60. O-ALKYL-O-METHYLCHLORFORMIMINOPHENYL PHOSPHONATES DELAYED NEUROTOXICITY RISK ASSESSMENT. *IN VITRO* AND *IN VIVO* STUDIES.

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INTRODUCTION

Organophosphorus compounds (OP) with anticholinergic properties are widely used as insecticides. Some highly toxic OPs are also chemical warfare agents. These compounds are believed to be included in the arsenals of several nations and terrorist groups, as witnessed in 1995 when sarin was used against Japanese civilians during a terrorist attack on the Tokyo subway system, resulting in over 5500 casualties [1].

The immediate hazard associated with OPs, the acute toxic effect, arises from their inhibition of acetylcholinesterase (AChE) at the nerve ending [2]. The cholinergic syndrome appears at approximately 50% AChE inhibition whereas death is believed to occur at > 90% if adequate treatment is not provided [2,3].

Certain OPs, including compounds with low acute toxicity can also produce OP-induced delayed neurotoxicity (OPIDN) in man and other susceptible species [4-10]. OPIDN is a distal degeneration of sensory and motor axons that occurs 2-5 weeks after an acute poisoning [10]. Stringent regulations and rigorous premarket testing of OPs in the U.S. and in Western Europe have resulted in few neuropathic OPs reaching the market. However, there is a real possibility of OPIDN from incidental OP exposure in the States of the Former Soviet Union and developing countries [11]. Moreover, it is possible that OPIDN could arise from terrorists using neuropathic OPs to cause OPIDN intentionally instead of cholinergic toxicity, which is the conventional endpoint of standard nerve agents.

The high human susceptibility to OPIDN, its insidious onset and usually permanent debilitating effects make the problem of risk assessment for this aspect of OP neurotoxicology highly important.

Considerable evidence suggests that a neuronal protein, known as Neuropathy Target Esterase (neurotoxic esterase, NTE), is the primary target molecule in OPIDN. The disease is thought to be initiated by organophosphorylation and subsequent specific modification (aging) of the inhibited enzyme [6-9]. In the experimental animals OPIDN is associated with > 70% brain NTE inhibition after single exposures [2,6,8]. The threshold in man is not known, although there are indications that it is similar.

NTE has proven to be an excellent tool for *in vitro* assessment neuropathic potential of OPs [9,12]. The relative potency of an OP or its active metabolite to inhibit NTE *versus* AChE *in vitro*, k_i(NTE)/k_i(AChE), was shown to correlate with the ratio between LD_{50} and neuropathic dose and can be used as a convenient index of probable neuropathic potential of the compound [6,9,13]. Values of the ratio k_i(NTE)/k_i(AChE) > 1 indicate that the dose required to produce OPIDN is less than LD_{50}, whereas values < 1 correspond to doses greater than LD_{50} being required to produce OPIDN [6,9,13]. According to M.Johnson [6], compounds for which the ratio k_i(NTE)/k_i(AChE) ≥ 0.05 should be subjected to careful toxicological studying because neuropathies caused by intoxication with such compounds may develop after successful curing acute cholinergic poisoning.

A stable preparation of hen brain NTE, that is the lyophilized (P_2+P_3) hen brain membrane fraction preinhibited with paraoxon, was developed in our laboratory [14]. This preparation retains inhibitor features of the native enzyme during a year and rather high

specific activity of NTE and can be used as a readily available «off-the-shelf» source of NTE for assessing the anti-NTE activity of OP compounds [14]. Based on stable preparation of hen brain NTE the convenient methods for kinetics studies of NTE inhibition were elaborated as well as a method of *in vitro* express testing OPs for delayed neurotoxicity by comparing its inhibitory potency against two primary targets of OPs: NTE and AChE.

In the present work we report an *in vitro* study of NTE and AChE inhibition by a series of model phenylphosphonates of the general formula $(RO)C_6H_5P(O)ON{=}CClCH_3$ (PhP), the assessment of neuropathic potential for the investigated OPs using ratios k_i(NTE)/k_i(AChE) and comparison the obtained results with the ratios of the median effective doses (ED_{50}) for hen brain NTE and AChE inhibition obtained *in vivo*.

