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COMPARISON OF INHIBITORY ACTIVITY OF VARIOUS ORGANOPHOSPHORUS COMPOUNDS AGAINST ACETYLCHOLINESTERASE AND NEUROTOXIC ESTERASE OF HENS WITH RESPECT TO DELAYED NEUROTOXICITY

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Abstract—A variety of organophosphorus (OP) compounds with and without delayed neurotoxicity were examined for inhibitory power against neurotoxic esterase (NTE) and acetylcholinesterase (AchE) of hen brain *in vitro* and *in vivo*. Generally, delayed neurotoxicity induced by OP compounds correlated with high inhibition of NTE *in vivo*, whereas *in vitro* studies comparing I_{50}s for both enzymes did not provide a guide to evaluation of delayed neurotoxicity. Single oral administration of delayed neurotoxic EPN, leptophos and TOCP resulted in more than 80 per cent inhibition of brain NTE at neurotoxic doses, whereas non-delayed neurotoxic methyl parathion, fenitrothion and cyanophos caused weak inhibition at near lethal doses which gave rise to severe inhibition of brain AchE. A delayed neurotoxic dose of (−)-EPN caused more severe inhibition of brain NTE as compared with the same dose of the non-delayed neurotoxic (+)-isomer. However, a few compounds produced severe inhibition of NTE at non-delayed neurotoxic doses. Hens paralysed by repeated administration of a low level of leptophos showed significant decreases in NTE activity of the brain and spinal cord.

A number of organophosphorus (OP) compounds cause delayed neurotoxicity in man, hen and several other animal species. These compounds produce ataxia and paralysis of the legs in hens 8–21 days after dosing. The neurotoxic lesions involve the long motor and sensory axons of the peripheral and central nervous systems. This effect is not related to acute cholinergic effects, and is proposed by Johnson to be initiated by inhibition of neurotoxic esterase (NTE) in the nervous system [1]. Moreover, Johnson [2] suggested that measurements of the degree of inhibition of both acetylcholinesterase (AchE) and NTE in hen brain are useful in predicting the potential delayed neurotoxicity of OP compounds.

This report deals with measurements of inhibitory activity of a variety of OP compounds, including the chiral isomers of EPN against AchE and NTE of hens *in vivo* and *in vitro*, and discusses the relationship between NTE inhibition and delayed neurotoxicity of OP compounds.

MATERIALS AND METHODS

Chemicals. The following thiophosphoryl (P = S) compounds were prepared by reacting phenolic precursors and alkyl or phenyl thiophosphoryl chlorides; leptophos (4-bromo-2,5-dichlorophenyl methyl phenylphosphonothionate), Et-leptophos (4-bromo-2,5-dichlorophenyl ethyl phenylphosphonothionate), DeBr-leptophos (2,5-dichlorophenyl methyl phenylphosphonothionate), DeBr-Et-leptophos (2,5-dichlorophenyl ethyl phenylphosphonothionate), EPN (ethyl 4-nitrophenyl phenylphosphonothionate), cyanofenphos (4-cyanophenyl ethyl phenylphosphonothionate), cyanophos (4-cyanophenyl dimethyl phosphorothionate), fenitrothion (dimethyl 3-methyl-4-nitrophenyl phosphorothionate) and methyl parathion (dimethyl 4-nitrophenyl phosphorothionate).

