MOLECULAR PHARMACOLOGY, 17, 56–60

Inhibition of Fast Axoplasmic Transport by Delayed Neurotoxic Organophosphorus Esters: A Possible Mode of Action

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Received February 22, 1979; Accepted July 26, 1979

SUMMARY

REICHERT, B. L. AND M. B. ABOU-DONIA. Inhibition of fast axoplasmic transport by delayed neurotoxic organophosphorus esters: A possible mode of action. *Mol. Pharmacol.* **17:** 56–60 (1980).

Several insecticides known to cause delayed neurotoxicity were examined for antitransport activity. The rat optic nerve served as the means to measure fast axoplasmic transport of proteins when the following chemicals were injected: *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate (leptophos); *O*-methyl *O*-2,5-dichlorophenyl phenylphosphonothioate; *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate; *O*-ethyl *O*-4-cyanophenyl phenylphosphonothioate (cyanofenphos); and *O*-methyl *O*-2,4-dichlorophenyl phenylphosphonothioate (S-Seven). Tri-*o*-cresyl phosphate and *O,O*-diethyl *O*-4-nitrophenyl phosphorothioate (parathion) were used as positive and negative controls, respectively. L-[^{3}H]Proline (50 μCi) and 5 μl of the compound of interest (0.3 μmol) were injected into the vitreous humor of one eye (in dimethyl formamide/saline solution v/v) and a similar solution lacking the drug was introduced into the contralateral eye as a control. Marked inhibition of fast axoplasmic transport was observed by all phenylphosphonothioate esters and tri-*o*-cresyl phosphate. No antitransport activity was observed with parathion. The 40–50% lower transport activity in animals treated with delayed neurotoxins, as compared with activity in the negative control (parathion-treated) animals, offers preliminary support to the hypothesis that impairment of fast axoplasmic transport may be involved in the mechanism of action of neurotoxic organophosphorus esters.

INTRODUCTION

Some pathological conditions have been attributed to impairment of axonal transport. Such axoplasmic transport of proteins from the neuron cell body has been thoroughly studied. Two main rates have been established: a slow rate, about 1–5 mm/day (1, 2), and a fast rate ranging from 100 to 500 mm/day (3, 4). Neurofibrillary degeneration induced by colchicine and other metaphase-blocking antimitotic agents has been related to abnormal axonal transport (5, 6). Neuropathy caused by acrylamide was reported by Pleasure *et al.* (7) to be associated with similar changes in transport, but Bradley and Williams (8) were unable to substantiate these results. Recently, Rasool and Bradley (9) have reported that the absolute transport rate of acetylcholinesterase (AChE)[2] (based on measurements of the mobile fraction of the enzyme) was decreased in acrylamide-treated rat sciatic nerve. Also the amount of AChE activity transported was decreased. Such a combined effect of changes in the rate and amount of transport caused a decrease in the distal delivery of AChE.

Organophosphorus pesticides may cause acute cholinergic poisoning with temporary muscle weakness. A few of these compounds have been demonstrated to produce a more persistent effect, delayed neurotoxicity (10, 11). The delayed neurotoxic effect was first demonstrated in man; later, other species (cats, dogs, cows and chickens) were found to be susceptible (12). Rodents and some

A preliminary account of this work has been presented (30). This study was supported in part by NIEHS grant ES01186 and NIEHS Training grant in Toxicology ES07002.

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[2] The abbreviations used are: AChE, acetylcholinesterase; leptophos, *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate; DBL, *O*-methyl *O*-2,5-dichlorophenyl phenylphosphonothioate (desbromoleptophos); Cyanofenphos, *O*-ethyl *O*-cyanophenyl phenylphosphonothioate; *S*-Seven or EPBP, *O*-ethyl *O*-2,4-dichlorophenyl phenylphosphonothioate; EPN, *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate; TOCP, tri-*o*-cresyl phosphate; parathion, *O,O*-diethyl *O*-4-nitrophenyl phosphorothioate; TCA, trichloroacetic acid; DFP, diisopropyl phosphorofluoridate.

