Mini review

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Determination of acetylcholinesterase activity by the Ellman assay: A versatile tool for *in vitro* research on medical countermeasures against organophosphate poisoning

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ABSTRACT: Inhibition of acetylcholinesterase (AChE) is the main mechanism of action of organophosphorus compounds (OP), and AChE reactivators (oximes) are at present the only causal therapeutic approach. Being the key target of OP toxicity, AChE may serve as a valuable tool for diagnosis of OP exposure as well as for the investigation of the kinetics of interactions between OP and oximes. At present, the rapid, simple, and cheap spectrophotometric Ellman assay is widely used for diagnosis, therapeutic monitoring and *in vitro* kinetic investigations. Application of the assay for investigation of the interactions between AChE, inhibitors, and oximes requires the consideration of potential matrix effects (e.g. hemoglobin), side reactions (e.g. oximolysis of substrate) and other determinants (e.g. pH, temperature). By taking these factors into account, the Ellman assay allows the precise and reproducible determination of kinetic constants as a basis for the understanding of toxic OP effects and for the development of improved therapies against poisoning by OP. In addition, advanced applications of the Ellman assay, for example, in a dynamic *in vitro* model for the real-time activity determination of membrane-bound AChE, enables the proper investigation of relevant tissue, primarily respiratory muscle, and extends the applicability of this method.

Keywords: acetylcholinesterase; Ellman assay; organophosphorus compounds; oximes; enzyme kinetics

Introduction

Inhibition of acetylcholinesterase (AChE; E.C. 3.1.1.7) and butyrylcholinesterase (BChE; E.C. 3.1.1.8) by binding to the active site serine is the main mechanism of action of organophosphorus compounds (OP), a group of chemicals which include insecticidal pesticides and highly toxic nerve agents.[1] The pathophysiological consequences of decreased AChE activity are reduced hydrolysis and accumulation of acetylcholine in the synaptic cleft and subsequent overstimulation of cholinergic receptors. Finally, this may lead to severe disruption of numerous body functions and death by respiratory failure.[2]

The standard treatment of OP poisoning includes an antimuscarinic, primarily atropine, and an AChE reactivator (oxime).[3,4] Hereby, atropine serves as a symptomatic antidote against OP effects exclusively at muscarinic receptors while oximes may act as a causal treatment by reactivation of OP-inhibited AChE. This is of utmost importance for the restoration of neuromuscular transmission, especially at respiratory muscles.[5] However, numerous *in vitro* and *in vivo* studies showed a limited effectiveness of the clinically used oximes obidoxime and pralidoxime (2-PAM) against a variety of OP and in mega-dose OP poisoning.[4,6,7] Therefore, additional research is required to develop new and more effective oximes and to search for alternative therapeutic strategies.

Hence, AChE is the key target for OP toxicity and may serve as a valuable tool for diagnosis of OP exposure[8–10] as well as for the investigation of kinetic interactions between OP and oximes.[11] AChE is widely spread in different tissues and is also attached to erythrocyte membranes at high concentration.[8,12] In humans and in various animal species AChE is encoded by a single gene although multiple different molecular forms exist in different tissues.[13]

Data from numerous experimental and clinical studies support the notion that erythrocyte AChE has a high functional similarity with synaptic AChE and may serve as a valuable surrogate parameter for diagnostic and mechanistic investigations.[5,14–17]

A large variety of electrometric, titrimetric, tintometric, radiometric, and colorimetric methods for the determination of AChE activity in different matrixes has been developed in the past six decades.[8,18] At present, the spectrophotometric Ellman assay[19] is mostly used for *in vitro* kinetic investigations,[20] occupational health screening, [10,21,22] laboratory diagnosis of OP exposure, and therapeutic monitoring of OP poisoned patients.[23,24] Scheme 1 shows the principal reactions of the Ellman assay.

The advantages of the rapid, simple and cheap Ellman assay are accompanied by a number of drawbacks which have to be taken into account if used for different applications.[8] This paper will address the potential and limitations of the Ellman assay for kinetic interactions of AChE, OP, and oximes.

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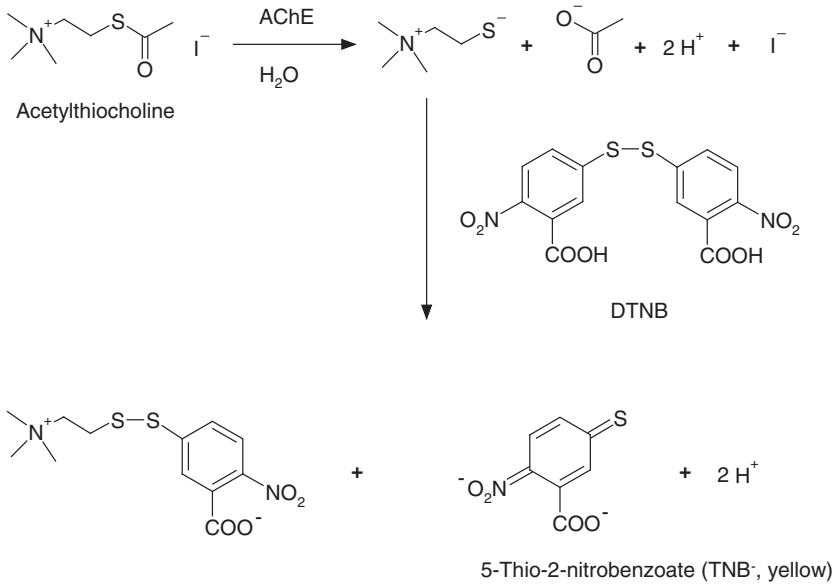
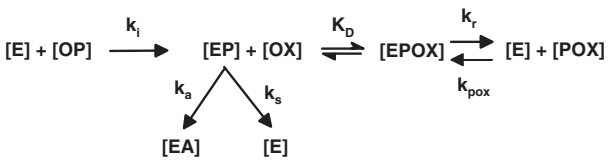


