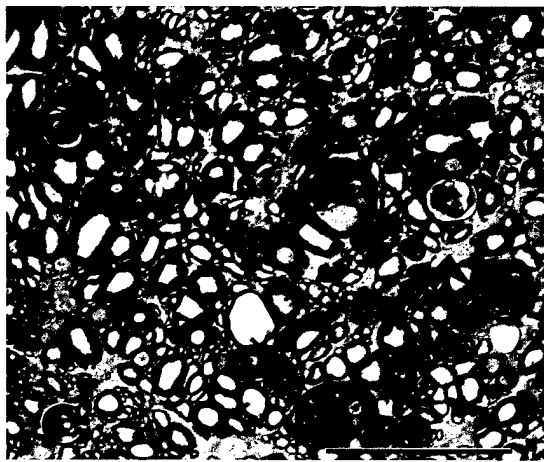
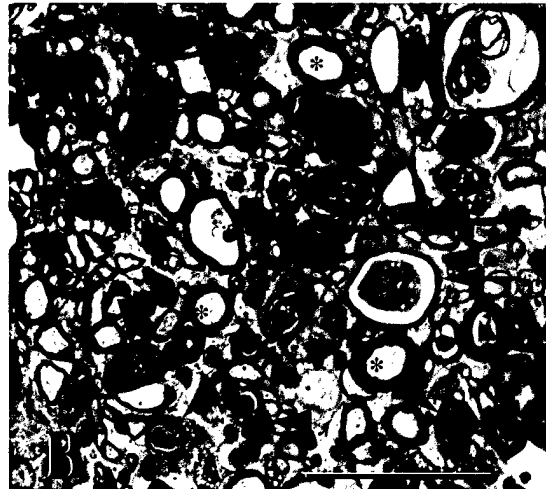
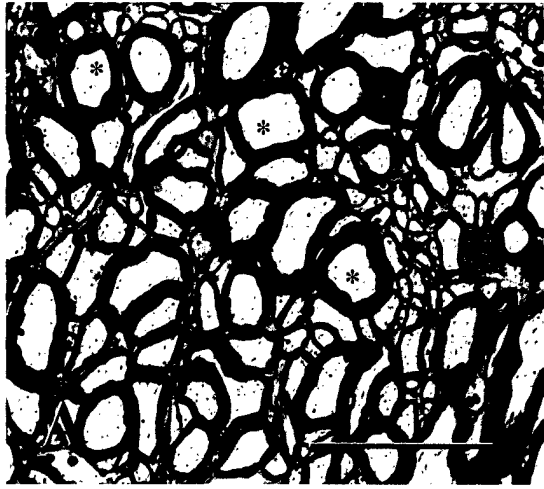
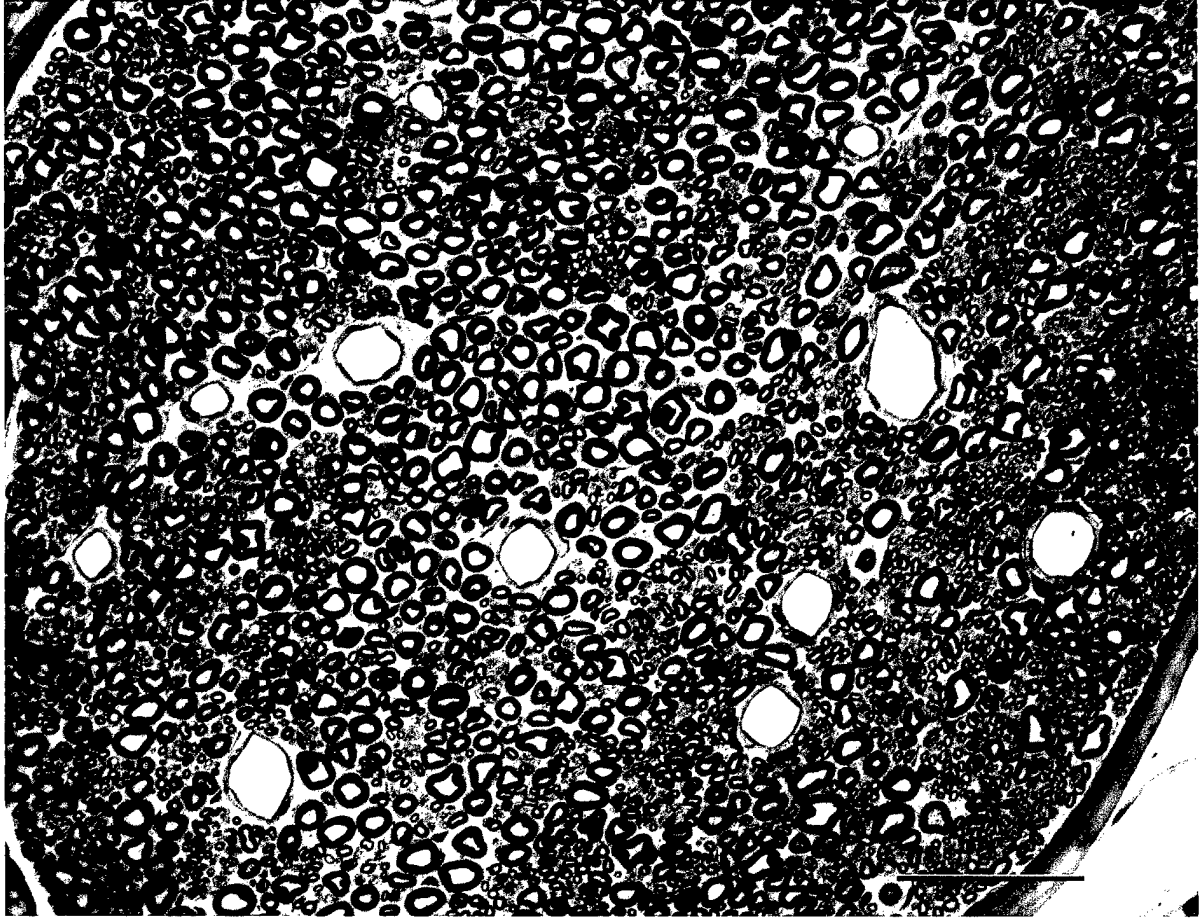


Figure 3. Cross section of the distal peroneal nerve of a hen (# 29) treated with 100  $\mu\text{g}/\text{kg}$  PB. The hen was given 21 i.m. injections (pectoral muscle) of 100  $\mu\text{g}/\text{kg}$  PB over a period of 29 days. The animal was perfused on the day after the last treatment. No pathology evident (Bar = 59  $\mu\text{m}$ ).



- Figure 4. Cross sections of the proximal (mid calf) sural nerves of hens treated with DFP.
- A. #43 dosed i.m (pectoral muscle) 20 times over 28 days with 100 mg/kg DFP. Hen was perfused 7 days after treatment ended. Nerve exhibits stage 2 pathology (Bar = 62  $\mu\text{m}$ ). Inset shows degenerating axons (arrows). Asterisks show preserved fibers (stage 2) (Bar = 40  $\mu\text{m}$ ).