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primates are not susceptible. The clinical condition, which first becomes apparent as ataxia, progresses to paralysis if sufficiently high doses are given. Lesions associated with the clinical condition are characterized by degeneration of axons with subsequent Wallerian degeneration of myelin. Although the mechanism of delayed neurotoxicity is unknown, the initial event is suspected to be phosphorylation of a protein (13). It has been suggested that this type of distal axonal neuropathy might result from impairment of axonal transport (10, 14, 15). Results of previous *in vivo* studies of axonal transport in animals treated with these insecticides have been conflicting (7, 8, 16). This paper investigates the possibility that alteration of fast axoplasmic transport of ^{3}H-labeled protein may underlie this axonal degeneration produced by neurotoxic organophosphorus esters.

MATERIALS AND METHODS

Chemicals. Pure *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate (leptophos) and desbromoleptophos (DBL) were supplied by Velsicol Chemical Company; cyanofenphos and technical grade (96.43%) S-Seven (EPBP) were supplied by Nissan Chemical Industries, Ltd., Tokyo, Japan; *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) was provided by E. I. du Pont de Nemours and Company, Wilmington, Delaware; tri-*O*-cresyl phosphate (TOCP), (99%), was obtained from Eastman Kodak Company, Rochester, New York; parathion was obtained from Pfaltz and Bauer, Inc., Stamford, Connecticut; sodium pentobarbital (Nembutal) was obtained from Abbott Laboratories, Chicago, Illinois; and colchicine was obtained from Sigma Chemical Company, St. Louis, Missouri.

Study protocol. Female rats (Sprague–Dawley, Madison, Wis.), weighing approximately 225 g were anesthetized with 15 mg/kg of sodium pentobarbital as previously described (17–19). The following mixture was injected into the vitreous humor of one eye: 5 μl 0.9% NaCl containing 50 μCi of L-[^{3}H]proline and 0.3 μmol of the test chemical in 5 μl of 0.9% NaCl and dimethylformamide (1:1, v/v). A similar solution, lacking the compound of interest, was introduced into the contralateral eye as a control. The injections were made by a single entry into the eye with a 50-μl Hamilton syringe (Hamilton Co., Reno, Nev.). The body temperature of the animals was monitored throughout the experiment using a YSI Electronic Tele-Thermometer Model 44 (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio), and was held constant at 35° by application of heat as necessary. Rats were killed by decapitation after 3 hr. The optic nerves were dissected and cut into 1-mm sections. The retinas were removed, weighed, homogenized in 18% trichloroacetic acid (TCA), and filtered. Radioactivity in the optic nerve sections and the TCA precipitate was determined in a Beckman LS-100 liquid scintillation spectrometer (Beckman Instruments, Inc., Irvine, Calif.), after preparation of the samples by oxygen combustion in Tissue Oxidizer Model 306B (Packard Instrument Co., Inc., Downers Grove, Ill.), using 10 ml of the trapping solution Monophase 40.

RESULTS

In the present study, the effect of a chemical on fast axoplasmic transport was determined by comparing the level of radioactivity found in the treated nerve with that found in the control nerve. Adjustments for differences in incorporation of radioactivity from eye to eye were carried out by the method of Paulson and McClure (17, 18). This method assumes that the appearance of radioactivity in the nerve is directly proportional to the amount of [^{3}H]proline incorporated into retinal protein (19). It is based on three parameters: the transport ratio, the protein incorporation ratio, and the net transport value.

The transport ratio (t_i) compares the amount of tritiated material found in the treated and control nerves:

$$t_i = \frac{(\text{dpm/mm treated nerve})_i}{(\text{dpm/mm control nerve})_i}.$$

A t_i value was calculated for each of the i pairs of 1-mm sections of the optic nerve taken at equal distances from each eyeball. The values of t_i were summed, and a mean (T) ± SE was obtained (to minimize sectioning error).

