

Eptastigmine–Phosphotriesterase Combination in DFP Intoxication

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A novel therapy against organophosphate exposure, the combination of a carbamate eptastigmine and an organophosphate hydrolase (phosphotriesterase) was studied in mice against diisopropylfluorophosphate (DFP) (1.75 mg/kg) exposure. Mice received eptastigmine (0.9 mg/kg; iv) 10 min prior to the ip injection of DFP. Phosphotriesterase (83 U/g body weight) was injected iv 10 min after DFP. Eptastigmine (1.5 mg/kg; iv) inhibited the acetylcholinesterase activities in brain and erythrocytes for a longer time than physostigmine. Eptastigmine caused only minor changes in the behavior and activity of the animals, whereas physostigmine clearly reduced their activity for about 30 min. The eptastigmine pretreatment clearly supplemented the protective effect of phosphotriesterase against DFP: the plasma butyrylcholinesterase activity was doubled and the activity recovered faster than in animals treated with phosphotriesterase alone. In lung, butyrylcholinesterase activity was initially lower after eptastigmine–phosphotriesterase than phosphotriesterase treatment alone. However, the activity returned 24 hr later to normal in eptastigmine–phosphotriesterase-treated groups. With phosphotriesterase only, it recovered only to 75% of the control level. Presumably eptastigmine, by preventing the binding of DFP to cholinesterases, caused an elevation of free DFP levels in body fluids and promoted phosphotriesterase hydrolysis of DFP. © 1996 Academic Press, Inc.

Several different approaches have been tried in the therapy of organophosphate (OP) poisoning. Oximes like 2-PAM, toxogonin, and HI-6 can actively reactivate the OP-inhibited cholinesterases (ChEs). Carbamates like physostigmine reversibly inhibit the ChEs and thus prevent access and subsequent binding of OPs to the active site (Somani and Dube, 1989). A potentially novel alternative to OP antidotes is the use of exogenous enzymes such as purified acetylcholinesterase (AChE) (Ashani *et al.*, 1991; Wolfe *et al.*, 1987, 1991), butyrylcholinesterase (BChE) (Ashani *et al.*, 1991; Broomfield *et al.*, 1991), carboxylesterase (CaE) (Maxwell, 1992), or OP hydrolases (Raveh *et al.*, 1992; Kaliste-Korho-

nen *et al.*, 1993; Tuovinen *et al.*, 1994, 1996). Exogenous AChE, BChE, and CaE provide alternative binding sites for OPs and reduce the inhibition of endogenous body enzymes. OP hydrolases can actively hydrolyze OPs. This reduces the amount of OP in the body and prevents the inhibition of body esterases.

In our previous studies, we have examined the ability of phosphotriesterase to protect mice against the toxicity of different OPs. Phosphotriesterase is an enzyme capable of hydrolyzing several OPs such as paraoxon, diisopropylfluorophosphate (DFP), sarin, and soman. Our findings showed that phosphotriesterase-treated mice tolerated 33-fold higher paraoxon doses than control animals (Tuovinen *et al.*, 1994). The iv-injected phosphotriesterase protected the serum and brain ChE activities in mice, when it was administered before or immediately after paraoxon (Kaliste-Korhonen *et al.*, 1993; Tuovinen *et al.*, 1994, 1996). Moreover, we found that recovery of nonaged paraoxon-inhibited ChEs was more rapid in phosphotriesterase-treated mice (Tuovinen *et al.*, 1994).

The aim of the present study was to investigate whether a combination of carbamate–phosphotriesterase could protect body ChEs against DFP better than phosphotriesterase alone. Theoretically, carbamate should increase the amount of OP in a free form, and phosphotriesterase should have greater potential to hydrolyze the OP molecules before they can inhibit the esterases. The carbamate used was eptastigmine, a new compound with lower toxicity and a longer duration of action at ChEs than physostigmine. Furthermore, the effects of eptastigmine and physostigmine on body esterases and behavior of the animals were compared.