  - B. #34 dosed i.m. (pectoral muscle) 25 times over 35 days with 100 mg/kg DFP & 100  $\mu\text{g/kg}$  PB. Hen was perfused 7 days after treatment ended (Bar = 60  $\mu\text{m}$ ). Inset shows degenerating myelinated nerve fibers (arrows). Note the myelinated nerve fibers that appear normal (asterisks) (Bar = 42  $\mu\text{m}$ ).
  - C. #32 dosed i.m (pectoral muscle) 12 times over 16 days with 200 mg/kg DFP. Hen was perfused 6 days after treatment ended. Nerve exhibits stage 4 pathology (Bar = 75  $\mu\text{m}$ ). Inset shows degenerating myelinated nerve fibers (arrows). Note the number of myelinated nerve fibers that appear normal (asterisks) (Bar = 42  $\mu\text{m}$ ).
  - D. #36 dosed i.m (pectoral muscle) 12 times over 16 days with 200 mg/kg DFP & 100  $\mu\text{g/kg}$  PB. Hen was perfused 6 days after treatment ended. Nerve exhibits stage 4 pathology. Note axonal degeneration (Bar = 87  $\mu\text{m}$ ). Inset shows degenerating myelinated nerve fibers (arrows). Note the number of myelinated nerve fibers that appear normal (asterisks) (Bar = 29  $\mu\text{m}$ ).

