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Alteration in Neurofilament Axonal Transport in the Sciatic Nerve of the Diisopropyl Phosphorofluoridate (DFP)-Treated Hen

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ABSTRACT. Diisopropyl phosphorofluoridate (DFP) is an organophosphorus ester that produces organophosphorus ester-induced delayed neurotoxicity (OPIDN) in hens 7–14 days after a single s.c. dose of 1.7 mg/kg. In this study, hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) 24 hr after [^{35}S]methionine injection into the sacrolumbar region of their spinal cord, and killed 3, 7, 14, or 27 days post-DFP treatment. The rates of transport of labeled high (NF-H), medium (NF-M), and low (NF-L) molecular weight neurofilaments, and tubulin were faster in DFP-treated birds than in controls after 3 days. Subsequently, the rate of transport of these proteins started falling, so that the peaks of labeled proteins in control and DFP-treated hens were overlapping after 7 days. At 14 days, the peaks of NF-H, NF-M, and NF-L in treated hens were distinctly behind the corresponding peaks in control hens. This was again followed by an increase in transport of NF-H and NF-L, but not of NF-M, so that the labeled NF-H and NF-L showed the same pattern in control and treated hens after 27 days. The transient decrease in NF-H and NF-L axonal transport rate, and recovery correlated in a temporal manner with the previously reported increase of Ca^{2+}/calmodulin-dependent protein kinase-mediated phosphorylation of neurofilament proteins and inhibition of calpain activity in the sciatic nerve in OPIDN. Proteinase inhibition has been reported recently to result in enhanced phosphorylation of neurofilaments in some cells. The present study suggests that the enhanced phosphorylation of neurofilaments by DFP-increased Ca^{2+}/calmodulin-dependent protein kinase activity may be contributing toward alteration in NF axonal transport and the development of OPIDN. BIOCHEM PHARMACOL 53;12:1799–1806, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. neurofilament proteins; diisopropyl phosphorofluoridate; hen; axonal transport; tubulin; OPIDN

A number of organophosphorus esters [e.g. TOCP,† *N,N'*-diisopropyl phosphofluoroamidate (mipafox), *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate (leptophos), and DFP] produce OPIDN in humans and other sensitive species, such as cows, chickens, cats, dogs, and water buffaloes [1]. Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate], an organophosphorus ester that produces delayed neurotoxicity at lethal doses only, is able to produce delayed neurotoxicity at a lower dose when it is administered along with safrotin, (*E*)-1-methylethyl 3-[[(ethylamino)methoxyphosphinothioyl]oxy]-2-butenoate [2]. Neither compound alone produces OPIDN at the given doses. Clinically, OPIDN manifests itself as hind-limb ataxia followed by paralysis 7–14 days post-administration. A characteristic feature of the pathology of OPIDN is the distally located swellings in large axons of the spinal cord and peripheral nerves, with the subsequent degeneration of axons. The axonal swelling mainly consists of aggregated and accumulated neurofilaments and microtubules, along with proliferated agranular endoplasmic reticulum. With the progression of the pathology, neurofilaments are partially matted and comparatively rarefied, while the microtubules are preserved for a still longer time [3, 4].

Some non-organophosphorus compounds (e.g. 2,5-HD and its derivatives, acrylamide, carbon disulfide, and IDPN) also produce neurofilamentous axonopathy, which is associated with axonal swelling in the distal or proximal parts of axons [5]. While numerous studies have been conducted to find the effects of these chemicals on the modification of cytoskeletal proteins, enzymes, and receptors, efforts have also been directed toward investigating the effects of these chemicals on axonal transport of proteins. The regulation of axonal transport is necessary for the maintenance of axonal membrane, axonal structure, and nerve endings, and alteration in the supply of essential materials is likely to

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† *Abbreviations:* DFP, diisopropyl phosphorofluoridate; 2,5-HD,2,5-hexanedione; IDPN, β,β'-iminodipropionitrile; NFs, neurofilaments; NF-H, neurofilament high molecular weight protein; NF-M, neurofilament middle molecular weight protein; NF-L, neurofilament low molecular weight protein; OPIDN, organophosphorus ester-induced delayed neurotoxicity, TCA, trichloroacetic acid, and TOCP, tri-*o*-cresyl phosphate.

