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Comparison of the oxime-induced reactivation of erythrocyte and muscle acetylcholinesterase following inhibition by sarin or paraoxon, using a perfusion model for the real-time determination of membrane-bound acetylcholinesterase activity

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ABSTRACT

The purpose of these experiments was to compare oxime-induced reactivation rate constants of acetylcholinesterase from different human tissue sources inhibited by organophosphorus compounds. To this end, preliminary testing was necessary to generate a stable system both for working with erythrocytes and musculature. We established a dynamically working *in vitro* model with a fixed enzyme source in a bioreactor that was perfused with acetylthiocholine, Ellman's reagent and any agent of interest (e.g. nerve agents, oximes) and analyzed in a common HPLC flow-through detector. The enzyme reactor was composed of a particle filter (Millex®-GS, 0.22 μm) containing a thin layer of membrane-bound acetylcholinesterase and was kept at constant temperature in a water bath. At constant flow the height of absorbance was directly proportional to the enzyme activity. To start with, we applied this system to human red cell membranes and then adapted the system to acetylcholinesterase of muscle tissue. Homogenate (Ultra-Turrax® and Potter–Elvehjem homogenizer) of human muscle tissue (intercostal musculature) was applied to the same particle filter and perfused in a slightly modified way, as done with human red cell membranes. We detected no decrease of acetylcholinesterase activity within 2.5 h and we reproducibly determined reactivation rate constants for reactivation with obidoxime (10 μM) or HI 6 (30 μM) of sarin-inhibited human muscle acetylcholinesterase ($0.142 \pm 0.004\ min^{-1}$ and $0.166 \pm 0.008\ min^{-1}$, respectively). The reactivation rate constants of erythrocyte and muscular acetylcholinesterase differed only slightly, highlighting erythrocyte acetylcholinesterase as a proper surrogate marker.

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Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); AU, absorbance units; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HI 6, 1-[[[4-(aminocarbonyl)-pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride; Obi (obidoxime), 1,1′-(oxybis-methylene)-bis[4-(hydroxyimino)methyl] pyridinium dichloride; Sarin, isopropyl methylphosphonofluoridate.

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1. Introduction

The acute toxicity of organophosphorus compounds (OP), i.e. pesticides and nerve agents, in mammals is primarily due to their irreversible binding to the esteratic site of acetylcholinesterase (AChE) in the nervous system and a subsequent accumulation of acetylcholine, leading to cholinergic crisis. Standard treatment of OP poisoning includes a combination of an antimuscarinic agent (e.g. atropine) and a cholinesterase reactivator (oxime; e.g. obidoxime or pralidoxime). Oximes free the inhibited enzyme from the organophosphorus compound and thus restore the enzyme function. However, AChE inhibited by different OP, e.g. soman or tabun, is rather resistant towards reactivation by oximes [1]. Therefore, numerous oximes have been prepared and tested for their efficacy in OP poisoning in the past decades. For ethical reasons the antidotal efficacy of oximes against nerve agent poisoning cannot be investigated in humans. Hence, the assessment of oxime efficacy is essentially based on data from animal experiments which are mainly done with rodents [2]. Several studies indicate substantial species differences in the ability of oximes to reactivate OP-inhibited AChE [3–5]. Structural and functional differences between animal and human AChE may result in a different affinity and reactivity of oximes. Therefore, data from animal experiments and exact analysis of species differences are crucial for the assessment of oxime efficacy in humans. Erythrocyte AChE is often used as enzyme source since this enzyme form is believed to be an adequate surrogate parameter for neuromuscular AChE [6] and an appropriate marker for diagnosis and monitoring [7]. AChE is encoded by a single gene in mammals, although a wide variety of molecular forms exists. The molecular diversity is believed to reflect post-transcriptional mechanisms and post-translational modifications of the membrane-anchoring tail rather than the catalytic unit. Therefore, it is generally assumed that the active site of mammalian AChE is identical in all tissues at all ages [8]. From this, reaction rate constants are expected to be similar with erythrocyte and muscle AChE within the same species.