The following phosphoryl (P = O) compounds were also prepared from phenolic precursors and alkyl or phenyl phosphoryl chlorides; paraoxon (diethyl 4-nitrophenyl phosphate), EPN-oxon (ethyl 4-nitrophenyl phenylphosphonate), Me-EPN-oxon (methyl 4-nitrophenyl phenylphosphonate), cyanofenphos-oxon (4-cyanophenyl ethyl phenylphosphonate), Me-cyanofenphos-oxon (4-cyanophenyl methyl phenylphosphonate), leptophos-oxon (4-bromo-2,5-dichlorophenyl methyl phenylphosphonate); Et-leptophos-oxon (4-bromo-2,5-dichlorophenyl ethyl phenylphosphonate), DeBr-leptophos-oxon (2,5-dichlorophenyl methyl phenylphosphonate), DeBr-Et-leptophos-oxon (2,5-dichlorophenyl ethyl phenylphosphonate), TC1MPP (methyl 2,4,5-trichlorophenyl phenylphosphonate), TC1EPP (ethyl 2,4,5-trichlorophenyl phenylphosphonate), DC1MPP (2,4-dichlorophenyl methyl phenylphosphonate), DC1EPP (2,4-dichlorophenyl ethyl phenylphosphonate), fenitrooxon (dimethyl 3-methyl-4-nitrophenyl phosphate) and cyanophos-oxon (4-cyanophenyl dimethyl phosphate).

Phenthoate [dimethyl S-(α-ethoxycarbonyl)benzyl phosphorothiolothionate] and phenthoate-oxon [dimethyl S-(α-ethoxycarbonyl)benzyl phosphorothiolate] were prepared as reported previously [3, 4].

TOCP (tri-*o*-cresyl phosphate) was prepared by refluxing a mixture of *o*-cresol and phosphoryl chloride. Salithion (2-methoxy-4H-1,3,2-benzodioxaphosphorin-2-sulfide), salioxon (2-methoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide), M-1 (2-*o*-tolyloxy-4H-1,3,2-benzodioxaphosphorin-2-oxide) and K-1(2-phenyl 4H-benzodioxaphosphorin-2-oxide) were prepared by the method of Eto and Oshima [5]. The preparation of S2571 (ethyl 3-methyl-6-nitrophenyl *N*-isopropylphosphoramidothionate), S2571-oxon (ethyl 3-methyl-6-nitrophenyl *N*-isopropylphosphoramidate), S2571-oxon-CH_2OH (ethyl 3-hydroxymethyl-6-nitrophenyl *N*-isopropylphosphoramidate), S2571-oxon-CHO (ethyl 3-formyl-6-nitrophenyl *N*-isopropylphosphoramidate) and S2571-oxon-COOH (ethyl 3-carboxyl-6-nitrophenyl *N*-isopropylphosphoramidate) was reported previously [6, 7]. Mipafox (*N*,*N*′-diisopropylphosphorodiamidic fluoride) and phenyl valerate were prepared according to Johnson [8].

The prepared compounds were each purified by column chromatography on silica gel with appropriate elution solvents prior to use. The structure and purity were checked by thin-layer chromatography, NMR, IR and MS spectroscopy.

The chiral isomers of EPN, EPN-oxon and cyanofenphos-oxon were the same samples as used previously [9–11].

The following chemicals were purchased; acetylthiocholine iodide, 5-5′-dithiobis(2-nitrobenzoic acid) (DTNB), eserine sulfate, 4-aminoantipyrine, sodium dodecyl sulfate and heparin sodium salt from Wako Pure Chemical Industries Ltd., Osaka, Japan; atropine sulfate from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan.

Hens. Adult White Leghorn hens (12–18 months old) weighing 1.5–2.4 kg were purchased from Nihon Dobutsu Co., Osaka, Japan and acclimatized for at least 1 week prior to use. The birds were housed 1 or 2 per cage and fed a diet of standard layer's mash (Osaka Shinko Shiryo Co., Ltd., Osaka, Japan).

Treatment of hens. Test chemicals were each dissolved in 10% (w/v) or 20% (w/v) Tween 80 solution, or corn oil, by sonication with an Ultrasonic Disruptor Model UR-200P (Tomy Seiko Co., Ltd., Tokyo, Japan). A 2–23 ml portion of each solution per kg body wt was given by oral intubation to groups (2–12 birds each) of hens. Control hens were also given equivalent amounts of carrier vehicle. Hens showing symptoms of acute cholinergic poisoning were given oral doses of 15 mg/kg of atropine sulfate in saline at 10–15 min before the OP compound as needed for survival. The dosed birds were monitored for up to 6 hr following administration of OP compounds for symptoms of acute cholinergic poisoning. Additional protective doses of atropine were given to the treated hens when necessary. The chiral isomers of EPN were dissolved in dimethyl sulfoxide and given subcutaneously to atropinized hens at the rate of 50 $mg.kg^{-1}.ml^{-1}$. However, most of the compounds produced no acute symptoms at the doses used and no atropine was administered in these cases. The treated birds were returned to their cages and supplied with food and water *ad lib.* All birds were observed daily for mortality and possible neurotoxic symptoms throughout test periods.

