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# Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes

Franz Worek[a,*], Horst Thiermann[a], Ladislaus Szinicz[a], Peter Eyer[b]

[a]*Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany*
[b]*Walther-Straub-Institute of Pharmacology and Toxicology, Ludwigs-Maximilians-University, Nussbaumstrasse 36, 80336 Munich, Germany*

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**Abstract**

The wide-spread use of organophosphorus compounds (OP) as pesticides and the availability of highly toxic OP-type chemical warfare agents (nerve agents) underlines the necessity for an effective medical treatment. Acute OP toxicity is primarily caused by inhibition of acetylcholinesterase (AChE, EC 3.1.1.7). Reactivators (oximes) of inhibited AChE are a mainstay of treatment, however, the commercially available compounds, obidoxime and pralidoxime, are considered to be rather ineffective against various nerve agents. The antidotal efficacy of new oximes is primarily tested in animals for ethical reasons. However, the various interactions between AChE, OP and oximes can be investigated with human AChE which enables the direct assessment of oxime potency, thus excluding species differences. The kinetics of inhibition, reactivation and aging were investigated with human erythrocyte AChE, various structurally different OP (organophosphates, -phosphonates and phosphoramidates) and oximes (obidoxime, pralidoxime, HI 6, HLö 7). The inhibitory potency of OPs, reactivating potency of oximes and spontaneous reactivation and aging were strongly affected by the structural characteristics of the OPs and of the phosphyl–AChE-complex. The kinetic data emphasize the superior inhibitory potency of organophosphonates. AChE inhibited by various phosphoramidates was mostly resistant towards reactivation by oximes while phosphonylated AChE was easily reactivated. HLö 7 was most potent with phosphonylated AChE and obidoxime with AChE inhibited by organophosphates and phosphoramidates. With the exception of soman, OP-inhibited AChE aged rather slowly ($t_{1/2}$ 3–231 h) and reactivated spontaneously with some compounds. These results indicate that there is obviously no direct structure-activity relationship for the various interactions of human AChE, OPs and oximes.


*Keywords:* Acetylcholinesterase; Organophosphates; Oximes; Inhibition; Reactivation; Aging

## 1. Introduction

The extensive use of organophosphorus pesticides for pest control and for attempting suicide causes worldwide huge numbers of intoxications and several hundreds of thousands of fatalities per year [1,2]. Besides, great stocks of highly toxic organophosphate-based chemical warfare agents ('nerve agents') are still available and present a real threat to the population. The use of nerve agents during military conflicts [3] and by terrorists [4] underlines the necessity to develop an effective medical treatment regimen for the whole range of nerve agents. The toxic effects of organophosphates (OPs) are mainly due to a progressive inhibition of cholinesterases by phosphylation (denotes phosphorylation and phosphonylation) of their active center serine leading to an inactive enzyme species [5,6]. The inability of inhibited AChE to hydrolyze acetylcholine

*Abbreviations:* AChE, acetylcholinesterase (E.C. 3.1.1.7); BChE, butyrylcholinesterase (E.C. 3.1.1.8); ATCh, acetylthiocholine iodide; BTCh, *S*-butyrylthiocholine iodide; DTNB, 5,5′-dithio-bis(-2-nitrobenzoic acid); DFP, diisopropylfluorophosphate; tabun, ethyl *N*-dimethylphosphoramidocyanidate; diethyltabun, ethyl *N*-diethylphosphoramido cyanidate; soman, pinacolylmethylphosphonofluoridate; sarin, isopropylmethyl phosphonofluoridate; butylsarin, n-butylmethylphosphonofluoridate; cyclosarin, cyclohexylmethylphosphonofluoridate; VX, O-ethyl *S*-[2-(diisopropylamino)ethyl) methylphosphonothioate; VR, S-[2-(diethylamino)isobutyl) methylphosphonothioate; paraoxon-ethyl, diethyl-*O*-4-nitrophenylphosphate; paraoxon-methyl, dimethyl-*O*-4-nitrophenylphosphate; fenamiphos, ethyl 3-methyl-4-(methylthio) phenyl isopropylphoshoramidate; methamidophos, *O*,*S*-dimethyl phosphoramidothioate; obidoxime, 1,1′-(oxybis-methylene)bis[4-(hydroxyimino)methyl] pyridinium dichloride; pralidoxime, 2-[hydroxyimino methyl]-1-methylpyridinium chloride; HI 6, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)-methyl]pyridinium dichloride monohydrate; HLö; 7, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2,4-bis-[(hydroxyimino)methyl] pyridinium dimethanesulfonate)

* Corresponding author. Tel.: +49 89 3168 2930; fax: +49 89 3168 2333.
*E-mail address:* franzworek@bundeswehr.org (F. Worek).

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results in accumulation of the transmitter and subsequently in over-stimulation of cholinergic receptors followed by paralysis of neuromuscular function.

Standard treatment of OP poisoning includes a muscarinic antagonist, e.g. atropine, and an AChE reactivator (oxime). Presently, the oximes obidoxime and pralidoxime are approved as antidotes against OP poisoning but are considered to be rather ineffective against different nerve agents, which led to the synthesis of numerous new compounds [7]. For ethical reasons, the efficacy of nerve agent antidotes cannot be investigated in humans in vivo. Therefore, testing is primarily performed with animals in vitro and in vivo [8]. However, substantial species differences in the toxicokinetics of inhibitors [9], pharmacokinetics, dosing of antidotes [8,10,11] and reactivating potency of oximes [12,13] hamper the extrapolation of animal data to humans.

Removal of the phosphyl moiety from the AChE active site serine (reactivation) is considered to be the primary mechanism of oxime action [14]. Recent clinical data from OP pesticide poisoned patients provide strong evidence for the validity of this assumption [15]. Moreover, it was shown that kinetic data generated with human erythrocyte AChE in vitro [16,17] correlated well with the in vivo cholinesterase status in these patients and could be used to optimize oxime treatment [15,18].

