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THE BISPYRIDINIUM-DIOXIME HLö-7

A POTENT REACTIVATOR FOR ACETYLCHOLINESTERASE INHIBITED BY THE STEREOISOMERS OF TABUN AND SOMAN

LEO P. A. DE JONG,*† MARCEL A. A. VERHAGEN,* JAN P. LANGENBERG,*
ILSE HAGEDORN‡ and MARLENE LÖFFLER†

*Prins Maurits Laboratory TNO, Lange Kleiweg 137, 2288 GJ Rijswijk, The Netherlands; and
‡Chemical Laboratory, University of Freiburg, Freiburg, Federal Republic of Germany

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Abstract—Purification of (+)-tabun was accomplished by treatment with electric eel acetylcholinesterase (AChE) in order to bind contaminating (−)-tabun, the more potent enantiomer with respect to AChE inhibition. Electric eel AChE inhibited with (−)-tabun and with purified (+)-tabun show similar properties in reactivation reactions with oximes (pH 7.5, 25°). The bispyridinium-2,4-dioxime HLö-7 is a substantially active reactivator for these inhibited enzymes as well as for human erythrocyte AChE inhibited with (−)-tabun. In contrast, the corresponding bispyridinium-2-monooxime HI-6 does not show any activity at similar reaction conditions. HLö-7 is also much more active than HI-6 when used as a reactivator for electric eel AChE inhibited by some *N*-unsubstituted derivatives of tabun. Surprisingly, HLö-7 is highly active in reactivating human erythrocyte and rat diaphragm AChE inhibited by C(+)P(±)- and C(−)P(±)-soman, i.e. at least as active as HI-6, which is the most potent reactivator for soman-inhibited AChE reported so far. To our knowledge, HLö-7 is the first compound reported in literature that shows a potent reactivating activity towards both tabun-inhibited AChE and soman-inhibited AChE.

Recently, the enantiomers of the nerve agent tabun (ethyl *N*,*N*-dimethylphosphoramidocyanidate, see Table 1 for chemical structures of organophosphates) were isolated and their LD_{50} values in mice and rate constants for the inhibition of acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7), the target enzyme for the toxic effects of this agent and related organophosphates, were determined [1]. In continuation of these investigations we now report a study on oxime-induced reactivation of (+)- and (−)-tabun-inhibited AChE. Reactivation of organophosphate-inhibited AChE counteracts the cause of organophosphate intoxication by displacing the organophosphate moiety from the active site of the enzyme. Consequently, this process is the basis of causal treatment of the intoxication.

One of the factors on which the efficacy of the reactivation process depends is the structure of the organophosphate moiety bound to the enzyme. AChEs inhibited with the nerve agents (±)-tabun and C(±)P(±)-soman§ (1,2,2-trimethylpropyl methylphosphonofluoridate) are notorious for their refractoriness against oxime-induced reactivation [2, 3]. Unfortunately, oximes that show a high efficacy in reactivation of both inhibited enzymes have not been reported so far. For instance, the most potent reactivators for C(±)P(±)-soman-inhibited AChE, the bispyridinium-2-oxime HI-6 (see Table 1 for chemical structures of oximes) and some other bispyridinium-2-oximes [3], are very weak reactivators for (±)-tabun-inhibited AChE [4]. Interestingly, we found in this study that the related bispyridinium-2,4-dioxime HLö-7, recently synthesized by one of us (M.L.) [5], is an effective reactivator for both (+)- and (−)-tabun-inhibited AChE. Therefore, we also studied this oxime as a reactivator for AChE inhibited by three *N*-unsubstituted derivatives of tabun and for C(+)P(±)- and C(−)P(±)-soman-inhibited AChE.

† To whom correspondence should be addressed.

§ Due to chirality in the 1,2,2-trimethylpropyl group and at phosphorus, soman consists of four stereoisomers denoted as C(+)P(+), C(+)P(−), C(−)P(+) and C(−)P(−); C stands for the 1,2,2-trimethylpropyl moiety and P for phosphorus.