In a separate experiment, 10 birds were given daily a single oral dose of 5 mg/kg of leptophos for 16 days as corn oil solution packed in gelatin capsules. Control birds were given corn oil.

Enzyme preparations. Hens were decapitated, and whole brains, spinal cords and/or sciatic nerves were immediately dissected out and rinsed with cold 0.9% NaCl solution. The whole brains and spinal cords were each homogenized with a Potter–Elvehjem glass homogenizer in ice-cold 0.9% NaCl solution or buffer (50 mM Tris–HCl buffer, pH 8.0, containing 0.2 mM EDTA) (1 g tissue/6.5 ml). Sciatic nerves were homogenized (1 g tissue/6.5 ml). in cold Tris buffer (50 mM Tris–HCl–0.2 mM EDTA, pH 8.0) using a Polytron (Kinematica GmbH, Luzern, Steinhofhalde, Switzerland) prior to homogenization with a glass homogenizer. The resulting homogenates were diluted with a 9 × volume of 50 mM Tris–HCl–0.2 mM EDTA buffer, pH 8.0, for NTE assay, or with a 29 × volume of 0.1 M phosphate buffer, pH 7.4, for AchE assay.

In time-course studies, one control and groups (2–4 hens each) of the treated hens were killed at various time intervals, and homogenates of the tissues were prepared in the manner described above.

Blood was obtained from the wing vein by a syringe and heparinized immediately. The heparinized blood was centrifuged at 400 *g* for 10 min to separate plasma and red cells.

Enzyme assays. NTE activity was mostly assayed according to Johnson [8] using phenyl valerate as substrate. Paired samples of enzyme preparations (0.1–0.6 ml) were preincubated at 37° for 20 min with paraoxan (40 μM) plus either (a) buffer or (b) mipafox (50 μM) in a final volume of 2 ml of 50 mM Tris–HCl–0.2 mM EDTA buffer, pH 8.0. After incubation, 2 ml of phenyl valerate solution (0.242 mg/ml of a 30:1 mixture of 0.03% Triton X-100 in water and dimethylformamide) was added to the mixtures and the incubation was continued for up to 20 min at 37°. The reaction was stopped by addition of 2 ml of 1% (w/v) sodium dodecyl sulfate in buffer containing 0.025% (w/v) 4-aminoantipyrine, followed by addition of 1 ml of 0.4% (w/v) $K_3Fe(CN)_6$ in water. The resultant red colour was read at 510 nm. In the *in vitro* assay, inhibitors were added to the preincubation medium as stock solutions in acetone (~ 10 μl) and compared against samples containing only solvent. Measurements of NTE (Figs. 2 and 3) were also made by the method of Johnson [12].

AchE activity was determined according to the method of Ellman *et al.* [13] with slight modifications: a mixture of 0.1 ml of enyme preparation and 0.1 ml of 10 mM DTNB in a final volume of 3.8 ml of 0.1 M phosphate, pH 7.4, was preincubated for 5 min at 37°. Reaction was initiated by addition of 0.1 ml of 15 mM acetylthiocholine iodide and continued for up to 15 min at 37°. The reaction was stopped by addition of eserine solution at the final concentration of 38 μM and immediate cooling in an ice bucket. Readings were made at 412 nm.

For determination of I_{50} values for OP compounds *in vitro*, the inhibitor was deposited on the bottom of test tubes by evaporation of aliquots of acetone solution immediately before addition of enzyme.

