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Efficacy of Trimedoxime in Mice Poisoned with Dichlorvos, Heptenophos or Monocrotophos

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Abstract: The aim of the study was to examine antidotal potency of trimedoxime in mice poisoned with three direct dimethoxy-substituted organophosphorus inhibitors. In order to assess the protective efficacy of trimedoxime against dichlorvos, heptenophos or monocrotophos, median effective doses and efficacy half-times were calculated. Trimedoxime (24 mg/kg intravenously) was injected 5 min. before 1.3 LD50 intravenously of poisons. Activities of brain, diaphragmal and erythrocyte acetylcholinesterase, as well as of plasma carboxylesterases were determined at different time intervals (10, 40 and 60 min.) after administration of the antidotes. Protective effect of trimedoxime decreased according to the following order: monocrotophos > heptenophos > dichlorvos. Administration of the oxime produced a significant reactivation of central and peripheral acetylcholinesterase inhibited with dichlorvos and heptenophos, with the exception of erythrocyte acetylcholinesterase inhibited by heptenophos. Surprisingly, trimedoxime did not induce reactivation of monocrotophos-inhibited acetylcholinesterase in any of the tissues tested. These organophosphorus compounds produced a significant inhibition of plasma carboxylesterase activity, while administration of trimedoxime led to regeneration of the enzyme activity. The same dose of trimedoxime assured survival of experimental animals poisoned by all three organophosphorus compounds, although the biochemical findings were quite different.

The primary molecular mechanism of action of the organophosphorus compounds is inhibition of acetylcholinesterase (AChE, EC 3.1.1.7.) (Koelle 1975). Inhibition of AChE results in accumulation of acetylcholine (ACh) at the synaptic cleft of the cholinergic neurones, leading to overstimulation of cholinergic receptors. In addition to the inhibition of AChE, organophosphorus compounds clearly have a potential to interact with other esterases such as carboxylesterases (EC 3.1.1.1.). Carboxylesterases play an important role in the detoxification of organophosphorus compounds via hydrolysis of ester bond in these compounds and binding of the compounds at the active site of carboxylesterases that reduces the free concentration of organophosphate potentially available to inhibit acetylcholinesterase (Jokanović *et al.* 1996; Sogorb & Vilanova 2002).

Together with atropine pyridinium oximes (pralidoxime, trimedoxime, obidoxime, HI-6, HLö-7) represent the most important component of the specific therapy used against organophosphate poisoning (Bismuth *et al.* 1992; Karallieddе & Szinicz 2001). Since the resulting inhibited AChE is generally considered very stable and is only slowly reactivated by spontaneous hydrolysis of the phosphate ester, the essential role of the oximes is their ability to reactivate inhibited AChE (Worek *et al.* 1997; Thiermann *et al.* 1999). Other effects attributed to the oximes (in very high doses) are: weak ability to inhibit cholinesterase, direct reaction with organophosphates, anticholinergic effect similar to that of atropine, sympathomimetic effect potentiating the pressor effect of adrenaline, depolarising effect at the neuromuscular junction and direct influence on synaptic transmission by mechanisms that are not clear at present (Bošković & Stern 1963; Clement 1981; Busker *et al.* 1991; Melchers *et al.* 1991; Van Helden *et al.* 1991 & 1996; Becker *et al.* 1997). However, some failures of the standard therapy in organophosphate poisoning are due to the unequal effectiveness of oximes in the treatment of intoxications caused by different organophosphorus compounds (Bismuth *et al.* 1992; Dawson 1994; Thiermann *et al.* 1999; Worek *et al.* 1996, 1997, 1998, 1999, 2002 & 2004). Many *in vitro* and *in vivo* studies have been focused on the efficacy of the oximes in organophosphate poisoning. Although some of them were very difficult to interpret due to the differences in experimental design, it could be summarised that trimedoxime (TMB-4) and obidoxime (LüH-6) were superior to the other oximes used in organophosphorus insecticide poisoning (Sanderson & Edson 1959; Jokanović & Maksimović 1995; Worek *et al.* 1996, 1997 & 1999). It is also well known that TMB-4 and LüH-6 (differing only in the structure of the bridge between the two pyridinium rings) have a great reactivating potential, which explains their antidotal efficacy against a number of organophosphorus insecticides (Hobbiger & Vojvodić 1966; De Jong *et al.* 1982; Worek *et al.* 1996). Generally, the antidotal effect is highly dependent on the type of the anticholinesterase and the oxime used.