MATERIALS AND METHODS

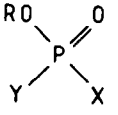
Materials

Membrane bound human erythrocyte AChE and electric eel AChE were preparations type XIII and type VI-S, respectively, from Sigma Chemical Co. (St. Louis, MO).

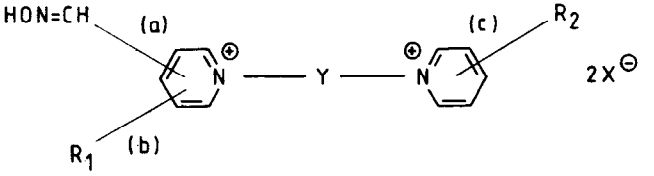
Rat diaphragm AChE was isolated as follows. A 10% suspension of diaphragm tissue pulverized by using a dismembrator was made in 0.01 M phosphate buffer, pH 7.5, containing 1% Triton X-100, 1.5 M NaCl, and the protease inhibitors *N*-ethylmaleimide (5 mM), benzamidine (2 mM), EGTA (10 mM), pepstatin (20 μg/ml) and bacitracin (1 mg/ml) to prevent degradation of the enzyme [6]. After sonication, the suspension was centrifuged at 30,000 *g* for 30 min and the supernatant was collected. This enzyme preparation was stable for several months when stored at −20°.

^{3}H-acetylcholine chloride (9.25 MBq/μmol) was purchased from Amersham (U.K.). Ethopropazine (10-(2-diethylamino-2-methylethyl)phenothiazine hydrochloride) was a gift of Rhone-Poulenc Nederland (The Netherlands). C(+)P(±)- and C(−)P(±)-

Table 1. Chemical structures of organophosphates and oximes used



Name	R	Y	X
Tabun	CH_3CH_2	CN	$N(CH_3)_2$
Soman	$(CH_3)_3CCH(CH_3)$	F	CH_3
Ethyl *p*-nitrophenyl phosphoramidate	CH_3CH_2	OC_6H_4-4-NO_2	NH_2
2-Fluoroethyl *p*-nitrophenyl phosphoramidate	CH_2FCH_2	OC_6H_4-4-NO_2	NH_2
2,2,2-Trifluoroethyl *p*-nitrophenyl phosphoramidate	CF_3CH_2	OC_6H_4-4-NO_2	NH_2



Code name	(a)	(b)	R_1	Y	(c)	R_2	X
TMB4	4		H	$(CH_2)_3$	4	CH=NOH	Br
Obidoxime	4		H	CH_2OCH_2	4	CH=NOH	Cl
HI-6	2		H	CH_2OCH_2	4	$C(O)NH_2$	Cl
HLö-7	2	4	CH=NOH	CH_2OCH_2	4	$C(O)NH_2$	I
P2S*	2		H	CH_3	—	—	CH_3SO_3
Benzyl-P2A*	2		H	$CH_2C_6H_5$	—	—	Br

* Monopyridinium compound.

soman and (+)- and (−)-tabun were prepared according to Benschop *et al.* [7] and Degenhardt *et al.* [1], respectively. The preparation of the *p*-nitrophenyl esters of ethyl, 2-fluoroethyl and 2,2,2-trifluoroethyl phosphoramidate was previously described [8]. P2S (2-hydroximinomethyl-1-methyl-pyridinium methanesulphonate) was purchased from Dr. Raschig GmbH, F.R.G., and obidoxime (1,1′-oxybis(methylene)bis(4-hydroxyiminomethyl-pyridinium) dichloride) from E. Merck, (Darmstadt, F.R.G.). The preparation of benzyl-P2A (1-benzyl-2-hydroxyiminomethyl-pyridinium bromide) was previously described [9]. TMB4 (1,1′-(1,3-propanediyl)bis(4-hydroxyiminomethyl-pyridinium) dibromide) was prepared according to Poziomek *et al.* [10], whereas HI-6 (1-(4-aminocarbonylpyridinio)methoxymethyl - 2 - hydroximinoethyl - pyridinium dichloride) was prepared according to Stark [11]. The preparation of HLö-7 (1-(4-aminocarbonylpyridinio)methoxymethyl - 2,4 - bis(hydroxyiminomethyl-pyridinium diiodide) has been described by Löffler [5].