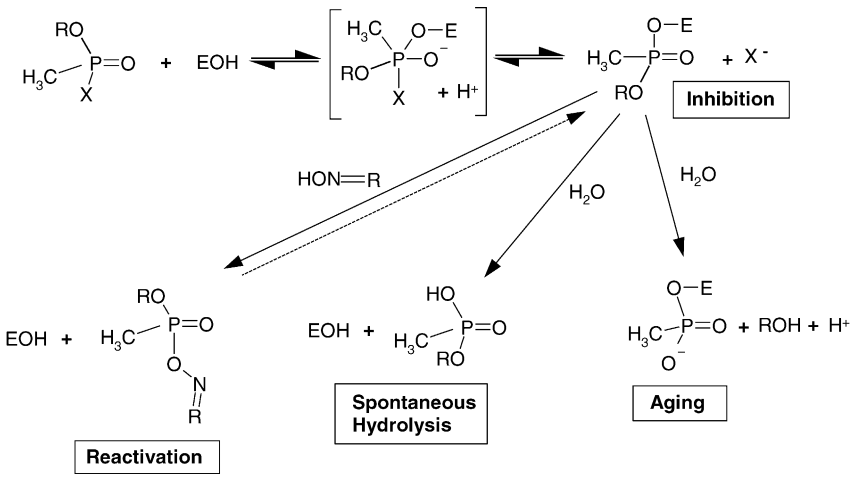
Phosphylated AChE underlies post-inhibitory processes which may affect oxime effectiveness [19]. According to Scheme 1 a phosphyl–AChE-complex may undergo spontaneous dealkylation through alkyl–oxygen bond scission ('aging'), resulting in an irreversibly inactivated enzyme, or spontaneous dephosphylation ('spontaneous reactivation'), a process which can be accelerated by several orders of magnitude by the addition of a strong nucleophile such as an oxime. Oxime-induced reactivation inevitably leads to the formation of highly reactive phosphylated oximes [20,21] which may re-inhibit reactivated AChE [22–24].

The different reactions of AChE with organophosphates were investigated in the past by using different compounds, enzyme sources and experimental conditions, making a proper assessment of oxime effects difficult. In addition, recent data indicate substantial species differences in oxime potency [13]. In order to provide a kinetic basis for the various interactions between human AChE, different OPs and oximes we initiated this study using standardized conditions for all experiments. The conventional oximes obidoxime and pralidoxime and the experimental compounds HI 6 and HLö 7, which showed to be promising antidotes against nerve agents [8,25–28], were selected as test compounds. Determination of the various kinetic constants of these oximes, nerve agents and pesticides should enable a proper assessment of the reactivating potency of oximes. By using different OPs, i.e. organophosphonates, organophosphates and phosphoramidates, a basis for the analysis of structure-activity relationship should be provided for a closer understanding of the great differences in oxime potency (Fig. 1).

## 2. Materials and methods

### 2.1. Materials

Acetylthiocholine iodide (ATCh), *S*-butyrylthiocholine iodide (BTCh), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), diisopropylfluorophosphate (DFP) and pralidoxime chloride (2-PAM) were obtained from Sigma and obidoxime dichloride (obidoxime) was purchased from Duphar. HI 6 was kindly provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada) and HLö 7 was a custom synthesis by J. Braxmeier. Sarin, butylsarin, cyclosarin, soman, tabun, diethyltabun, VX and VR (>98% by GC–MS, $^{1}$H NMR and $^{31}$P NMR) were made available by the German Ministry of Defence. Paraoxon–ethyl, paraoxon–methyl, methamidophos and fenamiphos were from Labor Dr. Ehrenstorfer, all other chemicals from Merck Eurolab GmbH.




Scheme 1.

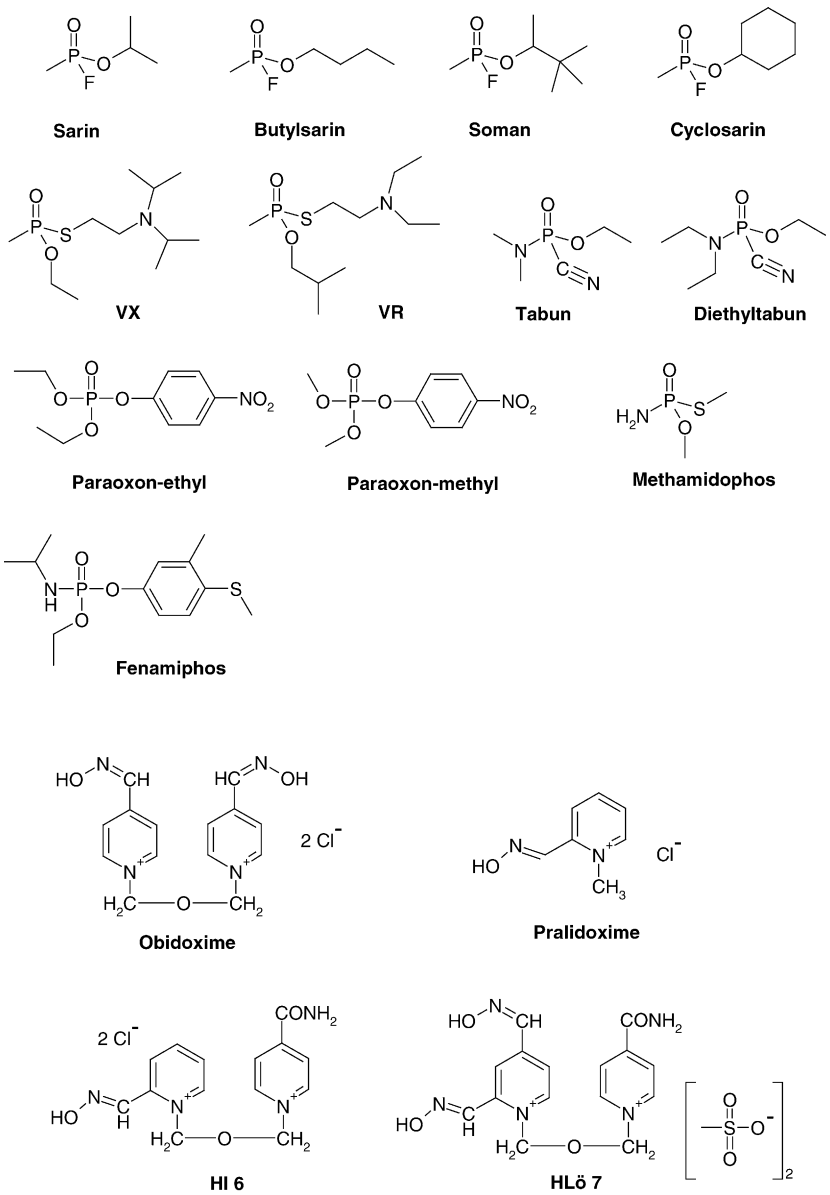



Fig. 1. Structures of organophosphorus compounds and oximes used in this study.

Stock solutions of sarin, butylsarin, cyclosarin, soman, tabun, diethyltabun, VX and VR (0.1% v/v), paraoxon–ethyl and –methyl (1% v/v) and methamidophos and fenamiphos (10% v/v) were prepared weekly in 2-propanol, stored at 4 °C and appropriately diluted in distilled water just before the experiment. Oximes (50 and 200 mM) were prepared in distilled water, stored at −60 °C and diluted daily as required in distilled water at the day of the experiment. All solutions were kept on ice until the experiment.