Concerning all these data on oxime efficacy, the aim of

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the present study was (1) to examine and compare antidotal potency of TMB-4 in mice poisoned with three direct dimethoxy-substituted inhibitors: dichlorvos, heptenophos and monocrotophos, and (2) to correlate the protective efficacy and reactivating ability of TMB-4 in central and peripheral tissues of experimental animals.

Materials and Methods

Experimental animals. Male albino mice (18–24 g) were obtained from the Military Medical Academy, Belgrade. The animals were rested at least one week before the experiment and received food and tap water *ad libitum.*

The study protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro).

Chemicals. Trimedoxime (1,1′-trimethylene bis(4-hydroxyimino methyl) pyridinium dichloride), was obtained from the Military Medical Academy, Belgrade and was 99% pure when examined by HPLC. Dichlorvos (2,2-dichlorovinyldimethylphosphate) (94.0%), heptenophos ((7-chlorobicyclo[3.2.0]hepta-2,6-dien-6-yl)dimethylphosphate) (92.0%) and monocrotophos (*O,O*-dimethyl-*O*-(1-methyl-2-methyl-carbamoylvinyl) phosphate) (82.8%) were purchased from the domestic commercial sources. Structural formulae are presented in fig. 1.

Stock solutions of organophosphates were prepared in isopropanol. Oxime was dissolved in distilled water and diluted to the required concentration immediately before use. All the solutions were administered intravenously via the tail vein at a volume of 0.1 ml/20 g of body mass.

Experimental procedures.

Protection. In the studies of antidotal protection, experimental animals were pretreated intravenously with increasing doses of TMB-4 at different time intervals (5–60 min.) before 1.3 LD50 intravenously of organophosphates. Median lethal doses of the tested compounds (LD50) and median effective doses of oxime ($ED50_t$) were calculated according to the method of Litchfield & Wilcoxon (1949), with 95% confidence limits. Median effective doses of the oxime along with the corresponding time intervals were used to calculate ED50 at null time ($ED50_0$) and efficacy half-time ($t_{1/2}$ efficacy) (Schoene *et al.* 1988).

Biochemistry. In the biochemical experiments, the brain, diaphragmal and erythrocyte AChE as well as plasma carboxylesterase activities were determined. TMB-4 (24 mg/kg; 0.3 LD50 intravenously) was injected 5 min. before 1.3 LD50 intravenously of poisons. Mice were decapitated and exsanguinated at different time intervals (10, 40 and 60 min.) after the antidote administration. Diaphragms and brains were removed and homogenised in isotonic saline. The brain and diaphragmal enzyme activities were measured by the spectrophotometric method (Ellman *et al.* 1961; Wilhelm 1968) using acetylcholine iodide as substrate. Erythrocyte AChE activity was determined titrimetrically (Augustinsson 1971), while acetylthiocholine iodide was added as substrate. Plasma carboxylesterase activity was assayed by the titrimetric pH-stat method (Clement 1982) using tributyrin (glycerin tributyrate) as a substrate. In order to determine the enzyme activities in animals treated with organophosphate alone (1.3 LD50 intravenously) (organophosphate control groups) and keep them alive until sacrifice, atropine 10 mg/kg intravenously had to be injected immediately before the poisons.

