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The Correlation between the Recovery Rate of Neurotoxic Esterase Activity and Sensitivity to Organophosphorus-Induced Delayed Neurotoxicity

The Correlation between Recovery Rate of Neurotoxic Esterase Activity and Sensitivity to Organophosphorus-Induced Delayed Neurotoxicity. CARRINGTON, C. D., AND ABOU-DONIA, M. B. (1984). *Toxicol. Appl. Pharmacol.* **75**, 350–357. Neurotoxic esterase (NTE) has been proposed to be the initiation site of organophosphorus compound-induced delayed neurotoxicity (OPIDN). There are two apparent problems associated with this hypothesis: NTE activity in the brain returns to nearly normal levels before the onset of the neuropathy, and NTE is present in and inhibited by organophosphorus compounds in young animals and other species which are relatively insensitive to the neurotoxic effects of these compounds. This paper presents data suggesting that differences in the recovery rates of NTE activity may account for some of these discrepancies. First, the onset of recovery of NTE activity following sc administration of 1.7 mg/kg of *O,O*-diisopropylphosphorofluoridate (DFP) in the hen sciatic nerve occurred several days later than in the brain. Furthermore, recovery was slower in distal than proximal parts of the nerve. This information indicates that NTE activity is depressed for a longer period at the site of the neuropathy than it would appear from the measurement of NTE activity in brain. Second, the rate of recovery of NTE activity was faster in the brains of chicks, of rats, and of hens treated with a daily po dose of 15 mg/kg cortisone acetate than it was in untreated hens. However, there was no significant increase in the NTE recovery rate in the peripheral nerves of the chicks or the cortisone-treated hens. Thus, it appears that although slower distal recovery could account for the greater sensitivity of longer axons to OPIDN, other factors are operating in chicks and cortisone-treated hens.

A large number of organophosphorus compounds (OPs) induce a neuropathy in many species, including man, 2 to 3 weeks after a single dose (Smith *et al.*, 1930; Abou-Donia, 1981). This phenomenon is termed organophosphorus compound-induced delayed neurotoxicity (OPIDN). Although the mechanism of OPIDN is still unknown, Johnson (1969, 1982) proposed that a protein assayed by phenylvalerate hydrolysis, called either neurotoxic esterase or neuropathy target esterase (NTE), is the initiation site for the neurotoxic effect. The validity of the NTE hypothesis has been questioned (Abou-Donia, 1981) because of the following observations: (1) NTE activity returns in brain, spinal cord, and sciatic nerve before the development of the neuropathy (Johnson, 1974; Caroldi and Lotti, 1982). While it is generally accepted that NTE activity is not important in the development of OPIDN, the recovery of activity, e.g., the brain and proximal peripheral nerves, may also signal the return of some function impaired by the binding of the OP to NTE which is involved in the development of the neuropathy. (2) OPs can inhibit NTE and aging in rats and chicks without inducing OPIDN (Johnson, 1975). (3) A physiological role for NTE is unknown. Nevertheless, NTE is currently the only candidate for the site at which OPIDN is initiated.

It is important to determine the rate at which NTE activity returns to normal levels following inhibition along the sciatic nerve of the hen for two reasons: First, previous determinations of the rate of the return of NTE activity have been performed with tissue samples which were predominately composed of nerve cell components in which the toxic response is not observed. OPIDN has been characterized as a distal neuropathy (Cavanagh, 1954) in which degeneration begins at the distal portions of the axons in the central and peripheral nervous systems. Consequently, measurements of NTE in brain, spinal cord, and whole sciatic nerve may

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mostly reflect activity in healthy nerve. The measurement of NTE activity in the distal parts of the hen sciatic nerve would fill this gap in the literature. Second, the ability of a shorter nerve to recover from damage caused by the binding of an OP to NTE in a shorter time period might account for differences in susceptibility that have been observed; the longest axons tend to be the most susceptible to OPIDN (Cavanagh, 1964). Furthermore, the species that are insensitive to OPIDN (i.e., rats, mice, and Japanese quail; Abou-Donia (1981)) tend to be considerably smaller than those that are sensitive (chickens, cattle, and humans).