Hemoglobin-free erythrocyte ghosts were prepared according to Dodge et al. [29] with minor modifications [13]. In brief, heparinized human blood was centrifuged (3000 × *g*, 10 min) and the plasma removed. The erythrocytes were washed three times with 2 volumes of phosphate buffer (0.1 M, pH 7.4). Then, the packed erythrocytes were diluted in 20 volumes of hypotonic phosphate buffer (6.7 mM, pH 7.4) to facilitate hemolysis followed by centrifugation at 50,000 × *g* (30 min, 4 °C). The supernatant was removed and the pellet re-suspended in hypotonic phosphate buffer. After two additional washing cycles the pellet was re-suspended in phosphate buffer (0.1 M, pH 7.4) and the virtually hemoglobin-free erythrocyte ghosts were concentrated by centrifugation at 100,000 × *g* (30 min, 4 °C). Finally, the AChE activity was adjusted to the original activity (i.e. 4–5 U/ml) by appropriate dilution with phosphate buffer (0.1 M, pH 7.4). Aliquots of the erythrocyte ghosts were stored at −60 °C until use. Prior to use, aliquots were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenator (Bandelin electronic, Berlin, Germany), three times for 5 s with 30 s intervals, to achieve a homogeneous matrix for the

kinetic studies. The hemoglobin content of the samples was determined by a modified cyanmethemoglobin method [30].

In order to prevent AChE denaturation during long-term experiments at 37 °C AChE was stabilized by addition of plasma with totally blocked BChE [16]. Plasma was obtained as described above and inhibited by soman (100 nM) for 30 min at 37 °C to ensure complete inhibition and aging of BChE. The inhibited plasma was dialyzed (phosphate buffer, 0.1 M, pH 7.4) overnight at 4 °C to adjust pH and to remove residual inhibitor.

### 2.2. Enzyme assays

AChE and BChE activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt) with a modified Ellman assay [30,31]. The assay mixture (3.16 ml) contained 0.45 mM ATCh (AChE) or 1.0 mM BTCh (BChE) as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37 °C.

### 2.3. Determination of inhibition rate constants ($k_i$)

At $t = 0$, appropriately diluted OP was added to temperature-equilibrated (37 °C) human erythrocyte ghosts, an aliquot was removed after specified time intervals (5 s–20 min) and transferred to a cuvette for the determination of residual AChE activity. In case of highly reactive OP's (soman, sarin, cyclosarin, butylsarin, VX, VR), ghosts were pre-diluted 1:40 in phosphate buffer (0.1 M, pH 7.4). The final concentrations of the inhibitors were sufficiently high to establish pseudo-first-order reaction conditions. $k_i$ was calculated from Eq. (1)

$$k_i = \frac{1}{[\mathrm{OP}]t} \ln \frac{v_0}{v_t} \quad (1)$$

where [OP] is the initial concentration of the tested OP, $v_0$ and $v_t$ are the reaction rates at time zero and at time $t$, respectively.

### 2.4. Determination of rate constants for aging ($k_a$) and spontaneous reactivation ($k_s$)

OP-inhibited AChE was prepared by incubating erythrocytes and ghosts with appropriate OP concentrations for 15 min at 37 °C resulting in an inhibition of 95–98% of control activity. In case of excess OP after inhibition (VX, VR, tabun, diethyltabun, fenamiphos, methamidophos) the samples were dialyzed and the absence of inhibitory activity was tested by incubation of treated and control enzyme (15 min, 37 °C). OP-treated samples were stored in aliquots at −60 °C until use.

OP-treated erythrocytes and ghosts were mixed with equal volumes of soman-treated human plasma to prevent denaturation of AChE during long-term experiments at 37 °C. Aliquots were taken after various time intervals for determination of AChE activity (spontaneous reactivation) and of the decrease of oxime-induced reactivation (aging, Table 1). Data were referred to control activities and the percentage reactivation (% react) was calculated [32]. The pseudo first-order rate constants $k_s$ (spontaneous reactivation) and $k_a$ (aging) were calculated by a non-linear regression model [16,33,34], where $[E_0]$ is the control AChE activity, $[E]$ and $[EA]$ are the activities of spontaneously reactivated and aged AChE, respectively.

$$[E] = \frac{k_s[E_0]}{k_s + k_a}(1 - e^{-(k_s+k_a)t}) \quad (2)$$

$$[EA] = \frac{k_a[E_0]}{k_a + k_s}(1 - e^{-(k_s+k_a)t}) \quad (3)$$

$[EA]$ can only be determined indirectly by the decrease in reactivatability of inhibited AChE. According to $[EA]$ =

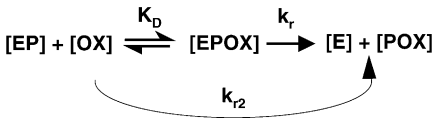
Table 1
Experimental conditions for the determination of aging kinetics of inhibited AChE

| OP | Matrix[a] | Oxime | [Oxime] (μM)[b] | $t_{reac}$ (min)[c] | $t_{obs}$ (h)[d] |
|---|---|---|---|---|---|
| Tabun | Erythrocytes | TMB-4 | 1000 | 60 | 0–63 |
| Sarin | Erythrocytes | HLö 7 | 1000 | 10 | 0–10 |
| Cyclosarin | Erythrocytes | HLö 7 | 1000 | 10 | 0–24 |
| VX | Erythrocytes | HLö 7 | 1000 | 30 | 0–44 |
| VR | Erythrocytes | HLö 7 | 100 | 10 | 0–53 |
| Butylsarin | Ghosts | HLö 7 | 1000 | 10 | 0–28 |
| Paraoxon-ethyl[e] | Erythrocytes | Obidoxime | 100 | 30 | 0–72 |
| Paraoxon-methyl[f] | Erythrocytes | Obidoxime | 20 | 30 | 0–4 |
| DFP | Ghosts | Obidoxime | 2000 | 30 | 0–30 |
| Methamidophos | Ghosts | HLö 7 | 500 | 10 | 0–8 |
| Fenamiphos | Ghosts | Obidoxime | 1000 | 35 | 0–96 |

[a] Erythrocytes and ghosts were mixed with an equal volume of soman-treated human plasma for stabilizing AChE activity.
[b] Oxime concentration used for testing maximum reactivatability.
[c] Reactivation time.
[d] Total observation time.
[e] From [17].
[f] From [16].