MATERIALS AND METHODS

Chemicals: Phenyl valerate (PV), mipafox (*N,N'*-diisopropylphosphorodiamido fluoridate), *O*-Alkyl-*O*-methylchloroformiminophenyl phosphonates were synthesized and characterized in the Institute of Physiologically Active Compounds Russian Academy of Sciences (Russia) [15]. The purity of all substances was not less than 99% (by spectral, chromatographic and elemental analysis data). Paraoxon (*O,O*-diethyl-4-nitrophenyl phosphate) was from Sigma Chemical Co. (St. Louis, Missouri USA).

Animals: Adult white Leghorn hens (18 months old, 1.5-2 kg) were from Noginsk poultry farm (Noginsk, Russia). Hens were kept 3 to a cage with food and water *ab libitum*. The hens were kept in room with 12-hr light cycle in which the temperature was controlled (20-23°C).

Enzymes. A stable lyophilized preparation of paraoxon-pretreated (P_2+P_3) hen brain membrane fraction was used as a source of NTE. The preparation was obtained according to [14,15]. Whole brains were removed from hens and homogenized in buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0, 4°C) with a Potter homogenizer. Paraoxon was then added to achieve a concentration of 40 μM to inhibit esterases other than NTE. Homogenate was centrifuged at 1000 x ***g*** for 10 min. The supernatant was removed and centrifuged at 105,000 x ***g*** for 30 min. The two centrifuging procedures were carried out at 25°C and took about 45 min. The 105,000 x ***g*** pellet was then resuspended in a cold twice-distilled water; after that it was frozen quickly in liquid nitrogen and lyophilized in ampoules on a freeze dryers system LGA-5 (Germany). The lyophilized membranes stored in sealed ampoules under vacuum, at –20 °C.

A lyophilized (P_2+P_3) hen brain membrane fraction prepared as above except the paraoxon pretreatment [14,15] was used as a source of the hen brain AChE.

NTE assay. NTE activity was assayed colorimetrically according to Johnson [16] with slight modifications. Before the experiment the lyophilized preparations were suspended in the assay buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0, 25°C) in Potter homogenizer with a Teflon pestle. The assay was carried out at 37°C in a final volume 1ml, protein concentration 0.1-0.14 mg/ml, using PV as substrate. NTE activity was measured as a difference between PV hydrolyzing activity of the used enzyme preparation, presenting PO-resistant esterase activity, and its activity after 20 min. incubation with 250 μM Mipafox.

NTE activity in 9S supernatant of whole brain homogenate was determined as the difference in PV hydrolyzing activity, between paired samples in the presence of either paraoxon (50μM), or 50 μM paraoxon plus 250 μM Mipafox.

Protein was assayed by microbiuret methods with bovine serum albumin as a standard.

AChE assay. AChE activity was determined with the method of Ellman [17] using acetylthiocholine as substrate.

Inhibitor activity determination. For kinetic studies of NTE and AChE inhibition a sample of corresponding enzyme was incubated with studied OP (acetone concentration 1%) for different times. The residual NTE or AChE activity was then assayed, each value was determined in duplicate. The slopes (k') of each semi-log plot were calculated by a linear regression procedure applied to each set of data points. These values of k' were then plotted against inhibitor concentration [I], and the slope (k'') of the line was derived by linear regression as above. Then the bimolecular rate constant of inhibition (k_i) was calculated using the relationship: $k_i = 2.303\ k' / [I] = 2.303\ k''$ [18]. Each value of k' was obtained from a line through 4-6 points.

The relationship between structure of PhP and their selectivity to NTE and, correspondingly, their neuropathic potential, was analyzed with multiple regression analysis using ORIGIN 5.0 software. Additive hydrophobicity Hansch's constants (πCH_2= 0.5) for substituents were used for modelling [19]. QSAR models for neuropathic potential of PhP were developed. A significance of the obtained equations was estimated with values of r – coefficient of multiple correlation, s – standard deviation of the fit, and F – Fisher's criterion, which characterizes a significance level of r under the selected confidence interval, and depends on a number of data points n and a number of basic functions in the applied equation.

