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Triphenyl phosphite neurotoxicity in the hen: inhibition of neurotoxic esterase and of prophylaxis by phenylmethylsulfonyl fluoride

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Abstract. The neuropathic syndrome resulting in the cat and the rat from single or multiple doses of the phosphorous acid ester tiphenyl phosphite (TPP) has been reported to differ from the syndrome caused by numerous phosphoric acid esters, which is known as organophosphorous compound-induced delayed neurotoxicity (OPIDN). Since the hen is used to test compounds for OPIDN, we chose to study the neurotoxicity of single subcutaneous doses of TPP using this animal model. TPP (1000 mg/kg) produced progressive ataxia and paralysis which began to develop 5–10 days after dosing. Similar signs were observed when subcutaneous doses of the OPIDN-causing agents tri-*o*-cresyl phosphate (TOCP) or diisopropyl phosphorofluoridate (DFP) were administered. The minimum neurotoxic dose of TPP was 500 mg/kg. Prior administration of phenylmethylsulfonyl fluoride (PMSF) prevented the development of a neuropathy induced by DFP, but did not fully protect the hens from TPP or TOCP. PMSF slowed, but did not prevent, the neuropathy caused by TOCP. PMSF reduced the neurotoxicity of 500 mg/kg TPP, but increased the neurotoxicity of 1000 mg/kg TPP. TPP was found to be a very potent inhibitor of neurotoxic esterase (NTE), the putative target site for OPIDN, in vitro, with a k_i of about 2.1×10^5 $M^{-1}min^{-1}$. Equimolar doses of either TPP (1000 mg/kg) and TOCP (1187 mg/kg) caused over 80% inhibition of neurotoxic esterase (NTE) in brain and sciatic nerve. This high level of NTE inhibition persisted for several weeks. This prolonged inhibition probably accounts for the inability of PMSF to block the neurotoxicity of TOCP. The dose-response curve for NTE inhibition 48 h after dosing indicated that a level of 70% inhibition correlated with the neurotoxicity of TPP.

Subneurotoxic doses of TPP and DFP were found to have an additive effect which could be blocked by PMSF. These results indicate that TPP can cause OPIDN in the hen. The synergism between PMSF and the higher dose of TPP suggests the presence of a second neurotoxic effect as well.

Key words: Triphenyl phosphite – Hen – Neurotoxic esterase – OPIDN – Tri-*o*-cresyl phosphate

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Introduction

A number of organophosphorous compounds induce a neuropathy in humans and animal models which begins to develop 6–14 days after a single exposure (Smith et al. 1930; Abou-Donia 1981). This effect is known as organophosphorous compound-induced delayed neurotoxicity (OPIDN) and results from exposure to a number of phosphonic and phosphoric acid esters. Despite the fact that these compounds are widely used as plasticizers (US EPA 1985) and that triphenyl phosphite (TPP) and several aryl phosphites have been known to be neurotoxic (Smith et al. 1932, 1933), the esters of phosphorous acid have received relatively little attention,

In the studies that have been conducted on the neurotoxic effects of aryl phosphorous acid esters, results atypical of OPIDN have been obtained. Differences from OPIDN in the clinical effect of TPP have been noted in the cat (Smith et al. 1933) and rat (Veronesi et al. 1986). Histopathological damage not characteristic of OPIDN has been noted in the cat after tri-*o*-cresyl phosphite (Lillie and Smith 1932), and in the rat (Veronesi et al. 1986) and hen (Carrington et al. 1988a, b) following exposure to TPP. Finally, there are structure-activity differences between the phosphites and those compounds which cause OPIDN. Triphenyl phosphite, tri-*o*-cresyl phosphite, tri-*m*-cresyl phosphite, and tri-*p*-cresyl phosphite are all neurotoxic (Smith et al. 1933). Of the corresponding phosphate isomers, only TOCP causes OPIDN (Smith et al. 1930, 1932).