Scheme 1. Principal reactions of the Ellman assay for the determination of AChE activity. The substrate acetylthiocholine is hydrolyzed by AChE and the generated thiocholine reacts with the chromogen DTNB under formation of TNB^- which is monitored at 412 nm. Hereby, the formation of TNB^- is directly proportional to the hydrolysis of acetylthiocholine.

Determination of AChE activity – basic considerations

For ethical reasons, the toxicity of OP pesticides and nerve agents and the therapeutic effect of antidotes have to be investigated in animal models.[25] However, the extrapolation of data from such studies to humans is hampered, in part, by substantial species differences regarding OP toxicokinetics, antidote pharmacokinetics, and AChE kinetics.[26] Hence, the investigation of *in vitro* kinetic interactions between OP, oximes, and AChE from human and animal origin is of utmost importance to provide a basis for the proper evaluation of in vivo animal data (Scheme 2).

For this purpose, isolated electric eel, *Torpedo californica* and recombinant AChE or erythrocytes, erythrocyte membranes ('ghosts') or brain homogenate from different species are commonly used as AChE source.[27–29] The original Ellman assay was designed for use with simple filter or spectrophotometers and a wavelength around 412 nm.[19,30] However, this wavelength does not allow the exact and sensitive determination of AChE activity if erythrocyte lysates are used since the absorption of the indicator TNB^- coincides with the Soret band of hemoglobin (Figure. 1A).[31,32] To overcome this drawback, a wavelength of up to 470 nm can be used which results in a more favorable ratio of TNB^- and hemoglobin absorption (Figure 1B). In fact, the highest TNB^-/hemoglobin ratio was determined at a wavelength of 462.5 nm. However, using longer wavelengths results in a marked decrease of TNB^- absorption, for example, at 462.5 nm only one-third of the maximum absorption is reached (Figure 1C) and requires the use of higher sample concentrations. Alternatively, hemoglobin-free AChE preparations are suitable for kinetic studies. Using such material the interference of hemoglobin can be avoided and the annoying side reaction between the Ellman reagent DTNB and hemoglobin sulfhydryl groups can be reduced.[30]



Scheme 2. Reaction scheme for the kinetic interactions of AChE with OP and oximes. The respective concentrations are denoted [E] the active AChE, [OP] the OP inhibitor, [EP] the phosphylated AChE, [OX] the reactivator, [EPOX] the Michaelis-type phosphyl-AChE-oxime-complex and [POX] the phosphylated oxime. k_i describes the bimolecular inhibition rate constant, k_a the dealkylation (aging) rate constant and k_s the rate constant for the spontaneous reactivation of EP. K_D is equal to the ratio [EP]*[OX]/[EPOX]) and describes the dissociation constant which is inversely proportional to the affinity of the oxime to [EP], and k_r denotes the rate constant for the displacement of the phosphyl residue from [EPOX], indicating the reactivity of the oxime. k_{pox} resembles the inhibition rate constant for the re-inhibition of reactivated AChE by POX.

Further modifications of the Ellman method were directed to the development of high-throughput assays, either by using microplate-based systems or fully automated robot-assisted liquid handling system and such systems were successfully implemented for different applications.[33–35]

Numerous studies investigated the effect of different experimental conditions on AChE activity and identified a number of determinants that have to be considered. Substrate hydrolysis by AChE is pH sensitive, having its optimum at pH 8.0,[36] and is dependent on the buffer composition used during the assay.[37] In addition, AChE activity is moderately affected by the assay temperature being approximately 30% lower at 25 °C compared to 37 °C (Figure 2).

Hence, such factors have to be considered for the determination of reproducible and comparable AChE activities with the Ellman assay.

Ellman assay application: OP inhibition kinetics

The determination of the inhibition kinetics of OP with human and animal AChE enables an initial assessment of the toxic