Protein content was measured by the method of Lowry *et al.* [14] with bovine serum albumin as standard.

All measurements were made in duplicate and repeated two or more times. I_{50} values were obtained from the straight line through four points in the range of 20–80 per cent activity of control. The statistical significance of the data, shown in Table 3, were determined using Student's *t*-test.

RESULTS

NTE activity in tissues of hen. The NTE activity was detectable in homogenates of whole brain, spinal cord and sciatic nerve of hens, but not in whole blood, as shown in Fig. 1. The enzyme was most active in the brain among the four tissues tested. The activities in the spinal cord and sciatic nerve were 24 and 4 per cent of the brain, respectively. Based on these data, the homogenate of whole brain was mainly used for subsequent studies as a source of the enzyme.

Inhibitory activity of OP compounds against AchE and NTE of hen brain in vitro. Table 1 compares the inhibitory activity of a number of OP compounds against AchE and NTE of hen brain *in vitro*. The most potent inhibitors against NTE were compounds 13, 14, 15, 16, 17, 18, 20, 22 and 23, the I_{50} values of which were below 1 μM. These phosphoryl compounds except compounds 20 and 22 were also strong inhibitors of hen brain AchE.

Compounds 5, 6, 7, 8, 10, 11, 12, 19 and 21 were fairly potent inhibitors of NTE. Also, compounds 9, 26 and 27 were less active against NTE, these I_{50} values being between 1 and 10 μM. On the other hand, compounds 5, 6, 7, 8, 9, 10, 11 and 21 were potent against AchE.

NTE was hardly inhibited by compounds 2, 3, 4, 24, 25 and 28; AchE was actively inhibited by compounds 2, 3 and 4.

As the ratio I_{50}'s for AchE/I_{50}'s for NTE indicated, compounds 15, 16, 19, 20, 22, 23, 25 and 26 were relatively selective inhibitors against NTE, whereas the other compounds tested were more potent inhibitors against AchE in comparison with NTE *in vitro*.

Inhibition of AchE and NTE activities in hen brain in vivo. Figure 2 shows the time-dependent change of the activity of NTE and AchE in hen brain after a single oral administration of 500 mg/kg of TOCP and 800 mg/kg of leptophos. The degree of inhibition of NTE reached maximum levels 2 days after administration of both compounds. The activity was gradu-

Table 1. Inhibitory activity of organophosphorus compounds against acetylcholinesterase (AchE) and neurotoxic esterase (NTE) of hen brain

No.	Compound	I_{50} (μM) AchE	I_{50} (μM) NTE	AchE/NTE
Group A (phosphates and phosphorothiolate)				
1	Paraoxon	0.011	—	—
2	Fenitrooxon	0.53	>102(16)*	<0.005
3	Phenthoate-oxon	0.026	>103(3)	<0.001
4	Cyanophos-oxon	0.79	>119(7)	<0.007
Group B (phosphonates)				
5	Me-EPN-oxon	0.019	6.81	0.003
6	EPN-oxon	0.030	2.14	0.014
7	(+)-EPN-oxon	0.020	2.56	0.008
8	(−)-EPN-oxon	0.077	1.68	0.046
9	Me-Cyanofenphos-oxon	0.46	19.0	0.024
10	Cyanofenphos-oxon	0.70	4.63	0.151
11	(+)-Cyanofenphos-oxon	0.36	5.78	0.062
12	(−)-Cyanofenphos-oxon	1.77	3.68	0.481
13	Leptophos-oxon	0.008	0.094	0.055
14	Et-Leptophos-oxon	0.012	0.077	0.156
15	DeBr-Leptophos-oxon	0.492	0.204	2.41
16	DeBr-Et-Leptophos-oxon	0.669	0.059	11.3
17	TC1MPP	0.016	0.104	0.154
18	TC1EPP	0.037	0.090	0.411
19	DC1MPP	1.38	1.11	1.24
20	DC1EPP	2.02	0.422	4.79
Group C (TOCP-related compounds)				
21	Salioxon	0.48	5.16	0.093
22	K-1	5.68	0.041	139
23	M-1	0.74	0.029	25.5
24	TOCP	>521(13)	>990(10)	—
Group D (S2571-related compounds)				
25	S2571-oxon	943	518	1.82
26	S2571-oxon-CH_2OH	133	18.7	7.11
27	S2571-oxon-CHO	13.2	17.2	0.77
28	S2571-oxon-COOH	>1300(18)	>986(23)	—

* The values in parentheses represent percentage inhibition at the indicated concentrations.