Data analyses. Twenty-four-hr LD50 and $ED50_t$ of investigated compounds (tables 1 & 2) were calculated by probit analysis according to the method of Litchfield & Wilcoxon (1949).

Protective effect of TMB-4 based on $ED50_0$ and $t_{1/2}$ efficacy in mice treated with organophosphorus compounds (table 2) was calculated by means of 2×2 assay (Pharm-Pharmacological Calculation System, v.4.0, Springer-Verlag, New York, USA, 1986).

Statistical significance between groups was determined by Mann-Whitney U-test (fig. 2) and Student's t-test (fig. 3, 4 & 5). The differences were considered significant when P value was less than 0.05. Statistical analyses were performed with commercial statistical software for PC, Stat for Windows R. 5.0.

Table 1.

Intravenous median lethal doses of TMB-4 and organophosphorus compounds.

Compounds	LD50 value (μmol/kg)	95% confidence interval
TMB-4	238.61	199.92–284.78
Monocrotophos	20.34	15.60–25.95
Dichlorvos	22.17	16.24–30.22
Heptenophos	196.68	163.72–208.41

Results

Intravenous LD50s of investigated compounds are given in table 1. It is evident that LD50 of heptenophos is about 10 times higher than in the other two organophosphorus compounds tested. This finding should be ascribed to complex interactions of numerous factors such as electronic effects, hydrophobicity, steric effects, ionic bonds, toxicokinetic properties and non-anticholinesterase effects (Maxwell & Lenz 1992).

Toxicity signs were typical of cholinesterase inhibition. Time to their onset was short and death usually occurred within 5–10 min. after administration of a lethal dose. This very short survival of mice that were not pretreated with TMB-4 imposed use of an experimental model based on administration of the oxime at various times before intravenous injection of organophosphorus compounds.

Use of TMB-4 against monocrotophos protected success-

Table 2.

Protective effects of TMB-4 in mice treated by 1.3 LD50 intravenously of organophosphorus compounds.

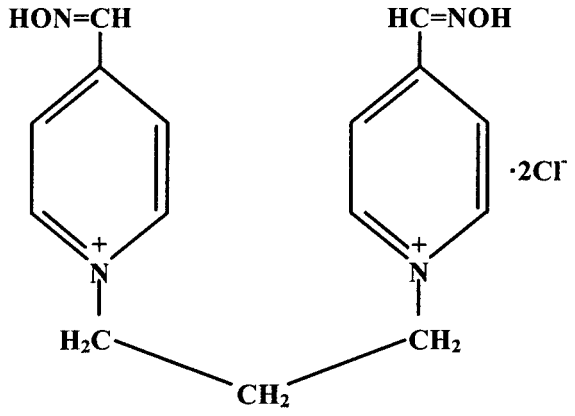
Time (min.)	$ED50_t$, μmol/kg (95% confidence interval)		
	Monocrotophos	Dichlorvos	Heptenophos
5	2.21 (1.18–4.11)	50.49 (36.39–70.03)	16.46 (11.06–24.49)
12.5		66.34 (59.12–74.43)	
20	3.27 (1.99–5.37)	88.81 (72.28–107.65)	73.81 (66.00–82.51)
27.5		114.11 (96.97–134.58)	
40	4.81 (2.88–8.09)		157.42 (86.91–285.11)
60	8.56 (4.39–16.77)		
		$ED\text{-}50_0$ (μmol/kg)	
	1.95	42.18	14.66
		$t_{1/2}$ eff. (min.)	
	28.67	18.98	10.98

fully the experimental animals along the whole time interval tested. The dose ($ED50_0$) of TMB-4 necessary to protect 50% of animals after the simultaneous administration of monocrotophos and oxime was 21.6 and 7.5 times lower than the corresponding doses calculated for the protocols with dichlorvos and heptenophos, respectively. Additionally, half-time of efficacy in monocrotophos poisoning was longer than in the case of dichlorvos or heptenophos intoxication indicated that TMB-4 was the most effective in protection against monocrotophos (table 2). $ED50_0$ of the oxime obtained in heptenophos poisoning was about 2.9 times lower than in experiments where dichlorvos was applied. However, half-time of efficacy in heptenophos poisoning was almost two times shorter than in the case of dichlorvos-treated animals. Therefore, considering both parameters it seemed that TMB-4 was more effective in protection against heptenophos.