To determine the rate of NTE recovery at the site of the development of OPIDN, we measured the rate at which NTE recovers in proximal and distal segments of hen sciatic nerve and its branches. In addition, we measured NTE recovery along a shorter nerve in the wing of the hen, the sciatic nerve of 6-week-old chicks, and the brains of hens, chicks, and rats. We also compared the rate of recovery of NTE in proximal and distal segments of hen sciatic nerve following the administration of a neurotoxic agent (*O,O*-diisopropylphosphorofluoridate; DFP) to the recovery rate observed following dosing with a non-neurotoxic compound (phenylmethylsulfonyl fluoride; PMSF). We also investigated the effect of steroids, which have been reported to prevent OPIDN in chickens (Glees, 1961) and cats (Baker *et al.*, 1982), on NTE recovery rate in hen brain and peripheral nerve.

METHODS

Animals. White leghorn hens, 18 months old, were obtained from Featherdown Farms, Raleigh, North Carolina. They weighed between 1.5 and 2.0 kg. Chicks were obtained from FCX, Durham, North Carolina. The chicks were 6 weeks old, female, and weighed about 250 g at termination. Male Sprague-Dawley rats weighing 200 to 250 g at the time of dosing were obtained from Holtzmann, Madison, Wisconsin.

Chemicals. *O,O*-diisopropylphosphorofluoridate, phenylmethylsulfonyl fluoride, *O,O*-diethyl-*p*-nitrophenyl phosphate (Paraoxon), atropine sulfate, physostigmine (Eserine), and cortisone acetate were obtained from Sigma Chemical Company (St. Louis, Mo.). Diazepam was obtained from Hofman–LaRoche (Nutley, N.J.). *N,N'*-Diisopropylphosphorodiamidic fluoride (mipafox) was synthesized by the Midwestern Research Institute (Kansas City, Mo.).

Dosing. All compounds were administered sc, except diazepam which was given im. The hens and chicks were dosed with either 1.7 mg/kg DFP in propylene glycol or 30 mg/kg PMSF in dimethyl sulfoxide. Hens given DFP were first protected with 1.0 mg/kg physostigmine and 0.2 mg/kg atropine sulfate. Chicks were given 0.2 mg/kg atropine sulfate and 0.6 mg/kg physostigmine. Rats were pretreated with 1 mg/kg physostigmine and 25 mg/kg atropine sulfate, as well as 5 mg/kg diazepam since an anticonvulsant was necessary to ensure survival. The animals dosed with cortisone acetate received daily po doses of 15 mg/kg in gelatin capsules from the day preceding DFP administration to termination. No solvent vehicle was given to control animals.

Tissue preparation. Animals were killed by asphyxiation with CO_2. The entire sciatic (*nervus ischiadicus*), tibial (*n. tibialis*), and peroneal (*n. peronaeous*) nerves were removed from 12 control animals and from groups of four hens killed at 0, 1, 2, 4, 6, 8, 10, and 14 days following dosing with DFP and at 0, 4, 7, 10, and 14 days following dosing with PMSF.

In a second experiment, the brain, the radial nerve (*n. radialis*) in the wing, and the lateral branch of the sciatic nerve (the peroneal, and the peroneal portion of the sciatic nerve) were removed from three control hens and five groups of three hens killed 0, 3, 6, 10, and 14 days after DFP administration. The brains and sciatic nerves were removed from three control chicks and five groups of three chicks killed at the same time intervals as the hens.

In a third experiment, the lateral branch of the sciatic nerve and brains were removed from four control hens, four hens which had received 2 days of cortisone acetate treatment, and five groups of eight hens (half of which received cortisone acetate) killed 0, 3, 6, 9, and 12 days after DFP administration. In addition, six groups of four rats were killed at the same time intervals as the hens.