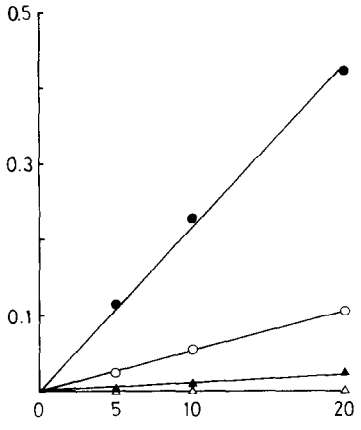

Fig. 1. Comparison of neurotoxic esterase (NTE) activity in homogenates of hen tissues: ●——●, whole brain; ○——○, spinal cord; ▲——▲, sciatic nerve; △——△, whole blood. The protein content (mg/100 mg wet tissue) was 14.6 in the brain, 12.1 in the spinal cord and 8.2 in the sciatic nerve. Vertical axis: NTE activity (moles hydrolysed/mg protein). Horizontal axis: incubation time (min).

ally recovered thereafter and returned to the levels of about 60 per cent of the control after 16 days. The AchE activity was also severely inhibited 6 hr to 2 days after administration of leptophos and then returned to control levels after 16 days. On administration of TOCP, AchE activity showed a small decrease after 4 days and returned to normal levels thereafter. In connection with this experiment, two hens given a single dose of 500 mg/kg TOCP produced weak ataxia, and also two hens treated with 800 mg/kg leptophos developed severe ataxia about 2 weeks after administration.

Table 2 shows the inhibitory activity of a number of OP compounds against NTE of hen brain at 2 days after a single oral administration. Compounds 29, 30, 31, 32 and 33 were given at near lethal doses to hens. For compounds 34, 35, 37, 22, 23 and 24, delayed neurotoxic levels were chosen. Also, the doses of compounds 36 and 38 were adjusted to the levels of the corresponding methyl analogs. The NTE activity was inhibited to the extents of more than 80 per cent by oral administration of compounds 34, 35, 37, 22, 23 and 24, which all had delayed neurotoxic effects at the indicated doses. Of these, compounds 34 and 35 also severely inhibited AchE activity of hen brain, whereas the other four compounds were less active in the inhibition of AchE. Compounds 37 and 24 were relatively selective for NTE *in vivo*. With compounds 32 and 33, NTE was inhibited to the extents of 72–75 per cent, and the degree of inhibition of both NTE and AchE was nearly the same. Compounds 36 and 38 were rather selective for NTE, although they did not produce ataxia in hens at the indicated dose. Compounds 29, 30 and 31, which did not show delayed neurotoxic effects, hardly inhibited NTE but actively inhibited AchE at the indicated doses.

Inhibition of NTE and AchE activities by the chiral isomers of EPN in vivo. The activity of brain NTE and AchE was determined for up to 2 days after single subcutaneous administration of each of the racemic, (+)- and (−)-EPN to atropinized hens at the rate of 50 mg/kg (Fig. 3). Among these three compounds, the delayed neurotoxic (−)-isomer inhibited NTE activity to the extent of 75 per cent 2 days after administration. In contrast, the non-delayed neurotoxic (+)-isomer inhibited about 50 per cent of the enzyme activity after 2 days. The racemic EPN caused about 60 per cent inhibition during the same period. On the other hand, more than 90 per cent of AchE activity was inhibited by these three compounds 16 hr to 2 days after administration.