Recently, we introduced a perfusion model [9] to examine inhibition and reactivation kinetics of erythrocyte AChE. This model was created to overcome specific drawbacks of the conventional static methods of AChE determination: (i) many inhibitors rapidly liberate active enzyme when the enzyme source is diluted in the analytic system. This occurs notoriously with carbamates and is even more annoying with the true reversible inhibitors such as used in Alzheimer disease. (ii) In the presence of AChE, OP and an oxime, highly reactive inhibitors such as phosphyloximes may accumulate which disturb the proper assessment of reaction kinetics. This problem can be partly overcome by working with highly diluted enzyme preparations. However, (iii) it was our goal to imitate the *in vivo* situation as close as possible for reliable prediction of the course of poisoning and the effect of therapeutic oximes. To this end we intended to change the concentrations of the reactants according to *in vivo* pharmacokinetics and to follow the AChE activity in real-time. We already succeeded in applying this model to the carbamate prophylaxis in soman-poisoned erythrocyte AChE [11]. Now we attempted to adapt the perfusion model from human erythrocyte to human muscle tissue AChE for real-time determination of membrane-bound AChE activity. Stable enzyme reactors with suspensions of homogenized intercostal muscle tissue had to be created and tested for long-term stability. Next, reactivation rates of paraoxon- and sarin-inhibited erythrocyte and muscle AChE were determined with HI 6 and obidoxime in order to investigate the potential similarity between both enzyme sources.

2. Materials and methods

Acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (Deisenhofen, Germany) and obidoxime dichloride from Duphar (Amsterdam, Holland). HI 6 dichloride monohydrate was provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada). Paraoxon-ethyl was from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and freed from disturbing *p*-nitrophenol as described in detail elsewhere [10].

Sarin (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR) was made available by the German Ministry of Defence (Bonn, Germany).

All other chemicals were from Merck Eurolab GmbH (Darmstadt, Germany) at the purest grade available. Particle filters employed were Millex®-GS, 0.22 μm (Millipore; Eschborn, Germany).

Sarin stock solutions (0.1%, v/v) were prepared in dimethylsulfoxide, stored at 20 °C and appropriately diluted in distilled water just before the experiment. Oximes (200 mM) were prepared in distilled water, stored at −60 °C and diluted as required in phosphate buffer on the day of the experiment. All solutions were kept on ice until the experiment.

Human intercostal muscle strips (approximately 3 cm × 1 cm × 0.5 cm) were used from excised tissue of three patients undergoing cancer surgery (oesophagus carcinoma). The tissue was stored frozen at −60 °C (up to 1 month) until the preparation of muscle homogenates. The study protocol was approved by the Ethics Committee of the Medical Faculty of the Technical University Munich. Written informed consent was obtained from the patients.

2.1. General experimental procedure

Experimental procedure was performed using the recently described dynamic perfusion model [9,11]. In short, human erythrocytes or human muscle homogenates were layered onto a particle filter (Millex®-GS, 0.22 μm, ∅33 mm) resulting in a stable enzyme reactor. Control AChE activity was determined by continuously perfusing the enzyme reactor with the medium, consisting of acetylthiocholine (0.45 mM), DTNB (Ellman's reagent, 0.3 mM), and phosphate buffer (0.1 M, pH 7.4). The reactor was immersed into a water-bath with the temperature set to 37 °C. The total flow rate through the enzyme reactor was 0.5 mL min^{-1}. The effluent passed a photometer set at 470 nm with the digitized absorbance values being collected at 1.6 s intervals. The perfusion system consisted of two HPLC pumps with integrated quaternary low-pressure gradient formers that were programmed by a computer using commercial HPLC software.

2.2. Preparation of the enzyme reactor

2.2.1. Human erythrocytes

Erythrocytes were prepared from freshly drawn heparinized whole blood following centrifugation at 3000 rpm for 10 min. Plasma was discarded by suction and the cells were washed five times with a threefold volume of 0.1 M phosphate buffer. The final sediment was re-suspended in the phosphate buffer to give a hemoglobin concentration of 50 g/L and kept in ice water in the cold room. Hemoglobin content was determined as cyanomethemoglobin [12]. Of this preparation, 80 μL were diluted to 5 mL with phosphate buffer of which 3.2 mL were slowly layered onto the Millex filter within 10 min by means of a peristaltic pump.