All other reagents were commercial products of an analytical grade.

Methods

Purification of (+)-tabun. The contamination of 10–15% (−)-tabun present in the preparation of (+)-tabun as determined by enantioselective gas chromatography [1] was removed by binding to an approximately equimolar amount of electric eel AChE. The molar concentration of the enzyme active site was determined from the final activity remaining after inhibition with C(+)P(±)-soman, when using excess enzyme with respect to the C(+)P(−)-soman concentration in the reaction mixture. For the calculations of the enzyme concentration, C(+)P(−)-soman was assumed to be the only inhibiting isomer in C(+)P(±)-soman, as was shown in a previous study [7].

(+)-Tabun (10 μM) was incubated in an AChE solution (2 μM of active sites; *ca.* 6 mg/ml) in 0.05 M veronal buffer, pH 7.5, at 25° for 30 min. Next, the solution (1 ml) was centrifuged through a membrane cone (Amicon centriflo, CF 50,000) for 45 min at 1000 *g* for removal of the enzyme. In this period, 0.75 ml of the purified (+)-tabun solution passed through the filter. The final tabun concentration was determined by gas chromatographic assay of a hexane extract (1.5 ml) from a sample (50–100 μl). Gas chromatography was performed on a fused silica column coated with the achiral phase CP Sil 8 CB (length, 51 m; i.d., 0.32 mm; film thickness, 1.3 μm; procured from Chrompack, The Netherlands) in a gas chromatograph (Carlo Erba HRGC 5160) equipped with an alkali flame ionization detector, using diisopropyl phosphorofluoridate (DFP) as an internal standard. For a chromatographic run, the column was heated from 80 to 200° at 7°/min. Carrier gas (helium) was used at a flow rate of 1.5 ml/min.

Reactivation of inhibited human erythrocyte and rat diaphragm AChE. Inhibited human AChE was formed by incubation of an enzyme solution (2.5 U/ml) in 0.01 M phosphate buffer, pH 7.5, with 20 nM C(−)P(±)-soman or 8 nM C(+)P(±)-soman at pH 10, or with 25 nM (−)-tabun at pH 7.5, for 1 hr at 25°. Inhibition of the rat AChE was achieved by incubation of a 10% diaphragm homogenate diluted threefold with 0.01 M phosphate buffer, pH 7.5, with 8 nM C(−)P(±)-soman or 5 nM C(+)P(±)-soman at pH 9.0 for 10 min (25°). Excess inhibitor was removed by further incubation for 1 hr at pH 10 (tabun) or by three extractions with an equal volume of hexane (soman). At these conditions, the human enzyme was inhibited for at least 95%, whereas the rat enzyme was inhibited for about 65% and 85% by C(−)P(±)- and C(+)P(±)-soman, respectively. Reactivation was started by addition of 4 vol. (human AChE) or 3.3 vol. (rat AChE) of an oxime solution in 0.05 M phosphate buffer, pH 7.5, to one volume of the inhibited enzyme solution. After incubation (25°) for 30 min (soman) or for various times (tabun), the reaction mixture of the human enzyme was diluted 10 times with the 0.05 M phosphate buffer. Next, enzyme activity (AIR_t) was assayed in triplicate in a 1.5-ml sample by using acetylthiocholine as a substrate, as described previously [12]. Reactivation of the rat enzyme was allowed to proceed for 45 min (25°). Then, 30 μl of a solution of the specific butyrylcholinesterase inhibitor ethopropazine (0.8 mg/ml 50% ethanol) was added to the reaction mixture (0.65 ml) and enzyme activity (AIR_t) was radiometrically determined in triplicate in a 200-μl sample by using ^{3}H-acetylcholine as a substrate. Blanks for the activity of the enzyme (A), of the enzyme in the presence of oxime (AR), and of the inhibited enzyme (AI) were run simultaneously.