AchE and NTE activities in the brain, spinal cord and plasma of hens after cumulative doses (5 mg.kg^{-1}.day^{-1}) of leptophos. When 10 adult hens

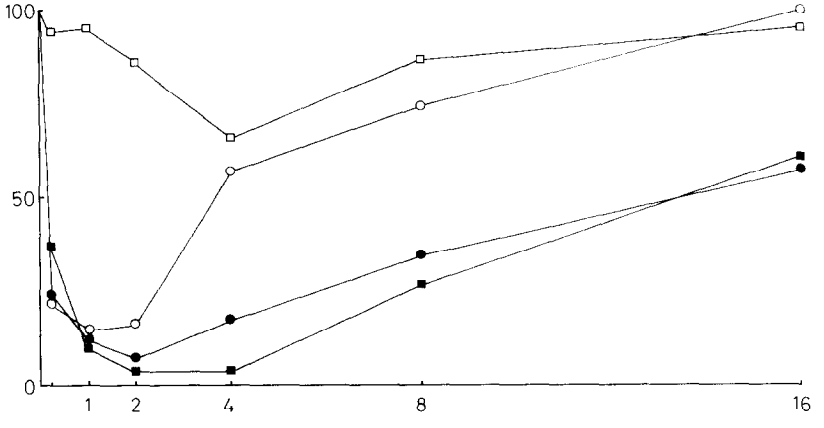

Fig. 2. Effect of oral administration of 500 mg/kg TOCP and 800 mg/kg leptophos on brain acetylcholinesterase (AchE) and neurotoxic esterase (NTE) of hens: ●——●, NTE (leptophos); ○——○, AchE (leptophos); ■——■, NTE (TOCP); □——□, AchE (TOCP). Values are means of 2–4 hens, expressed as the percentage of the enzyme activity in a control hen killed at the same time. The control activities [nmoles hydrolysed/mg protein/min, mean ± S.D.] of this experiment were 36.9 ± 3.9 for NTE and 228 ± 6 for AchE. Vertical axis: enzyme activity (% of control). Horizontal axis: days after administration.

Table 2. Inhibition of acetylcholinesterase (AchE) and neurotoxic esterase (NTE) in the brain of hens at 2 days after oral administration of organophosphorus compounds

No.	Compound	Dose (mg/kg)	DNT*	Enzyme inhibition‡ (%) AchE	NTE
29	Methyl parathion	100‡	—	85	12
30	Fenitrothion	500	—	78	8
31	Cyanophos	20	—	76	0
32	Salithion	100	—	76	72
33	Cyanofenphos	100	—	74	75
34	EPN	100‡	+	90	81
35	Leptophos	200	+	74	89
36	Et-Leptophos	200	—	54	67
37	DeBr-Leptophos	50	+	37	86
38	DeBr-Et-Leptophos	50	—	34	72
22	K-1	800	+	65	89
23	M-1	250	+	59	98
24	TOCP	500	+	14	97

* Positive (+) or negative (−) signs show clinical symptoms of delayed neurotoxicity (DNT) in hens at indicated doses. Each compound was singly administered to a group of 5–10 birds. Of these, 2 or 4 birds were examined for NTE activity 2 days after administration, and the remaining birds were observed for up to 4 weeks for delayed neurotoxic symptoms according to Ohkawa *et al.* [10]. The results were drawn from unpublished data on delayed neurotoxicity of OP compounds in hens.

‡ Each value is the mean of 2–4 hens, expressed as the percentage inhibition calculated on the basis of 1 or 2 control hens and treated groups.

‡ Hens were administered protective doses of atropine sulfate.

were given a daily oral dose of 5 mg/kg of leptophos for up to 16 days, all the hens produced ataxia and/or paralysis from day 11 to day 14. Of these, the five paralysed hens were analysed for esterase activities on day 17, as shown in Table 3. About 70 and 60 per cent of the NTE activity in the brain and spinal cord, respectively, were inhibited. Although AchE activity in the brain decreased to about 50 per cent of the control, the enzyme in the spinal cord was not affected. In addition, plasma AchE was hardly inhibited by the repeated administration.