The enzyme reactor was inserted at $t = 0$ min and flushed with buffer containing 0.2% gelatin (w/v; for experiments with erythrocytes only) for 5 min, followed by a pulse of distilled water ($t = 5$) for 5 min and further flushing with gelatin buffer ($t = 10$) for 5 min. At $t = 15$ DTNB and acetylthiocholine were added for the determination of the baseline enzyme activity ($t = 30$).

2.2.2. Human muscle tissue

Human intercostal muscle (about 1 g) was weighed into a 15 mL glass test-tube, mixed with 3 mL phosphate buffer (0.1 M; pH 7.4) and minced with an Ultra-Turrax®-homogenizer four times for 10 s each on ice. This suspension was transferred into a 5 mL Potter test-tube for further processing with a Potter–Elvehjem homogenizer. The suspension was homogenized six times for 10 s each at 1100 rpm and diluted with further 17 mL of phosphate buffer in the ice-bath. The resulting muscle homogenate was frozen in aliquots at −60 °C until further use. Of this preparation, 150 μL were diluted to 5 mL with phosphate buffer of which 4.5 mL were slowly layered onto the Millex filter within 14 min by means of a peristaltic pump.

The dynamic model's experimental procedure for erythrocytes was slightly changed adapting the conditions to muscle homogenate as enzyme source. The phosphate buffer used for flushing the muscle enzyme reactor contained no gelatin, which was indispensable for enzyme stabilization of red cells.

The enzyme reactor was inserted at $t = 0$ and flushed with buffer for 2 min. In order to saturate muscle tissue sulfhydryls, DTNB was added for 60 min before acetylthiocholine was added at $t = 60$ for the determination of the maximum enzyme activity ($t = 75$).

2.3. Perfusion protocol for inhibition of acetylcholinesterase and reactivation with oximes

A working solution of sarin (300 nM) in distilled water or paraoxon-ethyl (4.4 μM) in phosphate buffer passed through the system at 0.05 mL min^{-1} and 0.113 mL min^{-1}, respectively.

2.3.1. Human erythrocyte AChE

Starting at baseline enzyme activity ($t = 30$), erythrocyte AChE was inhibited with 30 nM sarin and 1 μM paraoxon-ethyl for 60 min and 30 min, respectively, followed by a 10 min washout. For reactivation 44.4 μM obidoxime or 133.2 μM HI 6 were added to the perfusion medium at a flow of 0.113 mL min^{-1}, resulting in end concentrations of 10 μM obidoxime or 30 μM HI 6. At $t = 160$ the oxime was discontinued and at $t = 180$ the enzyme reactor was replaced by a plain filter without enzyme source to determine the blank value of the complete perfusion medium (acetylthiocholine, DTNB and gelatin buffer) and of buffer only.

2.3.2. Human muscle AChE

Starting at baseline enzyme activity ($t = 75$), muscle AChE was inhibited with 30 nM sarin for 60 min or 1 μM paraoxon-ethyl for 30 min followed by a 10 min washout. For reactivation 44.4 μM obidoxime or 133.2 μM HI 6 were added to the perfusion medium at a flow of 0.113 mL min^{-1}, resulting in end concentrations of 10 μM obidoxime or 30 μM HI 6. At $t = 210$ the oxime was discontinued and at $t = 230$ the enzyme reactor was replaced by a plain filter without enzyme source to determine the blank value of the complete perfusion medium (acetylthiocholine, DTNB and buffer) and of buffer only.

2.4. Calculations

Processing of experimental data was recently described [9]. Briefly, absorbance data collected at 1.6 s intervals were analyzed by curve fitting programs using Prism™ Vers. 3.0 (GraphPad Software, San Diego, CA). The time-dependent reactivation of inhibited AChE by obidoxime and HI 6 was calculated by using Eq. (1).

$$A_{(t)} = A_{(0)}(1 - e^{-kt}) \quad (1)$$

First-order kinetics were assumed when the goodness of fit exceeded $R^2 = 0.995$.

Means are presented along with standard deviation (S.D.) and the number of experiments. Differences in the half-time of reactivation of inhibited erythrocyte and muscle AChE were analyzed for significance with the two-tailed Mann–Whitney U-test with Prism™ Vers. 3.0 Software. A $p < 0.05$ was considered to be significant.