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have a detrimental effect on the structure and functions of the axon. Axonal transport has been divided into three main categories: fast anterograde transport [6], fast retrograde transport [7], and slow transport [8].

The slow component a (SCa) apparently transports assembled neurofilaments and consists mainly of tubulin, neurofilaments, and fodrin. The slow component b (SCb) transports tubulin, actin, clathrin, fodrin, calmodulin, and glycolytic enzymes. Actin, clathrin, and calmodulin are also present in the SCa of spinal cord, and tubulin has been found to be absent from the SCb of optic nerve [9]. The transported proteins are finally digested in the synaptic terminal by proteinases. The movement of neurofilaments is fairly coherent, but tends to spread and slow with passage from the proximal to the distal end. Neurofilament subunits (200, 145, and 68 kDa) are synthesized in the cell body and transported down the axon in the form of assembled neurofilaments [10].

The correlation between alteration in the axonal transport of a specific protein and morphological changes may help in understanding the pathogenesis of axonal lesions. Neurofilament axonal transport has been studied invariably in chemically induced axonopathies to reveal the underlying mechanism of axonal swelling containing neurofilaments and degeneration. It has been proposed that atrophy of the proximal axon and accumulation of 10-nm NFs in the distal axon may be explained by increased NF transport in the proximal and slow transport in the distal part of the axon [5]. The decrease in neurofilament transport down the axon and the eventual arrest of flow and accumulation of neurofilaments in 2,5-HD-intoxicated animals were suggested to be due to an increase in intermolecular cross-linking between NFs down the axon. However, changes in interlinking of NFs are still not established in the case of other chemicals, such as carbon disulfide, acrylamide, and IDPN, which also produce accumulation of neurofilaments [5, 11]. This observation led to the suggestion that these chemicals may neutralize a negative (Glu or Asp) or positive (Lys or amino group) charge on the neurofilament [5]. The latter could affect the interaction of neurofilaments with the microtubules and other components of axoplasm and their phosphorylation by different protein kinases, and could result in abnormal NF transport and accumulation.

In this study, we investigated slow axonal transport in DFP-treated chickens to find whether OPIDN is associated with the alteration of slow axonal transport. The clinical signs of delayed neurotoxicity produced by DFP are similar to those of giant axonal neuropathy at least as far as the ataxia and axonal degeneration are concerned. We also attempted to correlate abnormal NF transport with our previous observations of alterations in Ca^{2+}/calmodulin-dependent protein kinase activity, which is increased in DFP- and TOCP-treated chickens [12, 13]. We suggest that abnormal phosphorylation of NFs and microtubules may produce an alteration in slow axonal transport as well as segregation of NFs from microtubules, resulting in their disorganization or aggregation, which has been noticed in chemically induced axonopathies [5].

MATERIALS AND METHODS

Materials

The radiochemicals [^{35}S]methionine (1000 Ci/mmol) and [^{125}I]-protein A (2–10 μCi/μg), and Enhance for fluorography were purchased from DuPont NEN (Boston, MA). The electrophoresis reagents and protein molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA), and the nitrocellulose membrane was obtained from Schleicher & Schuell, Inc. (Keene, NH). The monoclonal antibody anti-NF-H (Cat. No. RPN1103) was obtained from Amersham Life Sciences Inc. (Arlington Heights, IL), and monoclonal antibodies anti-NF-M (Cat. No. 814334) and anti-NF-L (Cat. No. 814326) were obtained from Boehringer Mannheim (Indianapolis, IN). The polyclonal antibody anti-tubulin, DFP, atropine, and eserine were purchased from the Sigma Chemical Co. (St. Louis, MO), and dithiothreitol was from Bachem California Inc. (Torrance, CA).