Inhibition of NTE and AChE in a hen brain after acute i.m. administration of increasing doses of Me and Bu derivatives. Inhibition of NTE and AChE in a hen brain was studied in 24 hrs after acute i.m. hens injecting with increasing doses of Me (MePhP) and Bu (BuPhP) derivatives. MePhP was administered in doses from 0.6 to 40 mg/kg, BuPhP - in doses from 0.6 to 60 mg/kg. Compounds were administered i.m. to groups of 3 hens per every dose. All hens were pretreated with atropine sulfate, 20 mg/kg, 20 min before PhP was administered. Control animals received atropine sulfate only. In 24 hrs after PhP administration hens were decapitated and brains were removed for determination of NTE and AChE activities. Brain of every hen was homogenized at +4°C in 5 vol of buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0) with Potter homogenizer and centrifuged for 15 min at 9000 x g at +4°C. The brain 9S supernatant was used for enzyme analysis [20].

Esterase activity in brain samples from hens treated with atropine and PhP was determined and compared to activity in tissue samples from animals treated with atropine only (control). Dose-response curves were analyzed with a four-parameter logistic function using ORIGIN 5.0 software.

RESULTS AND DISCUSSION

Structure of the studied phenylphosphonates is presented in Fig.1.

All compounds investigated were shown to be irreversible progressive inhibitors of both esterases. The time-course of NTE and AChE inhibition was found to follow first-order kinetics. For each OP the slopes of lines were proportional to inhibitor concentrations. The derived bimolecular rate constants of inhibition (k_i) are presented in Table 1.

Fig.2 presents the dependence of inhibitor activity of the studied phenylphosphonates to NTE and AChE on hydrophobicity of alkyl radicals. The differential effect of changing hydrophobicity on anti-NTE and anti-AChE activity suggests differences in the structure of the active sites of the two target esterases of the OP compounds, and correspondingly, their different inhibitor specificity.

Most of PhP were more potent inhibitors of NTE than AChE. Both antiNTE activity, selectivity for NTE and, correspondingly, the propensity of PhP to cause OPIDN raised with the increasing hydrophobicity (Table 1, Fig.2).

The relationship between structure of PhP and their selectivity to NTE was analyzed

with the multiple regression analysis. QSAR models were developed. The dependence of selectivity PhP to NTE on hydrophobicity of alkyl radicals (Fig.2) was found to be described with the equation:

$$\log [k_i(\text{NTE})/k_i(\text{AChE})] = (-1.89 \pm 0.17) + (3.22 \pm 0.25)\,\Sigma\pi - (0.65 \pm 0.08)\,(\Sigma\pi)^2 \quad (1)$$

(n=7, r=0.997, s=0.086, $F_{2,4}$ =326.95) $P < 0.001$

High values of the ratio k_i(NTE)/k_i(AChE) (Table 1) suggest that the studied PhP would have the potential to cause OPIDN at doses above (R = Me) or lower (R = Et – Pent) than LD_{50}.

To assess the validity of the obtained *in vitro* data on OP neuropathic potential prediction two series of experiments *in vivo* were carried out. Taking into account that activities of brain NTE and AChE are biomarkers for the respective toxic effects of OPs, OPIDN and acute cholinergic toxicity [9, 21], inhibition of NTE and AChE in a hen brain was studied in 24 hrs after acute i.m. administration of increasing doses of Me and Bu derivatives. These two PhP were chosen for studies *in vivo* because according to the data obtained *in vitro* (Table 1) they essentially differ in magnitude of neuropathic potential: BuPhP (ratio k_i(NTE)/k_i(AChE) = 104.8) far exceeds Me derivative (ratio k_i(NTE)/k_i(AChE) = 0.4) in OPIDN hazard.

Data obtained in both series of *in vivo* experiments are presented in Table 2.

Inhibition of both NTE and AChE in every series of experiments was dose-dependent (Fig.3). Inhibition of NTE to 70% that is known to be critical for OPIDN initiation [2,6,8] was achieved by Me-analogue in dose 35 mg/kg and by Bu-analogue - in dose 4 mg/kg.

Dose-dependence curves for NTE and AChE inhibition by MePhP were relatively similar, whereas the detectable AChE inhibition by Bu derivative was seen at considerably more high doses than NTE inhibition (Fig. 3), as is consistent with the results obtained *in vitro* (Table 1, Fig.2) and indicates to the potential delayed neurotoxicity of BuPhP in doses much lower than LD_{50}.

