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STEREOSPECIFIC REACTIVATION BY SOME HAGEDORN-OXIMES OF ACETYLCHOLINESTERASES FROM VARIOUS SPECIES INCLUDING MAN, INHIBITED BY SOMAN

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Abstract—Reactivation by bispyridinium mono-oximes (Hagedorn-oximes) and some classical oximes (0.03 or 1 mM) was studied *in vitro* of rat, bovine and human erythrocyte acetylcholinesterase and of electric eel acetylcholinesterase inhibited by soman. Relative reactivating potencies of the oximes are similar for the three inhibited erythrocyte enzymes. In general, Hagedorn-oximes are more potent than the classical oximes. Among the Hagedorn-oximes, HI-6 is the most potent reactivator for the three inhibited enzymes. Relative reactivating potencies for the inhibited erythrocyte acetylcholinesterases and electric eel acetylcholinesterase, however, clearly differ.

Since the reactivation experiments were carried out with racemic soman, a mixture of the two inhibited enzymes may be formed, which may cause additional problems in the comparison of various results. In order to get more detailed information on differences between human erythrocyte and electric eel acetylcholinesterase, reactivation of these enzymes inhibited with the P(−)-isomers of C(+)- and C(−)-soman were studied separately. Reactivation appeared to be dependent on the chirality of the α-carbon atom in the pinacolyl group. HI-6 is by far the most potent reactivator for the human enzyme inhibited by the two P(−)-isomers. It is suggested that electric eel acetylcholinesterase is not a reliable model for *in vitro* testing of therapeutic potencies of oximes against soman intoxication in mammals.

Rate constants of aging of the four acetylcholinesterases inhibited with racemic soman and of the human and eel enzyme inhibited by the P(−)-isomers of C(+)- and C(−)-soman were also determined. The aging of the inhibited rat enzymes proceeds remarkably slowly ($t_{\frac{1}{2}}$ = 21 min). The rate of aging is not affected by the chirality on the α-carbon atom in the pinacolyl group.

Consequences of the present results are discussed in view of extrapolation of reactivation data of a series of reactivators to their relative therapeutic effect, ultimately in man. It is speculated that the more rapid aging of the human inhibited enzyme may hamper oxime-therapy in man more seriously than in rat.

The mammalian toxicity of certain organophosphates, such as organophosphorus pesticides and the so-called nerve agents, is mainly based on their ability to inhibit acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by phosphylating the enzyme at its active site. Reactivation of the inhibited enzyme by displacing the phosphyl moiety affords a causal therapy of the organophosphate intoxication. Potent reactivators, such as the pyridinium oximes P2S, TMB4 and obidoxime (see Fig. 1 for chemical structures of the oximes), have been developed for acetylcholinesterase inhibited by various organophosphates.

Acetylcholinesterase inhibited by soman (1,2,2-trimethylpropyl methylphosphonofluoridate, see Fig. 2) is less accessible to reactivation by these oximes, which is at least partially due to a rapid transformation of the inhibited enzyme into a non-reactivatable form (aging). Some years ago, a group of bispyridinium mono-oximes (see Fig. 1 for chemical structures of some representatives) was synthesized by Hagedorn and co-workers which proved to be effective in therapy in rodents and dogs [1–10], and in reactivation of soman-inhibited acetylcholinesterase *in vitro* [11, 12]. Some of the most potent representatives of the series, HI-6, HS-6 and HGG-52, although being quite effective in restoring the neuromuscular transmission in rat diaphragm and intercostal muscle *in vitro* after soman administration, were found of negligible activity in restoring the neuromuscular function of intercostal muscle of man after soman administration *in vitro* [13, 14].

Differences in reactivatability of soman-inhibited cholinesterases of the muscle preparations was reported as one of the possible underlying mechanisms for the discrepancy between the two species. This finding prompted us to initiate an *in vitro* study on the reactivation of soman-inhibited acetylcholinesterases isolated from various species. As a first approach a study was carried out with the erythrocyte enzyme of rat, man and cow and with acetylcholinesterase from electric eel. The two latter acetylcholinesterases were included as these are most frequently used in *in vitro* studies. In addition to Hagedorn-oximes some well-known "classical" oximes were also tested as reactivators.