Animal Treatment

One-year-old White Leghorn laying female chickens (*Gallus gallus* domesticus) weighing 1.6 to 2.0 kg were purchased from Featherdown Farms (Raleigh, NC). The birds were anesthetized with Equithesian (pentobarbital and chloral hydrate) and were maintained with 0.25% halothane inhalation. The proteins were labeled by intraspinal administration of 1 mCi [^{35}S]methionine in the sacrolumbar region. Three sites on each side of the spinal cord were injected with 3 μL (0.056 μCi/μL) of this isotope diluted in phosphate-buffered saline. Twenty-four hours after the administration of [^{35}S]methionine, half of the animals received a single s.c. injection of 1.7 mg/kg DFP in propylene glycol. Atropine (1 mg/kg in normal saline, s.c.) and eserine (1 mg/kg in DMSO, s.c.) were also injected 15 min before DFP to counteract the acute cholinergic effects. Control birds received atropine, eserine, and the vehicle propylene glycol. The hens were examined daily after the DFP treatment for their general condition and ataxia. All of the hens were able to stand 24 hr after surgery. Control as well as DFP-treated birds developed a normal gait by day 3 and displayed no evidence of motor disturbance at this time. The DFP-treated hens showed evidence of leg weakness by day 10 and paralysis by day 23.

Determination of Axonal Transport

Groups of three hens treated with DFP/[^{35}S]methionine and corresponding time-matched control hens injected with only [^{35}S]methionine were killed at 3, 7, 14, and 27 days following DFP administration. The sciatic nerves including the ventral roots were rapidly excised, frozen in liquid nitrogen, and stored at −70°. Subsequently, individ-

ual nerves were thawed, cut into 5-mm segments, and homogenized in SDS/urea. The SDS/urea solution consisted of SDS-sample buffer [14] containing 60% (w/v) urea. Aliquots of homogenates were used to determine the radioactivity by liquid scintillation spectrometry, and aliquots containing 50 μg protein from each sample were resolved by SDS–PAGE. Gels were stained with Coomassie Blue, destained, and impregnated with Enhance. The dried gels were exposed to Imaging plates, and radioactivity was determined by Phosphor-image analysis using the Fuzix Bio-imaging Analyzer System (BAS 1000).

Individual neurofilament triplet proteins were identified by their mobility on the gels and western blotting using monoclonal antibodies against each protein. Slow axonal transport of each protein was compared by determining the advancing front of radioactivity or the distance of peak of the curve from the spinal cord in millimeters. The segment showing the termination of the major peak was taken as the advancing front of radioactivity. For example, the advancing front of NF-H radioactivity for control sciatic nerve was at segment 3 and that of treated hens was at segment 5 after 3 days (see Fig. 1).

SDS–PAGE and Immunoblotting

The 5-mm pieces of axons were homogenized in SDS-urea solution and heated at 90° for 2 min before loading for electrophoresis. The proteins were separated by discontinuous gel electrophoresis in 4% stacking gel and 7.5% polyacrylamide resolving gel [14], and electrophoretically transferred onto nitrocellulose membranes. The proteins were immunostained by treating the membranes with monoclonal or polyclonal antibodies followed by [^{125}I]protein A [15]. The blots treated with monoclonal antibodies were first treated with rabbit anti-mouse immunoglobulins (1:1000) for 1 hr before processing for [^{125}I]protein A treatment. The blots finally were subjected to autoradiography in the presence of two DuPont intensifying screens. The neurofilament subunits NF-H, NF-M, and NF-L were identified by using the monoclonal antibodies anti-NF-H (1:2500), anti-NF-M (1:2500), and anti-NF-L (1:2500), respectively, and tubulin was identified by a polyclonal antibody against tubulin (1:750).

Other Procedures

The sciatic nerve solutions were precipitated with 1 mL of 13.5% ice-cold TCA for 30 min, centrifuged for 20 min at 20,000 *g*, washed once with cold ethanol, and lyophilized for 10 min. The pellets were dissolved in 50 μL of 0.1 N NaOH, and aliquots were used for protein estimation by the method of Smith *et al.* [16] using bovine serum albumin as the standard. The bovine spinal cord neurofilaments were purified by the procedure of Dahl *et al.* [17], and they were heated in boiling water for 5 min to inactivate any endogenous protein kinase activity.

Statistical Analysis

The results obtained in millimeters of distance from the spinal cord were compared by Student's unpaired two-tailed *t*-test, and $P \leq 0.05$ was considered significant. The results have been expressed as means ± SEM.

RESULTS

General Observations

Hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) and examined daily for acute and delayed neurotoxicity. They showed acute cholinergic signs shortly after DFP administration, despite prophylactic pretreatment with atropine sulfate. The cholinergic signs disappeared within 24 hr of DFP dosing, and the birds developed a normal gait by day 3 after the surgery for [^{35}S]methionine injection in the sacrolumbar region. The hens started showing leg weakness from 8–10 days after DFP administration, developed ataxia by 12–14 days, and were paralyzed by day 23.