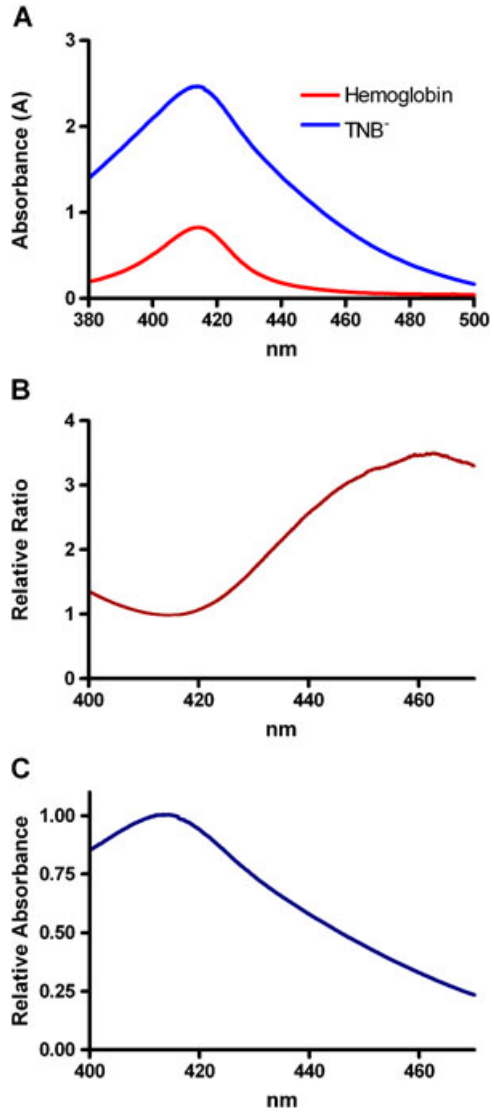


Figure 1. Spectral changes of hemoglobin upon subsequent addition of DTNB and glutathione. Human whole blood hemolysate was diluted 300-fold in phosphate buffer (red line) followed by addition of 300 μM DTNB and 1 mM glutathione (blue line; A). Hemoglobin and TNB^- absorption were normalized to 412 nm and the ratio of TNB^- and hemoglobin absorption was calculated (B) and TNB^- absorption was normalized to 412 nm (C).

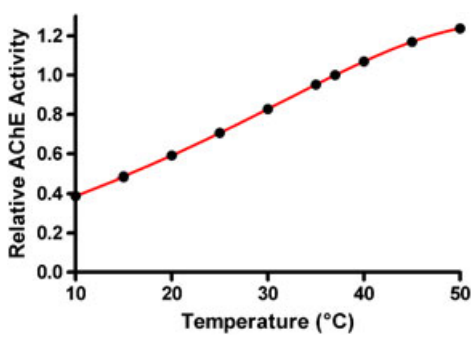


Figure 2. Temperature dependence of human erythrocyte AChE activity determined in whole blood dilutions according to standard procedures [31] with the conversion factor set to unity at 37 °C.

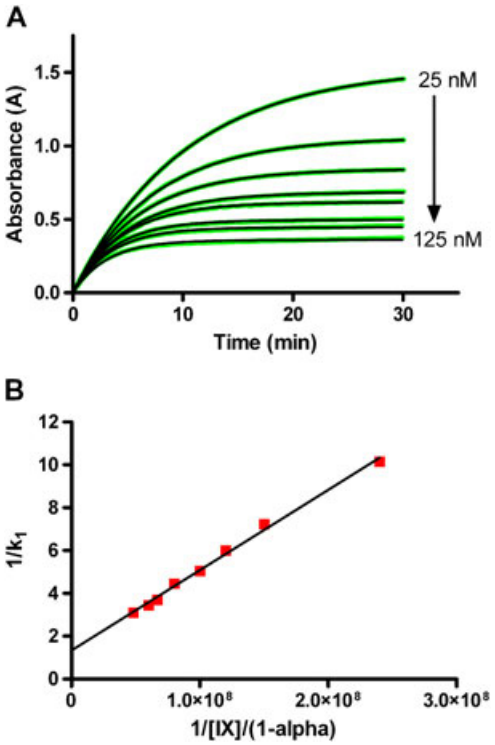


Figure 3. Inhibition kinetics of chlorpyrifos-oxon with human AChE. Enzyme was incubated with phosphate buffer, DTNB, substrate (acetylthiocholine) and OP (in nM final concentration) as indicated, substrate hydrolysis was continuously monitored at 412 nm and the first order rate constants (k_1) were calculated by non-linear regression analysis (A). $1/k_1$ was plotted against 1 / [IX](1- α) (B). See Appendix for details.

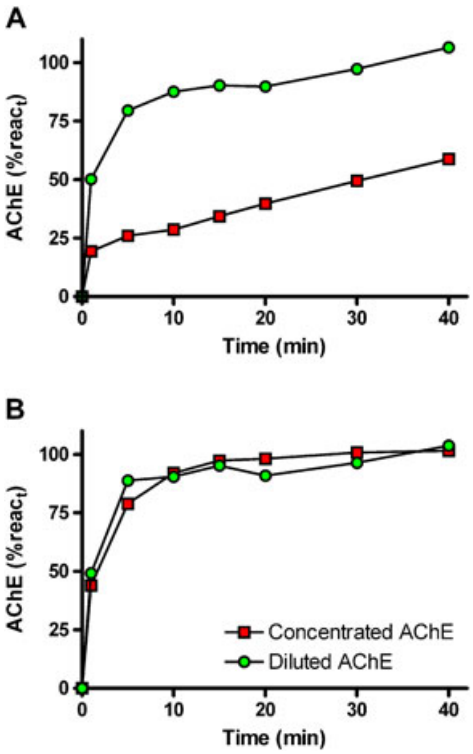


Figure 4. Reactivation of sarin-inhibited human AChE by 100 μM obidoxime (A) or HI-6 (B). The experiments were performed with concentrated AChE (approx. 20 nM active binding sites) or AChE diluted 100-fold with phosphate buffer (AChE activity determined at 436 nm). Data are given as means of four experiments.

potential of an OP and allows a preliminary extrapolation of *in vivo* data from animal studies to humans.[38,39] Various procedures for the determination of the inhibition rate constants have been proposed and were applied with different OP and AChE species.[40–42]