3. Results

Human erythrocyte and muscle AChE were inhibited with 30 nM sarin for 60 min leading to a completely inhibited enzyme in all the experiments, with a residual activity <0.1%. After a 10 min washout of the nerve agent, reactivation was started by administration of 10 μM obidoxime or 30 μM HI 6. Reactivation was followed until activity reached steady-state conditions, with optical changes <0.5 mAU/min. The reaction curves were analyzed, applying a mono-exponential association function to the data points and starting analysis 8 min after addition of the oximes.

As an example Fig. 1 shows the time course of AChE activity both for human erythrocyte and muscle AChE upon inhibition with 30 nM sarin for 60 min and reactivation with 10 μM obidoxime. The x-axis values of the erythrocyte experiment were transformed ($x + 50$) to shift them to the x-values of the muscle experiment, due to the different time programs of perfusion.

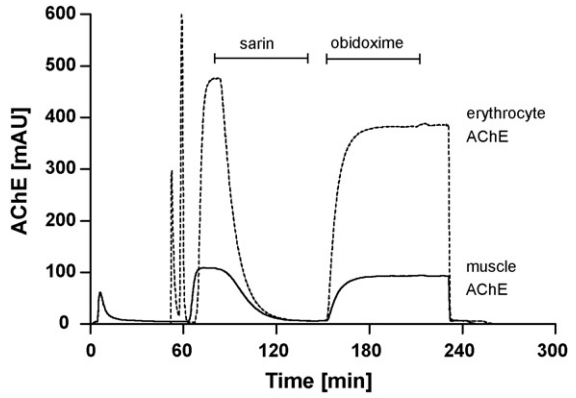

Fig. 1 – Time course of human AChE activity following inhibition with 30 nM sarin and reactivation with 10 μM obidoxime. Solid line: the human muscle enzyme reactor was flushed with 0.3 mM DTNB for 60 min before 0.45 mM acetylthiocholine was added. After reaching maximum activity 30 nM sarin was applied for 60 min. After a 10 min washout, reactivation was started with 10 μM obidoxime (*t* = 150). Hatched line: the human erythrocyte enzyme reactor was flushed with gelatin buffer, distilled water (for hemolysis of erythrocytes) and gelatin buffer for 5 min each before 0.3 mM DTNB and 0.45 mM acetylthiocholine were added for determination of maximum activity. Inhibition and reactivation followed as described above. The *x*-axis of this graph was transformed by *x* + 50 to align values with the muscle experiment for comparison. (The first sharp peak results from insertion of the red cells containing filter and flushing with gelatin buffer at *t* = 50 min, the second sharp peak stems from a distilled water pulse to ensure complete hemolysis; for details see [9].) Blank values of the perfusion medium (DTNB, acetylthiocholine, buffer) were determined by removing the enzyme reactor and replacing it with a filter without enzyme source.

The observed rate constants of reactivation for sarin-inhibited human AChE are given in Table 1.

Similar experiments were repeated for obidoxime-induced reactivation of human erythrocyte and muscle AChE inhibited by 1 μM paraoxon. After analysis of the individual curves, observed rates were calculated and are given in Table 2.

The statistical analysis of the reactivation rate constants revealed a significant difference between erythrocyte and muscle AChE in all cases.

Table 1 – Rates of reactivation (k_{obs}) of sarin-inhibited human AChE with oximes

AChE	k_{obs} (min^{-1})	
	Obidoxime (10 μM)	HI 6 (30 μM)
RBC	0.171 ± 0.009	0.211 ± 0.008
Muscle	0.142 ± 0.004	0.166 ± 0.008

The data are means of 6 experiments and given ±S.D. Substrate concentration, 0.45 mM. The goodness of fit of the individual curves was $R^2 > 0.995$.

Table 2 – Rates of reactivation (k_{obs}) of paraoxon-inhibited human AChE with obidoxime

AChE	k_{obs} (min^{-1})
RBC	0.182 ± 0.014
Muscle	0.114 ± 0.003

The data are means of six experiments and given ±S.D. Substrate concentration, 0.45 mM. The goodness of fit of the individual curves was $R^2 > 0.995$.