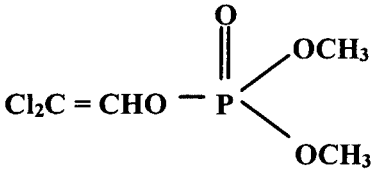
Biochemical results showed that mice that received only dichlorvos had no detectable erythrocyte AChE activity (fig. 2). However, in mice pretreated with TMB-4, the level of erythrocyte AChE recovered gradually over a 60 min. time period up to 92.00% of the total activity. After administration of dichlorvos, mice still had 8.3% of their brain and 35.10% of diaphragmal AChE functional. In the presence of TMB-4, AChE activity increased in both tissues, reaching its maximal level within 40 min., i.e. 37.80% and 85.63% of total brain and diaphragmal AChE activity, respectively (fig. 3 & 4).

After heptenophos intoxication, some tissue AChE activity (3.33% in erythrocytes, 3.37% in brain and 13.05% in diaphragm) remained uninhibited. After the 40 min. time interval, mice that received TMB-4 pretreatment still had no detectable erythrocyte AChE activity. However, erythrocyte AChE activity, measured at 60 min. after antidotal treatment, was about 10.67% of the control activity (fig. 2). One hour after administration of TMB-4, AChE activity recovered to 29.43% and 69.57% of the control brain and diaphragmal AChE activity, respectively (fig. 3 & 4). Unexpectedly, TMB-4 was not successful in restoring the AChE inhibited by monocrotophos (fig. 2, 3 & 4).

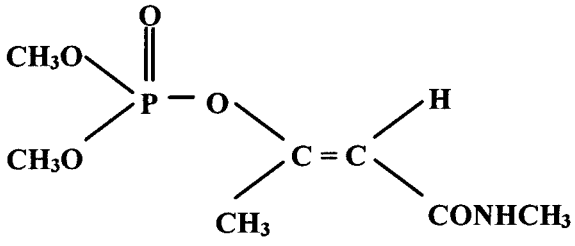
All the organophosphorus compounds tested produced significant inhibition of carboxylesterase activity in plasma.



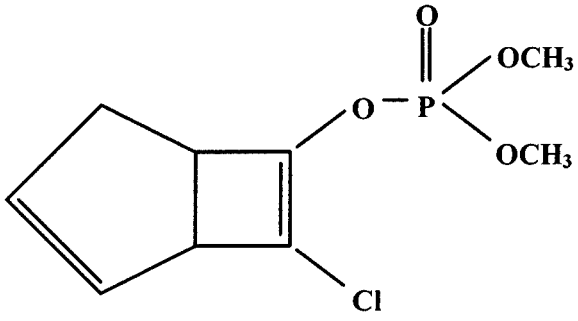
TMB-4



Dichlorvos



Monocrotophos



Heptenophos

Fig. 1. Structural formulae of oxime and organophosphorus compounds.