Reactivation of (+)- and (−)-tabun-inhibited electric eel AChE. Inhibition (>95%) of AChE was achieved by incubation of the enzyme (final concentration 0.3 mg/ml, i.e. 0.1 μM of active sites) in a diluted solution of purified (+)-tabun (final concentration 2 μM) or by incubation of an enzyme solution (0.05 mg/ml) with (−)-tabun (0.1 μM) for 45 or 60 min, respectively, at 25°. The incubation medium was a 0.05 M veronal buffer, pH 7.5, containing 0.1 mg bovine serum albumine/ml. Excess inhibitor was removed by passing 0.5 ml of the incubate through a Sephadex G 25 column (30 × 0.9 cm). The column was eluted with 0.01 M phosphate buffer, pH 7.5, containing 0.1 M KCl. The fractions containing the enzyme were collected and filled up to 15 ml with the elution buffer to which albumin was added (final concentration 0.2 mg/ml). Reactivation was started by addition of 29 vol. ((+)-tabun) or 4 volumes ((−)-tabun) of an oxime solution in the elution buffer, containing 0.2 mg albumin/ml, to one volume of the inhibited enzyme solution. After various times of incubation (25°), samples were taken, diluted three times, and assayed for enzyme activity (AIR_t) in triplicate, as described for the experiments with the human enzyme. Blanks for activity of the enzyme (A), of the enzyme in the presence of oxime (AR), and of the inhibited enzyme (AI) were run simultaneously.

Reactivation of electric eel AChE inhibited by N-unsubstituted phosphoramidates. The reactivation experiments were carried out according to the methods described previously by Langenberg *et al.* [8].

Radiometric activity assay. The radiometric AChE activity assay was performed according to Johnson

and Russell [13] with some minor changes. The substrate ^{3}H-acetylcholine was purified before use by extracting a stock solution (0.2 ml, 7.4 MBq/ml water) diluted with water, pH 4.0 (0.8 ml), and acetic acid (0.01 ml), three times with toluene/isoamylalcohol (9:1, v/v; 5 ml) and next three times with ether (5 ml). Residual ether was removed by passing a stream of air through the acetylcholine solution. Subsequently, 0.03 M acetylcholine perchlorate (3 ml) was added. An aliquot (0.02 ml) of this substrate solution was mixed with an enzyme sample (0.2 ml) and incubated in a scintillation vial for 45 min at 30°. The enzymatic reaction was stopped by addition of 10 M acetic acid (0.1 ml). The liberated ^{3}H-acetate was extracted into scintillation mixture (4 ml of 0.5% PPO and 0.05% POPOP in toluene/isoamylalcohol, 9:1) added to the vial and counted in a liquid scintillation system (Tri-carb 4430, United Technologies Packard).

Calculations. Percentages of reactivation at time t (% react$_t$) were calculated according to

$$\% \text{ react}_t = \frac{\text{AIR}_t\,(\text{A/AR}) - \text{AI}}{\text{A} - \text{AI}} \times 100 \qquad (1)$$

The first-order rate constant (k) for reactivation of (+)- or (−)-tabun-inhibited AChE and the percentage of reactivation at infinite time (% react$_{max}$) were calculated together with their standard deviations by fitting the equation

$$\% \text{ react}_t = \% \text{ react}_{max}\,(1 - e^{-kt}) \qquad (2)$$

to the data obtained for % react$_t$ at 0.5, 1, 2, 3, 5 and 24 hr by least squares criteria.

RESULTS

Optical purity of (+)-tabun

The (+)-enantiomer isolated from (±)-tabun contained 10–15% (−)-tabun according to chiral gas chromatographic analysis. As the (−)-enantiomer is the more active inhibitor for AChE [1], a considerable part of the inhibited AChE formed by incubation with this (+)-tabun preparation may consist of enzyme inhibited by the (−)-enantiomer, preventing a proper study on oxime-induced reactivation of (+)-tabun-inhibited AChE. It was attempted to remove the (−)-enantiomer by binding to electric eel AChE. For this purpose, 10 μM (+)-tabun was treated with 1.8 μM AChE. The initially fast inhibition of the enzyme, 60% within 1 min, proceeds similarly to the initial course of the inhibition of the same enzyme concentration by 1.3 μM (−)-tabun (see Fig. 1). This result is in accordance with an original contamination by 10–15% (−)-tabun, which is rapidly bound to the enzyme. After incubation for 30 min, the enzyme was removed by ultrafiltration, yielding a purified (+)-tabun solution.