4. Discussion

4.1. *Preparation of a stable muscle enzyme reactor*

It was an intriguing question, whether it would be possible to adapt the well-established dynamic model [9,11,13] from human erythrocyte AChE to AChE from human intercostal muscle homogenates.

Preparing stable enzyme reactors with muscle homogenates turned out to be a tricky task, as the homogenized solutions contained many visible muscle fibres and there was a great tendency of blocking the filter membrane. A good balance between the degree of dilution of the applied homogenate and the measurable level of AChE activity had to be found. The continuous progress of blocking a filter could easily be identified by a continuous increase in pressure in worst cases leading to a burst of the filter housing.

Finally, the optimized human muscle enzyme reactor contained about 6.5 mg of muscle (wet weight per filter) resulting in a maximum AChE activity of ca. 120 mAU.

The stability of AChE in the enzyme reactor during the experiment was measured over the relevant time interval of about 2.5 h after addition of substrate and no decrease in activity was detected.

With erythrocytes, resulting in a maximum AChE activity of approximately 500 mAU, gelatin was added to the phosphate buffer solution in order to stabilize enzyme activity. This was no longer necessary when using muscle homogenate as the long-time stability could not be increased and use of gelatin further accelerated the tendency of blocking the filter membrane causing pressure problems.

Whereas the general procedure in working with the erythrocyte reactor required a short pulse of distilled water to ensure complete hemolysis of the red blood cells this pulse could be omitted when working with muscle material. Upon addition of DTNB and acetylthiocholine for determination of maximum AChE activity there was a problem of an overshooting 'activity' before reaching rather slowly steady state conditions. To separate the reaction of free SH-groups in the muscle homogenate from the actual AChE reaction we changed the routine to flushing the reactor with DTNB alone before adding acetylthiocholine. This resulted in masking the free SH-groups in the muscle homogenates. After acetylthiocholine was added the maximum activity was reached without retardation.

To be sure that the assay only registered AChE activity and not butyrylcholinesterase (BChE) activity in the homogenates we added 20 μM of ethopropazine, a selective inhibitor of BChE, to the reaction mixture after reaching maximum

enzyme activity. There was a very slight decrease in activity but that was way below 3% of the total activity level. Hence, it can be assumed that the soluble BChE was washed out and did not contribute to the hydrolysis of substrate.

4.2. *Inhibition of muscle AChE with sarin*

Since sarin is unstable in buffer it was dissolved in distilled water, resulting in different densities of the various solvents which were more prone to lateral diffusion and hence contamination of the neighbouring solvent lines. To avoid small amounts of sarin contaminating the dynamic system after purging the individual channels with the solutions, we chose to purge the line of sarin only to an extent where the solution is not yet touching the four-way joint in the pump. The pump was programmed in that way that the sarin channel is flushed into the system starting from $t = 50$ but sarin reaching the enzyme reactor with a delay (about at $t = 80$). According to the pump program flushing took longer but inhibition did not start until $t = 80$ min and 60 min were required until maximum inhibition was achieved. For this reason it was not possible to calculate proper rate constants of inhibition as it had taken an undefined time within the 60 min for reaching the nominal end concentration. This was not a problem since the focus of the study was on the determination of reactivation rates upon administration of oximes to the totally inhibited enzyme. Contamination was no problem when using paraoxon, the line for paraoxon was completely purged before starting an experiment and delayed flushing was not necessary.

4.3. *Reactivation of AChE inhibited by sarin or paraoxon with oximes*

With the same system it was possible to determine reactivation rate constants for both erythrocyte and muscle AChE inhibited by sarin or paraoxon and reactivated by obidoxime or HI 6.

Reactivation with 10 μM obidoxime resulted in reactivation half-times of 4.1 ± 0.2 min ($n = 6$) with sarin-inhibited human erythrocytes and 4.9 ± 0.1 min ($n = 6$) with sarin-inhibited human muscle tissue. For 30 μM HI 6 reactivation half-times determined were 3.3 ± 0.1 min ($n = 6$) and 4.2 ± 0.2 min ($n = 6$) for sarin-inhibited erythrocyte and muscle AChE, respectively.