MATERIALS AND METHODS

Chemicals and Materials

The derivative of physostigmine (molecular weight 275), heptylphysostigmine (eptastigmine; MF-201 tartrate; molecular weight 359), was purchased from Mediolanum (Milano, Italy) and physostigmine from Sigma Chemical Company (St. Louis, MO). Both carbamates were dissolved in 0.9% NaCl (saline) solution. The phosphotriesterase enzyme was dissolved in 50 mM Hepes buffer, pH 8.5, 1.0 mg/ml with 12,000 U/ml (12,000 U/mg prot.) of paraoxon hydrolyzing activity. The phosphotriesterase coding gene from *Pseudomonas diminuta* was expressed in *Escherichia coli*

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and a protein preparation was purified by Omburo *et al.* (1992) at Texas A&M University. DFP was purchased from Fluka AG (Buchs, Switzerland), and it was dissolved in olive oil. Substrates such as acetylthiocholine, butyrylthiocholine and propionylthiocholine iodide, bovine albumin, atropine sulfate, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), 4,4-dithiopyridine (PDS), and Triton X-100 were purchased from Sigma Chemical Company. Paraoxon (diethyl-*p*-nitrophenylphosphate), the substrate of paraoxonase, was purchased from Ehrenstorfer (Augsburg, Germany). Astra 1397-HCl (10-diethylaminopropionyl phenothiazine-HCl), the specific inhibitor for BChE, was purchased from Astra (Södertälje, Sweden).

Animals

The animals used were 11-week old CD₂F₁ male mice (26–30 g) (National Laboratory Animal Center, Kuopio, Finland). They were housed in groups of six animals in stainless steel cages with aspen bedding (Tapvei Co., Kaavi, Finland). The mice were housed in a controlled environment: temperature 22 ± 1°C, the relative humidity 50 ± 10%, and light period of 12 hr (07.00–19.00). Pellets of mouse diet (R36, Lactamin AB, Södertälje, Sweden) and water were freely available. The study was approved by the Ethical Committee for Animal Experiments of the University of Kuopio.

Experiments

Comparison of physostigmine and eptastigmine. Physostigmine (0.09 mg/kg in volume of approximately 0.2 ml saline; 1.64 mM), or eptastigmine (1.5 mg/kg in volume of approximately 0.2 ml saline; 20.9 mM), or saline were injected into the tail vein. Immediately after the administrations, the animals were placed into cages in groups of two or three ($n = 5$ /group). The groups to be studied for 2 and 4 hr were video recorded for 2 hr. The number of animals being active with or without locomotion were recorded from the videotape once per minute. At the time points of 10 min, 2, 4, 6, and 24 hr the animals were sacrificed by carbon dioxide; blood samples (approximately 1.0 ml) were drawn by cardiac puncture into heparinized tubes. The 25 μ l of blood were diluted with 20 volumes of 0.1% Triton in 50-mM sodium phosphate buffer. After that the brains were, quickly dissected, and immediately frozen on dry ice. Plasma was separated with a hematocrit centrifuge at 1000g for 10 min at 4°C. The samples were stored at -75°C until analyzed.

Effect of eptastigmine–phosphotriesterase combination. Eptastigmine (0.9 mg/kg in volume of approximately 0.2 ml saline; 12.5 mM) was injected into the tail vein 10 min prior to the ip injection of DFP (1.75 mg/kg in volume of approximately 0.5 ml olive oil). Phosphotriesterase (83 U and 6.9 μ g/g body weight in volume of approximately 0.2 ml) was injected iv 10 min after DFP. The control animals received saline and/or olive oil. To avoid possible signs of poisoning the mice received sc atropine (37.5 mg/kg in a volume of approximately 0.15 ml saline) immediately after the OP injection. At 1, 3, 6, and 24 hr after the DFP exposure, the mice were sacrificed by carbon dioxide and blood samples (approximately 1.0 ml) were drawn by cardiac puncture into heparinized tubes. Blood, brain, and lung were removed, dissected, handled, and frozen as above.