The course of enzyme inhibition taking place during AChE treatment suggests a high optical purity of the purified (+)-tabun. Its relatively low concentration did not allow a gas chromatographic analysis [1] of the optical purity. Additional information was obtained in the following manner. At the conditions to be used in the reactivation experiments, the inhibition of electric eel AChE (0.1 μM) with purified (+)-tabun was followed. Inhibition of the same enzyme concentration with an equimolar concentration of (−)-tabun occurs much faster (Fig. 1). This indicates that in the former experiment the concentration (−)-tabun, if present as a contaminant in purified (+)-tabun, is much lower than the concentration used of AChE. Consequently, the optical purity of the purified (+)-tabun is sufficiently high to form mainly (+)-tabun-inhibited AChE. Since hydrolysis of tabun takes place to a small extent only (half-life time: 198 ± 6 min) under these circumstances, the latter reaction will hardly affect the courses of the inhibition processes.

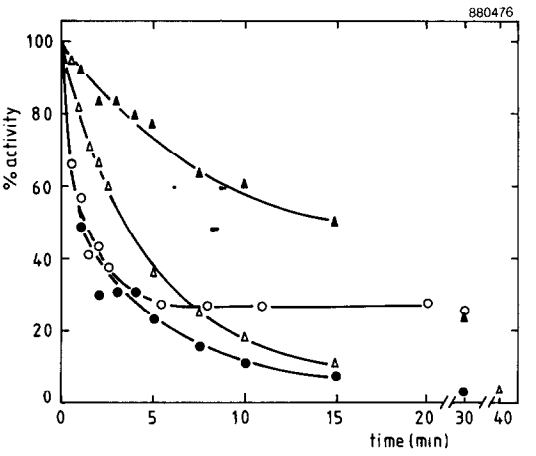


Fig. 1. Decrease of electric eel AChE activity with time, when incubating 1.8 μM enzyme with 10 μM non-purified (+)-tabun (●), 1.8 μM enzyme with 1.3 μM (−)-tabun (○), 0.11 μM enzyme with 1.5 μM purified (+)-tabun (▲), and 0.13 μM enzyme with 0.13 μM (−)-tabun (△), in 0.05 M veronal buffer, pH 7.5, at 25°.

Reactivation of (+)- and (−)-tabun-inhibited AChE

The data obtained for reactivation of both (+)- and (−)-tabun-inhibited electric eel AChE obey a description according to first-order kinetics (Eqn 2). Examples for reactivation by HLö-7 are given in Fig. 2. The parameters evaluated from these data are collected in Table 2. The calculated percentages of reactivation at infinite time indicate that complete reactivation is not achieved at the conditions used. We made similar observations in previous studies on (±)-tabun-inhibited bovine erythrocyte AChE [9, 14]. This phenomenon could be ascribed to simultaneous reactivation and conversion into a non-reactivatable form (the so-called aging reaction) of the inhibited enzyme taking place in the presence of oxime [14]. The susceptibilities to reactivation by various oximes of the two inhibited enzymes hardly differ. Benzyl-P2A shows a higher activity than the well-known reactivators TMB4 and obidoxime, similarly to previous results obtained for reactivation of (±)-tabun-inhibited bovine erythrocyte AChE [9]. At the oxime concentration used (3 μM), a reactivating effect is just measurable for P2S, but is not observed for HI-6. Interestingly, the recently developed HLö-7 shows substantial activity.