Worek et al. [5] determined reactivation constants of sarin-inhibited erythrocyte AChE in a static system. From these values observed half-times of 3.1 min and 2.7 min can be calculated for 10 μM obidoxime and 30 μM HI 6.

The results are in good agreement with each other regarding the two different applied methods –dynamic perfusion system and static cuvette system –of determination.

Reactivation with 30 μM HI 6 is superior to 10 μM obidoxime by factors of 1.21, 1.17, and 1.15 for human erythrocytes, human muscle tissue in the dynamic system and for human erythrocytes in the static system [5].

Inspection of Fig. 1 clearly indicates that reactivation of erythrocyte and muscle AChE following sarin inhibition was incomplete. This is to be expected given that the ageing half-life of sarin-inhibited AChE is approximately 3.0 h [1]. Hence, inhibition of the enzyme for 1 h on average results in a fraction of 20% aged enzyme resistant to reactivation.

Reactivation of paraoxon-inhibited human muscle tissue with 10 μM obidoxime resulted in reactivation half-times of 6.1 ± 0.1 min ($n = 6$), while human erythrocytes showed 3.8 ± 0.3 min ($n = 6$), values comparable to 4.3 min as reported by Worek et al. [14]. Earlier experiments with the dynamic model gave a half-time of 4.0 ± 0.2 min [9].

The difference of reactivation rate constants of inhibited erythrocyte and muscle AChE was significant in all cases. However, the practical relevance of this difference is minimal. By using the determined reactivation rate constants the time-dependent increase in AChE activity was calculated. As shown in Fig. 2 the increase in enzyme activity was comparably fast for both enzyme sources.

The perfusion model for the investigation of dynamic changes of erythrocyte AChE activity was modified successfully to investigate human intercostal muscle AChE. For the first time, we are able to show that human erythrocyte AChE and human muscle AChE are reactivated with oximes at a similar rate following inhibition by the nerve agent sarin or the pesticide paraoxon. Hence, these data support the assumption that erythrocyte AChE may serve as a proper surrogate for muscle AChE in OP poisoning. Furthermore, we think that this model is very versatile, offers much flexibility and operates with high precision and we are optimistic that this system is also applicable to erythrocytes and muscle tissue of other species. Preliminary experiments with prophylactic pyrido-

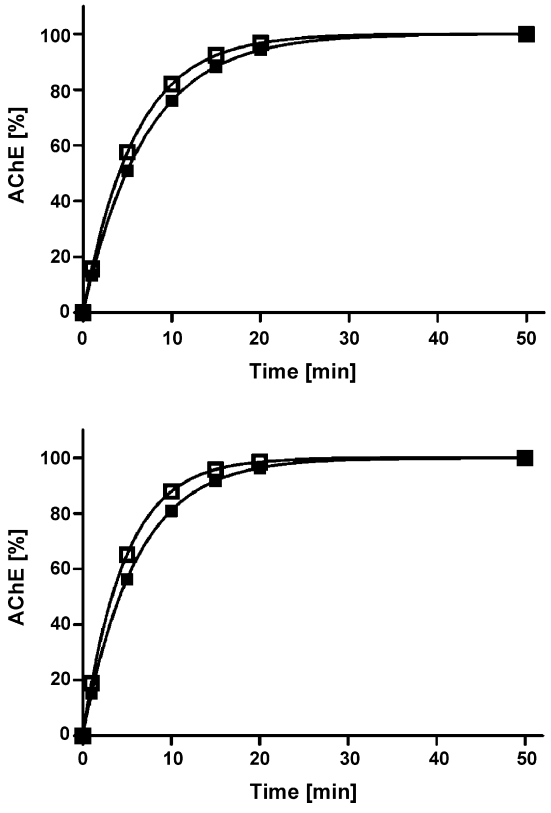

Fig. 2 – Calculated time-dependent reactivation of sarin-inhibited AChE by obidoxime (10 μM; top) and HI 6 (30 μM; bottom). AChE activities of muscle (solid squares) and erythrocyte AChE (open squares) were calculated by using the reactivation rate constants (Table 1) and Eq. (1).

stigmine followed by soman showed that the residual AChE activity of muscle homogenate was higher when both inhibitors were simultaneously present compared to the absence of the carbamate (to be published).

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