$[E_0]-[E_{reac}]$, Eq. (3) may be rearranged to Eq. (4)

$$[E_{reac}] = [E_0] - \frac{k_a[E_0]}{k_a + k_s}\left(1 - e^{-(k_s+k_a)t}\right) \quad (4)$$




Scheme 2.

### 2.5. Oxime reactivation of OP-inhibited AChE

The ability of oximes to reactivate OP-inhibited AChE was tested in pilot experiments by adding oxime (10, 100, 1000 μM) to enzyme samples and measuring the residual activity at specified time intervals (1–30 min). In case of expected high reactivating potency the reactivation kinetics were determined with the continuous procedure presented by Kitz et al. [13,35]. Hereby, 10 μl OP-inhibited AChE was added to a cuvette containing phosphate buffer, DTNB, ATCh and specified oxime concentrations (final volume 3.16 ml). ATCh hydrolysis was continuously monitored over 5 min (Fig. 2A). Activities were individually corrected for oxime-induced hydrolysis of ATCh. In order to reduce the effect of oxime-induced AChE inhibition the maximum concentration was 30 μM (HI 6, HLö 7) and 100 μM (obidoxime, pralidoxime), respectively.

In case of low reactivating potency a discontinuous procedure was applied (Fig. 2C) [16,36] which allowed use of higher oxime concentrations (up to 5 mM). 60 μl OP-inhibited AChE was incubated with 2 μl oxime solution (different concentrations) and 1 μl ATCh (450 μM final concentration) and 10 μl aliquots were transferred to cuvettes after specified time intervals (1–9 min).

### 2.6. Kinetics of oxime reactivation

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]/[*E*POX]) 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 [*E*POX] by the oxime, indicating the reactivity.

In case of complete reactivation and with $[OX] \gg [EP]_0$ a pseudo-first-order rate equation can be derived for the reactivation process [37]

$$k_{obs} = \frac{k_r[OX]}{K_D + [OX]} \quad (5)$$

$k_{obs}$ 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 [38] and $k_r$ and $K_D$ follow Michaelis–Menten kinetics [39].

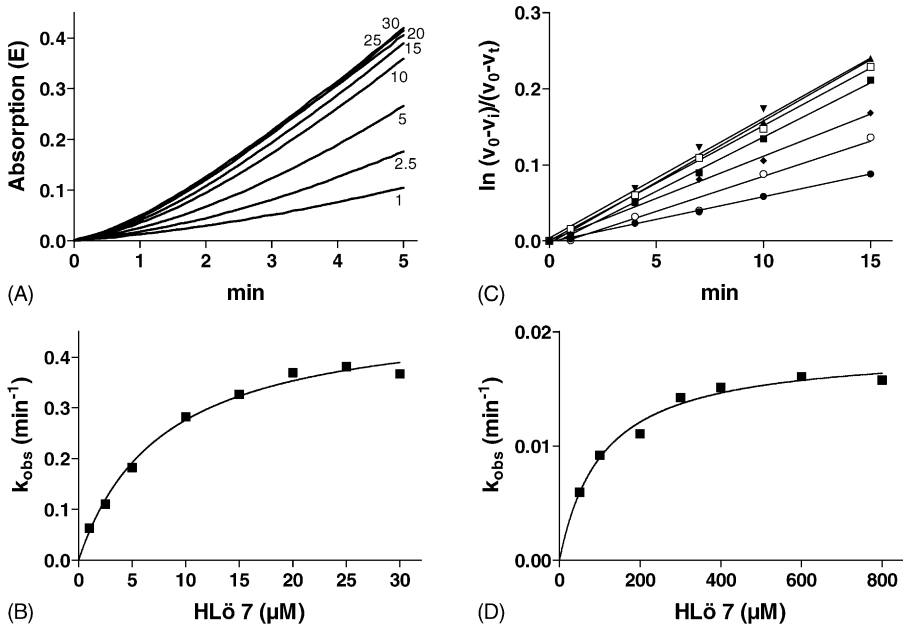



Fig. 2. Reactivation kinetics of VX- (A, B) and tabun-inhibited human AChE (C, D) by HLö 7. (A) Continuous recording of absorption change in the Ellman assay after addition of HLö 7 (1–30 μM, indicated by numbers). (C) Single data points indicate calculated AChE activities by Eq. (8) after the designated time of reactivation with 50 (●), 100 (○), 200 (◆), 300 (■), 400 (□), 600 (▲), and 800 μM HLö 7 (▼). $k_{obs}$ was calculated using Eq. (10). Secondary plot of $k_{obs}$ vs. [HLö 7] and the line fitted using Eq. (5) for VX (B) and tabun (D).

When [OX] « $K_D$, Eq. (5) simplifies to

$$k_{obs} = \left(\frac{k_r}{K_D}\right)[OX] \quad (6)$$

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

$$k_{r_2} = \frac{k_r}{K_D} \quad (7)$$

From experiments with discontinuous determination of enzyme activity after different reactivation times, $k_{obs}$ values were calculated at each oxime concentration by linear regression analysis, applying Eq. (8)

$$\ln\left(\frac{v_0 - v_t}{v_0 - v_i}\right) = -k_{obs}t \quad (8)$$

Alternatively, $k_{obs}$ was calculated from the continuous recording of d[*S*]/d*t*. 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

$$v_t = v_0(1 - e^{-k_{obs}t}) \quad (9)$$

with $v_t$: velocity at time t and $v_0$: maximum velocity (control)

Integration of (9) results in

$$-d[S] = \int_0^t v dt = v_0 t + \frac{v_0}{k_{obs}}(e^{-k_{obs}t} - 1) \quad (10)$$

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

$k_r$ and $K_D$ were obtained by the nonlinear fit of the relationship between $k_{obs}$ versus [OX] (Fig. 2B and D), $k_{r2}$ was calculated from Eq. (7).

## 3. Results

### 3.1. Inhibition kinetics of OPs with human AChE

The bimolecular rate constants for the inhibition of human AChE in the absence of substrate are summarized in Table 2. Accordingly, organophosphonates were substantially more potent inhibitors than organophosphates. For example, the rate constants of sarin, VX and cyclosarin were 12-, 55- and 222-fold higher, respectively, when compared to paraoxon-ethyl. The most potent compound tested in this study was butylsarin with a $k_i$ of $6.1 \times 10^8$ $M^{-1}$ $min^{-1}$. Notably, the size of the *O*-alkyl substituent of organofluorophosphonates had a great effect on the $k_i$. The replacement of the isopropyl group (sarin) by an *n*-butyl (butylsarin) or cyclohexyl group (cyclosarin) resulted in an 18- and 22-fold higher $k_i$, respectively.