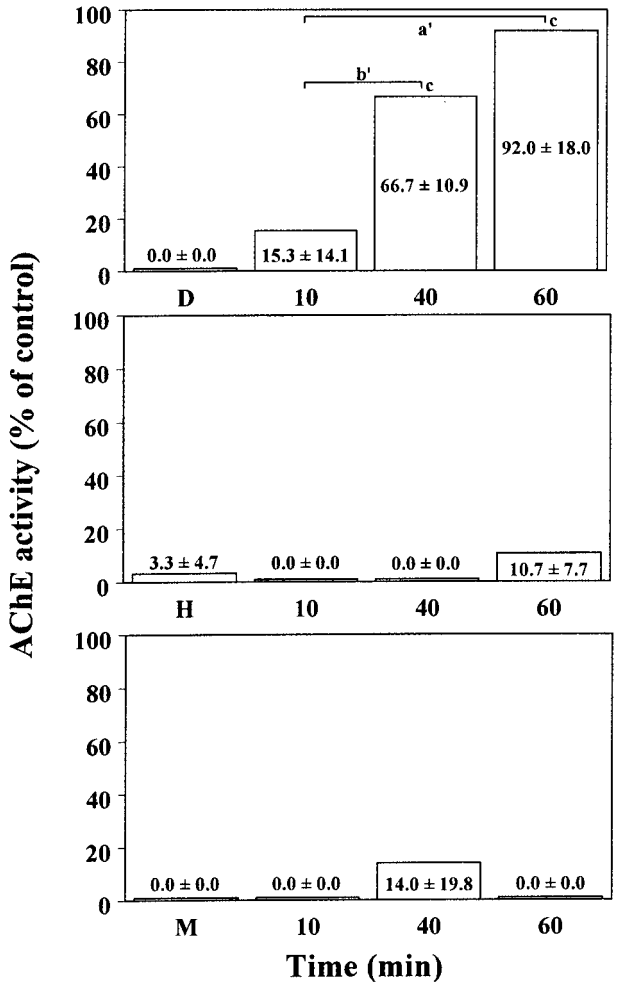


Fig. 2. The effect of TMB-4 on erythrocyte AChE activity in mice poisoned with 1.3 LD50 intravenously of dichlorvos (D), heptenophos (H) or monocrotophos (M). Data are means±S.D. of nine animals. c – $P<0.001$ versus poisoned group. a′,b′ – $P<0.05$, 0.01.

When the oxime was administered to the animals poisoned with dichlorvos, heptenophos or monocrotophos, carboxylesterase activity recovered to its maximum of 37.23%, 75.62% and 54.28% of the control activity, respectively (fig. 5).

Discussion

Trimedoxime is already well known as a potent reactivator of diethoxy, ethylethoxy-, methylethoxy-, methylisopropoxy- or diisopopoxy-phosphorylated cholinesterase (Reiner 1965; Dirks *et al.* 1970; Schoene & Strake 1971; Patočka 1973). Hobbiger & Sadler (1958) showed that TMB-4 was a significantly better reactivator than a pralidoxime methyliodide in mice poisoned with phospholine. In 1966, Hobbiger & Vojvodić showed that TMB-4 was a better reactivator of diethyl- and diisopropyl-phosphorylated acetylcholinesterase of human erythrocytes than LüH-6. Similar results were obtained by Bajgar *et al.* (1971) in experiments with equimolar doses of these two bispyridinium dioximes. In the experiments of Jokanović & Maksimović (1995), where pyridinium oximes (pralidoxime, TMB-4, LüH-6, HI-6) were administered along with atropine and diazepam to rats poisoned with 2 LD50 of organophosphorus insecticides including dichlorvos, heptenophos and monocrotophos, better protection was achieved when TMB-4 and LüH-6 were used. All these data clearly indicate that TMB-4 or LüH-6 can be accepted as the oximes of choice against the majority of organophosphorus insecticide poisonings.

In our experiment, along the investigated time interval, protective effect of TMB-4 decreased according to the following order: monocrotophos > heptenophos > dichlorvos (table 2). Administration of trimedoxime produced a significant reactivation of central and peripheral AChE inhibited with dichlorvos and heptenophos, with the exception of erythrocyte AChE inhibited by heptenophos (fig. 2, 3 & 4). Surprisingly, although TMB-4 was the most effective in protection of monocrotophos-poisoned mice, it did not induce reactivation of inhibited AChE in any of the tissues