By analyzing dose-response curves (Fig.3) we assessed ED_{50} values - median effective doses for inhibition of NTE and AChE in hen brain by Me and Bu derivatives: for MePhP ED_{50}(NTE) = 15.06±5.13 mg/kg, ED_{50}(AChE) = 12.91±0.91 mg/kg; for BuPhP ED_{50}(NTE) = 2.53±1.16 mg/kg, ED_{50}(AChE) = 55.82±39.5 mg/kg.

To characterize neuropathic safety of OP compounds an index based on the *in vivo* susceptibility of the relevant targets, NTE and AChE, to OP inhibitors has been supposed by Richardson [9]. This index was called the neuropathy target index (NTI):

NTI = ED_{50}(NTE) / ED_{50}(AChE).

Because we analyzed the *in vitro* data as ratios k_i(NTE)/k_i(AChE), according to Johnson [6], we consider in our study the reverse values: 1/NTI = ED_{50}(AChE) / ED_{50}(NTE) – the larger is this value, the more hazardous is the OP compound with respect to its ability to produce OPIDN. The obtained ratio ED_{50}(AChE) / ED_{50}(NTE) for MePhP is equal to 0.86 and for BuPhP is equal 22.1 that shows Bu derivative to be much more neuropathic than Me-analogue. The results, which reflect *in vivo* susceptibility of the target enzymes, NTE and AChE, agree with those obtained *in vitro* (Table 1) and provide support for *in vitro* approach to OPIDN prediction.

Use for *in vitro* testing a stable lyophilized preparation of hen brain NTE allows essentially to lower the charge of experimental animals (hens), reduces the price and accelerates and standardizes a biochemical estimation of neuropathic potential of OPs. The obtained with the lyophilized NTE data on anti-NTE activity and selectivity of OPs are homogeneous that makes possible creation of valid and predictive QSAR models [22,23].

The study was supported by ISTC (Project #1055.2) and RFBR (Projects #98-04-48831 and #00-04-48797).

SUMMARY

The inhibition of the two target esterases acetylcholinesterase (AChE) or neurotathy target esterase (NTE) by organophosphorus compounds (OPs) is followed by distinct neurological consequences in exposed subjects - acute cholinergic toxicity or OP induced delayed neurotoxicity (OPIDN). Ability of some OPs along with acute cholinergic toxicity initiate OPIDN may to be of interest for different terrorist groups. The relative potency of an OP to react with NTE or with AChE *in vitro* suggested to be predictive for its capability to produce OPIDN. The kinetics of NTE and AChE inhibition by phenyl phosphonates $(RO)C_6H_5P(O)ON{=}CClCH_3$ (PhP, R = Me, Et, iPr, Pr, iBu, Bu, Pent) was studied with stable lyophilyzed preparations of hen brain NTE and AChE, neuropathic potential of PhP was assessed using ratios k_i(NTE)/k_i(AChE) and compared with ED_{50} ratios obtained *in vivo*. Most of PhP was more potent inhibitors of NTE than AChE. Both antiNTE activity, selectivity for NTE and, correspondingly, the propensity of PhP to cause OPIDN raised with the increasing hydrophobicity. High values of the ratio k_i(NTE)/k_i(AChE): 0.6(Me), 3.6(Et), 15(iPr), 36(Pr), 69(iBu), 104(Bu), 124(Pent) suggest that the studied PhP would have the potential to cause OPIDN at doses above or lower than LD_{50}. Inhibition of NTE and AChE in hen brain was studied in 24 hrs after acute i.m. hens injecting with increasing doses of methyl and buthyl derivatives. Inhibitions of both NTE and AChE were dose-dependent. By analyzing dose-response curves ED_{50} values - median effective doses for inhibition of NTE and AChE in hen brain by Me and Bu derivatives were assessed and ratios ED_{50}(AChE)/ED_{50}(NTE) were calculated: for MePhP ED_{50}(NTE) = 15.06±5.13 mg/kg, ED_{50}(AChE) = 12.91±0.91 mg/kg, ratio = 0.86; for BuPhP ED_{50}(NTE) = 2.53±1.16 mg/kg, ED_{50}(AChE) = 55.82±39.5 mg/kg, ratio = 22.1 that shows Bu derivative to be much more neuropathic than Me-analogue. The results, which reflect *in vivo* susceptibility of the target enzymes, NTE and AChE, agree with those obtained *in vitro* and provide support for *in vitro* approach to OPIDN prediction.