Phosphoramidates showed marked differences in their inhibitory potencies, which were lower compared to those of organophosphonates. Again, small changes in the *N*-alkyl group had a great impact on the $k_i$, i.e. an eight-fold lower value of the diethyl analogue of tabun when compared to the parent compound.

### 3.2. Aging and spontaneous reactivation of OP-inhibited AChE

Phosphylated AChE is susceptible to spontaneous hydrolysis of an alkyl–ester bond, resulting in a negatively charged residue which is resistant towards nucleophilic attack [19]. The kinetics of aging and spontaneous reactivation of human AChE inhibited by different OPs followed first-order kinetics (Table 2). No essential qualitative differences between organophosphates, organophosphonates and phosphoramidates could be observed. Aging half-times between 3 (sarin) and 231 h (butylsarin) were determined. Small structural variations of the alkoxy residue had a dramatic impact on the aging half-time. Changing the ethyl group (VX) by a butyl group (butylsarin) resulted in a six-fold increase of aging half-time, and the replacement of an isopropyl (sarin) by an isobutyl group (VR) increased the half-time by a factor of 46. A comparable effect was observed with dimethyl– and diethyl–phosphoryl–AChE ($t_{1/2}$ 3.7 versus 32 h).

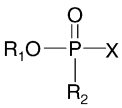
The kinetics of spontaneous reactivation of OP-inhibited AChE showed a different pattern (Table 2). With several compounds (sarin, cyclosarin, tabun, diethyltabun, DFP, fenamiphos) no spontaneous reactivation could be observed. The half-times of aging and spontaneous reactivation were almost identical with paraoxon–ethyl- and VX-inhibited AChE. Interestingly, the kinetics were also very similar for both compounds, despite structural differences of the phosphylated enzyme, diethylphosphoryl versus ethylmethylphosphonyl. In case of methamidophos-, paraoxon–methyl-, VR- and butylsarin-inhibited AChE the velocity of spontaneous reactivation outweighed aging, resulting in a substantial recovery of AChE activity. For example, the AChE activity increased to approximately 90% with VR- and butylsarin-inhibited AChE.

The comparison of the dealkylation and dephosphylation kinetics of all tested compounds gave no correlation between $k_a$ and $k_s$ ($R^2$ = 0.1159).

### 3.3. Reactivation kinetics of OP-inhibited AChE by oximes

The determination of reactivation rate constants of oximes with human AChE inhibited by a number of structurally different OP resulted in marked differences of affinity (Table 3) and reactivity (Table 4) depending on the OP and the oxime. HLö 7 was the most effective reactivator of phosphonylated AChE while obidoxime was roughly equipotent to HLö 7 with AChE inhibited by organophosphates and phosphoramidates. In general, phosphonylated AChE was far more susceptible towards

Table 2
Rate constants for the inhibition of AChE by OP ($k_i$) and for the spontaneous dealkylation ($k_a$) and reactivation of OP-inhibited AChE ($k_s$)[a]



| OP | $R_1$ | $R_2$ | $X$ | $k_i$ ($M^{-1}$ $min^{-1}$)[b] | $k_a$ ($h^{-1}$)[b] | $k_s$ ($h^{-1}$)[b] |
|---|---|---|---|---|---|---|
| VX | $C_2H_5$ | $CH_3$ | $SC_2H_4N(CH(CH_3)_2)_2$ | $1.2 \pm 0.002 \times 10^8$ | $0.019 \pm 0.001$ | $0.021 \pm 0.001$ |
| Butylsarin | $C_4H_9$ | $CH_3$ | F | $6.1 \pm 0.02 \times 10^8$ | $0.003 \pm 0.001$ | $0.079 \pm 0.003$ |
| Sarin | $CH(CH_3)_2$ | $CH_3$ | F | $2.7 \pm 0.1 \times 10^7$ | 0.228 | Ø[g] |
| VR | $CH_2CH(CH_3)_2$ | $CH_3$ | $SC_2H_4N(C_2H_5)_2$ | $4.4 \pm 0.006 \times 10^8$ | $0.005 \pm 0.001$ | $0.039 \pm 0.002$ |
| Soman | $CH(CH_3)C(CH_3)_3$ | $CH_3$ | F | $9.2 \pm 0.4 \times 10^7$ | 6.6[e] | Ø |
| Cyclosarin | Cyclohexyl | $CH_3$ | F | $4.9 \pm 0.006 \times 10^8$ | $0.099 \pm 0.003$ | Ø |
| Methamidophos | $CH_3$ | $NH_2$ | $SCH_3$ | $1.9 \pm 0.1 \times 10^3$ | $0.071 \pm 0.009$ | $0.239 \pm 0.011$ |
| Fenamiphos | $C_2H_5$ | $NHCH(CH_3)_2$ | *O*-3-methyl-4-methylthiophenyl | $0.2 \pm 0.01 \times 10^2$ | $0.005 \pm 0.002$ | Ø |
| Tabun | $C_2H_5$ | $N(CH_3)_2$ | CN | $7.4 \pm 0.2 \times 10^6$ | $0.036 \pm 0.001$ | Ø |
| Diethyltabun | $C_2H_5$ | $N(C_2H_5)_2$ | CN | $8.8 \pm 0.2 \times 10^5$ | N.D.[f] | Ø |
| Paraoxon-methyl[c] | $CH_3$ | $OCH_3$ | *O*-4-nitrophenyl | $1.2 \times 10^6$ | 0.186 | 1.01 |
| Paraoxon-ethyl[d] | $C_2H_5$ | $OC_2H_5$ | *O*-4-nitrophenyl | $2.2 \times 10^6$ | 0.022 | 0.022 |
| DFP | $CH(CH_3)_2$ | $OCH(CH_3)_2$ | F | $1.3 \pm 0.04 \times 10^5$ | $0.221 \pm 0.002$ | Ø |

[a] For structural details see Fig. 1.
[b] Mean ± S.E. of 2–5 determinations.
[c] From [16].
[d] From [17].
[e] At pH 8.0 and 24 °C [50].
[f] Aging kinetics could not be tested due to failure of oximes to reactivate diethyltabun-inhibited AChE.
[g] No spontaneous reactivation of AChE activity during the observation period.

reactivation by oximes than enzyme inhibited by phosphoramidates (Fig. 3), methamidophos being an exception. Diethyl– and dimethylphosphoryl–AChE was moderately reactivatable, while diisopropylphosphoryl–AChE showed to be rather resistant.