Assay of ChE Activities

ChE activities were measured spectrophotometrically at 410 nm at 37°C with the method of Ellman *et al.* (1961) using acetylthiocholine iodide as the substrate for brain and lung and butyrylthiocholine iodide for plasma. AChE activity in the blood was measured using propionylthiocholine iodide as the substrate according to the method of Augustinsson *et al.* (1978). AChE activity was measured after the inhibition of BChE with a specific inhibitor Astra 1397-HCl. The blood samples were preincubated with Astra-compound for 2 min before addition of the substrate.

Assay of Plasma CaE Activity

CaE activity was measured from plasma samples according to the method of Zemaitis and Greene (1976) using 0.02 M *p*-nitrophenylacetate as the

substrate. The kinetics of the enzyme reaction was spectrophotometrically followed at 400 nm at 22°C.

Assay of Plasma Paraoxon Hydrolyzing Activity

Paraoxon hydrolyzing activity of phosphotriesterase was measured from plasma using paraoxon as the substrate as described by Tuovinen *et al.* (1994). The activity was spectrophotometrically monitored at 405 nm at 37°C.

Protein

Protein levels were determined according to Lowry *et al.* (1951), using bovine serum albumin as a standard.

Statistical Analysis

The enzymatic data are presented as means ± SD. Comparison of enzyme activities between groups was calculated using one way analysis of Kruskal–Wallis and followed by Mann–Whitney U. The *p*-value 0.05 or less was used as the level of statistical significance. The behavioral activity data are presented as number of animals being active at each minute. The differences between carbamate-treated and control groups were tested using chi-square test.

RESULTS

Eptastigmine (1.5 mg/kg) inhibited the plasma BChE activity almost totally during the first 10 min, but the activity recovered to the control level within 6 hr (Table 1). The physostigmine-inhibited activity had also recovered close to the control level within 6 hr. The blood AChE activity, illustrating the AChE activity in erythrocytes, was slower and less inhibited by eptastigmine than the plasma BChEs. Eptastigmine administration inhibited the blood AChE activity only by 20% in 10 min and the maximum inhibition (30%) was seen 2 hr after the administration. The AChE activity in brain was immediately maximally inhibited by eptastigmine, but the activity had recovered totally by 24 hr. The effect of physostigmine on the enzyme activities in erythrocytes and brain was shorter than that of eptastigmine.

The behavioral activity of eptastigmine-treated animals seemed to be slightly reduced between 10–25 min (Fig. 1). However, the differences between eptastigmine and control groups were only occasionally significant. In contrast, the activity of mice treated with physostigmine was rapidly though transiently reduced. This effect lasted for 26 min, after which there were no notable differences between the groups.

The half-life of phosphotriesterase was 4–5 hr in the mouse circulation (data not shown). Phosphotriesterase (83 U/g bw) protected both the blood AChE and the plasma BChE activities against DFP inhibition (Fig. 2A and B). The combined eptastigmine–phosphotriesterase treatment of mice did not increase the DFP-inhibited AChE activities until 24 hr after the exposure (Fig. 2A). The eptastigmine–phosphotriesterase combination clearly improved, however, the protection of plasma BChE com-

TABLE 1
The *In Vivo* Recovery of AChE and BChE Activities Inhibited by Physostigmine (PHY) or Heptylphysostigmine Tartrate (EPTA) in Mouse Plasma, Blood, and Brain