To correct for the amount of [^{3}H]proline incorporated into the treated and control retinas, a protein incorporation ratio (P) defined:

$$P = \frac{\text{dpm in treated TCA residue/mg treated retina}}{\text{dpm in control TCA residue/mg control retina}},$$

where TCA residue refers to the insoluble residue of the retinal tissue homogenized in TCA. P was applied as a correction to the value of T calculated for the same animal. With no inhibition of axoplasmic transport in the treated nerve, T should equal P (provided that there is no alteration in protein synthesis in the retinal ganglion cells). If, however, a chemical inhibits axoplasmic transport (without affecting incorporation of the precursor), T will be smaller than P.

Finally, the net transport value (NT) of the corrected [^{3}H]proline in the treated nerve was defined as:

$$NT = T/P.$$

An NT value of 1.00 would indicate no net inhibition of axoplasmic transport, while a value of 0.00 would indicate complete blockage. Intermediate values of NT would correspond to partial blocking action.

The crossover of radioactivity to the untreated eye has been examined in the rat optic system. McClure and Paulson (17, 18) injected L-[^{3}H]proline into one eye, and 0.9% NaCl in the other. The ^{3}H precursor level in the control eye was found to be less than 2% of that found in the eye treated with the radioactive substance.

A typical transport profile of protein labeled with tritiated proline following the administration of EPN is shown in Fig. 1. Similar profiles were obtained for other organophosphorus esters capable of causing delayed neurotoxicity. In all cases in which the chemical affected fast axonal transport the highest value of t_i was in the section attached to the eye. In the region up to 3 mm from the eye intermediate values were obtained. The value reached a plateau in all sections 5 mm or more from the

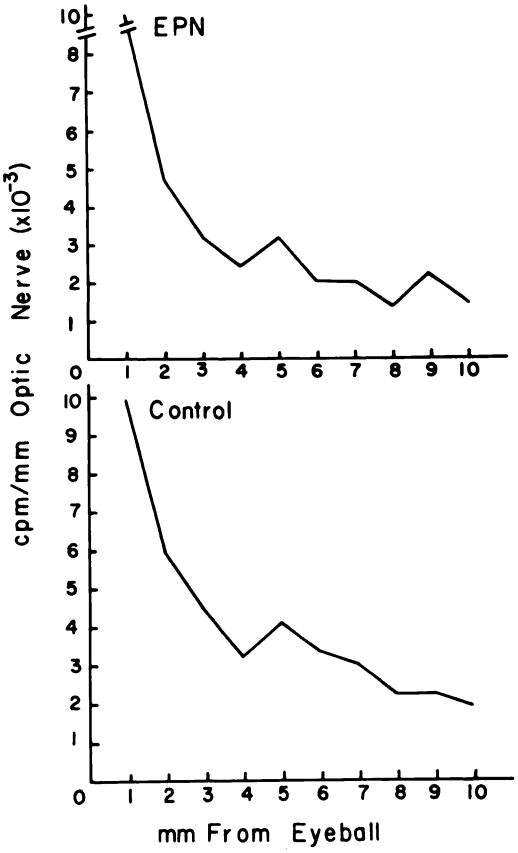


FIG. 1. *A typical axonal transport profile of protein labeled with tritiated proline in EPN-treated and control rat eyes*

EPN and control retinas contained 153,274 and 296,979 cpm/mg retina, respectively.

eye. The value of t_i in the plateau region was related to the dose of the chemical employed, while the values from sections of nerve at distances near the eye were not. For this reason, only the values from sections of nerve at distances greater than 4 mm from the eye were used to calculate T. To check this system, 12.5 μmol of colchicine, a drug known to inhibit fast axoplasmic transport, was injected into the treated eye. The resulting NT value (mean ± SE) 0.55 ± 0.04 (<0.001) was in good agreement with a previously published value (17, 18). Since T values for this compound ranged from 0.45 to 1.88 (as indicated by the relatively large SE, Table 1), it is clear that NT function satisfactorily corrected for uneven incorporation of L-[^{3}H]proline into retinal protein. The system also is capable of distinguishing protein inhibition from fast axoplasmic transport inhibition. The protein inhibitor drug, cycloheximide, has been injected into the eye at doses ranging from 10 to 250 mg. This procedure decreased the incorporation determined in the control retina which agreed with earlier results (18). NT values in these experiments, however, were approximately 1.0 or greater, indicating no apparent inhibition of fast axoplasmic transport.