The reactivating properties of HLö-7 were further

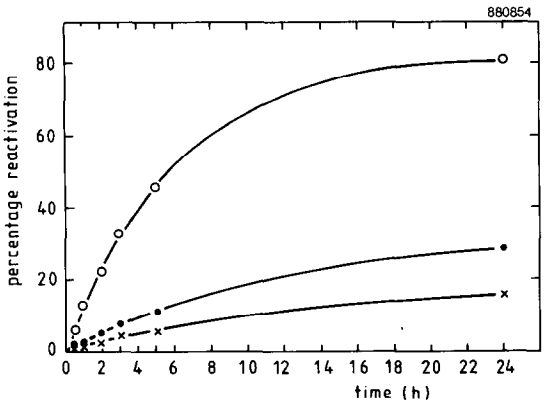


Fig. 2. Plots of percentages of reactivation of electric eel AChE inhibited by (+)-tabun (×) and (−)-tabun (●) and of human erythrocyte AChE inhibited by (−)-tabun (○) upon incubation with 3, 3, and 10 μM HLö-7, respectively, at pH 7.5 and 25°. The lines represent optimal fits of the equation for first-order kinetics (Eqn 2) to the data.

potency of TMB4 with that of the other oximes studied, the percentage of reactivation after 2 hr is used as a measure. The relative reactivating activities of most of the oximes for the inhibited human enzyme and for the inhibited electric eel AChE (Table 2) are similar. However, benzyl-P2A is more active than TMB4 for the inhibited electric eel enzyme, whereas these oximes show an equally high activity for the inhibited human enzyme.

Reactivation of AChE inhibited by N*-unsubstituted phosphoramidates*

Recently, Langenberg *et al.* [8] found that electric eel AChE inhibited by six *N*-unsubstituted derivatives of tabun is also much more rapidly reactivated by the classical oxime obidoxime than by HI-6, albeit that the differences in reactivating potencies of the oximes for these inhibited enzymes are less pronounced than for tabun-inhibited AChE. Therefore, in a second series of experiments on the reactivating potencies of HLö-7 its efficacy to reactivate these

Table 2. Calculated percentages of reactivation at infinite time (% $react_{max}$) and rate constants (k) for the reactivation of (+)- and (−)-tabun-inhibited electric eel AChE with 3 μM oxime at pH 7.5 and 25°

Oxime	(+)-Tabun-AChE % $React_{max}$	(+)-Tabun-AChE k (10^{-3} min^{-1})	(−)-Tabun-AChE % $React_{max}$	(−)-Tabun-AChE k (10^{-3} min^{-3})
Benzyl-P2A	78 ± 3	3.1 ± 0.3 (3)	86 ± 3	3.4 ± 1.1 (5)
TMB4	66 ± 3	1.5 ± 0.3 (3)	74 ± 5	1.8 ± 0.3 (5)
Obidoxime	45 ± 4	1.0 ± 0.1 (3)	50 ± 3	1.11 ± 0.05 (4)
HLö-7	23 ± 4	1.4 ± 0.1 (3)	31 ± 6	1.3 ± 0.1 (5)
P2S	5 ± 1*	— (3)	5 ± 1*	— (4)
HI-6	<5*	— (3)	<5*	— (3)

Mean values of the number of determinations denoted in parentheses are given with their standard deviations.
* Percentage of reactivation obtained after 24 hr of incubation.
—, Not calculated.

Table 3. Observed percentages of reactivation after 120 min (% $react_{120}$), calculated percentages of reactivation at infinite time (% $react_{max}$), and rate constants (k) for the reactivation of (−)-tabun-inhibited human erythrocyte AChE with 10 μM oxime at pH 7.5 and 25°

Oxime	% $React_{120}$	% $React_{max}$	k (10^{-3} min^{-1})
Benzyl-P2A	59 ± 14	95 ± 9	9.3 ± 2.8 (6)
TMB4	59 ± 5	—*	—* (4)
Obidoxime	39 ± 4	94 ± 10	4.8 ± 0.8 (6)
HLö-7	21 ± 3	77 ± 6	2.7 ± 0.3 (3)
P2S	3 ± 1	58 ± 5	0.58 ± 0.02 (3)
HI-6	<5†	—	— (3)

Mean values of the number of determinations denoted in parentheses are given with their standard deviations.
* No first-order kinetics.
† Percentage of reactivation obtained after 24 hr.
—, Not calculated.

investigated in three series of experiments. First, reactivation by 10 μM oxime of a human AChE inhibited by (−)-tabun was investigated (Table 3). Reactivation by TMB4 did not obey first-order kinetics. In order to compare the reactivating inhibited enzymes was studied. Results obtained for AChE inhibited by three representatives of these phosphoramidates are given in Table 4. Reactivation induced by the oximes proceeds much faster than the spontaneous reactivation shown by the inhibited enzymes. HLö-7 is at least as active as obidoxime. Similar results were obtained for reactivation of electric eel AChE inhibited by the three other phosphoramidates, i.e. 2-chloroethyl *p*-nitrophenyl phosphoramidate, *p*-nitrophenyl phosphorodiamidate, and *O*,*S*-dimethyl phosphoramidothioate (methamidophos).