It has been demonstrated that a number of compounds, particularly carbamates, sulfonyl fluoride, and phosphinates, are able to prevent the occurrence of OPIDN when given 24 h prior to the neurotoxic compound diisopropyl phosphorofluoridate (DFP; Johnson 1974). One such prophylactic compound, phenylmethylsulfonyl fluoride (PMSF), has also been found to protect from OPIDN induced by DFP, mipafox (N,N-diisopropyl phosphorodiamidic fluoride), 2-methylphenyl diphenyl phosphate, and TOCP (Johnson 1982; Carrington and Abou-Donia 1983).

Neurotoxic esterase (NTE), a protein characterized either by labelling with [^{3}H]-DFP or its esterase activity (Johnson 1982), is a putative target site for the induction of OPIDN. NTE is irreversibly inhibited by organophosphorous compounds through progressive phosphorylation (Carrington and Abou-Donia 1986). A criterion of 70% or greater inhibition of NTE has been found to correlate with

the induction of OPIDN by various compounds in the hen (Johnson 1982).

In this paper, we compare the neurotoxicity induced by TPP with OPIDN caused by either DFP or TOCP in the hen, which is the test animal commonly used for OPIDN. First, we investigated the ability of PMSF to prevent the neuropathies induced by various subcutaneous doses of either TPP or TOCP. Second, we monitored NTE activity over a 21-day period following neurotoxic subcutaneous doses of both TPP and TOCP. Third, we determined the dose-response relationship for the inhibition of NTE in vivo by TPP. Fourth, we measured the potency of TPP for NTE inhibition in vitro. Finally, we assessed the ability of TPP to prevent or potentiate the neurotoxicity of DFP.

Methods

Animals. White leghorn hens, 14 months old, weighing 1.5–2.0 kg were obtained from Featherdown Farm, Raleigh, NC.

Materials. DFP (*O,O*-diisopropyl phosphorofluoridate; 98% purity), TPP (triphenyl phosphite; 97%), diphenyl phosphite (85%), and paraoxon (*O,O*-diethyl *p*-nitrophenyl phosphate) were obtained from Aldrich Chemical Co., Milwaukee, WS. The impurities contained in the TPP generated ^{31P}-NMR peaks which corresponded to diphenyl phosphite and triphenyl phosphate (Carrington et al. 1988a). Physostigmine, phenylmethylsulfonyl fluoride (PMSF; 99%) and atropine sulfate were obtained from Sigma Chemical Company, St Louis, MO. Mipafox (*N,N*-diisopropyl phosphorodiamidic fluoride) was synthesized by the Midwestern Research Institute, Kansas City, Mo.). Tri-*o*-cresyl phosphate (TOCP; 99%) was obtained from Eastman Kodak Co., Rochester, NY.

Dosing. Hens were dosed with 1.7 mg/kg DFP (in propylene glycol); 250, 500, 750, 1000 mg/kg TPP; or 62.5, 125, 250, 500, 750, or 1187 mg/kg TOCP. All three organophosphorous compounds were administered subcutaneously at the side of the neck. The animals dosed with DFP were protected from the acute effects by the administration of 1.0 mg/kg physostigmine (s.c., in propylene glycol) and 300 mg/kg atropine (p.o.) 15 min prior to the organophosphorous compound. When given, the PMSF was administered 24 h prior to dosing with DFP, TPP, or TOCP at a dose of 30 mg/kg (s.c., 30% dimethyl sulfoxide solution). The prophylaxis of 250 mg/kg TPP was investigated with a challenge of 1.7 mg/kg DFP 48 h after the TPP. The additive effect of DFP and TPP was assessed by following a dose of 0.25 mg/kg DFP with 250 mg/kg TPP 24 h later. Control hens for the inhibition experiments were untreated.

Clinical assessment. Animals were initially rated on a 6-point scale (normal, T_1–T_4, or paralyzed; Abou-Donia 1978). For the purpose of comparing treatments, hens were given less equivocal ratings of normal (T_2 or less), ataxic (T_3 or T_4), or paralyzed (including moribund animals sacrificed). All the animals graded as paralyzed first progressed through a period of ataxia.