Several organophosphorus esters were examined for their effectiveness as inhibitors of fast axoplasmic transport in the rat optic system. Five of these chemicals were phenylphosphonothioate esters, which induce delayed neurotoxicity (Fig. 2). TOCP, an organophosphorus ester

TABLE 1

Effect of organophosphorus esters and colchicine on fast axoplasmic transport of proteins in the rat optic system

The dose used was 0.3 μmol of each compound tested in 5 μl dimethyl formamide and saline (1:1, v/v) as described under METHODS. Each value is the mean ± SE of ten determinations.

Compound	T^a	P^b	NT^c	p^d
Cyanofenphos	0.77 ± 0.08	1.32 ± 0.17	0.49 ± 0.06	<0.001
EPN	0.81 ± 0.16	1.26 ± 0.26	0.53 ± 0.07	<0.001
DBL	0.84 ± 0.10	1.37 ± 0.27	0.57 ± 0.05	<0.001
Leptophos	0.79 ± 0.13	1.14 ± 0.15	0.61 ± 0.08	<0.001
EPBP (S-Seven)	0.87 ± 0.12	1.09 ± 0.16	0.69 ± 0.04	<0.002
TOCP	1.10 ± 0.29	1.75 ± 0.47	0.57 ± 0.07	<0.001
Colchicine	0.87 ± 0.22	1.65 ± 0.50	0.55 ± 0.04	<0.001
Parathion	0.90 ± 0.11	0.93 ± 015	1.0 ± 0.05	
Control[e]	1.11 ± 0.16	1.05 ± 0.10	1.0 ± 0.04	

[a] T is the mean of t_i values which were calculated for each pair of 1-mm sections of the optic nerve taken at equal distances from control and treated eyeball:

$$t_i = \frac{(\text{dpm/mm treated nerve})_i}{(\text{dpm/mm control nerve})_i}.$$

[b] $P = \dfrac{\text{dpm in treated TCA residue/mg treated retina}}{\text{dpm in control TCA residue/mg control retina}}$.

[c] $NT = T/P$.

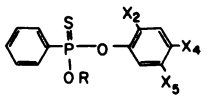
[d] Significant differences from parathion-treated animals.

[e] Both eyes were injected with 5 μl of dimethyl formamide and saline solution (1:1, v/v).

known to produce delayed neurotoxicity, and parathion, an organophosphate ester not capable of inducing neurotoxicity, were used as positive and negative controls. Table 1 shows that a dose of 0.30 μmol of each organophosphorus ester capable of inducing delayed neurotoxicity caused inhibition of fast axoplasmic transport in the rat optic system. By contrast, no antitransport activity was observed with parathion. Table 2 shows that a dose of 0.05 μmol of the neurotoxic organophosphorus esters caused less inhibition of fast axoplasmic transport. The order of effectiveness of these compounds as fast axoplasmic transport inhibitors was the same at both doses. The order from most effective to least effective was: cyanofenphos>EPN>DBL>leptophos>EPBP.

DISCUSSION

Most organophosphorus insecticides produce acute poisoning with temporary muscle weakness; a few organ-



COMPOUND	R	X_2	X_4	X_5
LEPTOPHOS	CH_3	Cl	Br	Cl
DBL	CH_3	Cl	H	Cl
EPBP (S-SEVEN)	C_2H_5	Cl	Cl	H
EPN	C_2H_5	H	NO_2	H
CYANOFENPHOS	C_2H_5	H	CN	H

FIG. 2. *Chemical structures of phenylphosphonothioate esters*

ophosphorus insecticides cause delayed neurotoxicity (10, 11). Recent studies have suggested that delayed neurotoxicity is a general property of phenylphosphonothioate insecticides (11, 20–22). We have observed in our ongoing studies in the chicken that leptophos caused an increase in plasma acid phosphatase levels. This suggested to us that an interruption of axonal transport may be involved in the mechanism of action of delayed neurotoxicity (14, 15). Although the initial event in delayed neurotoxicity might be inhibition of an esterase (13, 23), an interruption of the normal flow of essential metabolic factors (e.g., oxidation enzymes) from the cell body to the distal axon caused by organophosphorus compounds may explain the degenerative nerve process that brings about the typical clinical manifestations (10, 14, 15). Interruption in axoplasmic transport was suggested to explain the histologic features of Wallerian degeneration (24). A consequence of such abnormal transport would be the disruption of the lysosomal membranes, resulting in degeneration of the axis cylinders and ultimately of the enveloping myelin sheath.

