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Delayed Neuropathy and Inhibition of Soluble Neuropathy Target Esterase Following the Administration of Organophosphorus Compounds to Hens

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TIAN, Y., XIE, X.K., PIAO, F.Y. and YAMAUCHI, T. *Delayed Neuropathy and Inhibition of Soluble Neuropathy Target Esterase Following the Administration of Organophosphorus Compounds to Hens.* Tohoku J. Exp. Med., 1998, **185** (3), 161-171 —— Delayed neuropathy and inhibition of soluble neuropathy target esterase (NTE) and acetylcholinesterase (AChE) activities in different regions of brain and spinal cord of adult hens were studied after the intravenous administration of leptophos (30 mg/kg), tri-o-cresyl phosphate (TOCP 40 mg/kg) or dipterex (200 mg/kg). The level of NTE activity varied according to the regions of the central nervous system (CNS) of the control (normal) hen, being higher in the cerebrum (74.1 μmol of phenyl valerate hydrolyzed/10 minutes/mg protein) and in the cerebellum (68.7), and lower in the spinal cord (44.5 in cervical, 55.6 in thoracic and 50.0 in lumbar cord). Hens given leptophos and TOCP demonstrated delayed neuropathy with obvious inhibition of NTE, but the times of onset and the degrees of peak inhibition of NTE activity were different: 6-24 hours after dosing and 73-82% of normal activity for leptophos, and 24-48 hours and 45-80% for TOCP, respectively. Furthermore, the average inhibition of NTE during 6-48 hours after dosing, (called here 'period average inhibition') was also significantly different between the leptophos group (63-73%) and TOCP group (40-64%). Hens given dipterex did not demonstrate delayed neuropathy, and had the least peak inhibition and period average inhibition of NTE activity among the 3 groups. Ratios of NTE inhibition/AChE inhibition were higher in the leptophos group (0.91-1.24) and TOCP group (1.13-2.45) than in the dipterex group (0.25-0.79). These results indicate that the distribution of NTE in the soluble fraction of membrane proteins is different in different regions of the CNS, and that the degree of peak inhibition of NTE activity and the time of onset of peak inhibition induced by organophosphorus compounds (OPs) also differ for different OPs. Thus, practical and useful NTE measurements should identify the peak inhibition and period inhibition in several nervous tissue regions. —— delayed neuropathy; neuropathy target esterase (NTE); acetylcholinesterase (AChE); organophosphorus compounds; hen

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Organophosphorus compounds (OPs) are widely used in agriculture as pesticides and in industry as intermediates for chemical syntheses. The majority of these compounds inhibit acetylcholinesterase (AChE) and cause cholinergic poisoning. Cholinergic signs include excessive respiratory secretions, bradycardia, flaccid paralysis, and convulsions. In addition, some OPs also inhibit neuropathy target esterase (initially called neurotoxic esterase, NTE), and thus induce delayed neuropathy, which is characterized by progressive, irreversible paralysis.

The possibility that NTE is an initiating factor of organophosphorus ester-induced delayed neuropathy (OPIDN) in nervous tissues was predicted by Aldridge (1954, 1969), and Johnson (1969) proposed that an enzymatic activity in the hen brain initiates delayed neurotoxicity and designated it as NTE. NTE is a membrane-bound protein and exists mainly in nervous-system tissue. Johnson (1982) reported that clinical delayed neuropathy observed 10–14 days after OP dosing in hens corresponds to the inhibition (more than 70–80%) of NTE activity in the brain at 1 to 40 hours after dosing. The percentage of inhibition of NTE in animals exposed to OPs has, therefore, the potential to predict delayed neuropathy (Johnson 1993). To accurately assess the neuropathic potential of OPs, the maximal effect on NTE activity should be determined (Johnson 1995). With just a one-point assay (one time point or one tissue only) of NTE, it is difficult to identify the maximal inhibition of NTE, and the one-point assay has also a risk of underestimating delayed neuropathic potentials of the OPs. In addition, little information is available on the regional distributions of NTE and AChE activities in the central nervous system (CNS). The average inhibition levels of these two enzymes during the first 48 hours following the administration of OPs with different neurotoxicities are still unclear. In order to investigate practical and meaningful indicators of NTE inhibition, we used in the present study 3 neurotoxic Ops with different delayed neurotoxicities: leptophos and tri-*o*-cresyl phosphate (TOCP), which have typical, strong delayed neurotoxicity, and dipterex, which has partial, weaker delayed neurotoxicity. We determined the inhibitory alterations of the 2 enzymes following the administration of these OPs, and furthermore, we tried to determine if there is any integrative indicator of inhibition of NTE for predicting OPIDN, in order to overcome the limitations of one-point assays.