Hart and O'Brien introduced a procedure for the investigation of inhibition kinetics of cholinesterase inhibitors in the presence of substrate.[43] As an example, Figure 3 shows the recording of the inhibition curves in the presence of human AChE and different chlorpyrifos-oxon concentrations and the secondary plot for the calculation of the bimolecular inhibition rate constant k_i. This method was successfully adopted for the use of different OP and carbamates with AChE from different species and turned out to be a versatile tool for the rapid and precise determination of inhibition kinetics with compounds having a wide range of inhibitory potency.[44–46]

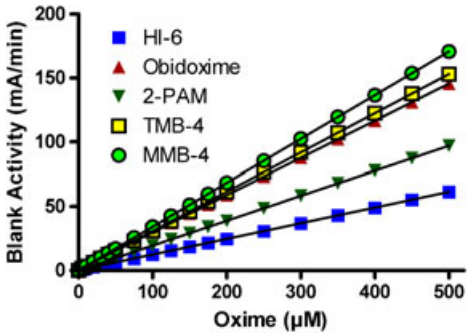


Figure 5. Apparent activity due to oxime-induced substrate hydrolysis ('oximolysis'; blank reaction) by obidoxime, 2-PAM, HI-6, TMB-4 and MMB-4. The blank reaction of the oximes was determined at different concentrations (1–50 μM) at 436 nm and was extrapolated up to 500 μM oxime.

Ellman assay application: Oxime reactivation kinetics

The investigation of the ability of oximes to reactivate OP-inhibited AChE is a major application for kinetic *in vitro* AChE assays.[4] Time-dependent reactivation of OP-inhibited AChE with different oxime concentrations allows the determination of the dissociation constant K_D and the reactivity constant k_r (Scheme 2) and enables the quantification and comparison of the reactivation potential of oximes. [20] The proper determination of the reactivation kinetics of oximes requires the consideration of various interfering side reactions:

1. The reactivation of OP-inhibited AChE by oximes requires an enzyme preparation where free inhibitor was effectively removed. Otherwise, excess inhibitor will re-inhibit reactivated AChE and will lead to falsified reactivation kinetics.
2. According to Scheme 2 OP-inhibited AChE is subject to ongoing aging (i.e. spontaneous dealkylation of the OP moiety resulting in a biologically irreversibly inhibited AChE) and spontaneous reactivation, which may interfere with reactivation by oximes. The rapid aging of soman-inhibited (t½ ~1-2 min[47]) and spontaneous reactivation of dimethyl-OP-inhibited human AChE (t½ ~45 min[48]) complicates the investigation of

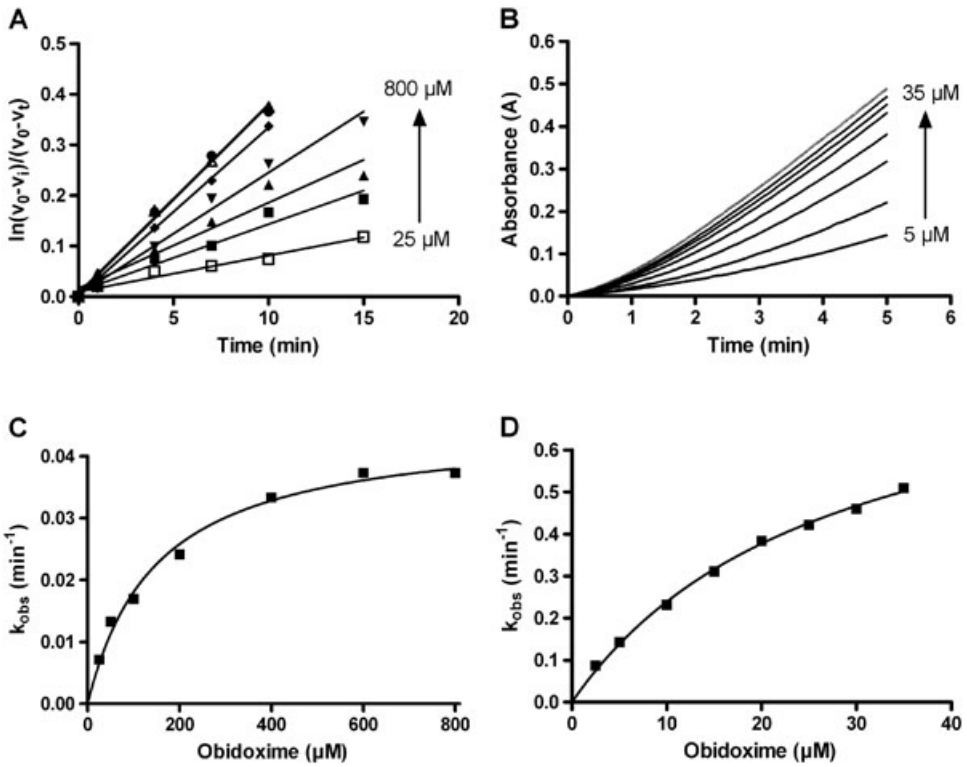


Figure 6. Reactivation kinetics of tabun- (A, C) and VX-inhibited human AChE (B, D) by obidoxime. (A) Semi-logarithmic plot of AChE activities determined discontinuously (436 nm) after incubation of tabun-inhibited AChE with 25–800 μM obidoxime. (B) Continuous recording of absorption change in the Ellman assay (436 nm) after addition of obidoxime (5–35 μM). Secondary plot of k_{obs} vs [obidoxime] for tabun (C) and VX (D). Linear and non-linear regression analysis of the data for the calculation of k_{obs}, K_D and k_r according to Worek *et al.* [20] See Appendix for details.