The results obtained in this study were collected from an experimental system based upon the optic pathway of the rat. This system has the advantages of yielding immediate and quantitative results. The disadvantage of this system is that rodents including rats do not show ataxia, paralysis or nerve damage normally associated with delayed neurotoxicity induced by organophosphorus esters in susceptible species (12). We believe our choice of this model is justified for two reasons. In this preliminary investigation we did not attempt to produce neurological signs of delayed neurotoxicity. Rather, we used the rat optic system as a tool to study the effect of these chemicals on fast axoplasmic transport of protein. Furthermore, our previous work has shown a correlation between species selectivity and differential pharmacokinetics and metabolism of organophosphorus esters (25, 26). Susceptible species, e.g., chickens and cats, fail to metabolize these chemicals quickly, while nonsusceptible species rapidly excrete organophosphorus esters as degradation products, principally in the urine (25, 27).

Using the *NT* parameter as a measure of the inhibition of fast axoplasmic transport for a given dose of the chemical, a structure–activity relationship can be drawn from the data of Table 1. The inhibitory effect of phenylphosphonothioate esters on fast axoplasmic transport in the rat optic system generally paralleled the potency of these esters to elicit delayed neurotoxic effect in hens: cyanofenphos>EPN>DBL>leptophos>EPBP (11).

TABLE 2

Effect of organophosphorus esters (0.05 μmol) on fast axoplasmic transport of proteins in the rat optic system[a]

Compound	*T*	*P*	*NT*	*p*
Cyanofenphos	0.90 ± 0.06	1.07 ± 0.09	0.66 ± 0.01	<0.001
EPN	0.83 ± 0.08	0.95 ± 0.09	0.70 ± 0.06	<0.001
DBL	0.88 ± 0.09	1.11 ± 0.10	0.77 ± 0.06	<0.001
Leptophos	0.79 ± 0.08	0.90 ± 0.10	0.83 ± 0.03	<0.001
EPBP (S-Seven)	0.74 ± 0.07	0.94 ± 0.09	0.88 ± 0.05	<0.001
TOCP	0.66 ± 0.28	0.80 ± 0.06	0.76 ± 0.04	<0.001

[a] Abbreviations are explained under Table 1.

The relative efficacy of these neurotoxic organophosphorus esters in provoked delayed neurotoxicity does not correlate well, in a quantitative sense, with the action of the same compounds upon fast axoplasmic flow. For example, the minimum single oral dose that caused delayed neurotoxicity in hens varied widely: cyanofenphos, 10 mg/kg; EPN, 25 mg/kg; DBL, 50 mg/kg; leptophos, 100 mg/kg; and EPBP, 800 mg/kg (11). The effectiveness of these esters as inhibitors of fast axoplasmic transport, however, varied only slightly from one compound to the other at the dose 0.3 μmol of each compound. In order to test whether a maximal effect was being observed at this dose level a smaller dose (0.05 μmol) was used to examine the effect of these compounds on fast axoplasmic transport. Again, the results demonstrated the same order of efficacy in inhibition of fast axoplasmic transport by these compounds (Table 2). Even though the comparisons are made between different species and using various criteria of effectiveness, a general correlation seems to exist between the relative effectiveness of these organophosphorus esters as neurotoxic and as inhibitors of fast axoplasmic transport. It is possible that the two effects are related.