Materials and Methods

Chemicals

Leptophos (96%) was prepared by the method of Konno et al. (1977). TOCP was obtained from the Eastman Kodak Co. (Rochester, NY, USA). Paraoxon was obtained from the Aldrich Chemical Co. (Milwaukee, WI, USA). Dipterex (*o*, *o*-dimethyl 2, 2, 2-trichlorohydroxy ethylphosphonate), 3-{(3-cholamidopropyl) dimethyl ammonio} propanesulfonate (CHAPS), acetylthiocholine iodide

(ATCH) and 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Industries Ltd. (Osaka). Mipafox and phenyl valerate were gifts from Dr. K. Kato, Fukushima Medical College (Fukushima). Other chemicals and reagents used were commercially available.

Animals and administration

Forty-eight adult hens (*Gallus gallus domesticus*, aged 20–24 months) were randomly divided into 3 groups (16 in each) and intravenously administered leptophos (30 mg/kg body weight), TOCP (40 mg/kg) or dipterex (200 mg/kg). To prevent these hens from acute death, each hen was subcutaneously given atropine sulfate (20 mg/kg) 10 minutes before and 15 minutes after the OP dosing. Four hens in each group were observed for clinical signs of delayed neuropathy after OP administration. The 6-point scale (Yamauchi et al. 1980) was used to estimate the signs of delayed neuropathy in these hens every 2 days. The remaining 12 hens in each group were used to measure the activities of NTE and AChE. Four hens each were sacrificed by decapitation at 6, 24, and 48 hours following the administration. Another group of 6 hens without OP dosing was used as a control to evaluate the activities of NTE and AChE in normal hens. Brain (cerebrum, midbrain and cerebellum) and spinal cord (cervical cord, thoracic cord and lumbar cord) samples were collected from the hens.

Tissue preparation

Membrane protein was solubilized according to the method of Pope and Padilla (1989) and Konno et al. (1994). Briefly, the tissue samples from the hens were homogenized in 10 volumes of ice-cold 2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer (50 mM, pH 7.4) using a Teflon-glass homogenizer. The homogenates were centrifuged twice at 1000 g (Type 26 centrifuge, Hitachi, Tokyo) for 5 minutes at 4°C. The supernatants were combined and centrifuged at 50 000 *g* (Twi 40, Beckman, California) for 10 minutes at 4°C. The pellet was resuspended in HEPES buffer, and sedimentated by centrifugation once again at 50 000 *g* for 10 minutes. The washed pellet was dispersed with a Polytron homogenizer (Kinematica, Kriens, Switzerland) in 10 volumes of sodium phosphate buffer (10 mM, pH 7.4), containing 1% CHAPS. The protein detergent suspensions were mixed gently using a magnetic stirrer at 4°C for 60 minutes and centrifuged at 108 000 *g* at 4°C for 60 minutes (Twi 40). The supernatant (soluble fraction) was collected, and aliquots (0.8–1.5 mg protein/ml for brain and 0.4–0.8 mg/ml for spinal cord) were stored at −80°C until the assays of NTE and AChE activities.

NTE and AChE assay

The NTE and AChE activities were spectrophotometrically determined according to the modified method of Johnson (Spraque et al. 1981) using paraoxon

and mipafox and modified method of Ellman (Gorun et al. 1978), respectively. The respective substrates for the two enzymes were phenyl valerate and acetylthiocholine; 200 μl and 400 μl of brain and spinal cord stored aliquots, respectively, were used for NTE activity assays and 25 μl and 50 μl of brain and spinal cord stored aliquots, respectively, were used for AChE activity assays. The peak inhibition of NTE activity means here the maximal inhibition of activity among the inhibitions at 3 time points: 6, 24, and 48 hours after the administration of OPs. The period average inhibition was calculated by averaging the 3 inhibitions at the same time points mentioned above. Inhibitions are expressed as percentages. The ratios between peak inhibition of NTE and AChE in brain and spinal cord were calculated by the method of Ehrich et al. (1995)

Protein concentrations were determined by the method of Lowry et al. (1951) using a protein assay kit (Sigma Diagnostics, St. Louis, MO, USA).