Time (hr)	10 min	2 hr	4 hr	6 hr	24 hr
Plasma BChE (% of control)					
PHY	69.7 ± 21.4	79.2 ± 2.0*	81.1 ± 6.7*	85.8 ± 5.8	106.2 ± 17.4
EPTA	12.1 ± 0.5*	41.2 ± 2.7*,†	74.1 ± 10.0*,†	101.3 ± 21.8	95.2 ± 3.3
Blood AChE (% of control)					
PHY	80.7 ± 8.2*	93.4 ± 4.8†	102.4 ± 11.2	83.4 ± 5.1*,†	97.6 ± 4.9
EPTA	80.4 ± 10.8*	68.8 ± 8.2*	78.8 ± 11.2*	71.4 ± 16.8*	89.0 ± 6.7
Brain AChE (% of control)					
PHY	59.6 ± 3.9*	104.0 ± 6.4†	98.3 ± 5.3	97.8 ± 10.1	101.2 ± 3.5
EPTA	37.2 ± 1.4*	47.4 ± 5.1*,†	52.5 ± 3.4*	63.2 ± 1.9*,†	94.0 ± 4.0†

Note. PHY (0.09 mg/kg)/EPTA (1.50 mg/kg) or saline was injected iv. Control activities: Brain AChE, 168.3 ± 6.3 nmol/min × mg protein; Blood AChE 1815.1 ± 200.5 nmol/min × ml; Plasma BChE, 4930.6 ± 852.1 nmol/min × ml. Means ± SD are given, $n = 5-7$.

* $p < 0.05$ compared with the control.

† $p < 0.05$ compared to the previous time point.

pared with phosphotriesterase treatment alone (Fig. 2B). DFP inhibited by 90% the plasma BChE activity within the first hour, from which the activity recovered spontaneously to about 30% at times between 6 and 24 hr. The phosphotriesterase treatment alone slightly protected this activity during the first 6 hr. On the other hand, in eptastigmine-phosphotriesterase-treated mice the activity of this enzyme was inhibited only by 55% at 1 hr, and it had recovered almost to the normal level by 24 hr.

Both the phosphotriesterase and the eptastigmine-phosphotriesterase treatments increased the plasma CaE activities measured at 6 hr after DFP exposure in mice (Fig. 3). In untreated mice, the activity had also partly recovered during

this time. At 24 hr, the enzyme activity had also spontaneously recovered to 60% of the control. The activity was slightly increased by eptastigmine-phosphotriesterase treatment. Compared with the plasma BChE, CaE activity seemed to be less inhibited.

Phosphotriesterase administration effectively protected the brain AChE activity against the DFP-induced inactivation (Fig. 2C). Eptastigmine inhibited the brain AChE activity by 45% in the first hour, and the activity returned to the 90% of control within 24 hr. The eptastigmine-phosphotriesterase combination also protected the enzyme activity against DFP. However, phosphotriesterase alone provided better protection for the brain enzymes at the 6-hr assessment. Twenty-four hours after the treatment, the activity was at the same level in both treatment groups.

Phosphotriesterase did not prevent the inhibition of lung AChE activity during the first hour. After that, however, it assisted the recovery of activity up to 6 hr. Twenty-four hours after the DFP exposure there was no difference in the activity between the DFP and DFP-phosphotriesterase treatments (Fig. 2D). The eptastigmine treatment reduced the activity more than DFP. The combination of eptastigmine with phosphotriesterase did not provide any additional protection for the enzyme during the first 6 hr. After 24 hr, however, there was a clear difference between the groups. The enzyme activity had recovered to normal in the eptastigmine-phosphotriesterase group, whereas in mice receiving only phosphotriesterase the activities were still inhibited by 25%.

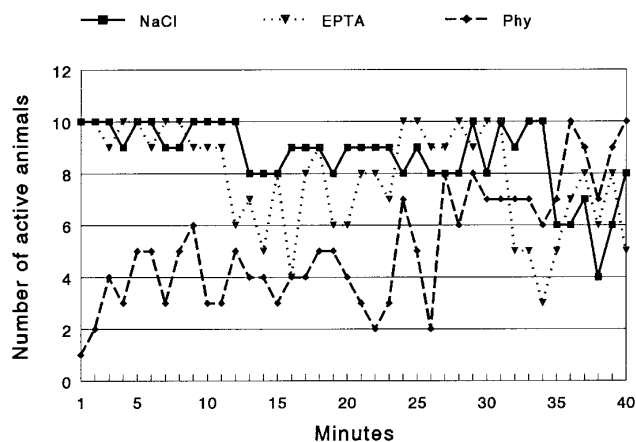


FIG. 1. The number of active mice after iv NaCl, physostigmine (PHY) (0.09 mg/kg), or eptastigmine (EPTA) (1.5 mg/kg) administrations, recorded once/minute. $N = 10$ /group. PHY-treated animals differed significantly from the controls during the first 26 min (chi-square test). EPTA-treated animals differed from controls at 12, 16, 33, and 34 min.