The affinity of oximes towards OP-inhibited AChE, reflected by $K_D$, was of huge difference (Table 3). With pralidoxime, $K_D$ values between 2 μM (methamidophos) and more than 3 mM (cyclosarin) were observed. The affinity was especially low with fenamiphos-inhibited AChE. In addition, the ratio of oxime $K_D$ (highest/lowest $K_D$) for individual OPs showed great variability. Values between 1.2 (paraoxon–methyl) and 176.5 (cyclosarin) were calculated.

The ability of oximes to remove the phosphyl residue from the active site of the enzyme, reflected by the reactivity constant $k_r$, showed also marked differences (Table 4). The range of $k_r$ was between 0.01 $min^{-1}$ (tabun) and 4.24 $min^{-1}$ (paraoxon–methyl). A remarkably low reactivity was recorded with AChE inhibited by tabun, DFP and fenamiphos. The differences between oximes, indicated by the ratio highest/lowest $k_r$, were less prominent when compared to affinity. A ratio between 2.7 (methamidophos) and 47.1 (paraoxon–methyl) was calculated.

The specific reactivity of oximes, quantified by the second-order reactivation rate constant $k_{r2}$ is dependent on affinity and reactivity. Therefore, $k_{r2}$ also reflects the

Table 3
Dissociation constants ($K_D$) for the oxime-induced reactivation of OP-inhibited AChE[a]

| OP | $R_1$ | $R_2$ | Obidoxime[b] | Pralidoxime[b] | HI 6[b] | HLö 7[b] | Ratio[c] |
|---|---|---|---|---|---|---|---|
| VX[d] | $C_2H_5$ | $CH_3$ | 27.4 | 28.1 | 11.5 | 7.8 | 3.6 |
| Butylsarin | $C_4H_9$ | $CH_3$ | 43.3 ± 5.4 | 138 ± 65 | 25.6 ± 2.6 | 15.3 ± 2.5 | 9.0 |
| Sarin[d] | $CH(CH_3)_2$ | $CH_3$ | 31.3 | 27.6 | 50.1 | 24.2 | 2.0 |
| VR | $CH_2CH(CH_3)_2$ | $CH_3$ | 106 ± 20 | 30.7 ± 3.4 | 9.2 ± 1.2 | 5.3 ± 1.1 | 20.0 |
| Cyclosarin[d] | Cyclohexyl | $CH_3$ | 945.6 | 3159 | 47.2 | 17.9 | 176.5 |
| Methamidophos | $CH_3$ | $NH_2$ | 7.5 ± 1.1 | 2.1 ± 0.3 | 9.1 ± 1.6 | 3.3 ± 0.4 | 4.3 |
| Fenamiphos | $C_2H_5$ | $NHCH(CH_3)_2$ | 615 ± 272 | 695 ± 127 | 889 ± 383 | 240 ± 64 | 3.7 |
| Tabun | $C_2H_5$ | $N(CH_3)_2$ | 97.3 ± 10.6 | 706 ± 76 | Ø[f] | 106.5 ± 15 | 7.3 |
| Paraoxon–methyl[e] | $CH_3$ | $OCH_3$ | 163 | 164 | 155 | 141 | 1.2 |
| Paraoxon–ethyl | $C_2H_5$ | $OC_2H_5$ | 32.2 ± 6.9 | 187.3 ± 19 | 548.4 ± 46 | 47.8 ± 6.9 | 17.0 |
| DFP | $CH(CH_3)_2$ | $OCH(CH_3)_2$ | 63.8 ± 9.6 | 847 ± 117 | 1935 ± 335 | 83.7 ± 13.9 | 30.3 |

[a] For structural details see Fig. 1.
[b] Mean ± S.E., $K_D$ in μM, 6–10 different concentrations were used for each oxime.
[c] Ratio of highest and lowest $K_D$ values.
[d] From [13].
[e] From [16].
[f] No reactivation of tabun-inhibited AChE up to 5 mM HI 6.

Table 4
Reaction rate constants ($k_r$) for the oxime-induced reactivation of OP-inhibited AChE[a]

| OP | $R_1$ | $R_2$ | Obidoxime[b] | Pralidoxime[b] | HI 6[b] | HLö 7[b] | Ratio[c] |
|---|---|---|---|---|---|---|---|
| VX[d] | $C_2H_5$ | $CH_3$ | 0.893 | 0.215 | 0.242 | 0.49 | 4.1 |
| Butylsarin | $C_4H_9$ | $CH_3$ | 0.24 ± 0.01 | 0.08 ± 0.02 | 0.72 ± 0.04 | 0.7 ± 0.06 | 9.0 |
| Sarin[d] | $CH(CH_3)_2$ | $CH_3$ | 0.937 | 0.25 | 0.677 | 0.849 | 3.7 |
| VR | $CH_2CH(CH_3)_2$ | $CH_3$ | 0.63 ± 0.09 | 0.06 ± 0.002 | 0.71 ± 0.03 | 0.84 ± 0.06 | 13.5 |
| Cyclosarin[d] | Cyclohexyl | $CH_3$ | 0.395 | 0.182 | 1.3 | 1.663 | 9.1 |
| Methamidophos | $CH_3$ | $NH_2$ | 0.84 ± 0.06 | 0.31 ± 0.01 | 0.39 ± 0.03 | 0.49 ± 0.02 | 2.7 |
| Fenamiphos | $C_2H_5$ | $NHCH(CH_3)_2$ | 0.09 ± 0.02 | 0.02 ± 0.001 | 0.02 ± 0.003 | 0.03 ± 0.002 | 4.5 |
| Tabun | $C_2H_5$ | $N(CH_3)_2$ | 0.04 ± 0.001 | 0.01 ± 0.0005 | Ø[f] | 0.02 ± 0.0007 | 4.0 |
| Paraoxon–methyl[e] | $CH_3$ | $OCH_3$ | 4.24 | 0.48 | 0.09 | 1.28 | 47.1 |
| Paraoxon–ethyl | $C_2H_5$ | $OC_2H_5$ | 0.81 ± 0.08 | 0.17 ± 0.007 | 0.2 ± 0.009 | 0.34 ± 0.02 | 4.0 |
| DFP | $CH(CH_3)_2$ | $OCH(CH_3)_2$ | 0.06 ± 0.003 | 0.05 ± 0.003 | 0.02 ± 0.002 | 0.02 ± 0.0006 | 3.0 |

[a] For structural details see Fig. 1.
[b] Mean ± S.E., $k_r$ in $min^{-1}$, 6–10 different concentrations were used for each oxime.
[c] Ratio of highest and lowest $k_r$ values.
[d] From [13].
[e] From [16].
[f] No reactivation of tabun-inhibited AChE up to 5 mM HI 6.