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KEYWORDS:
Neurotathy target esterase, acetylcholinesterase, organophosphates, OPIDN

FIGURES AND TABLES

Fig.1. The structure of O-alkyl-O-methylchlorformiminophenyl phosphonates (PhP).
R = alkyl group as listed in Table 1.

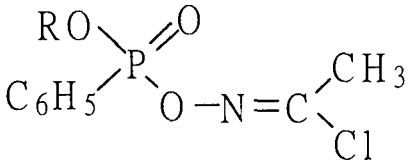

Fig. 2. The dependence of logarithm of inhibitory activity of PhP against NTE and AChE and the dependence of logarithm of selectivity PhP to NTE on hydrophobicity of alkyl radicals ($\Sigma\pi$). Data were derived from Table 1. The continuous line for NTE selectivity is calculated by equation 1.

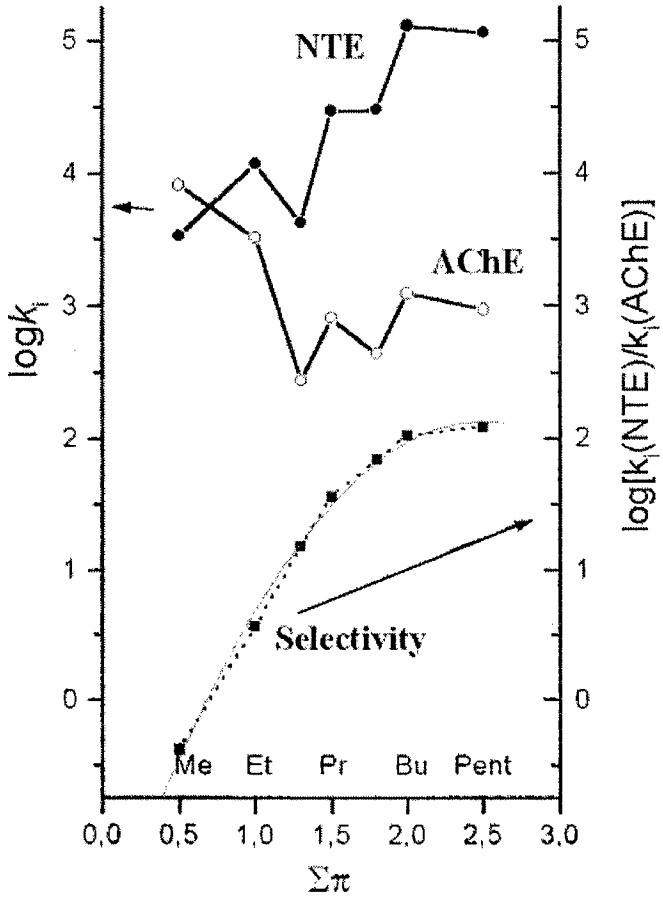

Fig.3. Dose-related NTE and AChE inhibition in hens given methyl (MePhP) and buthyl (BuPhP) derivatives of the studied phenylphosphonates. Results are expressed as means ± SEM, n=3.