All nerves were divided into 3-cm segments, yielding five segments from each of the pairs of hen sciatic nerves and branches. Although there were slight differences due to variation in the size of the animals, in general the two most proximal segments came from the sciatic nerve, the two most distal segments came from the peroneal nerve, and the central segment came from the point of bifurcation. Three segments were obtained from each of the pairs of radial nerves from the hens and the sciatic and peroneal nerves from the chicks. The segments were homogenized in 1 to 2 ml of 0.32 M sucrose–50 mM Tris, pH 8.0, with a Polytron for 5 sec. The brains were homogenized in Tris–sucrose (20% w/v) for 20 sec. All homogenates were then frozen at −20°C for 1 to 10 days before being assayed for NTE.

NTE assays. NTE activity was measured by the method of Johnson (1977). Three modifications in the assay for small segments of peripheral nerve were necessary. First, the volume of the reaction was reduced by 50%. Second, the incubation period was increased to 90 min. The reaction rate was linear over a 2-hr period. Third, the reaction was performed with a 1000*g* supernatant fraction rather than a whole homogenate; this fraction contained over 90% of the activity found in the whole homogenate. The first two modifications increased the sensitivity of the assay, which was necessary due to the much lower specific activity of the enzyme in peripheral nerve compared to brain. Centrifugation removed the relatively large particles of connective tissue that remained after homogenization.

Calculations and statistics. NTE activity was calculated on a per milligram of protein basis. Protein concentration was measured by the method of Bradford (1976), with bovine γ-globulin as a standard. Statistical differences in the rate of return of NTE due to either the distance from the cell body, the inhibitor used, the species, the age of the animal, glucocorticoid treatment, or the length of the nerve were examined by either single or multiple linear regression using the logarithms of the percentage of NTE inhibition. Inhibition was calculated as the difference between control values and NTE levels at 4 hr following the administration of the inhibitor. DFP inhibited 85 to 95% of the NTE activity in the tissue samples, while the PMSF treatment produced only about 80% inhibition. To prevent obscuring a delay in the onset of recovery, only the data from the last time point in each tissue group (brain or segment) which preceded a group with an average recovery of at least 15% were included in the analysis; data from earlier time points were omitted. In addition, groups containing values in excess of the control average, and all time points thereafter, were omitted from the multiple regression analysis. These alterations were necessary since the logarithm of a value which indicates negative inhibition, which results from an NTE value in excess of the mean control value, is not a real number.

RESULTS

Hens dosed with DFP, including those given cortisone acetate, and killed at 10 days were ataxic, while those killed at 14 days were nearly paralyzed. All other animals appeared normal at termination.

The NTE activities in the most proximal and distal segments of the different nerve preparations at varying time points following inhibition, expressed as a percentage of control, are given in Table 1. There was consistently less recovery in the distal portion, compared to the proximal portion, of the hen sciatic nerve, the hen radial nerve, and the chick sciatic nerve, 6 days following treatment with 1.7 mg/kg DFP.

While time and distance were both important in NTE recovery, the relationship between the two factors is not simple. The transformed data (log percentage inhibition) from sciatic nerves of DFP-treated hens could be fit almost equally well by models which utilized time and either distance ($r = 0.587$) or time $\times$ distance interaction ($r = 0.605$) factors. Both factors were significant in either model ($p < 0.001$). Utilizing both distance and time $\times$ distance factors did not significantly enhance the fit ($r = 0.606$). Analysis of variance of all the raw data suggested that the distance factor is important ($p < 0.001$) while the interaction factor is not ($p > 0.05$). On the other hand, multiple linear regression analysis of the group means (log percentage inhibition) indicated that only the interaction factor is significant ($p < 0.05$). Since the interaction model produced a slightly better fit, it is the model used in Figs. 1 and 2.