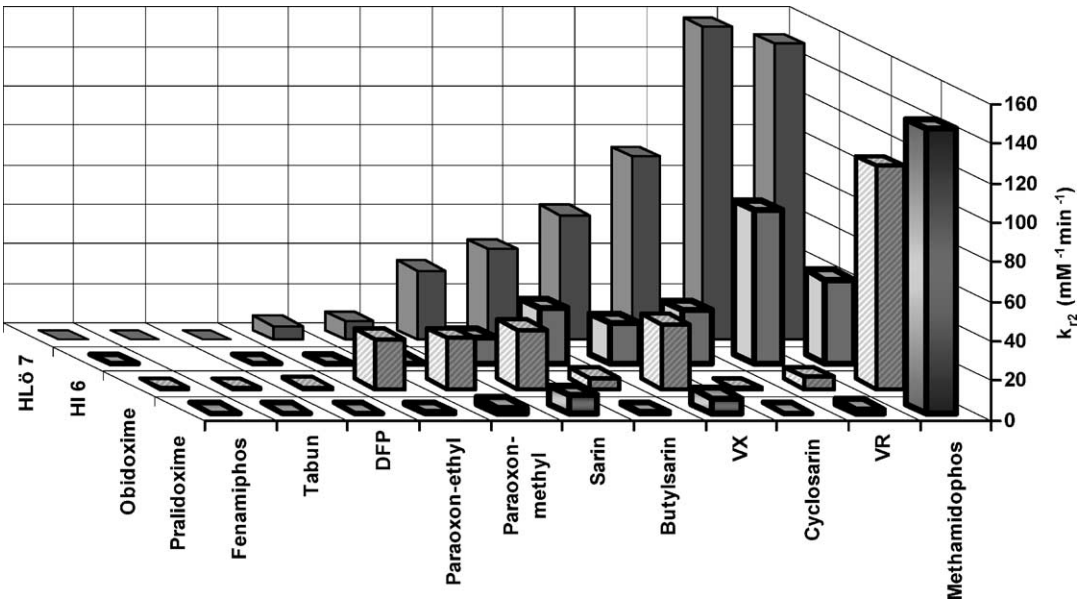



Fig. 3. Second-order rate constants $k_{r2}$ ($mM^{-1}$ $min^{-1}$) for the reactivation of OP-inhibited AChE by oximes.

inhibitor- and oxime-related differences of $K_D$ and $k_r$ (Fig. 3). The range of $k_{r2}$ was between 0.01 (DFP) and 158.7 $mM^{-1}$ $min^{-1}$ (VR) and the ratio of the most versus least potent oxime for individual OP's was between 3.5 (methamidophos) and 927 (cyclosarin).

Diethyltabun-inhibited AChE was completely resistant towards reactivation by the oximes tested. No increase in AChE activity could be observed even after addition of 5 mM oxime.

Nucleophilic attack at the phosphorus atom is the common mechanism of oximes and $H_2O$ for the dephosphylation of OP-inhibited AChE. However, a comparison of the kinetics of spontaneous hydrolysis ($k_s$) and oxime-induced reactivation $k_{r2}$ gave no correlation of the data ($R^2 = 0.002$, plotting $k_a$ versus $k_{r2}$ gave a $R^2$ of 0.007).

## 4. Discussion

### 4.1. Inhibition of AChE by OPs

The determination of inhibition kinetics of OPs with human AChE showed a superior inhibitory potency of organophosphonates compared to organophosphates and phosphoramidates. These marked differences are in agreement with previous studies using electric eel, fetal bovine serum, bovine erythrocyte and brain AChE [40–42]. Hereby, small structural modifications of the alkyl residue had a substantial impact on the $k_i$, as was demonstrated before for tabun analogues [43] and V-compounds [44]. Several organophosphonates (butylsarin, cyclosarin, VR) showed to be more potent inhibitors

of human AChE than the classical nerve agents soman, sarin and VX.

A common feature of the organophosphonates and phosphoramidates used in this study is the presence of a stereogenic phosphorus atom (Fig. 1), resulting in equal amounts of enantiomers in the racemic product. Biochemical and toxicological studies gave evidence that such stereoisomers have different toxicological properties [40,45–47]. For example, the ratio of inhibition rate constants of $P(-)$ versus $P(+)$ stereoisomers and $P(-)C(\pm)$ versus $P(+)C(\pm)$ in case of soman was found to be 200 (VX), 4200 (sarin) and >40,000 (soman) [40]. This difference may explain the mono-phasic inhibition kinetics of AChE which was observed in the present study with the racemic OPs.

### 4.2. Aging and spontaneous reactivation of OP-inhibited AChE

Phosphylated AChE underlies secondary reactions, i.e. spontaneous dealkylation (aging) and dephosphylation (reactivation) [19]. Aging proceeds through P–O bond scission (P–N bond scission in case of tabun [48,49]). This acid-catalyzed process results in formation of a negatively charged phosphyl–AChE-complex [50,51], thwarting nucleophilic attack by an oxime. Aging proceeds extremely rapid with soman-inhibited human AChE [50,52], the half-time being in the range of 2–3 min. The aging velocity was substantially slower with the OP's used in the present study, ranging from 3–231 h (Table 2), with the aging kinetics of organophosphonates being dependent on the structure of the alkoxy group. Previous studies suggested that aging is most pronounced with branched alkyl groups [50,53]. In agreement with this assumption, aging occurred much faster with sarin-inhibited AChE compared to VX- and butylsarin-inhibited enzyme. On the other hand, VR-inhibited AChE had a remarkably long aging half-time, indicating that the aging kinetics cannot be predicted univocally from the structure of the alkoxy group. In case of organophosphates (DFP, paraoxon–methyl and –ethyl) and phosphoramidates (tabun, fenamiphos) further factors may contribute to the differences in aging kinetics.

Spontaneous reactivation of OP-inhibited AChE was dependent on the structure of the phosphyl moiety but showed a different pattern compared to aging (Table 2). Spontaneous hydrolysis of dimethyl– and diethylphosphoryl–AChE as well as methamidophos-inhibited AChE was observed in vitro and in vivo [16,33,54,55] but its kinetics was not sufficiently investigated with phosphonylated AChE [53]. The data of the present study indicate that there is no real structure-activity relationship for the kinetics of spontaneous reactivation. AChE inhibited by sarin, cyclosarin, DFP, tabun and fenamiphos showed no increase in enzyme activity whereas a remarkable recovery of AChE activity was observed with VX, VR, butylsarin, methamidophos and both paraoxon analogues. Spontaneous reactivation proceeded even faster than aging with these agents (with the exception of paraoxon–ethyl having equal aging and reactivation half-times). Spontaneous dephosphylation of inhibited AChE may mimic the therapeutic effect of oximes in vivo in case of small OP doses, short residence times and rapid spontaneous reactivation. AChE inhibited by dimethyl–OPs or methamidophos may serve as an example [16,55]. The favourable ratio of $k_s/k_a$ of VR-inhibited AChE, resulting in almost 90% reactivated AChE, may be of minor importance in vivo. Only a small portion of inhibited AChE may recover in the initial phase of poisoning due to a half-time of spontaneous reactivation of approx. 18 h and the expected long systemic persistence of toxicologically relevant VR concentrations [56] may re-inhibit reactivated enzyme.