Additional problems in the comparison of reactivation results in different species may arise as a consequence of stereoisomerism in soman. Due to two chiral centres, the α-carbon atom of the pinacolyl group (C) and the phosphorus atom (P) (see Fig. 2), soman consists of four stereoisomers. Only the

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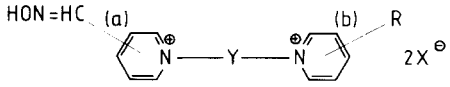

Code name	a	Y	b	R	X
HI-6	2	CH_2OCH_2	4	$C(O)NH_2$	Cl
HS-6	2	CH_2OCH_2	3	$C(O)NH_2$	Cl
HGG-12	2	CH_2OCH_2	3	$C(O)C_6H_5$	Cl
HGG-21	2	CH_2OCH_2	3	$C(O)CH_3$	I
HGG-42	2	CH_2OCH_2	3	$C(O)C_6H_{11}$	I
HGG-52	2	CH_2OCH_2	3	$C(O)CH_2CH(CH_3)_2$	Cl
CHS-6	2	$(CH_2)_3$	3	$C(O)NH_2$	Br
Obidoxime	4	CH_2OCH_2	4	CH=NOH	Br
TMB4	4	$(CH_2)_3$	4	CH=NOH	Cl
P2S	2	CH_3	—	— (monopyridinium compound)	CH_3SO_3

Fig. 1. Chemical structures of Hagedorn-oximes and of "classical" oximes used in this study.

two P(−)-isomers are potent inhibitors of acetylcholinesterase [15, 16]. Hence, upon inhibition of enzyme by treatment with racemic soman, a mixture of two [C(+)P(−)- and C(−)P(−)-soman-]inhibited enzymes may be generated. In order to make a more straightforward comparison of the reactivatability of the human erythrocyte and electric eel inhibited acetylcholinesterases, reactivation of these enzymes inhibited by the P(−)-isomers of C(+)- and C(−)-soman was studied separately with a limited number of oximes.

MATERIALS AND METHODS

Materials. Rat blood was obtained from male Wistar (WAG) rats, bred in the Medical Biological Laboratory TNO under SPF conditions, by heart puncture under nembutal anaesthesia. Blood was collected in a syringe containing 0.2 ml of a heparin solution (5000 IE/ml). Human blood was obtained from volunteers of our laboratory by venapuncture. Blood was collected in heparinized vessels. Blood pooled from at least three animals or individuals was used.

Bovine erythrocyte and electric eel acetylcholinesterase were preparation type I and preparation type VI-S, respectively, from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

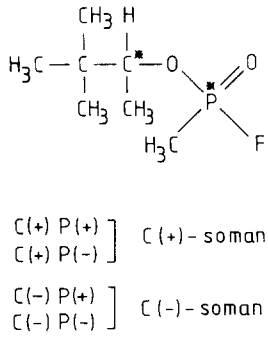

Fig. 2. Four stereoisomers of soman. C stand for the chiral α-carbon atom in the pinacolyl moiety and P for the chiral phosphorus atom.

Racemic soman, C(+)- and C(−)-soman were synthesized in this laboratory and were at least 99% pure (GLC). The optically pure C(+)- and C(−)-soman were synthesized from the (+)- and (−)-enantiomers of pinacolyl alcohol, respectively. The resolution of the alcohol and the subsequent synthesis of the organophosphates were described previously by Benschop *et al.* [16].

P2S was purchased from Dr. F. Raschig, GmbH (F.R.G.) and obidoxime from E. Merck (Darmstadt, F.R.G.).

The bispyridinium mono-oximes having an ether bridge were synthesized in this laboratory according to methods described by Schoene [17] and by Hagedorn [18]. CHS-6 was prepared by alkylating 2-hydroxyiminomethylpyridine with 1-(3-iodopropyl)-3-carboxamido-pyridinium iodide. TMB4 was prepared according to Poziomek *et al.* [19].

All other reagents were commercial products of an analytical grade.

Isolation of human and rat erythrocyte acetylcholinesterase. Erythrocyte ghosts were prepared according to the modified procedure of Dodge *et al.* [20] as described by Hanahan and Ekholm [21], except that phosphate buffers, pH 7.4, were used instead of Tris buffers. The solubilization of acetylcholinesterase was carried out by suspending erythrocyte ghosts obtained from 40 ml 50% erythrocyte suspension, in 10 ml 5 imOsM* phosphate buffer, pH 7.4, containing 1% Triton X-100, according to Miller [22]. The suspension was centrifuged for 1 hr at 100,000 *g* and the supernatant collected. The enzyme preparation obtained in this manner was stable for several months when stored at −20°.