reactivation kinetics while these reactions are of minor importance with most of the relevant pesticides and nerve agents.[20]

3. The reactivation of OP-inhibited AChE by oximes leads to the inevitable formation of phosphyloximes (POX) as reaction products (Scheme 2[49,50]). Use of oximes with an oxime function at position 3 or 4 at the pyridinium ring results in the formation of stable POX with an exceptionally high inhibitory potency and may lead to a substantial deviation of the reactivation kinetics.[51–53] This bi-molecular reaction is dependent on the concentration of the reaction partners, i.e. OP-inhibited AChE and oximes, and may be minimized even for testing of 4-oximes by adequate dilution of the AChE solution (Figure 4).
4. Oximes are reversible inhibitors of AChE and may lead to a concentration dependent inhibition of AChE if present during enzyme assay.[4,54,55] This effect needs the use of an appropriate experimental setup to minimize the inhibitory effect of oximes.
5. The determination of AChE activity in the presence of oximes results in a concentration dependent reaction with the substrate acetylthiocholine (ATCh; 'oximolysis'[56,57]). The resulting apparent activity is dependent on the structure and concentration of the oxime (Figure 5). The impact on reactivation kinetics can be minimized by reduction of the oxime concentration during assay and subtraction of the 'blank' reaction.

Huge differences in the affinity and reactivity of oximes require the use of adjusted experimental protocols for the optimized determination of reactivation kinetics. In principle, two different procedures were proposed.[58,59] High oxime concentrations are needed during reactivation with oximes having a low affinity to OP-inhibited AChE and removal of the oxime or extensive dilution of the incubate during AChE assay is necessary. Usually, OP-inhibited AChE is incubated with various oxime concentrations

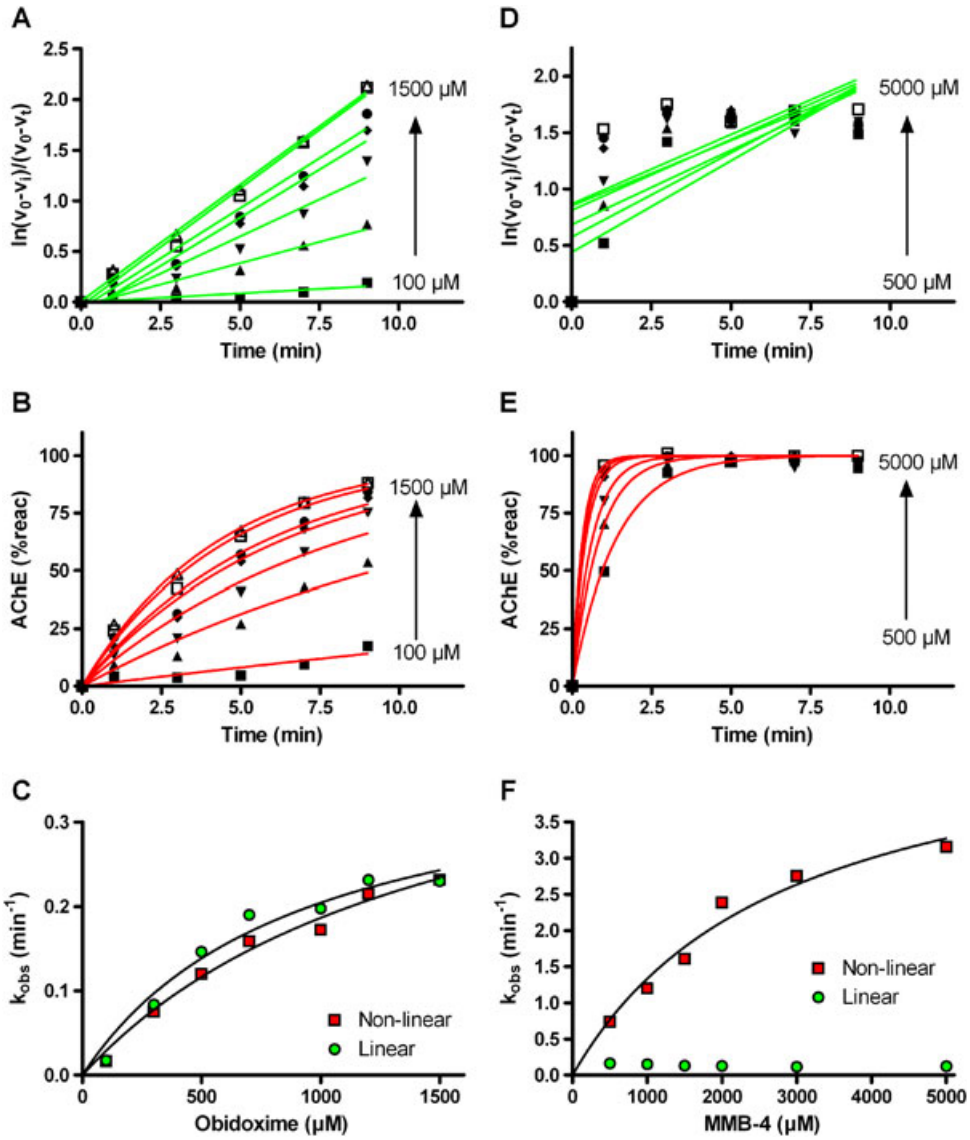


Figure 7. Reactivation kinetics of cyclosarin-inhibited human AChE by obidoxime (A–C) and MMB-4 (D–F). AChE activity was determined discontinuously (436 nm) after incubation of cyclosarin-inhibited AChE with 100–1500 µM obidoxime or 500–5000 µM MMB-4. (A, D) Semi-logarithmic plot of AChE activities and (B, E) linear plot of AChE activities. Secondary plot of k_{obs} vs [oxime] for obidoxime (C) and MMB-4 (F). Linear and non-linear regression analysis of the data for the calculation of k_{obs}, K_D and k_r according to Worek *et al.* [29] See Appendix for details.