NTE assays. The assays measuring the inhibition of NTE in vivo were conducted by measuring NTE activity in brain and sciatic nerve at 1–21 days after dosing with TPP using the method described by Johnson (1982). The sciatic nerve was prepared and assayed as described previously (Carrington and Abou-Donia 1984). The inhibition of NTE in vitro was measured using paraoxon preinhibited hen brain homogenate prepared as described previously (Carrington and Abou-Donia 1986). The protein was preincubated with 50 μM mipafox for 15 min prior to the addition of varying concentrations of TPP (10^{-8}–10^{-5} M) for 7–10 min. The preincubation step was conducted in a volume of either 2 or 0.1 ml. Following preincubation, either 2 ml substrate solution (1.4 mM phenylvalerate), or substrate solution plus 1.9 ml buffer (Tris HCl, pH 8.0) was added to bring the total volume to 4 ml. After 15 min, the reaction was stopped and substrate hydrolysis measured as described previously (Johnson 1982). The data were analyzed using weighted, nonlinear regression analysis as described previously (Carrington and Abou-Donia 1986), and a model assuming one enzyme and progressive phosphorylation with no significant Michaelis complex formation.

Protein concentrations for the experiments involving both in vivo and in vitro exposure to TPP were measured by the method of Lowry et al. (1951).

Statistics. Statistical tests for prophylaxis, protection or potentiation were performed using a chi-square test. Models assuming the incidence of ataxia or paralysis in the two treatment groups to be either identical or different were compared. All comparisons had 3 degrees of freedom.

Results

Clinical signs

Triphenyl phosphite produced clinical signs similar to those observed following exposure to neurotoxic phosphoric acid esters (Abou-Donia 1981). However, ataxia and paralysis tended to develop several days sooner when compared to animals treated with either TOCP or DFP. Early signs of neurotoxicity (T_1 or T_2) were observed more frequently before 7 days after TPP. This rarely occurred in animals treated with the classical OPIDN inducing agents. At 21 days after dosing, the ED_{50} for the production of paralysis in hens by subcutaneously administered TPP was found to be about 500 mg/kg, while the ED_{50} for ataxia was somewhere between 250 and 500 mg/kg. The ED_{50} for paralysis by subcutaneous TOCP was between 125 and 250 mg/kg, while ED_{50} for ataxia was between 62.5 and 125 mg/kg.

Prophylactic action of PMSF

Prior treatment with 30 mg/kg PMSF fully prevented the neuropathy induced by DFP, but did not protect the animals treated with either 1000 mg/kg TPP or an equimolar dose (1187 mg/kg) of TOCP (Table 1). We have previously found that PMSF does prevent the neuropathy caused by a smaller oral dose (750 mg/kg) of TOCP (Carrington and Abou-Donia 1983). To investigate the possibility that the difference between the oral dose and the subcutaneous doses of TOCP and TPP are due their persistance beyond the 3–6 day period during which protection is afforded by PMSF (Johnson 1974), we examined the prophylactic effect of PMSF using lower doses of the neurotoxic chemi-

Table 1. Prophylaxis of PMSF for neurotoxicity induced by TPP, TOCP, or DFP. Clinical condition of hens at 10 or 21 days after the administration of various doses of DFP, s.c., TOCP, s.c., or TPP, s.c. with or without the prior (24 h) administration of 30 mg/kg PMSF, s.c. All doses are given in mg/kg

Time/compound/dose			Clinical condition							
			No PMSF				+ PMSF			
			(n)	Normal	Ataxic	Paralyzed	(n)	Normal	Ataxic	Paralyzed
10	TPP	250	5	5	0	0	6	6	0	0
		500	10	6	4	0	9	8	0	1
		750	6	3	3	0	4	3	0	1
		1000	17	10	5	2	4	1	2	1
	TOCP	62.5	3	3	0	0	3	3	0	0
		125	3	3	0	0	3	3	0	0
		250	4	4	0	0	4	4	0	0
		500	4	4	0	0	4	4	0	0
		750	4	3	1	0	4	3	1	0
		1187	4	0	4	0	4	2	2	0
21	TPP	250	8	6	2	0	6	6	0	0
		500	13	1	4	8	9	6	2	1
		750	4	0	0	4	4	1	1	2
		1000	25	2	5	18	12	0	0	12
	TOCP	62.5	3	2	1	0	3	0	0	0
		125	3	0	2	1	3	0	0	0
		250	4	0	1	3	4	3	1	0
		500	4	0	0	4	4	2	1	1
		750	4	0	0	4	4	1	2	1
		1187	4	0	0	4	4	0	2	2
	DFP	0.25	3	2	1	0	0	0	0	0
	DFP	1.7	7	0	1	6	3	3	0	0
	DFP + TPP	0.25 + 250	4	0	0	4	4	4	0	0

cals and examined the time course for the onset and severity of the neuropathy (Table 1, Fig. 1).