DISCUSSION

A wide range of inhibitory power against NTE of hen brain was found among a variety of the phosphoryl compounds studied *in vitro*. This did not correlate with the anti-AchE power of these com-

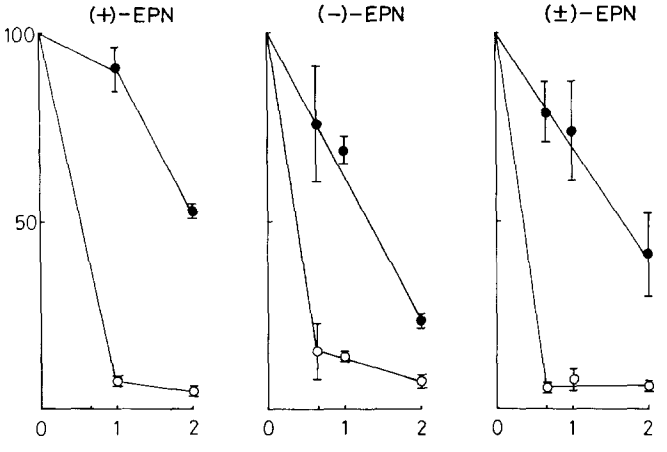

Fig. 3. Activities of neurotoxic esterase (NTE) and acetylcholinesterase (AchE) in the brain of hens after subcutaneous administration of each of racemic, (+)- and (−)-EPN at 50 mg/kg; ●——●, NTE and ○——○, AchE. Values are the means ± S.E. of 2–4 hens, expressed as a percentage of the enzyme activities of a control hen killed at the same time. The control activities [nmoles hydrolysed/mg protein/min, mean ± S.E.] of this experiment were 32.1 ± 0.8 for NTE and 118.2 ± 4.7 for AchE. Vertical axis: enzyme activity (% of control). Horizontal axis: days after administration.

Table 3. Acetylcholinesterase (AchE) and neurotoxic esterase (NTE) activities in tissues of hens after continuous oral administration of leptophos at 5 mg.kg^{-1}.day^{-1} for 16 days

	Enzyme activity (nmoles hydrolysed.mg protein^{-1}.min^{-1})*				
	AchE			NTE	
	Brain	Spinal cord	Plasma	Brain	Spinal cord
Control	111.3 ± 7.6	8.6 ± 1.1	6.9 ± 0.4	25.1 ± 4.1	6.4 ± 1.6
Leptophos-treated	49.2 ± 8.7†	9.8 ± 3.5	8.2 ± 3.0	6.9 ± 4.2†	2.6 ± 1.0†

* Mean ± S.D. of five hens.
† Significantly ($P<0.05$) different from control results.

pounds. Some compounds, such as K-1, M-1 and DeBr-leptophos-oxon, were selective inhibitors for NTE, and some others, including fenitrooxon, phenthoate-oxon and cyanophos-oxon, were selective for AchE. The comparison of inhibitory power of the phosphoryl compounds against NTE *in vitro* did not provide a guide to the delayed neurotoxic potential of the appropriate thiophosphoryl compounds *in vivo*; namely, leptophos was positive and Et-leptophos was negative in delayed neurotoxicity *in vivo*, although the oxon analogs of both compounds were almost equally potent against NTE *in vitro*. A similar trend was found with *in vivo* neuropathy of DeBr-leptophos and Et-DeBr-leptophos and *in vitro* inhibitory power of their oxon analogs against NTE. This inconsistency between *in vitro* and *in vivo* studies may be due in part to differences in metabolic disposal of the thiophosphoryl compounds in hens.