and the activity of reactivated AChE is determined discontinuously ('discontinuous procedure').[20] A semi-logarithmic plot of the enzyme activities *vs* time gives the observed first-order reactivation rate constant (k_{obs}) at any given oxime concentration (Figure 6A).

In case of oximes with high reactivity the discontinuous procedure results in a reactivation of OP-inhibited AChE, too rapid for proper handling, and leads to a progressive deviation of the data from a straight line. In order to overcome this problem Kitz *et al*. proposed a 'continuous procedure'.[59] Hereby, the oxime-induced reactivation is followed during the AChE assay and gives concentration-dependent progressive curves, which can be analyzed by non-linear regression analysis with correction of the substrate impact to give k_{obs} (Figure 6B).

With both procedures the dissociation constant K_D and the reactivity constant k_r can be determined by plotting k_{obs} vs oxime concentration followed by non-linear regression analysis (Figures 6C and 6D).[20]

As described, the use of these procedures is dependent on the specific properties of oximes and certain limitations have to be considered. Besides the difficulty of using highly reactive oximes the discontinuous procedure is also inappropriate if stable and reactive POX are formed during the reactivation of inhibited AChE.[26] On the other hand, the maximum oxime concentration is limited with the continuous procedure due to concentration-dependent oxime-induced inhibition and oximolysis.

In order to overcome these shortcomings a modified approach was introduced which allows the investigation of oximes with different affinities and reactivities.[29] Hereby, OP-inhibited AChE can be incubated with high oxime concentrations and the activity is determined in aliquots at different time. The major difference in the discontinuous procedure is the calculation of k_{obs} by non-linear regression analysis, which allows the precise calculation even if oximes with high reactivity and low affinity are investigated. Figure 7 demonstrates the application of the modified approach for the determination of reactivation kinetics of obidoxime and MMB-4 with cyclosarin-inhibited human AChE. With obidoxime, an oxime with a low affinity and reactivity towards cyclosarin-inhibited AChE, both linear (Figure 7A) and non-linear regression analysis (Figure 7B) are suitable and provide similar curves in the secondary plot (Figure 7C) while the high reactivity of MMB-4 leads to a rapid deviation of the data from a straight line (Figure 7D) and does not allow the calculation of k_{obs} by linear regression analysis. In consequence, only a non-linear analysis is suitable in case of MMB-4 (Figures 7E and 7F).

Thus, knowledge and consideration of the underlying mechanisms and determining factors enables the selection of a suitable method and the determination of meaningful kinetic constants.

Ellman assay application: Tissue AChE

Erythrocyte and recombinant AChE are mostly used for investigating kinetic interactions with OP and oximes[4,29] although synaptic AChE is the toxicologically relevant target of such compounds. There are various reasons for using preferentially surrogate AChE in kinetic studies. The availability of human brain and muscle AChE is limited for ethical reasons. More important is the low specific AChE activity in muscle tissue which hampers its use for kinetic investigations[60] and the annoying Tyndall effect if tissue homogenates are used in the photometric Ellman