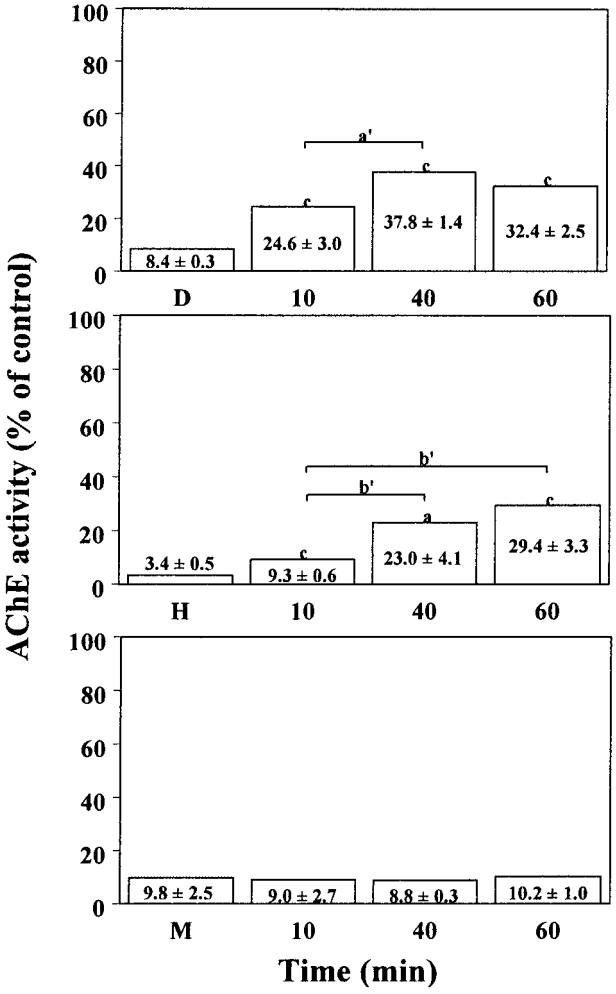


Fig. 3. The effect of TMB-4 on brain AChE activity in mice poisoned with 1.3 LD50 intravenously of dichlorvos (D), heptenophos (H) or monocrotophos (M). Data are means±S.D. of nine animals. a, c – $P<0.05$, 0.001 versus poisoned group. a′, b′ – $P<0.05$, 0.01.