Figure 1 is a plot of the log NTE inhibition in the most proximal segment (closed squares) of the hen sciatic nerve and the most distal segment (open squares) of its branches versus time after DFP administration. The lines denote the values predicted for the proximal and distal segments derived from the regression analysis of the data from all five segments. Figure 2 contains similar plots from sciatic nerves of PMSF-treated hens (A), sciatic nerves of DFP-treated chicks (B), sciatic nerves of DFP-treated hens with daily cortisone acetate treatment following OP administration (C), and radial nerves of DFP-treated hens (D). Neither the interaction model illustrated in Fig. 2 nor the independent factor model yielded any significant differences from control hen sciatic nerve in intercept, time, distance, or interaction factors due to the inhibitor used, the age of the animal, cortisone acetate treatment, or the peripheral nerve used. The most notable differences were a lower intercept following PMSF treatment than after DFP administration, a faster overall rate of recovery

TABLE 1

RECOVERY OF NEUROTOXIC ESTERASE IN PROXIMAL AND DISTAL PERIPHERAL NERVES

Days after administration		Hen				
		Sciatic DFP	Sciatic PMSF	Sciatic DFP + CA	Radial DFP	Chick Sciatic DFP
0	Proximal	10.1 ± 2.2 (11)	23.8 ± 11.1	−2.0 ± 2.6**	2.8 ± 3.2	24.3 ± 10.3
	Distal	7.5 ± 2.5	24.0 ± 2.9**	3.6 ± 3.6	6.5 ± 2.5	19.6 ± 9.8
1	Proximal	15.1 ± 1.0 (4)	—	—	—	—
	Distal	13.9 ± 2.4	—	—	—	—
2	Proximal	12.4 ± 4.7 (4)	—	—	—	—
	Distal	9.1 ± 2.4	—	—	—	—
3	Proximal	27.3 ± 4.8 (11)	—	38.5 ± 6.1	38.5 ± 11.4	32.3 ± 4.3
	Distal	21.4 ± 3.7	—	4.0 ± 4.0**	23.2 ± 8.5	34.8 ± 10.3
4	Proximal	38.6 ± 9.4 (4)	58.1 ± 4.4	—	—	—
	Distal	21.2 ± 1.0	31.3 ± 13.9	—	—	—
6	Proximal	63.1 ± 6.4 (11)*	—	52.6 ± 11.6*	49.2 ± 4.4*	73.2 ± 8.3*
	Distal	28.6 ± 5.8	—	11.9 ± 4.2**	20.1 ± 3.7	40.7 ± 3.6
7	Proximal	—	50.7 ± 4.0	—	—	—
	Distal	—	43.3 ± 3.4	—	—	—
8	Proximal	71.5 ± 4.0 (4)	—	—	—	—
	Distal	61.5 ± 3.8	—	—	—	—
9	Proximal	68.6 ± 15.1 (4)	—	53.8 ± 9.9	—	—
	Distal	54.7 ± 13.3	—	32.0 ± 5.0	—	—
10	Proximal	68.9 ± 6.1 (7)	82.9 ± 7.4	—	79.8 ± 19.2	94.2 ± 4.5*
	Distal	52.5 ± 5.5 (4)*	48.6 ± 14.9	—	58.4 ± 2.8	87.1 ± 3.9*
12	Proximal	115.4 ± 6.4*	—	95.1 ± 11.0	—	—
	Distal	55.4 ± 24.8	—	52.8 ± 20.1	—	—
14	Proximal	83.6 ± 8.4 (7)	103.7 ± 7.7	—	78.2 ± 6.0	78.1 ± 3.3
	Distal	67.1 ± 8.6	104.8 ± 3.8**	—	52.1 ± 9.9	71.3 ± 7.7