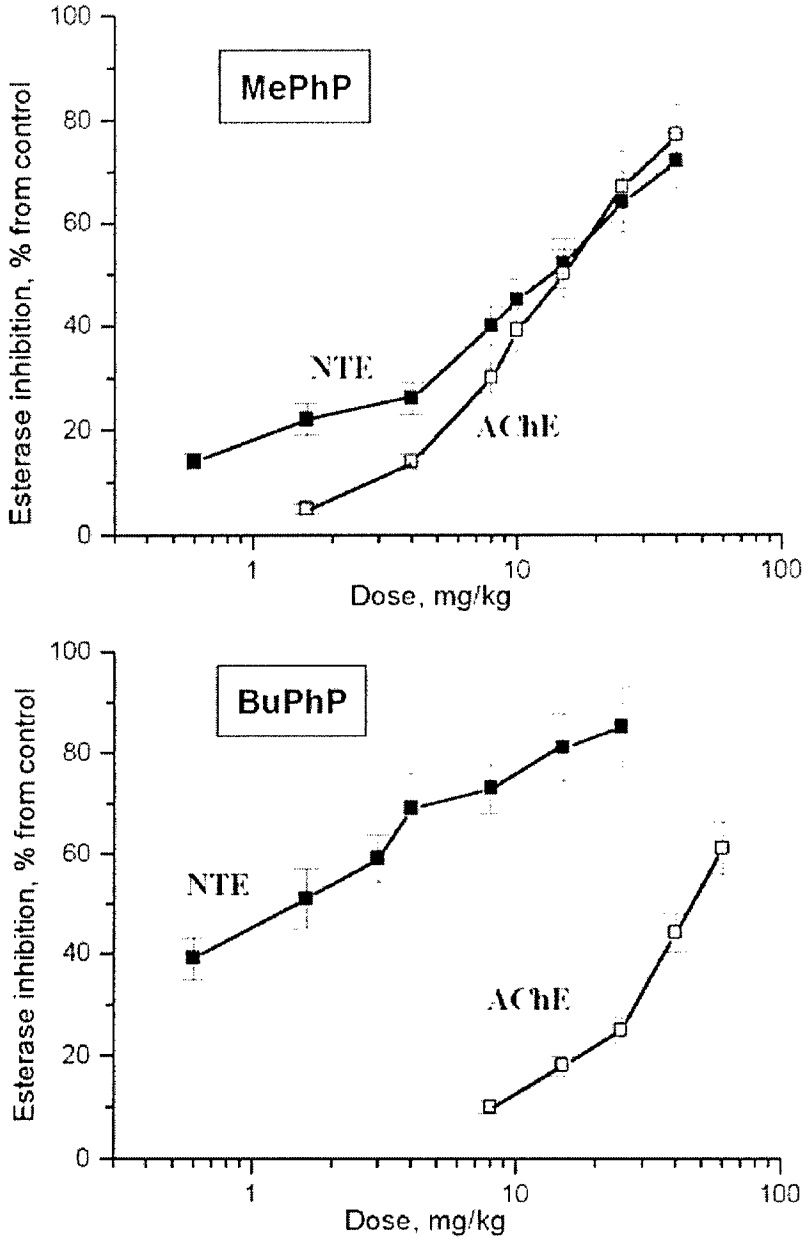

Table 1. Bimolecular rate constants of inhibition (k_i) of NTE and AChE by phenyl phosphonates (PhP), $(RO)C_6H_5P(O)ON{=}CClCH_3$, Hansch's hydrophobicity constants of alkyl substituents ($\pi CH_2 = 0.5$) and relative inhibitor potency of PhP against two OP primary targets - NTE and AChE

R	CH_3	C_2H_5	iC_3H_7	C_3H_7	iC_4H_9	C_4H_9	C_5H_{11}
π	0,5	1	1,3	1,5	1,8	2	2,5
$\log k_i$(NTE)	3,53	4,07	3,62	4,47	4,48	5,11	5,06
$\log k_i$(AChE)	3,91	3,51	2,44	2,91	2,64	3,09	2,97
k_i(NTE)/k_i(AChE)	0,4	3,6	15,1	36,3	69,1	104,8	123,8

Table 2. Neuropathy target esterase and acethylcholinesterase inhibition (% from control) in brains of hens dosed with MePhP and BuPhP.

Dose, mg/kg	MePhP		BuPhP	
	Brain NTE inhibition, % [a]	Brain AChE inhibition, % [a]	Brain NTE inhibition, % [a]	Brain AChE inhibition, % [a]
0,6	14,1±1,6	n/d	39,0±4,2	n/d [b]
1,6	22,2±3,1	5,1±0,9	51,1±6,0	n/d
3	-	-	58,9±4,8	n/d
4	25,9±2,9	14,0±1,5	69,0±7,0	n/d
8	40,0±3,9	29,8±2,9	73,2±4,9	10,0±1.2
10	44,8±4,1	39,1±4,0	-	-
15	52,1±4,8	49,9±4,7	81,1±6,6	17,8±1,9
25	64,1±5,8	67,0±7,1	85,0±7,9	25,1±2,5
40	72,0±5,4	77,1±5,9	n/d	44,0±3,9
60	-	-	n/d	61,0±5,3

a) Esterase inhibition in brain presented as the mean ± SEM (n=3). Control activities (100%): hen brain NTE = 30.9 ± 2.8 nmol PV/min/mg protein, Hen brain AChE = 215 ± 17 nmol ATCh/min/mg protein;
b) n/d – activity has not been determined