The results using TOCP are consistent with the view that high subcutaneous doses of this chemical persist beyond the prophylactic period of a single dose of PMSF. As the dose is lowered, the frequency and severity of the clinical signs of nerve degeneration are decreased by prior administration of PMSF (Fig. 1). In addition, there appears to be a delay in the onset of ataxia and paralysis even at high doses, since a greater degree of protection is afforded at 10 and 14 days than at 21 days. At 14 days, 15 out of 16 animals were ataxic, and 4 of out 12 animals were paralyzed without PMSF; with prior administration of PMSF, the corresponding figures are 4 out 16, and 0 out of 12 (Table 1).

The effect of PMSF on TPP-induced neurotoxicity appears to depend on the dose of TPP used. PMSF had a somewhat paradoxical effect 10 days after 500–750 mg/kg doses of TPP; although fewer animals were ataxic after PMSF (5 out of 10 without PMSF, 2 out of 10 with PMSF), the two animals that showed signs of neurotoxicity with PMSF were paralyzed even thoug none of the animals not given PMSF were affected so severely (Table 1). At 21 days, PMSF protected hens given 500 mg/kg TOCP (using ataxia as a criterion, χ-square = 8.5, $p < 0.05$; using paralysis, χ-square = 5.6, $p \approx 0.06$). At the highest dose (1000 mg/kg), PMSF apparently potentiates the neurotoxicity of TPP since a higher percentage of the animals became ataxic or paralyzed at all time points. Most significant was that 12 out of 12 animals given PMSF were paralyzed at 21 days, yet only 15 out of 21 were paralyzed when PMSF was not given (Table 1; χ-square ≈ 0.13).

Inhibition of NTE by TPP

Inhibition of NTE in vitro. Triphenyl phosphite was found to be a potent inhibitor of NTE in vitro. However, the measured potency of TPP as an inhibitor greatly depended on whether or not the TPP was removed by dilution prior to the addition of the substrate. Without dilution, TPP has an apparent k_i of $2.1 \times 10^5 \pm 0.6 \times 10^5$ $M^{-1}min^{-1}$ ($n = 6$). With dilution, the k_i was much lower ($2.5 \times 10^4 \pm 0.6 \times 10^4$ $M^{-1}min^{-1}$, $n = 4$). In addition, the slope of the inhibition curve observed without dilution was much steeper than would be predicted by a progressive phosphorylation model (Fig. 2).

Inhibition and recovery of NTE in vivo. Neurotoxic esterase activity was highly inhibited in vivo 24 h after the hens were doses subcutaneously with 1000 mg/kg TPP. NTE activity in brain was decreased by about 80%, and activity in sciatic nerve was completely inhibited (see Figs. 3 and 4). Even though brain levels showed recovery 14 days after TPP, NTE activity remained depressed in sciatic nerve for the full 21 days during which activity was monitored. An equimolar dose of 1187 mg/kg TOCP resulted in essentially complete inhibition of NTE in both brain and sciatic nerve which persisted for 21 days.