Axonal Transport of NF-H

The 5-mm segments of sciatic nerves of control and treated hens were used for SDS–PAGE and autoradiography using Phosphor-imaging plates. The bands of [^{35}S]methionine labeled NF-H are shown on the top of each panel of Fig. 1. The upper row represents the samples from the control chicken and the lower one from the DFP-treated chicken. The radioactivity related to each band was quantified by the Phosphor-image analyzer, and it was obtained by subtracting the radioactivity belonging to the background around the NF-H band. The radioactivity was finally plotted as percent of total radioactivity loaded in each lane for electrophoresis. The advancing front of the DFP-treated chicken NF-H reached a greater distance (14.2 ± 1.7 vs 24.2 ± 1.7 mm, $P < 0.02$) in 3 days, although there was no difference in peaks of the labeled NF-H from control and treated chickens during this period. The rate of NF-H transport started falling after this time, so that the profiles of NF-H from both control and treated hens were nearly the same at 7 days, and the peak of treated chicken NF-H was clearly behind (15.8 ± 1.7 vs 22.5 ± 0 mm, $P < 0.02$) that of control chicken NF-H by day 14 after dosing. The results showed that the rate of treated hen NF-H transport again increased after 14 days and approached that of NF-H from the control chickens.

Axonal Transport of NF-M

The profile of DFP-treated chickens' NF-M transport exhibited the same pattern as shown by the NF-H up to 14 days after DFP administration (Fig. 2). The advancing front of treated chicken NF-M was about 70% ahead (14.2 ± 1.7 vs 24.2 ± 1.7 mm, $P < 0.02$) of control NF-M in 3 days, but there was no difference between the two NF-M peaks. The profiles of NF-M from both control and treated

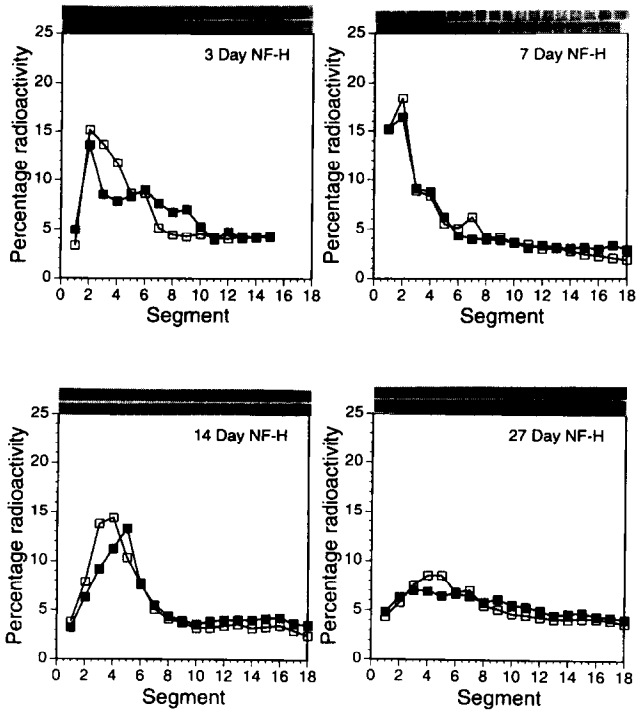

FIG. 1. Comparison of the distribution curves of radiolabeled NF-H in the sciatic nerve segments of control and DFP-treated hens. Twenty-four hens were injected with 1 mCi of [^{35}S]methionine into three sites on each side of the sacrolumbar region of the spinal cord. Half of them were administered a single dose of 1.7 mg/kg DFP 24 hr after the [^{35}S]methionine injection, and each set of DFP-treated hens and the time-matched control birds were killed 3, 7, 14, and 27 days post-DFP administration, as described in Materials and Methods. The sciatic nerves were excised from the spinal cord, cut into 5-mm segments, and used for SDS–PAGE and autoradiography. The percentage of radioactivity in the NF-H band was plotted against the number of 5-mm sciatic nerve segments. The representative curves showed an increase in axonal transport rate in DFP-treated chickens at day 3 and a decrease at day 14. The inset on the top of the figure shows the autoradiogram of the NF-H bands in the sciatic nerve segments from control (upper row) and DFP-treated (lower row) chickens. The closed and open symbols represent percent radioactivity in NF-H from the sciatic nerve segments of the control and the DFP-treated birds, respectively.

chicken were the same on day 7 and again the peak of treated chicken NF-M was about 37% behind (14.2 ± 1.7 vs 22.5 ± 0 mm, $P < 0.01$) that of control on day 14. In contrast to NF-H, the rate of DFP-treated chicken NF-M transport did not recover after 14 days, and it continued to lag behind control NF-M up to 27 days.