DISCUSSION

When compared with the phosphotriesterase treatment on its own, the combination of eptastigmine-phosphotriesterase

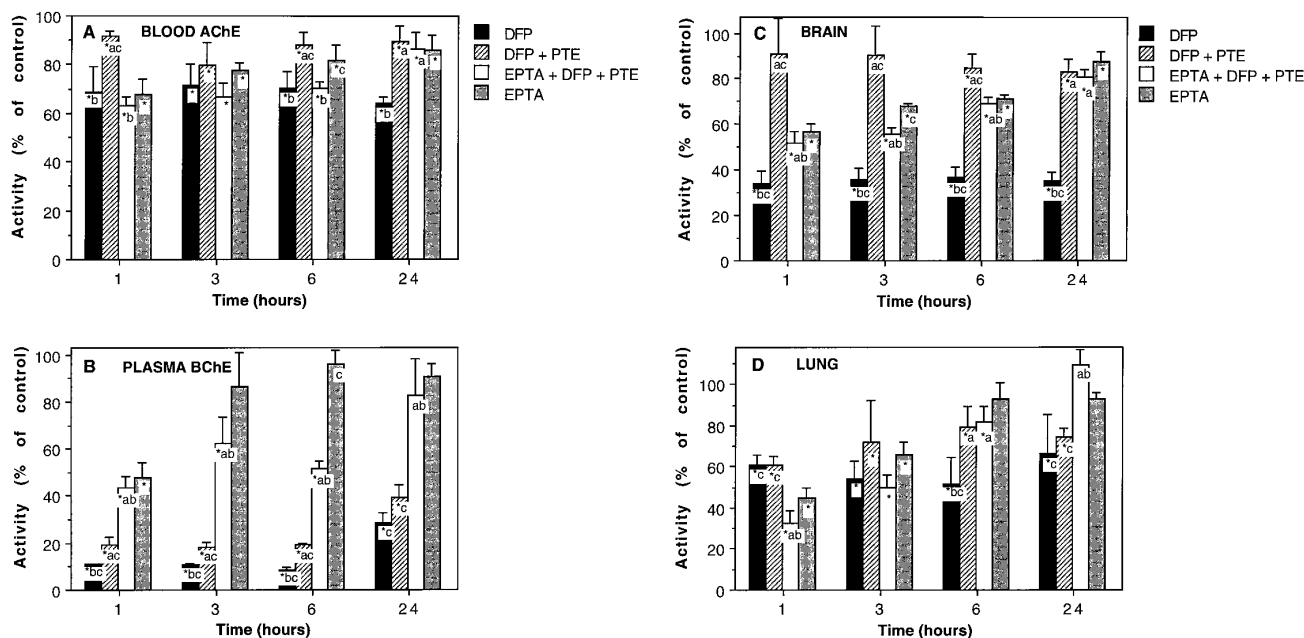


FIG. 2. The effect of iv phosphotriesterase (PTE) (approximately 83 U/g bw) and/or heptylphosphostigmine tartrate (EPTA) (0.90 mg/kg) on blood AChE (A), plasma BChE (B), brain AChE (C), and lung BChE (D) activities after DFP exposure in mice. EPTA was injected iv 10 min prior to the ip injection of DFP (1.75 mg/kg). PTE was injected iv 10 min after DFP. Furthermore atropine sulfate (37.5 mg/kg) was injected sc immediately after DFP. Control activities were in blood 2699 ± 124 nmol/min \times ml, in plasma 4141 ± 310 nmol/min \times ml, in brain 217 ± 17 nmol/min \times mg protein, and in lung 21 ± 2 nmol/min \times mg protein. Means \pm SD are given, $n = 3-5$. * $p < 0.05$ compared with the control. a, $p < 0.05$ compared with the DFP-treated mice. b, $p < 0.05$ compared with the DFP-PTE-treated mice. c, $p < 0.05$ compared with the EPTA-DFP-PTE-treated mice. EPTA-treated mice compared only with the control and EPTA-DFP-PTE-treated mice.