Reactivation experiments. Inhibited human and rat erythrocyte acetylcholinesterases were obtained by incubating a mixture of 50 vol of a 10-fold dilution of the enzyme preparation in 0.01 M veronal buffer, pH 10, with one volume of a solution of the soman preparation in water for 60 min at pH 10.0 and 25° in a Radiometer pH-stat equipment. The final concentrations of racemic, C(+)- and C(−)-soman were 5.5 and 9.5 nM, respectively.

* imOsM, ideal milliosmolarity (see Dodge *et al.* [20]).

Inhibited bovine erythrocyte and electric eel acetylcholinesterases were formed by incubating a mixture of 100 vol of a solution of 10 mg bovine enzyme/ml (approx. 50 nM of active sites) in 6.6 mM veronal buffer or of a solution of 55 μg eel enzyme/ml (approx. 20 nM of active sites) in 0.01 M phosphate buffer, containing 0.1 M potassium chloride and 2 mg bovine serum albumin/ml, with 1 vol of a solution of the soman preparation in isopropanol for 30 min in a Radiometer pH-stat equipment at 25° and pH 10.2 or 10.0, respectively. The final concentration of racemic soman was 300 (bovine) or 50 nM (eel); the final concentrations of C(+)- and C(−)-soman for the inhibition of the eel enzyme were 50 and 70 nM, respectively. At the conditions used premature aging of the inhibited enzymes is slowed down appreciably, and at the same time the excess of inhibitor is removed by hydrolysis. In control experiments without inhibitor incubation of the enzymes for 1 hr at pH 10.2 (bovine) or 10.0 and 25° did not influence their activities, except for the activity of the rat enzyme which decreased about 10%. The removal of excess inhibitor was checked from the absence of inhibitory effect of a 2-fold (rat, human) or 10-fold (bovine, eel) diluted inhibited enzyme solution on fresh acetylcholinesterase during incubation for at least 1.5 hr at pH 7.5 and 25°.

Rat, bovine and eel enzyme were inhibited for at least 90% by the treatment used, whereas the human enzyme was inhibited for 60–70% with racemic soman and C(+)-soman and for 50–55% with C(−)-soman. Reactivation was started by addition of 1 vol of the inhibited rat or human enzyme solution to 1 vol of an oxime solution in 0.05 M veronal buffer, pH 7.1, or by addition of 1 vol of the inhibited bovine or eel enzyme solution to 9 vol of an oxime solution in 0.05 M phosphate buffer, pH 7.5, or in 0.01 M phosphate buffer, pH 7.5, containing 0.1 M potassium chloride and 0.2 mg albumin/ml, respectively.

The enzymes were allowed to reactivate at pH 7.5 and 25° for 30, 45 or 60 min. Then, a sample of 0.2 ml (human), 0.8 ml (rat) or 60 μl (bovine, eel) was taken and added to 0.05 M veronal buffer, pH 7.5, containing 0.05% Triton X-100 (human, rat), to 0.05 M phosphate buffer, pH 7.5 (bovine) or to 0.01 M phosphate buffer, pH 7.5, containing 0.1 M potassium chloride and 0.2 mg albumin/ml, (eel) up to a final volume of 5 ml. Next, the restored enzyme activity (AIR) was determined in triplicate by incubating a 1.5 ml-sample of these mixtures with 4 ml of a solution of 1 mM acetylthiocholine iodide and 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.05 M phosphate buffer, pH 7.5, for 30 min (rat) or 10 min at 30° and by subsequently measuring the absorbance of this incubation mixture at 412 nm. The entire procedure, i.e. addition of substrate to sample, incubation at 30° and colorimetric determination, was performed with a Prestomatic analyser, model 8 (Meyvis and Co., Bergen op Zoom, The Netherlands). Enzyme activities were corrected for spontaneous hydrolysis and for oxime-induced hydrolysis of the substrate.