Johnson [1] reported that OP compound-induced delayed neurotoxicity occurs in association with high inhibition (more than 75 per cent) of NTE in the brain of hens, but not with inhibition of AchE. The present study revealed that a single oral administration of delayed neurotoxic EPN, leptophos, DeBr-leptophos, TOCP, K-1 and M-1 resulted in more than 80 per cent inhibition of NTE in the brain of hens at the neurotoxic doses, whereas non-delayed neurotoxic OP compounds such as methyl parathion, fenitrothion and cyanophos caused only weak inhibition of the enzyme even at near lethal doses that gave rise to severe inhibition of brain AchE. Generally, these findings appear to be in reasonable agreement with the results reported by Johnson [15] regarding structure–activity relationships for OP compound-induced delayed neurotoxicity. However, there were a few disputable examples among the tested compounds. Although near lethal doses of salithion and cyanofenphos showed more than 70 per cent inhibition of brain NTE, these did not produce any symptoms of delayed neurotoxicity at the doses tested in hens. It may be necessary to test at higher doses in atropinized hens, as reported with EPN [10], for further evaluation of delayed neurotoxicity of both compounds. Recently, Hollingshaus *et al.* [16] reported that Et- and DeBr-Et-leptophos did not show delayed neurotoxic effects even at 1000 mg/kg. In the present study, these compounds were found to be rather selective inhibitors for NTE *in vivo*, but less potent than the corresponding methyl analogs. Further studies on time course and dose dependency of NTE inhibition *in vivo* may elucidate such obscurity in correlation between delayed neurotoxicity and high NTE inhibition.

NTE activity was detected in homogenates of whole brain, spinal cord and sciatic nerve of hens, as reported by Olajos *et al.* [17]. The neurotoxic lesion occurs in the long fiber pathways of the spinal cord and the long axons of peripheral nerves but not in the brain [18, 19]. In the present study, the hens which produced paralysis and/or ataxia by the repeated administration of a low level of leptophos showed decrease in brain NTE activity to the extent of about 30 per cent of the control, decrease in spinal cord NTE activity to a lesser extent, decrease in brain AchE, but no decrease in spinal cord AchE. It may be considered that the brain is more susceptible to continued exposure of a low level of leptophos than the spinal cord, although NTE assays using preparations of spinal cord which is the site of injury may provide a more realistic estimate of the delayed neurotoxicity potential of OP compounds, as suggested by Johnson *et al.* [20]. In addition, monitoring NTE activity during chronic exposure could reveal the level and time of maximal inhibition.

As reported previously [10], (−)-EPN produced paralysis of the legs in hens, but (+)-EPN caused no delayed neurotoxic effects, although the (+)-isomer of EPN was about 4 times more acutely toxic to hens than the (−)-isomer. Moreover, the (−)-isomer of EPN-oxon was about three times more potent as an inhibitor against α-chymotrypsin than the (+)-isomer [11]. The present study revealed that (−)-EPN-oxon was a slightly more potent inhibitor against NTE than the (+)-isomer, although the (+)-isomer was about 4 times more potent against AchE than the (−)-isomer *in vitro*. In addition, the chiral isomers of EPN showed a slight, yet definite, difference in the inhibition of NTE of hen brain *in vivo*. It is likely that the delayed neurotoxic dose of (−)-EPN causes more severe inhibition of the brain NTE than the same dose of the non-delayed neurotoxic (+)-isomer.

The *in vitro* study demonstrated that S2571-oxon-CH_2OH and S2571-oxon-CHO were more potent inhibitors against both brain AchE and NTE than S2571-oxon. Therefore, it is likely that hydroxylation of the *m*-methyl group and subsequent oxidation of the hydroxymethyl group to the formyl group in the S2571 molecule results in activation in inhibition of both AchE and NTE. The previous study [17] revealed that metabolism of S2571 by rabbit liver microsomes fortified with NADPH produces S2571-

oxon-CH_2OH. The similar trend was found with fenitrothion, the *m*-hydroxymethyl and *m*-formyl derivatives of which were more acutely toxic to mice than the parent compounds [21]. In addition, *m*-hydroxymethyl and *m*-formyl fenitrooxon were also more potent inhibitors against AchE than fenitrooxon.

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