Note. NTE recovery at varying time points following inhibition, expressed as a percentage of control values (±SEM), of the most proximal and most distal segments of the hen sciatic nerve and peroneal branch (HSP) following treatment with DFP (the number of determinations is given in parentheses), the hen sciatic and peroneal branch following treatment with PMSF, in the hen sciatic and peroneal branch following DFP administration with daily cortisone acetate, in the hen radial nerve (HRN) following DFP, and in the chick sciatic nerve and peroneal branch (CSP) after DFP. Control values, in 10^{-10} M phenylvalerate hydrolyzed per mg/min, are 12.2 ± 0.8 (P) and 9.2 ± 0.9 (D) for HSP (n = 9), 12.3 ± 0.8 (P) and 13.8 ± 1.7 (D) for HR (n = 3), and 15.5 ± 1.6 (P) and 15.1 ± 3.6 (D) for CSP (n = 3).

* Percentage recovery in proximal segment differs significantly from distal segment ($p < 0.05$).

** Percentage recovery in group differs significantly from the comparable value in HSP-DFP ($p < 0.05$).

in chick nerve, and a lower intercept and a greater interaction factor in the radial nerve.

Figure 3 illustrates the recovery of NTE in chick brain, in rat brain, and in hen brain with and without cortisone acetate treatment. The rates of recovery in brains of the chicks, rats, and cortisone-treated hens were all significantly faster than that of DFP-treated hen

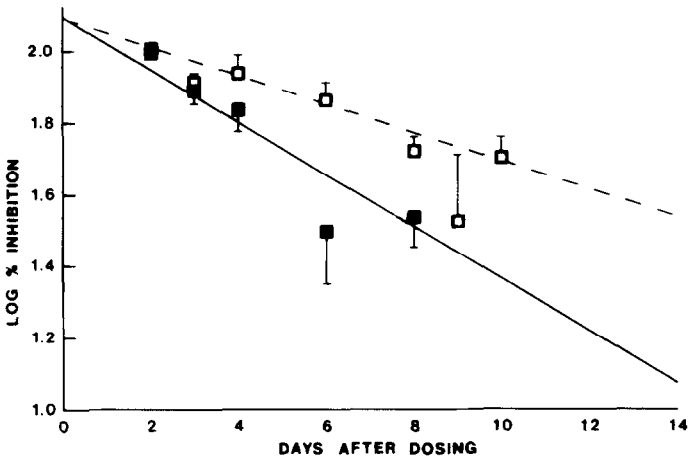

FIG. 1. Plot of the logarithm of the percentage inhibition of neurotoxic esterase activity in 3-cm segments of hen sciatic nerve vs time of termination following inhibition with DFP. The closed squares represent activity in the most proximal segment (0 to 3 cm from the spinal cord) while the open squares represent activity in the most distal segment (12 to 15 cm from the spinal cord). Each point is the average of 4 to 11 measurements (see Table 1). The error bars denote SE. The lines represent the predicted values resulting from multiple linear regression analysis of the data from the entire nerve. The differences in slope are significant ($p < 0.001$).

brains (Student's t test, $p < 0.001$). Recovery in rat brain was particularly rapid, with a return to control values after only 6 days. Comparison of recovery rate in hen brain to that observed in the most proximal segment of the sciatic nerve yielded significant differences in