Axonal Transport of NF-L and Tubulin

The pattern of NF-L transport was very similar to that of NF-H. Both the peak and the advancing front of DFP-treated chicken sciatic nerve NF-L were ahead of control NF-L on day 3, and about 37% behind (14.2 ± 1.7 vs 22.5 ± 0 mm, $P < 0.01$) that of control NF-L on day 14 (Fig. 3). The peaks of NF-L from control and treated hens were overlapping on days 7 and 27 when the animals were euthanized. This showed that the rate of NF-L transport increased after 14 days and came close to that of NF-L in control chickens. The peak of tubulin showed faster transport (9.2 ± 1.7 vs 15.8 ± 1.7 mm, $P < 0.05$) only after 3 days in DFP-treated chickens, and there was no difference in the rate of transport of tubulin from control and treated chicken at other time periods (7, 14, 27 days) of euthanasia (Fig. 4).

DISCUSSION

Most of the neurotoxic chemicals producing pathologic conditions in the central/peripheral nervous system are associated with abnormal fast and slow axonal transport. The slow axonal transport has been studied extensively in rats treated with 2,5-HD and its derivatives, as well as other chemicals such as acrylamide, carbon disulfide, and IDPN. However, very few fast and slow axonal transport studies have been reported in the case of organophosphorus compounds, such as TOCP and DFP, which are very neurotoxic, and even a single administration of these compounds is able to produce delayed neurotoxicity [6, 8, 18].

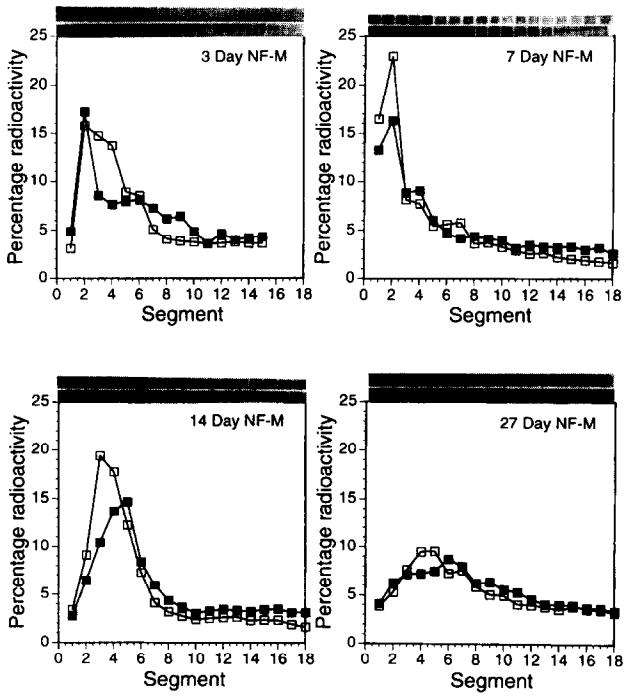

FIG. 2. Comparison of the distribution curves of radiolabeled NF-M in the sciatic nerve segments of control and DFP-treated hens. The percentage of radioactivity in the NF-M band was plotted against the number of 5-mm sciatic nerve segments. The inset on the top of the figure shows the autoradiogram of the NF-M bands in the sciatic nerve segments from control (upper row) and DFP-treated (lower row) chickens. The representative curves show an increase in axonal transport rate in DFP-treated chickens at day 3, and a decrease at days 14 and 27. The closed and open symbols represent percent radioactivity in NF-M from the sciatic nerve segments of the control and the DFP-treated birds, respectively.