Blanks for the activity of the enzyme (A), for the activity of the enzyme incubated with oxime (AR) and for the activity of the inhibited enzyme (AI) were run in a similar manner. The percentage of reactivation obtained (% react) was calculated according to

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

Aging experiments. A solution of inhibited enzyme was obtained as described for the reactivation experiments. Aging was started in the same way as described for the initiation of reactivation by mixing of an inhibited enzyme solution with a buffer solution, except that no oxime had been added to the buffer solution. The inhibited enzyme was allowed to age at pH 7.5 and 25°. Aging was determined from the decrease of attainable reactivation. Samples of 0.2 ml were taken every 0.5 min (human, eel) or every 1 min (bovine) during the first 4 or 6 min and subsequently at larger intervals up to 14 or 30 min, respectively. Ten samples of 0.8 ml of the rat inhibited enzyme were taken during 30 min reaction. The samples were added to 50 μl of an oxime solution. The oxime solutions used were 5 mM HI-6 or 1.7 mM HI-6 in 0.05 M veronal buffer, pH 7.5, containing 0.05% Triton X-100, or 5 mM HI-6 in 0.05 M phosphate buffer, pH 7.5, in case of human, rat or bovine enzyme, respectively. Reactivation of the eel enzyme inhibited with racemic, C(+)- or C(−)-soman was performed with 50 μl of a solution of 25 mM obidoxime, 5 mM HI-6 or 15 mM obidoxime, respectively, in 0.01 M phosphate buffer, pH 8.1, containing 0.1 M potassium chloride and 0.2 mg albumin/ml. After 30 min of incubation at 25° the reactivation mixtures of rat and human enzyme were filled up to 5 ml with 0.05 M veronal buffer, pH 7.5, containing 0.05% Triton X-100. A 75 μl-sample of the reactivation mixtures with bovine or eel enzyme was diluted to 5 ml with 0.05 M phosphate buffer, pH 7.5, or with 0.01 M phosphate buffer, pH 7.5, containing 0.1 M potassium chloride and 0.2 mg albumin/ml, respectively.

The determination of the restored enzyme activity (AIR) was carried out as described for reactivation experiments. Blanks for the activity of enzyme (A), of enzyme in the presence of the oxime (AR), and of the inhibited enzyme at the start of the aging reaction (AI) were run in a similar manner. Values of percentage of reactivation (% react) were calculated according to equation (1). Rate constants of aging were calculated from plots of ln(% react) vs time by means of the method of least-squares.

RESULTS

Although the formation of inhibited enzyme and the removal of excess soman were carried out at pH 10.0 or 10.2, part of the inhibited enzyme may be aged already at the start of the reactivation. This part of the inhibited enzyme was estimated from the rate of aging at pH 10.2 (bovine enzyme) or pH 10.0 and 25°. For that purpose the solution of inhibited enzyme was incubated after formation of the inhibited enzyme for a subsequent 2 hr at pH 10.2 or 10.0 and 25°. During this time the reactivatability of the inhibited enzyme was determined every 15 min. From the results it was estimated that at the start of reactivation less than 5% of the inhibited rat and bovine enzyme, but about 20% of the inhibited

human and eel enzyme were in a non-reactivatable form. So, about 80% of reactivation will be maximally attainable for the latter two inhibited enzymes. The order of relative potencies of the oximes for reactivation of these inhibited enzymes will not be affected by the percentage of aged enzyme present already at the start of reactivation.

Maximum reactivation was obtained within 30 min of oxime-induced reactivation of inhibited human and eel acetylcholinesterase and within 45 min of reaction with the inhibited rat and bovine acetylcholinesterase. Previously [12], we showed that incomplete reactivation of inhibited bovine enzyme is due to simultaneously proceeding aging. It is reasonable to assume that reactivation of the other inhibited acetylcholinesterases is also adversely affected by the simultaneous occurrence of this reaction.

The percentages of reactivation obtained with 0.03 mM of oxime for the three erythrocyte enzymes after incubation with racemic soman are given in Table 1. The relative potencies of the oximes for reactivation of the three inhibited erythrocyte enzymes are almost identical. The Hagedorn-oximes are more potent than the classical oximes, obidoxime, TMB4 and P2S. Among the Hagedorn-oximes HI-6 is by far the most active compound. In preliminary experiments the reactivation induced by 0.03 mM of some of the oximes in the inhibited eel enzyme was very low (5% or less). The reactivating potencies of the oximes for this inhibited enzyme were, therefore, tested at a higher concentration (1 mM). For comparison reactivation experiments of the inhibited human enzyme were also carried out with the oximes at this concentration (Table 1). The relative potencies of the oximes when used at the lower (0.03 mM) and at the higher (1 mM) concentration are similar for the reactivation of the human inhibited enzyme. The relative reactivating potencies of the oximes for this inhibited enzyme and for the inhibited eel enzyme, however, clearly differ. For instance, for the latter inhibited enzyme HGG-21 is the most potent reactivator, whereas HI-6, which is the most potent towards the human inhibited enzyme, shows the same activity as HS-6 and HGG-42.