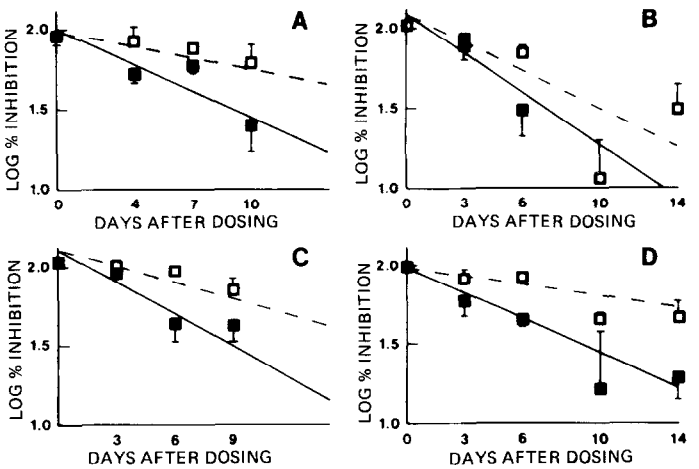

FIG. 2. Plot of the logarithm of the percentage inhibition of neurotoxic esterase activity in 3-cm segments of avian peripheral nerve vs time of termination following inhibition. The closed squares represent activity in the most proximal segment (0 to 3 cm from the spinal cord) while the open squares represent activity in the most distal segments 12 to 15 cm (A and C) or 6 to 9 (B and D) from the spinal cord. (A) Sciatic nerve from hens treated with PMSF; (B) sciatic nerve from chicks dosed with DFP; (C) sciatic nerve from hens dosed daily with cortisone acetate following the administration of DFP; (D) radial nerve from hens treated with DFP. Each point is the average of 3 to 4 measurements (see Table 1). Recovery was not significantly different from untreated hen sciatic nerve in any of these tissues.

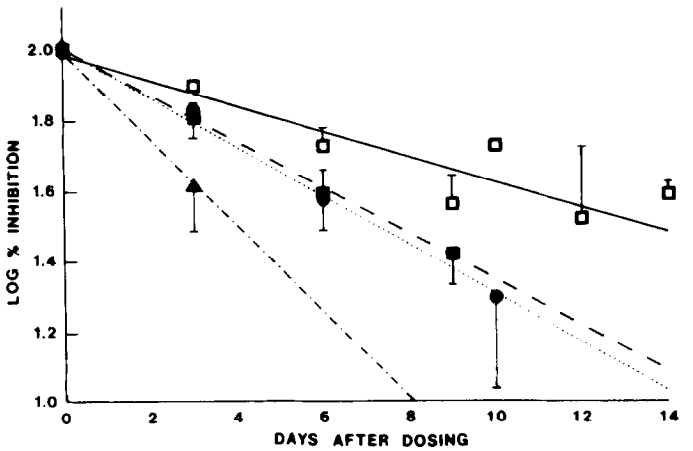

FIG. 3. Plot of the logarithm of the percentage inhibition of neurotoxic esterase activity in the brains of untreated hens (open squares), hens treated with cortisone acetate (closed squares and dashed line), chicks (circles and dotted line), and rats (triangles) vs time of termination after inhibition with DFP. Each point is the average of 3 to 7 measurements. The error bars denote SE. The lines are least squares fits. The slope of the line fitted to the brain data of untreated hens differs significantly from each of the other three (Student's t test, $p < 0.001$).

both slope ($p < 0.001$) and intercept ($p < 0.01$). The difference between slope and intercept were equally significant when brain recovery rate was compared to the most distal segment. Thus, the rate of recovery is faster in nerve than in brain, but there is a delay in onset.

DISCUSSION

The two most important points derived from the data follow: (1) There is a significant delay in the onset of the return of NTE activity, even in the most proximal segment of the hen sciatic nerve. Our data are similar to the results of Caroldi and Lotti (1982) with the exception that we found the difference in the rate of recovery between nerve and brain to be statistically significant. Since we used a compound which is rapidly cleared in the test animals' systems, the delay in onset is probably not due to continued inhibition by the organophosphate. More likely, there is a delay due to the axonal transport of NTE from the site of synthesis in the cell body and/or incorporation of the protein into the transport system. The faster rate of recovery in peripheral nerve is not particularly surprising in view of the much lower specific activity of NTE in the peripheral nerve. There may be preferential entry of newly synthesized NTE into the axonal transport system. (2) Recovery of NTE occurs later in the most distal portions of a nerve. While it is not clear whether this delay of recovery is due to a further delay in onset, a reduction in the rate of recovery, or both, a high level of inhibition (>75%) is maintained in the hen peroneal nerve for a week following a single dose of DFP. Presumably, this also occurs in the distal portions of long axons of the spinal cord. Consequently, any damage resulting from the binding of an organophosphorus compound may be incurred over a longer time period than would be apparent from the assay of NTE in brain.