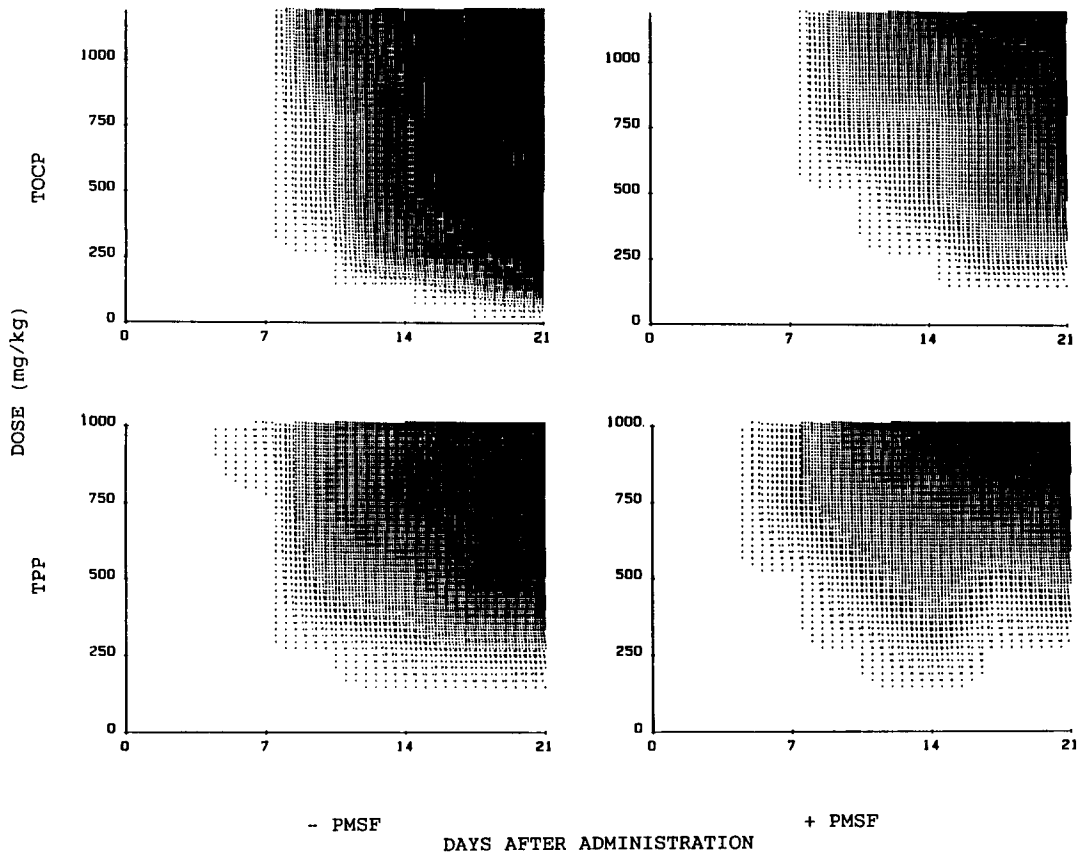

Fig. 1. Average behavioral deficit score as a function of time after administration (*X-axis*, in days) and dose (*Y-axis*, mg/kg). The top plots are for TOCP while the bottom two are for TPP. The left plots are without PMSF pretreatment, the right plots are for animals pretreated with 30 mg/kg PMSF. The shade density reflects the average clinical condition of the animals, where no shading denotes that all the animals were normal and the darkest shade indicates that all the animals were paralyzed

Dose-response relationship for inhibition of NTE by TPP. The minimum neurotoxic dose of 500 mg/kg TPP resulted in over 70% inhibition of NTE in both brain and sciatic nerve (Fig. 5). NTE levels following a 250 mg/kg dose of TPP were about one half the normal values in brain, and were close to what are considered threshold levels for OPIDN in sciatic nerve.

Additive neurotoxicity of TPP and DFP. A subneurotoxic dose of 250 mg/kg TPP did not prevent the neuropathy induced by 1.7 mg/kg DFP given 48 h later (data not shown), indicating that it is not prophylactic. This same dose of TPP did have an additive neurotoxic effect when combined with a borderline dose of DFP (0.25 mg/kg).

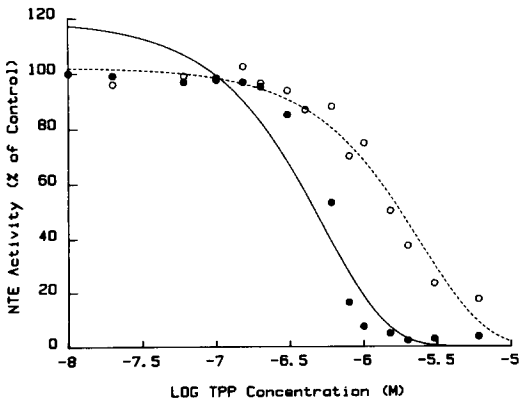

Fig. 2. The inhibition of neurotoxic esterase activity by TPP with either a 2:1 (*solid circles, solid line*) or 40:1 dilution upon the addition of substrate after a 10-min preincubation at 37° C. The lines represent the best fit (weighted least squares) of the data using a progressive phosphorylation model. The poor fir in the former case indicates that the model is inappropriate