It might be possible that upon incubation with racemic soman at pH 10 the eel enzyme is predominantly inhibited by one of the two P(−)-isomers, whereas the human enzyme mainly reacts with the other P(−)-isomer. If so, the different behaviour of the inhibited enzymes from the two species towards oxime-induced reactivation might be due to stereospecificity of the reactivation reaction rather than to different inherent properties of the acetylcholinesterases from the two species. In order to examine this possibility as well as to make a more straightforward comparison of the reactivatabilities of the human and eel inhibited enzymes these acetylcholinesterases were inhibited by C(+)- and C(−)-soman. In this way, the reactivation of C(+)P(−)-soman-inhibited and C(−)P(−)-soman-inhibited enzymes could be studied separately. The results of the studies with some of the oximes are given in Table 2.

Both acetylcholinesterases inhibited with C(−)-soman are much more refractory to oxime reactivation than the enzymes inhibited with C(+)-soman, except for the remarkably high percentage of reactivation induced by HI-6 in the C(−)-soman-inhibited human enzyme. Intermediate percentages of reactivation are obtained for the enzymes after inhibition with racemic soman. Apparently, a mixture of C(+)P(−)-soman- and C(−)P(−)-soman-inhibited enzymes is formed upon incubation with racemic soman at pH 10, reflecting the relative rate constants for inhibition by the two stereoisomers. The results clearly show the different reactivatabil-

Table 1. Percentages of maximum reactivation* of rat, bovine and human erythrocyte acetylcholinesterases and of electric eel acetylcholinesterase after inhibition with racemic soman on incubation with 0.03 or 1 mM of oxime at pH 7.5 and 25°

Oxime	% Reactivation by 0.03 mM oxime of soman-inhibited erythrocyte enzymes of			% Reactivation by 1 mM oxime of soman-inhibited enzymes of	
	Rat†	Cow†	Man‡	Human erythrocyte‡	Electric eel†
HI-6	70 ± 3	37 ± 2	46 ± 4 (4)	66 ± 1 (6)	30 ± 1
HS-6	35 ± 2	17 ± 1	26 ± 3 (5)	53 ± 2 (3)	30 ± 2
HGG-21	30 ± 2	16 ± 2	23 ± 1 (3)	47 ± 5 (5)	35 ± 3
HGG-52	27 ± 2	15 ± 3	24 ± 3 (3)	44 ± 5 (4)	23 ± 1
HGG-42	20 ± 1	14 ± 2	20 ± 2 (3)	37 ± 3 (4)	30 ± 1
HGG-12	17 ± 2	8 ± 1	15 ± 4 (4)	34 ± 4 (4)	23 ± 1
CHS-6	8 ± 1	3 ± 1	6 ± 1 (4)	27 ± 1 (3)	16 ± 1
Obidoxime	13 ± 2	3 ± 1	6 ± 2 (5)	29 ± 4 (4)	22 ± 1
TMB4	17 ± 1	4 ± 1	7 ± 2 (4)	32 ± 3 (3)	24 ± 1
P2S	2 ± 1	1 ± 1	0 ± 1 (4)	14 ± 3 (3)	15 ± 1

* Mean values ±standard error. The values are not corrected for the percentage of aged inhibited enzyme which was already present at the start of the reactivation due to premature aging taking place during the formation of the inhibited enzyme at pH 10.2 (bovine enzyme) or 10.0. These percentages are less than 5% for the rat and bovine enzyme and about 20% for the human and eel enzyme (see also text).

† Averages of three determinations.

‡ Number of determinations is given within parentheses.

Table 2. Percentages of maximum reactivation of human erythrocyte and of electric eel acetylcholinesterase after inhibition with C(+)-soman, C(−)-soman and racemic soman on incubation with 1 mM oxime at pH 7.5 and 25°

Oxime	Percentage reactivation* of Human enzyme inhibited with C(+)-soman	C(−)-soman	rac. soman	Electric eel enzyme inhibited with C(+)-soman	C(−)-soman	rac. soman‡
HI-6	73 ± 4	61 ± 4	66 ± 1†	45 ± 1†	7 ± 1	30 ± 1
HGG-42	55 ± 2	7 ± 3	37 ± 3	44 ± 2	7 ± 1	30 ± 1
Obidoxime	40 ± 3	11 ± 2	29 ± 4	28 ± 3	11 ± 1	22 ± 1

* Mean values ± standard error; averages of 4 determinations unless otherwise stated. The values are not corrected for the percentage of aged inhibited enzyme which was already present at the start of the reactivation (see Table 1).