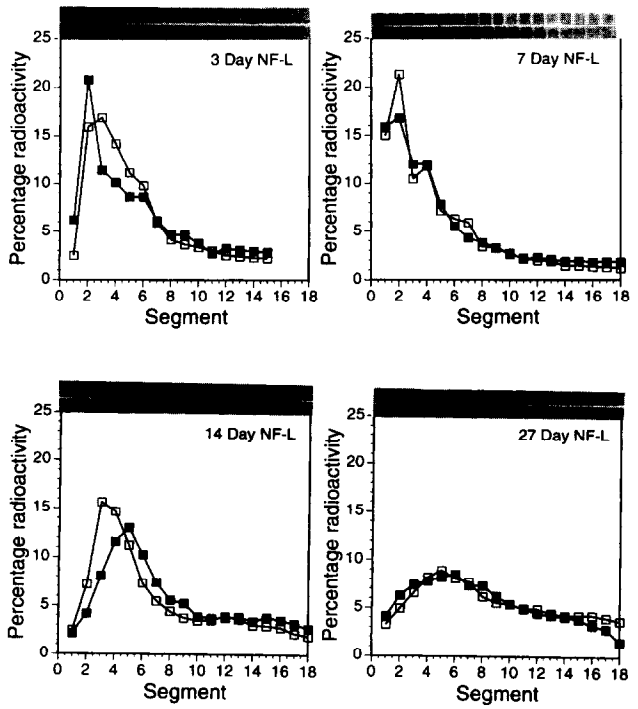

FIG. 3. Comparison of the distribution curves of radiolabeled NF-L in the sciatic nerve segments of control and DFP-treated hens. The percentage of radioactivity in the NF-L band was plotted against the number of 5-mm sciatic nerve segments. The inset on the top of the figure shows the autoradiogram of the NF-L bands in the sciatic nerve segments from control (upper row) and DFP-treated (lower row) chickens. The representative curves show an increase in axonal transport rate in DFP-treated chickens at day 3 and decrease at day 14. The closed and open symbols represent percent radioactivity in NF-L from the sciatic nerve segments of the control and the DFP-treated birds, respectively.

This study demonstrates an eventual impairment of the transport of neurofilament subunits and tubulin in DFP-treated chickens. The axonal transport of the studied proteins (i.e. NF-H, NF-M, NF-L, and tubulin) in treated chickens was initially increased at 3 days. However, this was followed by a substantial decrease in the transport of NF-H, NF-M, and NF-L such that there was a clear difference between the positions of peaks at day 14. However, in the case of tubulin, the transport decreased to control rate by 7 days post-DFP administration and then did not change afterward. In contrast to the effect induced by other toxic chemicals, the transport of NF-H and NF-L in the DFP-treated birds again increased and reached control rate at 27 days after DFP treatment. James and Austin [8] studied slow axonal transport of total protein in the sciatic nerve of DFP-treated chickens, but did not observe any difference in transport rate. This discrepancy may be ascribed to (1) the different dose (1 mg/kg) of DFP administered to the chickens, (2) counting of radioactivity of total proteins instead of separating them by SDS–PAGE, and (3) the use of 10-mm segments of sciatic nerve.

The above-mentioned profile of NF transport in DFP-treated chickens is different from that observed in rats treated with other neurotoxic chemicals (e.g. 2,5-HD, 3-methyl-2,5-HD, acrylamide, carbon disulfide). The animals treated with 2,5-HD, 3-methyl-2,5-HD, or carbon disulfide showed acceleration of transport of NF subunits in the proximal axons, when the transport was measured 25–28 days after [^{35}S]methionine injection [19–21]. The axonal transport was not measured in these studies at earlier time periods. Furthermore, DFP administration resulted in the acceleration of NF transport, when the birds showed no clinical signs of neurotoxicity and probably had no pathological signs of degeneration. The earliest sign of Wallerian-like degeneration has been observed only 7 days after DFP administration, when DFP (1 mg/kg) was injected daily for 4 days [22]. The neurotoxic chemicals dimethyl hexanedione (DMHD) and IDPN, which cause accumulation of NFs proximal to the sciatic nerve, inhibit NF transport when measured 12 days after [^{35}S]methionine injection [23]. The acrylamide treatment of rats reduces NF transport in optic axons [24] and increases transport in the sciatic nerve [25]. The transport was measured in the optic axons 5 and 11 days after L-[4,5-^{3}H]leucine injec-