The variations produced by a 0.3-μmol dose of these organophosphorus esters in the incorporation of L-[^{3}H]proline into proteins are interesting (Table 1). These compounds seemed to give a general negative correlation between their action upon protein synthesis and their ability to induce delayed neurotoxicity. For example, cyanofenphos, EPN, and DBL are all most active in causing delayed neurotoxicity, and all gave stimulation of protein synthesis; leptophos and EPBP are much less active in causing delayed neurotoxicity, and produce a much lower stimulation. However, at the small dose 0.05 μmol, these chemicals had no profound effect on protein synthesis (Table 2). In this regard, Paulson and McClure (17, 18) proposed the use of incorporation only as a "first order" correction to transport data. They obtained data that indicated that a more complex relationship existed between incorporation and the amount of radioactivity transmitted (e.g., for high doses of mescaline), and interpreted as real only those inhibitions in which *T* was significantly less than one, irrespective of the value of *P*. In the present study most of *T* values were less than one. These data indicate that the depression in *NT* was due to an inhibition of axoplasmic transport, rather than an increase in stimulation of protein synthesis.

Further evidence that delayed neurotoxicity may be related to inhibition of fast axoplasmic transport come from a comparison of TOCP and parathion. TOCP, an organophosphorus ester capable of inducing delayed neurotoxicity, caused inhibition of fast axoplasmic transport, but parathion, which does not cause delayed neurotoxicity, did not have any effect on fast axoplasmic transport. This differential effect on fast axoplasmic transport suggests that organophosphorus esters capable of causing delayed neurotoxicity may do so by inhibition of fast axoplasmic transport.

Substances synthesized in the cell bodies are continuously being transported to the axons. Cavanagh (10) suggested that if the nerve cell body becomes impaired

and the transport of materials—in particular proteins—to the peripheral branches of the axon is consequently decreased, distal axonal neuropathies can develop. If this is an accurate picture, we would expect delayed neurotoxicity caused by organophosphorus insecticides to reflect it. Studies on the effect of neurotoxic organophosphorus esters, however, have produced conflicting results. Pleasure *et al.* (7) found that axoplasmic flow "slow rate" was maintained in control and TOCP-treated cats, while it was absent in dorsal roots in acrylamide intoxication. James and Austin (16) reported that axoplasmic flow was the same in sciatic nerves of both DFP-treated and control chickens. Bradley and Williams (8) studied the waves of fast and slow axoplasmic flow in TOCP-treated and control cats. They concluded that the alterations that were demonstrated in the amount and rate of axoplasmic transport were insufficient to explain the axonal degeneration. The discrepancies between the work of James and Austin and the other studies cited may be due to the differences in animal models and toxicants used among the studies. Although there are conflicting results concerning what the abnormalities are in axonal transport as the result of organophosphorus esters intoxication, the evidence overall points to some defects in fast axonal transport that at least play a part in the mechanism of the delayed neurotoxicity induced by these compounds.

A generalized interruption of axoplasmic transport resulting from damage to nerve cell body is inconsistent with the observed axonal regeneration during intoxication and with the presence of distal and nonterminal distribution of multifocal degeneration. It is possible, however, that organophosphorus esters give rise to a "localized disruption" of axoplasmic transport in the distal axon. This could then cause "focal axonal degeneration", that spreads somatofugally to involve the entire distal axon. This hypothesis is in harmony with the conclusion in recent reports that DFP induced a focal distal, but not terminal, axonal degeneration. Wallerian degeneration of the more distal part is then precipitated by this "chemical transection" of the axon (28, 29).

This study has reported results showing that changes in fast axoplasmic transport in the rat optic system by organophosphorus esters paralleled the neurotoxicity of these compounds. Further studies are being carried out to resolve various proteins transported and check whether these effects are reversible or go on to axonal death. These studies also investigate species susceptible to delayed neurotoxicity.

ACKNOWLEDGMENTS

The discussion and advice of Dr. W. O. McClure of the Department of Biological Sciences, University of Southern California, and Dr. J. C. Paulson of the Department of Biochemistry, University of California, Los Angeles, are appreciated. The supply of organophosphorus esters by Velsicol Chemical Company, Nisson Chemical Industries, and E. I. du Pont de Nemours and Company is acknowledged.

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