† Average of 6 determinations.

‡ Averages of 3 determinations.

ities of the inhibited human and eel enzymes. HI-6 is more potent than the two other oximes in reactivating both inhibited human enzymes, whereas this oxime exhibits the same activity as HGG-42 towards both the C(+)- and the C(−)-soman-inhibited eel enzyme. For the latter inhibited enzyme obidoxime is even slightly more active.

A reaction counteracting reactivation, especially of soman-inhibited acetylcholinesterase, is aging. Rate constants of this reaction were determined in separate experiments. Aging of the bovine, human and eel enzyme inhibited by racemic soman proceeds according to first-order kinetics during at least three half-life times of reaction. From the reactivation studies it was shown that mixtures of the C(+)P(−)- and C(−)P(−)-soman-inhibited enzymes are present upon incubation of the human and eel enzyme with racemic soman at pH 10. The kinetic course of aging of these mixtures by first-order reaction implies aging of the C(+)P(−)- and C(−)P(−)-soman-inhibited enzymes at about the same rate. In agreement with this, the rate constants of aging of the C(+)P(−)- and the C(−)P(−)-soman-inhibited acetylcholinesterases of man and of electric eel are not significantly different nor deviate to a large extent from the rate constants obtained with the enzymes inhibited with racemic soman (Table 3). The aging of the soman-inhibited rat enzyme proceeds relatively slowly (Table 3). To evaluate the rate constant the reaction was followed for about 1.5 half-lives during which period no deviation from first-order kinetics was observed.

DISCUSSION

Reactivation and aging studies with the inhibited human and erythrocyte acetylcholinesterases were performed in the presence of 0.05% Triton X-100. The concentration of the detergent in the activity assays was 0.013%. At these conditions chosen on the basis of the work of Brodbeck and co-workers [23–26] properties of the solubilized enzymes may correspond more closely to those of the enzymes when bound to membranes of the erythrocytes. These investigators showed that human erythrocyte acetylcholinesterase is present as a dimer both in a solution of a detergent at concentrations above its critical micellar concentration (0.016% in case of Triton X-100) and in enzyme–lipid complexes. Moreover, they found that the enzyme exhibits its full catalytic activity in sufficiently concentrated detergent solutions (at least 0.006% in case of Triton X-100).

The Hagedorn-oximes generally exhibit a higher reactivating potency than the "classical" oximes towards the three erythrocyte acetylcholinesterases inhibited by soman. This finding corresponds to the reactivation results previously obtained for the soman-inhibited bovine enzyme with a limited number of these oximes [12]. HI-6 is by far the most potent oxime. The relatively high potency of this oxime is especially pronounced in reactivation of the human enzyme inhibited by C(−)-soman. The latter inhibited enzyme is more resistant to reactivation by HGG-42 and obidoxime than the corresponding C(+)-soman-inhibited enzyme, whereas only a slight stereospecificity is observed for reactivation by HI-6.

It should be expected that the efficacy of the reactivation reaction increases with decreasing rate of simultaneously proceeding aging reaction. Consistently, the inhibited rat enzyme is reactivated the most readily and at the same time exhibits the slowest aging of the inhibited enzymes from the four species

Table 3. Rate constants of aging at pH 7.5 and 25° of rat, bovine and human erythrocyte and electric eel acetylcholinesterase after inhibition with racemic soman, C(+)-soman and with C(−)-soman

Enzyme inhibited with	Rate constant* (min^{-1}) of inhibited acetylcholinesterase of Rat	Cow	Man	Electric eel
Rac. soman	0.033–0.033	0.13–0.13	0.54–0.66	1.0–1.1
C(+)-soman			0.50–0.59	1.0–1.2
C(−)-soman			0.41–0.51	0.9–1.1

* Values of duplicate experiments.

studied, whereas the opposite is found for the inhibited eel enzyme. However, a further correlation between the reactivatability of the inhibited enzymes (rat > human > bovine > eel) and their half-lives of aging (rat > bovine > human > eel) is not observed.