Reactivation of C(+)P(±)- *and* C(−)P(±)*-soman-inhibited AChE*

In a third series of experiments the reactivating potency of HLö-7 for soman-inhibited AChE was studied. C(+)P(±)- and C(−)P(±)-soman inhibited AChEs from human erythrocytes and from rat diaphragm were used. The maximum percentages of reactivation induced by HLö-7, by obidoxime and by HI-6, the most potent reactivator of soman-inhibited AChE reported so far [12, 15], were determined. The results given in Table 5 reveal that HLö-7 is at least as active as HI-6 towards the two C(+)P(±)-

Table 4. Calculated percentages of reactivation at infinite time (% $react_{max}$) and rate constants (k, corrected for spontaneous reactivation*) for reactivation of electric eel AChE inhibited by the *p*-nitrophenyl esters of ethyl (I), 2-fluoroethyl (II), and 2,2,2-trifluoroethyl phosphoramidate (III), with 5 μM HLö-7 at pH 7.5 and 25°

	I-AChE		II-AChE		III-AChE	
Oxime	% $React_{max}$	k (10^{-3} min^{-1})	% $React_{max}$	k (10^{-3} min^{-1})	% $React_{max}$	k (10^{-3} min^{-1})
HLö-7	99 ± 3	36 ± 8	97 ± 2	200 ± 40	73 ± 3	170 ± 30
HI-6*	92 ± 4	15 ± 1	99 ± 2	22 ± 2	79 ± 5	19 ± 2
Obidoxime*	99 ± 2	26 ± 3	97 ± 3	110 ± 40	74 ± 4	170 ± 60

Data obtained previously* for 5 μM obidoxime and HI-6 are given for comparison. Mean values of 3 determinations are reported with their standard deviations.

* Data from Langenberg *et al.* [8]. Rate constants obtained for spontaneous reactivation of I-AChE, II-AChE and III-AChE are 1.9 ± 0.2, 5.0 ± 0.6 and 9.0 ± 0.9 10^{-3} min^{-1}, respectively.

Table 5. Percentages of maximum reactivation* induced by oxime (pH 7.5, 25°) in human erythrocyte AChE and rat diaphragm AChE following inhibition with C(+)P(±)- and C(−)P(±)-soman

	Reactivation with 30 μM oxime of human AChE inhibited with		Reactivation with 60 μM oxime of rat AChE inhibited with	
Oxime	C(+)P(±)-soman	C(−)P(±)-soman	C(+)P(±)-soman	C(−)P(±)-soman
HLö-7	75 ± 4 (6)	46 ± 8 (8)	92 ± 1 (4)	74 ± 5 (4)
HI-6	67 ± 6 (6)	23 ± 4 (8)	72 ± 4 (7)	29 ± 5 (5)
Obidoxime	12 ± 2 (5)	4 ± 2 (8)	13 ± 4 (8)	9 ± 4 (4)

Mean values of the number of determinations denoted in parentheses are given with their standard deviations.

* Time of reactivation: 30 min.

soman-inhibited enzymes and even considerably more active than HI-6 towards the C(−)P(±)-soman-inhibited enzymes. The latter enzymes are more resistant to oxime-induced reactivation, analogously to previous reactivation results obtained for soman-inhibited human and mouse AChEs [12, 15, 16].