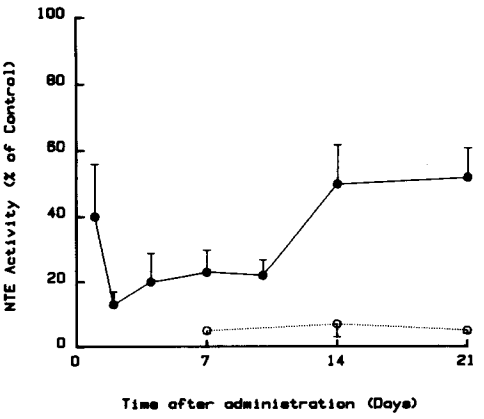

Fig. 3. Activity levels of neurotoxic esterase in hen brain at varying time points after the subcutaneous administration of either 1000 mg/kg TPP (*solid circles, solid line*), or 1187 mg/kg TOCP (*open circles, dotted line*). Control values were 16.4±2.0 nM phenylvalerate hydrolyzed per mg protein per min ($n = 6$). There were three animals at each time point, except for TPP at day 7 for which $n = 6$

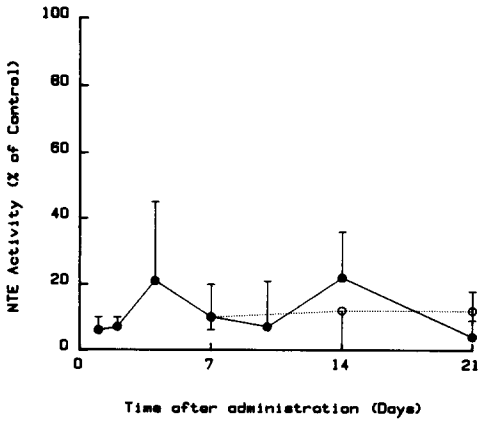

Fig. 4. Activity levels of neurotoxic esterase in hen sciatic nerve at varying time points after the subcutaneous administration of either 1000 mg/kg TPP (*solid circles, solid line*), or 1187 mg/kg TOCP (*open circles, dotted line*). Control values were 0.73 ± 0.18 nM phenylvalerate hydrolyzed per mg protein per min ($n = 6$). There were three animals at each time point, except for TPP at day 7 for which $n = 6$

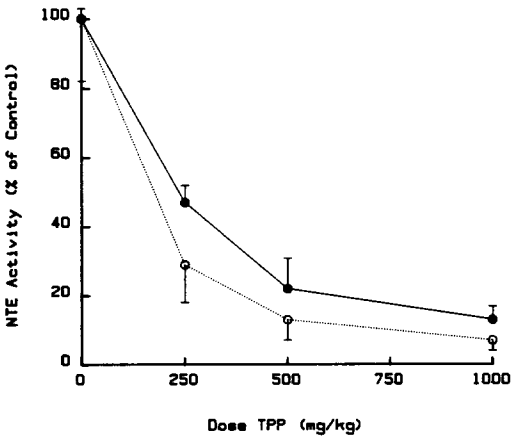

Fig. 5. Activity levels ($\pm$ SEM) of neurotoxic esterase in hen brain (*circles, solid line*) and sciatic nerve (*diamonds, dotted line*) 48 h after the subcutaneous administration of 0–1000 mg/kg TPP. Control values were 13.6 $\pm$ 0.4 nM phenylvalerate hydrolyzed per mg protein per min for brain ($n = 6$) and 1.10 ± 0.19 nM/mg-min for sciatic nerve ($n = 6$). There were three animals for each of the three doses of TPP

The combined effect of the two compounds was blocked by PMSF (Table 1).