The differences of clinical scores between the leptophos and TOCP groups were tested for significance with the Student's t-test. The effect of esterase inhibitors on the activities of NTE and AChE, the peak and period inhibition of NTE activities, and the NTE inhibition rates of tissue regions were analyzed using analyses of variance and the Kruskal-Wallis test to distinguish differences among groups of hens. $p<0.05$ was considered statistically significant.

Results

Clinical evaluation of delayed neuropathy

The clinical scores of delayed neuropathy following the administration of the 3 organophosphorus compounds are shown in Fig. 1. No delayed neuropathic signs were observed in hens administered dipterex (0 point) throughout the experimental period. In the TOCP group, however signs of delayed neuropathy (progressive ataxia) were observed on the 8th day after dosing, and then developed gradually into paralysis. The mean score in the TOCP group reached 2.75 on the 16th day and thereafter remained at that level throughout the experimental period. In the leptophos group, neuropathic signs appeared on the 10th day, and became gradually aggravated with time. The mean score reached 4.75 on the 20th day. The mean scores of delayed neuropathy were higher in the leptophos than in TOCP group after the 14th day ($p<0.05$).

NTE and AChE activity in the control hens

As shown in Table 1, the activity of the NTE in the brains of control hens was much higher than that in the spinal cords ($p<0.05$) and this was also true for the AChE activity. Furthermore, the activity of NTE in the brains of the control hens was highest in the cerebellum lower in the cerebrum, and lowest in the midbrain. In the spinal cord, the highest activity was in the thoracic cord and the lowest was in the cervical cord. On the other hand, a highly elevated level of AChE activity was found in the cerebellum, followed by the level in the

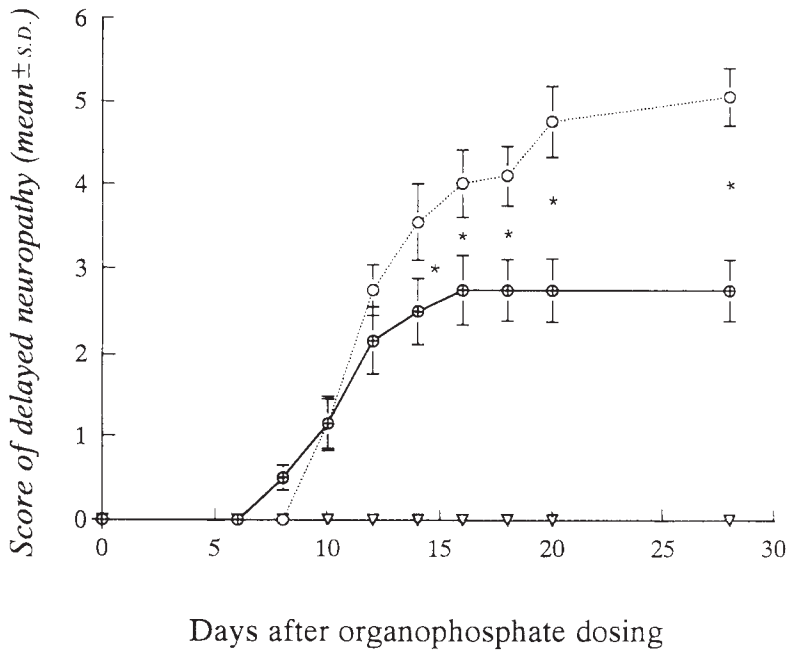

Fig. 1. The mean scores of delayed neuropathy following leptophos, TOCP and dipterex administration. Three groups of hens (4 hens each) were administered leptophos (○ ····· ○), TOCP (⊕ —— ⊕) and dipterex (△ —— △), respectively. The clinical evaluation of delayed neuropathy was estimated in hens by a 6-point scale method (Yamauchi et al. 1980) every 2 days for 28 days.
*significant difference of score between leptophos and TOCP groups ($p<0.05$).