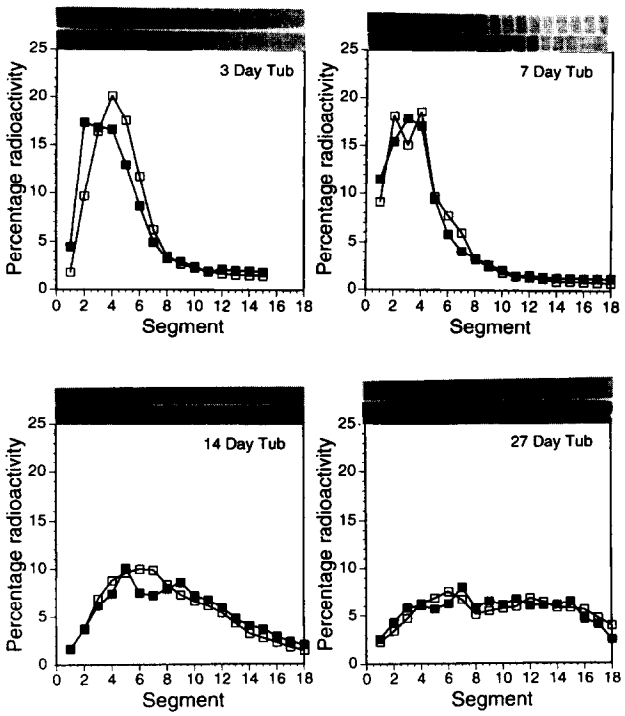

FIG. 4. Comparison of the distribution curves of radiolabeled tubulin in the sciatic nerve segments of control and DFP-treated hens. The percentage of radioactivity in the tubulin band was plotted against the number of 5-mm sciatic nerve segments. The inset on the top of the figure shows the autoradiogram of the tubulin bands in the sciatic nerve segments from control (upper row) and DFP-treated (lower row) chickens. The representative curves show an increase in axonal transport rate in DFP-treated chickens at day 3 and the same transport profile after that time period. The closed and open symbols represent percent radioactivity in tubulin from the sciatic nerve segments of the control and the DFP-treated birds, respectively.

tion and in the sciatic nerve 12 days after [^{35}S]methionine labeling.

DFP treatment of chickens caused a transient increase of tubulin transport after 3 days and the same rate of transport as control after 7, 14, and 27 days. In contrast, carbon disulfide and 2,5-HD treatment of rats did not alter the tubulin transport [20, 21], while acrylamide and 3-methyl-2,5-HD treatment increased tubulin transport [25, 26]. Thus, the transport profile of NFs and tubulin in DFP-treated hens is different from that seen with some other non-organophosphorus neurotoxic compounds, suggesting that the transporting neurofilaments, microtubules, or some other cytoskeletal proteins are modified in a different way during the development of OPIDN.

Although DFP directly phosphorylates serine residues in some enzymes (e.g. proteinases, cholinesterases) and inhibits their activity immediately, it is unlikely that a single small dose of DFP (1.7 mg/kg), which is not stable in an aqueous environment, can produce alterations in axonal transport depending upon the stage of development of OPIDN by one-step direct phosphorylation at the time of administration. Recent studies on the transport of neurofilament proteins in 2,5-HD-treated animals also suggest that pyrrole formation may not be necessary to alter the transport of neurofilament protein [27]. This became evident when the rats showed acceleration of neurofilament transport whether L-[^{35}S]methionine was injected before the 2,5-HD treatment or during the recovery period, when there was no 2,5-HD in the blood to react with the newly synthesized neurofilament.

Phosphorylation is the major posttranslational modification of NFs [28]. Phosphorylation of amino acid residues by protein kinases has been suggested to induce extension of the carboxy-terminal sidearms of NF-H and NF-M radially, and create steric drag, which may slow their axonal transport [29]. The extension of side-arms may expose potential binding sites and promote reversible interaction of NFs with the relatively stationary axonal structures (e.g. cortical skeleton) that could further slow the transport [29]. The hypothesis that the phosphorylation of NFs slows their transport is supported by the observation that a decrease in NF phosphorylation is accompanied by acceleration of NF transport [28].