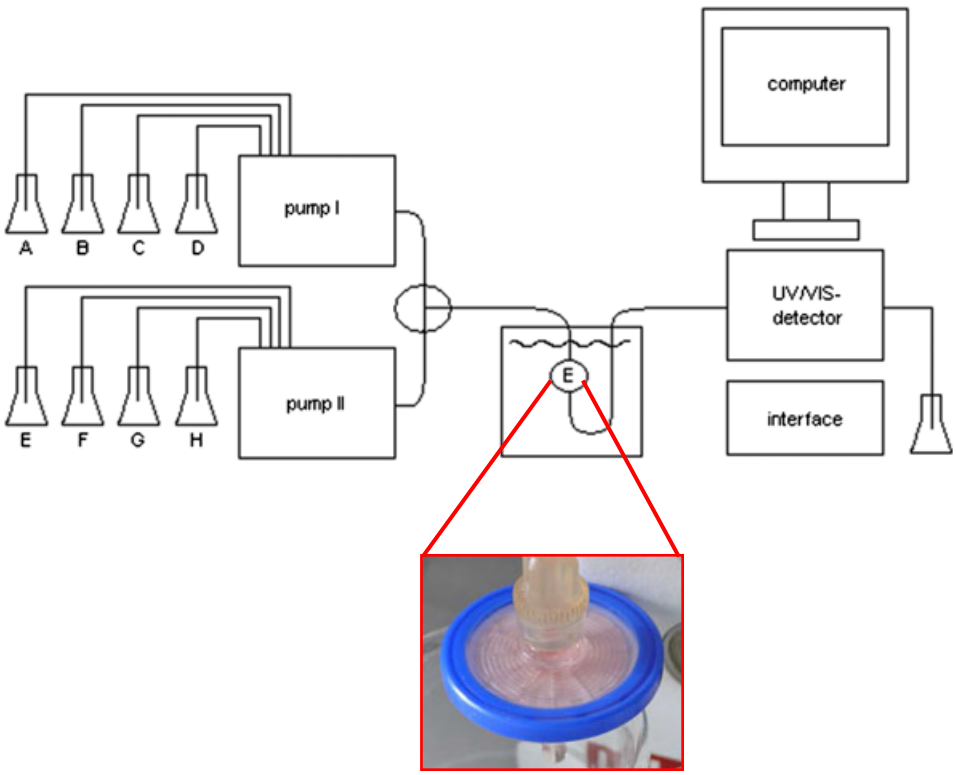


Figure 8. Scheme of the assembly of the dynamic in vitro model for the real-time determination of membrane-bound AChE. Eight different solvents (e. g. buffer, substrate, chromogen, OP, oximes) can be delivered consecutively or simultaneously by the pumps, are mixed and finally pumped through the enzyme reactor ('E'). The outflow is delivered to a UV/VIS-detector for online recording of absorbance changes. The insert presents the enzyme reactor 'E' after loading the filter with diluted human erythrocytes.

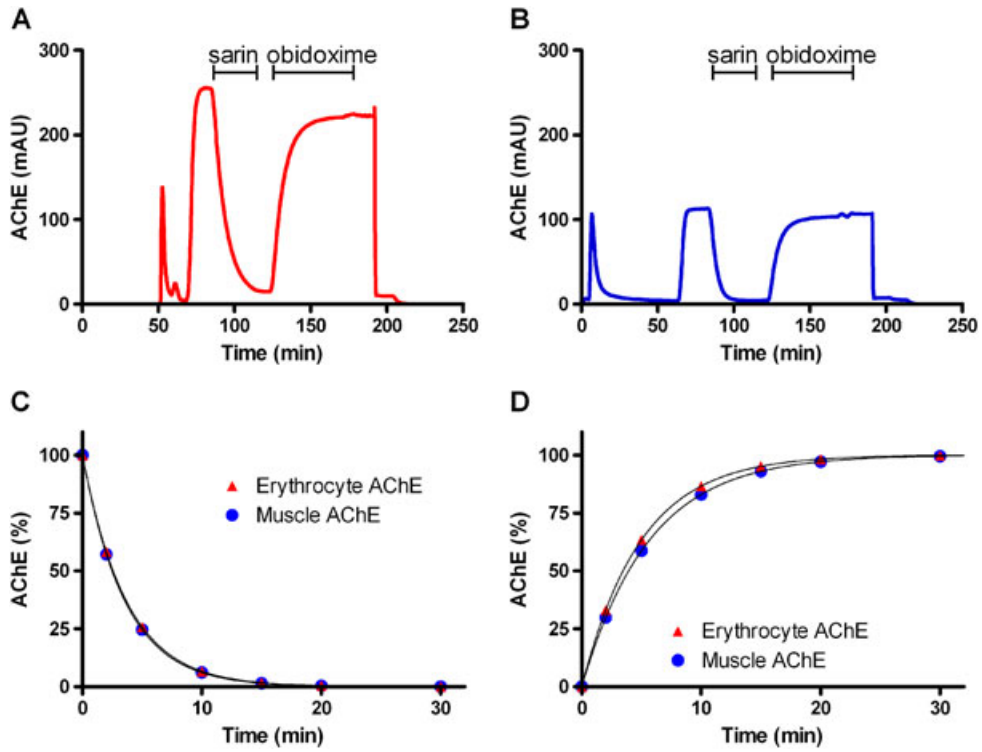


Figure 9. Time course of AChE activity from Rhesus monkey erythrocytes (A) and muscle tissue (B) after inhibition with sarin and reactivation with obidoxime. After having reached maximum activity at 470 nm in the presence of 0.3 mM DTNB and 0.45 mM acetylthiocholine, 50 nM sarin was added for 30 min and after a 10 min washout phase, reactivation was initialized by administration of 10 μM obidoxime. Blank values of the perfusion medium consisting of buffer, DTNB and acetylthiocholine were collected by removing the bioreactor and replacing it with a filter without any enzyme source. Time-dependent inhibition by 50 nM sarin (C) and reactivation by 10 μM obidoxime (D) of Rhesus monkey erythrocyte and muscle AChE was calculated using inhibition and reactivation constants generated with the dynamic model.[17]

assay. The latter effect could be overcome by solubilization and purification of the enzyme but studies showed that the most commonly used detergent Triton X-100 alters the kinetic properties of solubilized AChE.[61,62]

Recently, a dynamic *in vitro* model for the real-time determination of membrane-bound AChE was developed for use with human erythrocyte AChE (Figure 8) and was successfully adopted for kinetic investigations of OP, carbamates and oximes with muscle homogenates from different species.[15–17,63,64] This Ellman-based set-up enables the online recording of dynamic changes of AChE activities in the presence of inhibitors and reactivators and subsequent calculation of kinetic constants (Figure 9). Hereby, potentially annoying side reactions, for example, re-inhibition of reactivated AChE by phosphyloximes, are minimized. For the first time, the accordance of kinetic properties of erythrocyte and muscle AChE from different species could be verified (Figures 9C and 9D), providing a kinetic basis for the proper evaluation and species extrapolation of *in vivo* data from animal experiments and for the development of refined *in vivo* models. Future studies will elucidate whether this model can be adopted for use of other tissues.