TABLE 1. *The activities of NTE and (AChE) in brain and spinal cord of the control hens*

Region	NTE	AChE	NTE/AChE
Cerebrum	68.7±6.1	623.2±52.5	0.110±0.041
Midbrain	52.5±7.1	714.9±55.5	0.073±0.110
Cerebellum	74.1±8.2	983.1±99.6	0.075±0.002
Cervical cord	44.5±5.8	177.2±30.1	0.246±0.028
Thoracic cord	55.6±5.7	248.1±41.7	0.220±0.047
Lumbar cord	50.0±5.3	298.1±48.3	0.164±0.029

The numerals are mean±S.D. ($n=6$). NTE, neuropathy target esterase, AChE, acetylcholin esterase. The activities of NTE and AChE are expressed as μmol of phenyl valerate and acetylthiocholine hydrolyzed/10 minutes/mg membrane protein, respectively.

*$p<0.05$, comparing the tissue regions by the Kruskal-Wallis test and analyses of variance.

midbrain, and no significant difference was found among the 3 regions of spinal cord. The ratios of NTE to AChE activities were much lower in the brains than in the spinal cords of the control hens ($p<0.05$).

The percentage of inhibition of NTE and AChE after OP dosing

The percentages of inhibition of the NTE and AChE activities following the administration of OPs are shown in Table 2. In the leptophos group, both NTE and AChE activities clearly decreased following the treatment. The percent inhibitions of the NTE activities were 63-82%, 63-73% and 55-72% at 6, 24 and 48 hours after dosing, respectively. This was a much greater inhibition than those by either TOCP (32-57%, 43-80% and 45-66%) or dipterex (14-45%, 6-15% and 5-10%) ($p<0.01$). There were differences among the NTE inhibition rates of the various regions of the CNS for all the OP groups.

The peak and period average inhibitions of NTE

The peak and period average inhibitions of NTE activities following the OP administration are shown in Table 3. In the leptophos group, the peak inhibition of NTE occurred at 6 hours after dosing in cerebrum, cerebellum, and cervical and thoracic cord, while it occurred at 24 hours in midbrain and lumbar cord. It varied from 68 to 82%. The period average inhibition rate of NTE in this group ranged from 63% in midbrain to 73% in thoracic cord, and was the highest among the 3 OP groups ($p<0.01$).

In the TOCP group, the peak inhibition of NTE was observed at 24 hours in

TABLE 2. *The inhibition (%) of NTE and AChE activities in brain and spinal cord following organophosphate administration*

Region	Enzyme	Leptophos			TOCP			Dipterex		
		6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Cerebrum	NTE	82±2	63±2	72±1	32±1	43±2	45± 2	27±3	6±3	5±4
	AChE	90±4	93±5	71±4	42±3	65±4	23± 2	64±4	68±2	69±3
Midbrain	NTE	63±3	69±2	55±3	57±3	80±4	45± 3	45±4	13±5	10±2
	AChE	80±5	67±2	62±3	40±2	71±3	19± 2	57±3	64±2	49±2
Cerebellum	NTE	79±3	72±2	65±3	34±4	45±4	64± 3	14±2	9±2	7±4
	AchE	86±3	85±2	61±4	44±3	78±2	29± 2	56±2	65±3	25±2
Cervical	NTE	69±2	68±3	59±2	49±3	48±3	57± 3	17±3	15±3	7±1
cord	AChE	69±6	50±4	31±7	20±8	64±4	23±12	44±5	41±4	46±3
Thoracic	NTE	75±3	69±4	73±1	56±2	72±2	66± 3	36±1	11±6	8±3
cord	AChE	75±4	58±4	24±8	38±5	43±7	14± 5	54±4	57±4	58±8
Lumbar	NTE	71±4	73±3	62±3	42±2	58±4	56± 3	19±2	7±2	8±2
cord	AChE	77±5	59±7	41±4	37±5	49±5	20± 2	55±3	40±4	45±5

Esterase inhibition (%) is presented as the mean±S.D. ($n=4$), compared to control.
h; hours after administration

TABLE 3. *PI and PAI of NTE activities in CNS from 6 to 48 hours following administration of organophosphates to hens (%)*

Tissue section	Leptophos		TOCP		Dipterex	
	PI	PAI	PI	PAI	PI	PAI
Cerebrum	82 (6)	72±10	45 (48)	40± 9	27 (6)	13±11
Midbrain	68 (24)	63± 7	80 (24)	59±14	44 (6)	23±15
Cerebellum	79 (6)	72± 6	64 (48)	47±13	15 (6)	10± 4
Certical cord	69 (6)	65± 6	57 (48)	52±10	17 (6)	14± 6
Thoracic cord	76 (6)	73± 3	72 (24)	64± 9	36 (6)	18±15
Lumbar cord	73 (24)	69± 5	58 (24)	52± 9	19 (6)	11± 8

PI, the peak inhibition of NTE activities, was calculated as the percentage of maximally inhibited activity (OP group) relative to the control activity of NTE (the control group). The number in parenthesis was the time when the peak inhibition occurred following the OP administration.