proved to be more effective against DFP exposure in plasma, where the BChE activity had recovered somewhat already within 1 hour. It seems likely that, phosphotriesterase could more effectively hydrolyze DFP in plasma when eptastigmine prevented its binding to BChEs. When eptastigmine was released from the BChEs, the concentration of DFP was too low to inhibit the enzymes. The DFP inhibition of plasma CaE was also partly prevented by phosphotriesterase. Eptastigmine-phosphotriesterase combination did not increase these activities.

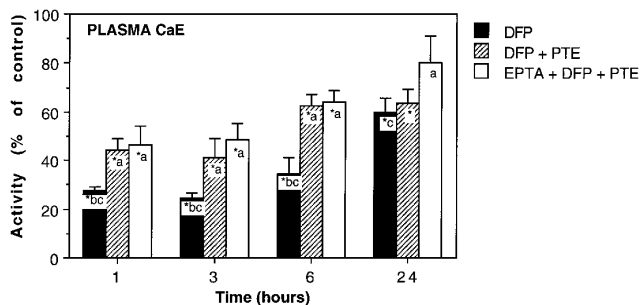


FIG. 3. The effect of iv phosphotriesterase (PTE) (approximately 83 U/g bw) and/or heptylphosphostigmine tartrate (EPTA) (0.90 mg/kg) on plasma CaE activities in mice. Control activity was 3843 ± 324 nmol/min \times ml. Means \pm SD are given, $n = 3-5$. For explanations see Fig. 2.

In lung, eptastigmine-phosphotriesterase administration decreased the AChE activity at first more than DFP. After 6 hr, however, the activity was at the same level as in phosphotriesterase-treated animals. After 24 hr, the activity had recovered to the normal level with the combined eptastigmine-phosphotriesterase treatment. Interestingly, in the lungs of animals treated with eptastigmine only, the activity remained lower than with the eptastigmine-phosphotriesterase combination. In brain and erythrocytes, the protective effect of phosphotriesterase was so great that eptastigmine could not supplement it further.

Physostigmine and its heptyl derivative eptastigmine are able to penetrate the blood-brain barrier and protect brain enzymes against OPs. Eptastigmine has greater lipophilicity and a longer duration of action than physostigmine (Marta *et al.*, 1988; Sramek *et al.*, 1995; Unni *et al.*, 1994). The longer duration of action may be due to the greater molecular size of eptastigmine ($C_{21}H_{33}N_3O_2$ vs $C_{15}H_{21}N_3O_2$). In the brain of rats, the reactivation time is approximately 20 min for physostigmine, while for eptastigmine it is seven times longer (Marta *et al.*, 1988). Unni *et al.* (1993) suggested that the eptastigmine-inhibited erythrocyte AChE would be a peripheral marker for AChE inhibition in the brain. According to our results both enzymes had recovered nearly to normal within 24 hr. The

maximal inhibition, however, was greater and faster in brain than in erythrocytes. In man, the eptastigmine dose of 30 mg inhibited maximally the plasma BChE in 3 hr and erythrocyte AChE activity by 4 hr (Auteri *et al.*, 1993; Cazzola *et al.*, 1993). The half-time of enzyme recovery was about 12 hr in plasma and 10.5 hr in erythrocytes. When eptastigmine or physostigmine was administered iv, the activity of AChE in erythrocytes was less inhibited than plasma BChE.