Discussion

The data presented in this report strongly indicate that TPP causes OPIDN in the hen. TPP inhibits NTE, a putative target site for OPIDN, both in vitro and in vivo. The dose-response curve for NTE inhibition correlated with the curve for neurotoxicity, with a level of about 70% inhibition corresponding to a threshold neurotoxic dose. PMSF, an agent known to be prophylactic with regard to OPIDN, protects hens from the minimum neurotoxic dose of 500 mg/kg TPP. TPP is clearly not prophylactic, since a non-neurotoxic dose of TPP which results in about 60% NTE inhibition will not prevent the neuropathic effect of DFP. On the other hand, subneurotoxic doses of TPP and DFP have an additive effect which is blocked by PMSF. The observation that all lesions characteristic of OPIDN are present after TPP administration (in addition to neuronal chromatolysis and necrosis), and that DFP administration after 1000 mg/kg TPP causes no additional damage (Carrington et al. 1988a) also indicates that TPP induces OPIDN.

One anomaly supports the histopathological evidence (Carrington et al. 1988a) that TPP causes a second syndrome in the hen as well; PMSF potentiates rather that protects hens from the highest dose (1000 mg/kg) of TPP studied. Since PMSF protects at lower but not higher doses of TPP, it is likely that the dose-response curve for clinically significant damage is lower for OPIDN than for the second syndrome.

The lack of complete protection by PMSF of the TOCP-dosed animals, and perhaps to some extent the TPP-dosed animals as well, is attributable to the persistence of the compound beyond the period during which prophylaxis is afforded. This is supported both by the observation that the onset of the neuropathy is at least delayed in the TOCP-dosed animals and that both TPP and TOCP or their metabolites persist at concentrations sufficient to inhibit NTE in hen brain and sciatic nerve to a substantial (>70%) degree for at least 10 days (21 days after TOCP). In contrast, NTE activity inhibited by DFP starts to recover in hen brain within 1 day (Johnson 1974; Carrington and Abou-Donia 1984) and in sciatic nerve after 2–3 days (Carrington and Abou-Donia 1984). An oral dose of 500 mg/kg TOCP has been found to result in essentially complete NTE inhibition for 4 days, with 25% recovery by the 8th day after dosing (Ohkawa et al. 1980). We recently provided evidence that this prolonged inhibition may be related to persistence of the dose at the subcutaneous injection site (Carrington et al, 1988b).

The NTE inhibition observed following in vitro exposure to TPP with dilution upon addition of substrate is due to progressive phosphorylation, since the amount of inhibition increased with the time of preincubation (Carrington, unpublished data). The additional inhibition which occurs if the TPP is not diluted may result from competitive inhibition in addition to the progressive phosphorylation. In a single experiment, diphenyl phosphonate was found to be about 50 times less potent as an inhibitor of NTE in vitro (Carrington, unpublished data), suggesting that the inhibition of NTE by TPP is not due to hydrolysis and tautomerization to a phosphonic acid.

TPP has previously been reported to be potent inhibitor of rat brain NTE in vitro (Padilla and Grizzle 1987). The results reported here with NTE from hen brain are essentially identical. However, the degree of NTE inhibition resulting from exposure to a neurotoxic regimen of TPP in vivo differs greatly between the two species. While two doses of 1000 mg/kg TPP result in less than 40% inhibition of NTE in the rat (Veronesi et al. 1986; Padilla and Grizzle 1987), a threshold dose of 250 mg/kg results in over 50% inhibition in the hen. The fact that NTE is not inhibited to a significant extent in the rat is consistent with the fact that TPP apparently does not cause OPIDN in this animal (Veronesi and Dvergsten 1987). The variation in NTE inhibition between species may due to differences in the rate of absorption from the site of injection, or to other pharmacokinetic or metabolic differences (Abou-Donia 1983). In any case, it appears that some process occurring prior

to the arrival of the neurotoxicant to the tissue accounts for the lack of sensitivity of the rat to OPIDN induced by TPP.

TPP is apparently neurotoxic by two independent mechanisms. Although OPIDN has been more fully characterized, neither mechanism has been fully elucidated. Each syndrome could contribute to risk from human exposure. The contribution of either mechanism might differ with chronic low-level dermal exposure, to which humans are more likely to be exposed (US EPA 1985). Further study of the risk associated with dermal exposure may be warranted in lieu of the finding that daily dermal application of TPP produces a neuropathy in hens (Abou-Donia, unpublished data).

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