PAI, the period average inhibition of NTE, represents the average inhibition rate of the NTE during the 6–48 hours after OP dosing, respectively (mean±S.D.).

midbrain, and in thoracic and lumbar cord, while it was observed at 48 hours in cerebrum, cerebellum and cervical cord, and the peak inhibition in the TOCP group was observed later than in the leptophos and dipterex groups. It ranged from 45% in cerebrum to 80% in midbrain. The period average inhibition rate in this group was 40–64%.

Compared with the leptophos and TOCP groups, weaker peak inhibition of NTE in the dipterex group occurred at 6 hours after dosing (only 15–44%) and the activity then recovered easily. Within the dipterex group, the inhibition was relatively higher in midbrain (44%) and thoracic cord (36%). The period average inhibition in the dipterex group varied from 10% in the cerebellum to 23% in the midbrain, and was the lowest among the OP groups ($p<0.01$).

Ratio of peak inhibitions of NTE and AChE

The ratios of peak inhibition of NTE and AChE are shown in Fig. 2. These were highest levels in most regions of the CNS in the TOCP group, intermediate in the leptophos group, and lowest in the dipterex group: 0.9–1.0 in brain and 1.0–1.2 in spinal cord of the leptophos group, 1.1–2.2 in brain and 1.7–2.5 in spinal cord of the TOCP group, respectively. In the dipterex group, the ratios were as low as 0.3–0.8 in brain and 0.3–0.7 in spinal cord.

DISCUSSION

Concerning the neurotoxicity of OPs, not only cholinergic crisis or delayed neuropathy, but also subacute neuropathy (the so-called 'third neuropathy') (Kinebuchi et al. 1994), or neuro-immunological disorder (Cowan et al. 1996)

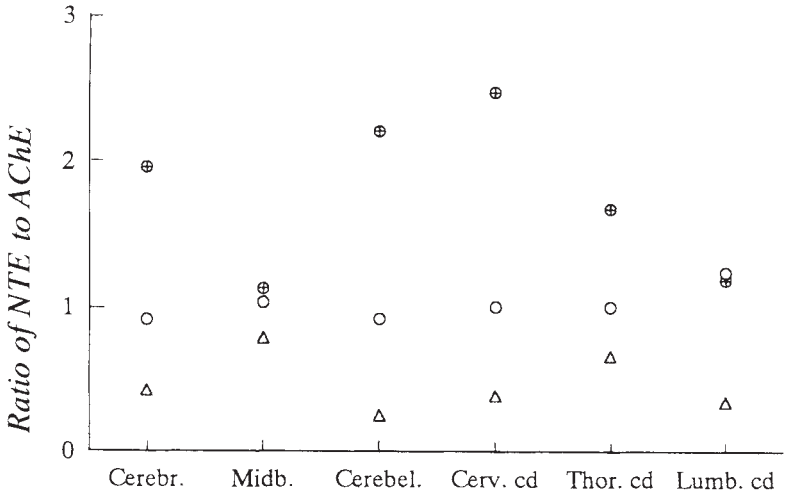

Fig. 2. The ratios between peak inhibition of neuropathy target esterase (NTE) and peak inhibition of acetylcholinesterase (AChE) in brain and spinal cord after leptophos ( ), TOCP (⊕) and dipterex (△) dosing. The soluble fraction of membrane protein was prepared from these samples and the activities of NTE and AChE were measured (see the text). The ratios between NTE inhibition and AChE inhibition in tissues were calculated by dividing the peak inhibition of NTE by the peak inhibition of AChE.