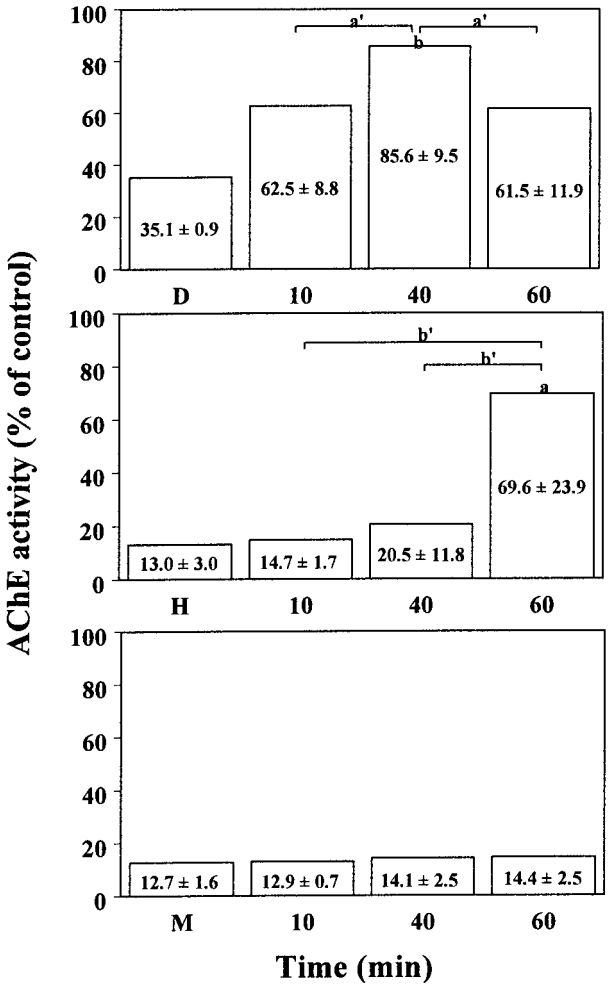


Fig. 4. The effect of TMB-4 on diaphragmal AChE activity in mice poisoned with 1.3 LD50 intravenously of dichlorvos (D), heptenophos (H) or monocrotophos (M). Data are means±S.D. of nine animals. a, b – $P<0.05$, 0.01 versus poisoned group. a′, b′ – $P<0.05$, 0.01.

tested. These conflicting results were probably due to the production of monocrotophos metabolites (N-methylhydroxy monocrotophos and N-desmethyl monocrotophos), known as analogues, the toxicities of which are comparable to the parent compound (Menzer & Casida 1965; Skripsky & Loosli 1994). Such biotransformation of monocrotophos probably led to a fast reinhibition of the previously reactivated AChE. Thiermann *et al.* (1997) had shown that a significant reactivation occurred only after the organophosphorus concentration in the body fell below a critical value, i.e. significant reactivation occurred only when the reactivation rate exceeded the inhibition rate. Another interesting feature was that the animals treated with TMB-4 were alive during the 60 min. time period albeit corresponding AChE activities did not differ significantly from the group which received monocrotophos only. The observed discrepancy indicated that TMB-4 had a therapeutic action independent of AChE-reactivation. One could conclude that the so called "direct" effects of TMB-4, i.e. pharmacological effects other than reactivation of AChE were essential for the survival of monocrotophos-intoxicated mice.

The beneficial effects of oximes are generally thought to be due to their ability to reactivate the inhibited enzyme, although the protective effect is attributed at least partly to action(s) other than enzyme reactivation. Therefore, contribution of pharmacological effects other than reactivation of AChE, including the direct interaction between the oxime and the active site of AChE, should not be excluded. Illustrative to this notion are several findings where HI-6 antagonised lethal soman poisoning of primates although activity of blood AChE was very low (Lipp & Dola 1980; Hamilton & Lundy 1989; Van Helden *et al.* 1992). It is well known from the literature that TMB-4 acts as weak AChE inhibitor (Bismuth *et al.* 1992). In our experiment the degree of AChE inhibition induced by TMB-4 should not differ among the mice subsequently poisoned with dichlorvos, monocrotophos or heptenophos. Despite the fact that the experimental design used could not differentiate between