### 4.3. Oxime-induced reactivation of OP-inhibited AChE

The reactivation of OP-inhibited AChE by oximes was strongly dependent on the structure of the phosphyl moiety (Tables 3 and 4, Fig. 3). Phosphonylated as well as dimethyl– and diethylphosphoryl–AChE were highly susceptible towards reactivation by oximes while diisopropylphosphoryl-, tabun- and fenamiphos-inhibited AChE were rather resistant. HLö 7 showed to be superior with phosphonylated AChE and obidoxime with AChE inhibited by organophosphates.

Various explanations have been presented for the different reactivating effectiveness of oximes. Some authors stressed the influence of the acidity of the oxime group [57,58] since the removal of the phosphyl moiety from the active site is caused by the oximate anion [59]. Accordingly, oximes with lower $pK_a$ values should have a higher reactivity. A comparison of the $pK_a$ values [25,57] of pralidoxime (7.68), HI 6 (7.28) and HLö 7 (7.04 for the 2-aldoxime function) seems to support this concept. However, the substantially higher $pK_a$ values [60] of obidoxime (7.9) and TMB-4 (8.2), being effective reactivators of phosphorylated AChE, indicates that the acidity of the methin proton of the oxime group, which induces the rapid decay of the phosphyloxime [57], is of importance, too.

De Jong emphasized the importance of the position of the oxime group in the pyridinium ring and suggested that an oxime group in position 2 is important for the reactivation of soman-inhibited AChE whereas an oxime group in position 4 may be favorable for the reactivation of tabun-inhibited AChE [61]. This view was supported by the comparison of the reactivating potency of obidoxime, HI 6 and HLö 7 with soman- and tabun-inhibited AChE [61]. In fact, the present study showed 4-oximes to be superior to 2-oximes with organophosphates (paraoxon, DFP) and with phosphoramidates with substituted amido groups (fenamiphos). However, recent data indicate that obidoxime may be also an effective reactivator of non-aged human phosphonylated AChE under conditions where re-inhibition by phosphonylated oxime is prevented [62].

Finally, the present study demonstrates that the assumption of the relationship between the position of the oxime group and the reactivation of soman-inhibited AChE cannot be generalized for all organophosphonates (Fig. 3).

The resolution of the three-dimensional structure of AChE [63], realizing that the catalytic triad, Ser–His–Glu, is at the bottom of a 20 Å deep and narrow gorge, was the starting point for detailed structural studies. By using site-directed mutagenesis and molecular modeling, a number of studies were undertaken in order to unravel the underlying mechanisms for inhibition and reactivation of the enzyme [64]. Experiments with recombinant mouse AChE and mutant enzymes using paraoxon and methylphosphonates as inhibitors and HI 6 and pralidoxime as nucleophiles gave further insight into the structural components involved in the interactions between the active center gorge, the inhibitor and the oxime [65–67]. These studies indicate that the orientation of the phosphyl moiety in the active center as well as spatial constraints and steric limitations affect the oxime entry to the point of optimal reaction and its ability for nucleophilic attack. Formerly, it was suggested that inhibitors with larger alkyl residues may be less amenable to oxime reactivation [67]. This assumption cannot be supported by the now available data. The higher reactivating potency of HI 6, compared to pralidoxime, was attributed to an interaction of the second pyridinium ring with the peripheral anionic site of AChE, facilitating the orientation of the oxime during reactivation [68]. Luo et al. suggested that HI 6 may take two different orientations in the reactivation of AChE inhibited by methylphosphonates and paraoxon [66]. Taken together, these studies provide new insight into the mechanisms of reactivation but do not present a convincing concept for a wider range of OPs and oximes. In view of substantial species differences in the reactivatability of OP-inhibited AChE [13] the presented data need to be verified by further studies with human AChE.

Tabun-inhibited AChE is notoriously resistant towards reactivation by oximes [69]. A comparison of the reactivatability of phosphoramidate-inhibited AChE may shed light upon the possible reasons. AChE inhibited by methamidophos, bearing an unsubstituted amido group [48], was highly susceptible towards reactivation. This may not be attributed to spontaneous reactivation ($t_{1/2}$ 2.9 h) but was due to a high affinity and reactivity of the tested oximes. In contrast, oximes had an extremely low reactivity with AChE inhibited by phosphoramidates with substituted amido groups, i.e. tabun (dimethylamido), diethyltabun (diethylamido) and fenamiphos (isopropylamido). It can be assumed that substituted amido groups reduce the electrophilicity of the phosphorus atom, thus preventing a nucleophilic attack by an oxime function. Further structural studies will eventually elucidate whether a differential orientation of methamidophos and tabun in the active center gorge may additionally affect the accessibility of oximes.

### 4.4. Conclusions

The investigation of interactions between human AChE, oximes and a variety of nerve agents and pesticides provides a kinetic basis for the evaluation of oxime efficacy and for the estimation of effective oxime concentrations in humans. The suitability of such in vitro data for optimizing oxime treatment was demonstrated in human pesticide poisoning [18,31,70]. The determined inhibition rate constants emphasize the substantial differences in the inhibitory potency of organophosphonates, organophosphates and phosphoramidates. No qualitative differences in aging kinetics were observed with these OP classes and aging half-times in the range of hours are not expected to impair initial oxime treatment. Marked differences in reactivating potency of the tested oximes were found, HLö 7 and obidoxime being the most potent reactivators of phosphonylated and phosphorylated AChE, respectively. According to the proposed mechanism of resistance of phosphoramidate-inhibited AChE towards oxime-induced reactivation it remains uncertain whether oximes can be developed being able to serve as effective reactivators. The available data on aging and reactivation kinetics indicate that further kinetic and structural studies are required for a better understanding of the underlying mechanisms of oxime-induced reactivation and for the development of potent and effective broad-spectrum oximes. Finally, the marked differences in the specific reactivity of the various oximes underline once more that the erroneously used concept of the effective oxime plasma concentration (4 mg/l) should be dismissed.

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