The physostigmine treatment clearly decreased the behavioral activity of the animals, this effect lasting about 30 min. With eptastigmine treatment, the behavior was only minimally influenced. Interestingly, the decreased behavioral activity did not correlate with the inhibition of AChEs in brain or in other tissues. In all tissues, eptastigmine, which caused only minor behavioral changes, induced greater inhibition of the enzyme activities than physostigmine. Hence, the decreased behavioral activity of mice was not due to the AChE inhibition. One explanation for the behavioral effects of physostigmine could be direct effects of carbamates on the nicotinic acetylcholine receptor ion channel and on glutamatergic neuromuscular junction (Albuquerque *et al.*, 1985; Aracava *et al.*, 1987). Eptastigmine seemed to be a generally more effective and longer-lasting inhibitor of body AChEs or BChEs than physostigmine. The absence of behavioral changes after eptastigmine treatment also supported its selection as the agent to be tested in combination with phosphotriesterase.

A potentially novel alternative OP antidote is to use exogenous enzymes such as purified AChE (Wolfe *et al.*, 1987, 1991, 1992; Ashani *et al.*, 1991), BChE (Ashani *et al.*, 1991; Broomfield *et al.*, 1991; Raveh *et al.*, 1993), CaE (Maxwell, 1992; Maxwell and Koplovitz, 1990), or OP hydrolases (Raveh *et al.*, 1992; Kaliste-Korhonen *et al.*, 1993; Tuovinen *et al.*, 1994, 1996). Exogenous AChE, BChE, and CaE provide alternative binding sites for OPs and reduce the inhibition of endogenous body enzymes. While the above-mentioned scavengers offer protection against the nerve agents, they have the disadvantage that they all have high molecular weights and react in a 1:1 ratio with the OPs. According to Broomfield (1992), about 500 mg of these enzymes must be active in the circulation for each milligram of OPs to be neutralized. Instead of simply acting as scavengers, OP hydrolases can actively hydrolyze OPs. This reduces the amount of OP in the body and prevents the inhibition of body esterases. The phosphotriesterase treatment could effectively prevent the inhibition of AChEs in erythrocytes and in brain. Its effect was also seen in the activities of lung and plasma BChEs and plasma CaE. These results are in accordance with our earlier studies with higher DFP doses (3.8–7.5 mg/kg) (Tuovinen *et al.*, 1994, 1996). The DFP dose used in this study (1.75 mg/kg) inhibited plasma BChE activity by 90%. With this DFP concentration, the detoxification ability

of phosphotriesterase was already seen after 1 hr in the recovery of plasma BChE activity.

The protection efficacy of carbamate against OPs depends on animal species. In mice and rats, physostigmine gives a protection ratio of 2- to 3-fold against soman, whereas in guinea pigs the protection ratio was better, up 6- to 8-fold (Lennox *et al.*, 1985). This has been thought to be due to the different elimination rates of physostigmine in different animals. The half-life of physostigmine in rats was 15 min (Somani and Khalique, 1986, 1987), in dogs 31 min (Giacobini *et al.*, 1987), and in guinea pigs 50 min (Somani and Dube, 1989). In man, the half-lives of physostigmine and eptastigmine were 22 min (Hartvig *et al.*, 1986; Marta *et al.*, 1988) and 140 min (Marta *et al.*, 1988), respectively. Eptastigmine, due to its long-lasting effect, may be more effective than physostigmine. On the other hand, the prolonged inhibition caused by eptastigmine may also be harmful in cases of severe OP intoxications. Our results showed that eptastigmine, when combined with phosphotriesterase treatment, was effective at least against DFP. By preventing the binding of DFP to BChEs, eptastigmine presumably led to an increase in the amount of free DFP in body fluids, and phosphotriesterase could hydrolyze this free OP more effectively.

One advantage of eptastigmine over physostigmine is also its lower toxicity. Our results showing minimal changes in the behavioral activity in mice after eptastigmine treatment indicate also that eptastigmine may have less detrimental effects in the central nervous system than physostigmine.

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