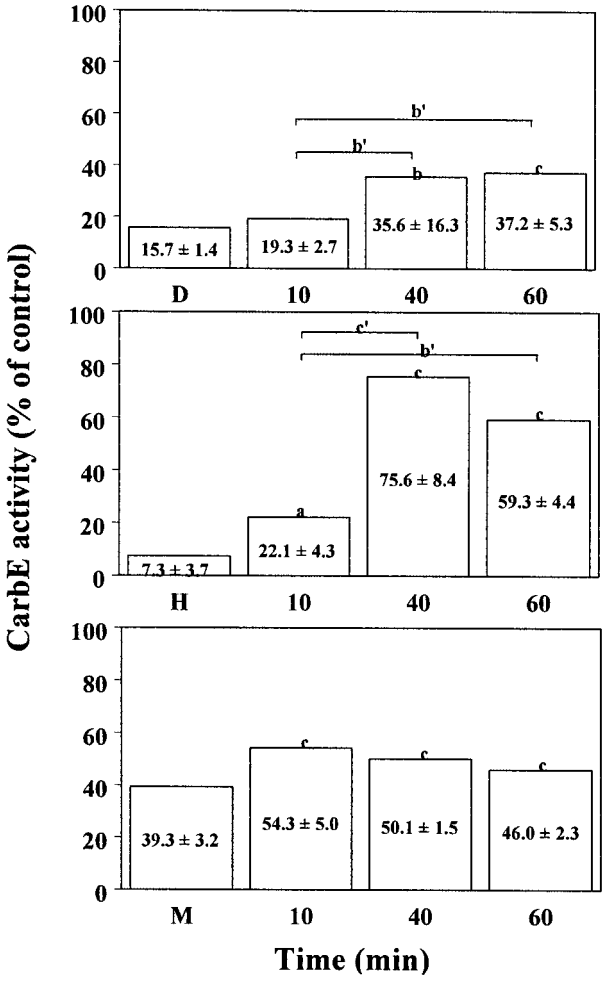


Fig. 5. The effect of TMB-4 on plasma carboxylesterase (CarbE) activity in mice poisoned with 1.3 LD50 intravenously of dichlorvos (D), heptenophos (H) or monocrotophos (M). Data are means±S.D. of nine animals. a, b, c – $P<0.05$, 0.01, 0.001 versus poisoned group. b′, c′ – $P<0.01$, 0.001.

the protection and reactivation of AChE it is reasonable to assume that the reactivating mechanism accounts for the major part of the overall protection obtained. On the other hand, it is well known that TMB-4 possesses curare- and atropine-like effects and exerts hypotension in experimental animals. Hobbiger & Sadler (1959) showed that TMB-4 reduced sensitivity of small intestine to acetylcholine in guinea pigs while Bošković & Stern (1963) proved its direct effect on the activity of the rat striated muscle. Results of Lindgren & Sundwall (1960) confirmed its strong parasympatholytic effect in animals not exposed to organophosphates.

The phosphorylation occurs by the loss of the "leaving group" of the organophosphorus compound and the formation of a covalent bond with AChE through the serine hydroxyl group. Differing only in their leaving groups, dichlorvos, heptenophos and monocrotophos generate the same type, i.e. dimethoxy-phosphorylated AChE. Interestingly, the extent of its reactivation was not equal. A similar finding was obtained in *in vitro* experiment where mevinphos, malaoxon and trichlorfon were used as inhibitors of human erythrocyte AChE by Worek *et al.* (1997), confirming that the contribution of a specific "leaving group" to the complexity of toxic response to organophosphates should be taken into account in attempt to assess the antidotal efficacy.

Dichlorvos, heptenophos and monocrotophos induced statistically significant inhibition of plasma carboxylesterase activity, while the administration of TMB-4 led to its significant reactivation (fig. 5). It is well known that carboxylesterase detoxify organophosphorus compounds so that they are considered a protective buffer for AChE. Regeneration of carboxylesterase produced by TMB-4 seems to be important additional mechanism contributing to its antidotal effects.

The reactivity of organophosphorus compounds varies depending upon their chemical structure. The organophosphorus pesticides or their active metabolites are electrophilic compounds with moderate to high potency for phosphorylating the serine hydroxyl group located at the active site of AChE. The same dose of TMB-4 kept experimental animals alive in poisonings caused by all three organophosphorus compounds, but at the same time biochemical findings were quite different. It was very difficult or even impossible to correlate protective effect of the oxime with its ability to reactivate the inhibited acetylcholinesterase. With the presumption that pharmacokinetic properties of TMB-4 (Milić *et al.* 1996) were not affected significantly by the tested organophosphates, as shown previously in experiments with HI-6 and HLö-7 (Göransson-Nyberg *et al.* 1995; Worek *et al.* 1995), the antidotal efficacy of TMB-4 was predominantly under the influence of toxicodynamic and toxicokinetic characteristics of organophosphates used in this experiment (Jokanović 2001; Karalliedde *et al.* 2003). Having in mind numerous factors that influence the antidotal potency of oximes, further investigations are necessary in order to optimise the antidotal therapy. Therefore, intentions aimed at synthesis an oxime with "universal" antidotal properties seem to be still a promising research field.

Acknowledgements

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