Previous studies in our laboratory have shown that DFP administration has caused increased *in vitro* neurofilament phosphorylation by the brain supernatant [12], and TOCP induces a transient increase of NF phosphorylation in the sciatic nerve, which peaks at day 6 after TOCP administration [13]. In the latter study, birds were treated with a single dose of TOCP and killed after 1, 6, 14, and 21 days. The increase in *in vitro* phosphorylation of neurofilaments (NF-H, NF-M, NF-L) at day 6 is consistent with the decrease in neurofilament transport after day 3. The Ca^{2+}/calmodulin-dependent protein kinase activity returned to normal by day 14, and the transport of neurofilaments NF-H and NF-L probably also increased after that time. However, in the present study the rate of NF-M transport in the DFP-treated chickens did not increase after 14 days. The reason for the continued slow transport of NF-M is not clear at this time, but it could be ascribed to a difference in the rate of dephosphorylation of abnormally phosphorylated NF-M compared with NF-H and NF-L in OPIDN.

The reason for the initial increase of NF transport rate observed on day 3 is not clear at present, but it could be due to some change in the conformation of NFs phosphorylated at specific sites in the initial stage of OPIDN, before extensive phosphorylation. The changed conformation of NFs may promote interaction with the moving cytoskeletal elements, resulting in acceleration of transport. The rate of NF axonal transport is probably an outcome of the NF interaction with the stationary axonal structures and the moving transport carrier [29]. The relative strength of interaction with stationary axonal structures on one hand and the moving structures on the other determine the period when the NF is moving or stationary.

Alternatively, increased synthesis of NFs may assist in accelerating the NF transport in the first 3 days, when they are not extensively phosphorylated. Recent studies in our laboratory have shown transient increase (50–200%) in NF transcript in the brain and spinal cord of DFP-treated birds in the first 24 hr, and that may result in increased synthesis of NFs (unpublished data). Immunohistochemical studies in our laboratory have found intense CaM kinase II immunoreactivity associated with the anomalous neurofilament aggregates in the spinal cord axons of TOCP-treated chickens (unpublished data). However, a role for some other Ca^{2+}-dependent (e.g. protein kinase C) [30] and -independent protein kinases [31–33] in increased NF phosphorylation in the axons of DFP-treated hens cannot be ruled out. The enhanced phosphorylation of some other cytoskeletal proteins such as tau could also participate in the abnormal transport of NFs and tubulin by altering the stability of microtubules [34].

The major phosphorylation of NF-H and NF-M takes place in their carboxy-terminal domain, which consists of many Lys-Ser-Pro motifs [35, 36]. The C-terminal domain is phosphorylated by second messenger-independent protein kinase(s), and some of them, which probably phosphorylate these sites *in vivo*, belong to the mitogen-activated protein kinase (MAPK) family [37]. A recent report indicated an increased phosphorylation of neurofilament subunits in the dorsal root ganglia by a cysteine proteinase inhibitor, *N*-acetyl-Leu-Leu-norleucinal [37]. Our results on neurofilament transport also show a good correlation with calpain (a cysteine proteinase) activity inhibition reported earlier [38] in the sciatic nerve of the DFP-treated hens. There were 23, 50, 52, and 29% decreases in millimolar calcium-activated neutral proteinase (mCANP) activity after 1, 7, 14, and 21 days of DFP administration, respectively. Thus, there was an increase in NF-H and NF-L transport on day 3 with 23% calpain activity inhibition, a fall in transport rate after 3 days with 50% activity inhibition, and again a recovery of transport back to normal at 29% activity inhibition. Since the

cysteine proteinase inhibitor treatment enhanced *in vivo* NF phosphorylation in dorsal root ganglion [37], it is feasible that the inhibition of calpain activity may stimulate some protein kinase(s) in the sciatic nerve of DFP-treated hens. The stimulated protein kinase could contribute toward increased phosphorylation of NFs, and the abnormal phosphorylation may result in the alteration of their rate of axonal transport.

In summary, we determined the rate of transport of NF subunits and tubulin in the sciatic nerves of DFP-treated chickens, and attempted to correlate it with the previously reported Ca^{2+}/calmodulin-dependent protein kinase activity and calpain activity inhibition in the sciatic nerve. A good correlation was found between alteration in axonal transport of NF-H and NF-L, our previously reported stimulation of *in vitro* CaM kinase activity and NF phosphorylation, and the calpain activity inhibition in the sciatic nerve [13, 38]. The results suggest that abnormal phosphorylation of NFs may be the underlying mechanism for alteration of the slow axonal transport in DFP-treated hens.

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