DISCUSSION

AChEs inhibited by the (+)- and the (−)-enantiomer of tabun show similar susceptibilities to reactivation by oximes. As (−)-tabun is the more potent inhibiting enantiomer, this result might be interpreted as evidence for inhibition of AChE by (−)-tabun in the (+)-isomer preparation. However, strong evidence that reactivation of (+)-tabun-inhibited AChE was indeed investigated is given by the results presented in Fig. 1, i.e. the time course of AChE inhibition taking place during purification of the (+)-tabun preparation by enzymatic removal of (−)-tabun as well as the time course of AChE inhibition with the purified (+)-tabun isomer, in relation to inhibition experiments carried out with the (−)-tabun isomer.

So far, oxime-induced reactivation of AChE inhibited by resolved enantiomers has been studied for three other organophosphates having phosphorus as a chiral centre. Stereoselectivity of reactivation was found for AChE inhibited by VX (ethyl *S*-diisopropylaminoethyl methylphosphonothioate) [17] and by fonofos oxon (ethyl *S*-phenyl ethylphosphonothioate) [18], but was absent for AChE inhibited by ethyl *S*-propyl methylphosphonothioate. The observed absence of stereoselectivity in reactivation of tabun-inhibited AChE can be explained by one of the following two mechanisms. First, the organophosphate moieties attached to the active site of the enzyme have opposite configurations around phosphorus due to a similar stereochemical course of AChE inhibition by the two enantiomers.* It has to be assumed then that the two differently inhibited enzymes show similar properties in oxime-induced reactivation reactions. Second, the stereochemical courses of AChE inhibition are opposite—inversion of configuration at phosphorus for reaction with one enantiomer, but retention for reaction with the other—which results in the formation of the same inhibited enzyme from the two enantiomers. Evidence for this phenomenon was reported by Harvey *et al.* [17] for reaction of AChE with (+)- and (−)-ethyl *S*-propyl methylphosphonothioate. Oxime-induced reactivation of ethyl methylphos-

* Probably, the inhibition reaction occurs with inversion of configuration at phosphorus, by analogy with the sterochemistry of nonenzymatic reactions of organophosphates.

phonylated AChE formed with these two enantiomers proceeds similarly, whereas stereoselective reactivation was observed for the ethyl methylphosphonylated AChEs formed by reaction with the enantiomers of VX.

In agreement with earlier results [9], benzyl-P2A, TMB4 and obidoxime are potent reactivators for (±)-tabun-inhibited AChE, whereas P2S is a weak reactivator. The bispyridinium-2-oxime HI-6 does not show any activity at the concentration used. The HI-6 analogue HLö-7, in which an additional oxime group has been introduced at the 4-position in the same pyridinium ring, is substantially active as a reactivator of this inhibited enzyme. Reactivating potencies of obidoxime, HI-6 and HLö-7 towards AChE inhibited by *N*-unsubstituted phosphoramidates show a similar ranking. In connection with these findings it is interesting to mention that pyridinium-4-oximes having two quarternary nitrogen atoms are often more potent reactivators for (±)-tabun-inhibited AChE than the corresponding 2-oximes [9, 19–23]. It should be mentioned, however, that a few examples have been reported for which the 2-oxime was as active as the 4-oxime [22, 24] or even more active [21, 24]. The present results together with the results reported by Langenberg *et al.* [8] indicate that reactivation of AChE inhibited by *N*-unsubstituted phosphoramidates is also achieved more rapidly with bispyridinium-4-oximes than with bispyridinium-2-oximes.

Remarkably, HLö-7 is also a potent reactivator for soman-inhibited AChE. To the best of our knowledge, HLö-7 is the first compound reported in literature which restores the activity of phosphylated AChEs that are notoriously resistant to oxime-induced reactivation, i.e. the soman-inhibited and tabun-inhibited enzymes. Bispyridinium-2-oximes such as HI-6, which are the most potent reactivators towards the former inhibited enzyme [3], do not show any activity for the latter inhibited enzyme [4], whereas the reverse relationship has been found for benzyl-P2A, TMB4 and obidoxime [2, 9]. It is tempting to assume that HLö-7 is a potent reactivator of both inhibited enzymes due to the presence of structural elements akin to both the bispyridinium-2-oxime HI-6 and the bispyridinium-4-oxime obidoxime. *In vivo* studies will be needed to evaluate the usefulness of this oxime as a general therapeutic agent for treatment of intoxications by the nerve agents.

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