Conclusions

In principle, the spectrophotometric Ellman assay is an excellent tool for the determination of AChE activity. Application of the assay for investigation of kinetic interactions between AChE, inhibitors and oximes requires the consideration of potential matrix effects (e.g. hemoglobin), side reactions (e.g. oximolysis) and other determinants (e.g. pH, temperature). By taking these factors into account, the Ellman assay allows the precise and reproducible determination of kinetic constants as a basis for the understanding of toxic OP effects and for the development of improved therapies against poisoning by OP. In addition, advanced applications of the Ellman assay; for example, dynamic *in vitro* model for the real-time determination of membrane-bound AChE, enables the proper investigation of relevant tissue, primarily respiratory muscle, and extends the applicability of this method.

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Appendix

Inhibition kinetics

The determination of the inhibition kinetics of OP in the presence of substrate was initially proposed by Hart and O'Brien [43] and by Forsberg and Puu.[44] In a slightly modified protocol [45] 10 µl AChE and 5 µl diluted OP (different concentrations) are added to a cuvette containing phosphate buffer, DTNB and ATCh (final volume 3.165 ml). ATCh hydrolysis is continuously monitored for up to 30 min. The recorded curves are analyzed by non-linear regression analysis (one phase exponential association) and used for the further determination of the bimolecular reaction constant $k_i = k_2/K_d$ (Equation 1).

$$\frac{\Delta t}{\Delta \ln v} = \frac{K_D}{k_2} * \frac{1}{[IX]\ (1-\alpha)} + \frac{1}{k_2} \quad (1)$$

with v: velocity of substrate hydrolysis; K_D: dissociation constant; k_2: unimolecular phosphylation rate constant; [IX]: OP concentration; α: [S] / (K_m + [S]) where [S] is substrate concentration and K_m is the species specific Michaelis constant.

Reactivation kinetics – general considerations

Oxime reactivation of OP-inhibited AChE proceeds according to Scheme 2. In this scheme [EP] is the phosphylated AChE, [EPOX] the Michaelis-type phosphyl-AChE-oxime complex, [OX] the reactivator, [E] the reactivated enzyme and [POX] the phosphylated oxime. K_D is equal to the ratio [EP]*[OX]/[EPOX]) and approximates the dissociation constant which is inversely proportional to the affinity of the oxime to [EP], and k_r the rate constant for the displacement of the phosphyl residue from [EPOX] by the oxime, indicating the reactivity.

In case of complete reactivation and with [Ox] » $[EP]_0$ a pseudo-first-order rate equation can be derived for the reactivation process (Equation 2)

$$kobs = \frac{kr * [OX]}{KD + [OX]} \quad (2)$$

k_{obs} is the observed first-order rate constant of reactivation at any given oxime concentration. The value of k_{obs} is not proportional to the oxime concentration but underlies a saturation kinetics and k_r and K_D follow Michaelis-Menten kinetics [20]. When [Ox] « K_D, Equation 2 simplifies to

$$kobs = \left(\frac{kr}{KD}\right) * [OX] \quad (3)$$

and finally the second order reactivation rate constant k_{r2}, describing the specific reactivity, can be derived

$$kr2 = \frac{kr}{KD} \quad (4)$$

Reactivation kinetics – continuous procedure

Kitz *et al.* developed a procedure to determine the reactivation kinetics of oximes with OP-inhibited AChE in the presence of substrate in a single run per concentration by continuously monitoring the AChE activity. [59] In an adopted protocol [20] 10 µl OP-inhibited AChE are added to a cuvette containing phosphate buffer, DTNB, ATCh and specified oxime concentrations (final volume 3.16 ml). ATCh hydrolysis is continuously monitored over 5–10 min and the activities are individually corrected for oxime-induced hydrolysis of ATCh ('oximolysis').k_{obs} can be calculated from the continuous recording of d[S]/dt. Hereby, the concentration of the reactivated AChE is proportional to the enzyme activity, i.e. the velocity of substrate hydrolysis (v) and may be expressed as pseudo-first-order process of reactivation

$$vt = v0 * \left(1 - e^{-kobs*t}\right) \quad (5)$$

with v_t = velocity at time t and v_0 = maximum velocity (control). Integration of Equation 5 results in

$$-d[S] = \int_0^t vdt = v0 * t + \frac{v0}{kobs}\left(e^{-kobs*t} - 1\right) \quad (6)$$

which is used for non-linear regression analysis of the data points from individual oxime concentrations.

Reactivation kinetics – discontinuous procedure

For the determination of reactivation rate constants with the discontinuous procedure [58] the following protocol proved its suitability. [20] Sixty µl OP-inhibited AChE are incubated with 2 µl oxime solution (different concentrations) and 1 µl ATCh (450 µM final concentration), then 10 µl aliquots are transferred to cuvettes after

specified time intervals (e.g. 1–15 min). AChE activities are referred to control activities and k_{obs} values are calculated at each oxime concentration by linear regression analysis, applying Equation 7

$$\ln\left(\frac{v0 - vt}{v0 - vi}\right) = -kobs * t \quad (7)$$

with v_i = velocity of inhibited AChE, v_t = velocity at time t and v_0 = maximum velocity (control).

Alternatively, k_{obs} can be calculated by non-linear regression analysis using Equation 5.

With all procedures k_r and K_D can be obtained by the non-linear fit of the relationship between k_{obs} vs [OX] and k_{r2} can be calculated using Equation 4.