should be discussed. Although complex symptoms may occur in cases of poisoning induced by OPs, each aspect of these neurotoxicities of OPs should also be analyzed by itself as discretely as possible. In the present study, OPIDN was studied by focusing mainly on alterations of NTE. Certain OPs can inhibit NTE and cause significant inhibition of this enzyme (more than 70–80%) followed by irreversible delayed neuropathy (progressive paralysis) in susceptible animals (Johnson 1982; Lotti 1992). The mechanism which initiates neuropathy has been thought to be a two-step process: Inhibition (phosphorylation) of NTE and aging of phosphorylated NTE (Johnson 1983). Recently, some studies have indicated that NTE is mainly present in particulate form bound to membranes of the microsomal fraction in the brain, whereas it is present in soluble form in the peripheral nerves of the hens (i.e., the sciatic nerve) (Vilanova et al. 1990). The proportion of soluble NTE relative to particulate NTE was higher in sensitive animals, such as hens and cats (Tormo et al. 1993). NTE inhibited by certain OPs can be reactivated in vitro or in vivo (Milatovic and Johnson 1993), and the initiation of delayed neuropathy depends not directly on the loss of catalytic activity of NTE in vivo, but on a particular change in the NTE molecule itself as the result of the covalent binding of the initiating agents (Johnson 1995). Therefore, although NTE is still an excellent means for assessment of neuropathic potential of OP compounds (Johnson 1983, 1993, 1995), the original one-time/whole brain regulatory test of NTE has clear limitations. First, the one-time point assay can not identify the peak effect of NTE inhibition, since it occurs at

different times for various agents. In the present study, for example, the peak inhibition of NTE in different regions was reached at 6 hours, 24 hours and 48 hours after dosing with dipterex, leptophos and TOCP, respectively (Table 2). Second, when percent inhibition of NTE following administration of certain neuropathic compounds (such as TOCP in the present study) is less than 70%, but delayed neuropathy is initiated by the compounds, a one-time point determination of NTE tends to underestimate the delayed neurotoxic potential of the compounds even though the peak effect is identified. To evaluate the delayed neurotoxicity of OPs more accurately, the period average inhibition of NTE described in this paper should be measured over a suitable period of time during which the peak inhibition can be identified. A plateau level (40-60%) of the period average inhibition is suggested to be a threshold. Finally, a general assay of NTE (usually in whole brain) may not thoroughly reflect the inhibition of NTE activity by OPs. Separate measurements of NTE activity in various regions, not only in whole brain but also in spinal cord or peripheral nerve, may offer detailed information concerning the mechanism of OPIDN. We have reported, for instance, that the activity of NTE in cerebrum and cerebellum of untreated hens was higher than that in midbrain, and also that NTE activity was not inhibited as greatly in cerebellum of hens after dipterex dosing as in other nervous-system tissues (Xie et al. 1998). Moreover, the results of this study indicated variation of NTE activity (Table 1) and its inhibition (Table 2) among various tissue regions. The measurement of NTE activity is especially important in spinal cord or peripheral nerves, because the spinal cord or peripheral nerves are the principle targets in initiation of delayed neuropathy (Johnson 1990). Although the NTE assay is easy to conduct in brain owing to the large amount of NTE in brain, it is recommended that the NTE level should be assayed in various tissues.

It is well known that the OPs inhibiting NTE activity also inhibit AChE activity which related to cholinergic toxicity as mentioned above. As severe AChE inhibition can result in lethality, the degree of inhibition of this enzyme could be used to predict whether animals will survive long enough to develop delayed neuropathy (Lotti and Johnson 1978; Richardson et al. 1993). Therefore, the ratios of NTE/AChE inhibition in treated animals may be more predictive of delayed neuropathic potential of OPs (Ehrich et al. 1995; Jianmongkol et al. 1996). In the present study, the ratios of NTE/AChE inhibition were around 1.0 or more in both the TOCP and leptophos groups, with typical signs of delayed neuropathy, whereas they were less than 0.8 in the dipterex group, without any symptoms (Fig. 2). These results indicate that leptophos or TOCP probably initiate the delayed neuropathy at the smaller doses which produce acute poisoning, whereas dipterex could not initiate delayed neuropathy even at the larger doses, and that the ratio of NTE/AChE inhibition is a valid index for evaluation of the delayed neuropathic potential of organophosphates.

In conclusion, the distribution of NTE and its inhibition following dosing of

OPs vary among nervous tissue regions, and the peak inhibition of NTE occurs at different times following administration of the OPs. In order to monitor the delayed neuropathic potential of OPs practically and usefully, it is suggested that a thorough NTE screening test would evaluate the peak level of inhibition and the period average of inhibition (1–48 hours) in several nervous tissue regions.

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