The fact that distance from the cell body is also a factor in the recovery of NTE following the administration of PMSF leads to two conclusions. First, the lack of neurotoxicity of PMSF is not attributable to a difference in recovery rate. Second, the delay in recovery observed in distal nerve is not a result of the delayed neurotoxic effects of DFP. Although the difference was not significant, the lower

intercept observed following DFP administration than after PMSF administration may reflect an effect of DFP on protein synthesis not shared by PMSF. DFP has been reported to inhibit protein synthesis in the spinal ganglia of the cat (Porcellati, 1971).

Cortisone acetate did not protect our hens from OPIDN, although it did accelerate the rate of NTE recovery in brain. Cortisone acetate may be effective only in borderline cases such as the low dose of tri-*ortho*-cresyl phosphate used by Glees (1961) and the local injections used by Baker *et al.* (1979). Since cortisone acetate is reportedly effective even after the development of the neuropathy (Glees, 1961), some other effect of cortisone acetate must account for any benefit derived from administration.

NTE recovery was faster in the brains of chicks and rats, animals which are relatively more insensitive to OPIDN than in hens. Johnson and Barnes (1970) found no significant difference between the rate of recovery of NTE in hen and chick brain. However, they used only six chicks which were 2 weeks older than the chicks used in this study. The fact that we found a lower specific activity in chick than in hen brain, whereas Johnson (1970) did not, is attributable to differences in the method of calculation. We measured activity per mg protein, instead of per gram of wet weight. The chick brains contained more protein per gram of wet weight.

As with the cortisone acetate hens, there was not a significantly greater overall recovery rate in the peripheral nerve of the chicks. However, there was a significantly greater percentage recovery in both the most proximal and distal segments of the chick sciatic nerve than in the hen sciatic nerve 10 days following administration. At this time point, the difference could either reflect the etiology or be a result of the neuropathy which is involved in the development of the neuropathy. The more rapid recovery of the activity observed in the insensitive animals may reflect a faster general metabolic rate which is helpful in responding to cellular damage after it has been initiated.

NTE recovery was monitored in the hen radial nerve to assess the importance of the distance factor in a shorter nerve. Clearly, recovery was slower in the distal than in the proximal portions of the radial nerve. Although the difference was not significant, distance appeared to attenuate NTE recovery to a greater degree in the radial nerve than in the sciatic and peroneal nerves. The transport rates of NTE in the two nerves may not be identical. Although we did not find any degeneration in this nerve 21 days after a neurotoxic dose of tri-*ortho*-cresyl phosphate (unpublished data), we do not have histopathological data for the radial nerve following DFP treatment. It is notable that the wing function of the DFP-treated hens did appear to be impaired, although this impairment may have been due to axonal degeneration in the spinal cord.

Our data suggest that a negative correlation between the rate of recovery of NTE activity and the distance from the cell body may account for the greater sensitivity of longer axons to OPIDN. Since the inhibition of NTE activity is apparently not responsible for OPIDN, it is not clear how a more speedy recovery of the activity would prevent the development of the neuropathy. However, it is possible that some function necessary for the maintenance of the axon is associated with NTE and is compromised upon the binding of an organophosphorus compound to NTE. This putative cellular component may be transported with NTE. If this theory is correct, then chronic exposure to OPs should be able to overcome at least some of the differences in sensitivity due to age, species, and axonal length. There is evidence that chronic dosing with OPs can produce neuropathies in so-called "insensitive" species (rat, Veronesi and Abou-Donia (1982) and mouse, Lapadula *et al.* (1983)). Alternatively, the recovery rate of NTE may be indicative of a more general correlation between distance from the cell body and the time required to restore a compromised function.

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