The fast aging of the inhibited human and eel acetylcholinesterase is in agreement with data reported by Harris *et al.* [27] and Michel *et al.* [28], respectively. The aging of rat erythrocyte acetylcholinesterase proceeds remarkably slowly ($t_{\frac{1}{2}}$ = 21 min). A relatively slow rate was also found for *in vivo* aging of inhibited rabbit erythrocyte acetylcholinesterase ($t_{\frac{1}{2}}$ = 16 min) [29] and for aging of the inhibited rat diaphragm enzyme ($t_{\frac{1}{2}}$ = about 30 min) [13].

The rate of aging is hardly dependent on the configuration at the α-carbon atom of the pinacolyl group in the phosphonyl moiety of the human as well as the eel inhibited enzyme. Similar results were reported by Keijer and Wolring [30] for soman-inhibited bovine erythrocyte acetylcholinesterase. Apparently, the orientation around the α-carbon atom of the pinacolyl group does not affect the course of the monomolecular aging reaction of the inhibited enzyme, but does affect the nucleophilic attack by an oxime on the phosphorus atom of the inhibited enzyme, in agreement with more strict stereochemical demands of bimolecular reactions.

One of the goals of reactivation studies *in vitro* is to find a reliable model in order to predict the relative efficacies of a series of compounds as therapeutic agents for organophosphate intoxication, ultimately in man. The present work shows that the relative reactivating potency *in vitro* of a series of compounds may depend on the species from which the acetylcholinesterase has been isolated. In this respect the eel enzyme clearly behaves differently from the mammalian enzymes studied. We, therefore, suggest that reactivation of inhibited eel acetylcholinesterase is not a reliable model for testing putative therapeutic agents for soman intoxication in mammals.

The soman-inhibited erythrocyte acetylcholinesterases of rat and man are substantially reactivated, especially by HI-6. The rate constants of aging of the two inhibited enzymes, however, differ at least one order of magnitude. It is tempting to speculate that the more rapid aging of the human inhibited enzyme may hamper oxime-therapy in man more seriously than in rat. Similar properties of the inhibited acetylcholinesterases of human and rat muscle preparations might explain the differences found for oxime-induced recovery of the neuromuscular transmission in the muscle preparations after soman administration *in vitro* [13, 14], as follows. Unlike the conditions used in the present study to preclude premature aging to a large extent (pH 10), *in vitro* inhibition of acetylcholinesterases of muscle preparations was carried out at physiological conditions. Then, the inhibited human muscle enzyme may be almost completely in a non-reactivatable form already at the start of oxime treatment due to its rapid aging, whereas a substantial part of the inhibited rat enzyme may still be reactivatable. However, up to now no data are available to establish whether the properties of inhibitied mammalian erythrocyte acetylcholinesterase are representative for other acetylcholinesterases of the same species, e.g. from muscles and brain. Some indications in support of this similarity have been reported in the literature. Both rat erythrocyte (present work) and rat diaphragm acetylcholinesterase [13] inhibited by soman age at comparably slow rates. A high level of homology was found between human erythrocyte and human intercostal acetylcholinesterase [31] and between human erythrocyte and human membrane-bound brain acetylcholinesterase [32] as revealed by immunological cross reactivity. However, the ability of soman-inhibited rat erythrocyte acetylcholinesterase to be readily reactivated *in vitro* (present work) on one hand and the absence of HI-6-induced reactivation *in vivo* of brain acetylcholinesterase in soman-poisoned rats [33, 34] on the other hand seem not be bear out the assumption of similar properties of various acetylcholinesterases of the same species. Further investigations to evaluate soman-inhibited acetylcholinesterases as a model for testing the efficacy of therapeutic reactivators are now in progress.

Recently, Benschop *et al.* [16] presented indications that mice challenged subcutaneously with 1 LD50 of racemic soman are killed primarily by the C(−)P(−)-isomer. In the present study it is found that upon *in vitro* inhibition with racemic soman a mixture of C(+)P(−)- and C(−)P(−)-soman-inhibited acetylcholinesterases is formed which have substantially different reactivatabilities. These findings suggest that data obtained from reactivation of acetylcholinesterases inhibited *in vitro* by racemic soman may not be adequate for extrapolation to an *in vivo* therapeutic effect of the reactivator. We therefore conclude that in addition to information about the dependence of the reactivatability on the acetylcholinesterase studied, data from separate reactivation studies of C(+)P(−)- and C(−)P(−)-soman-inhibited enzymes could be necessary when aiming at extrapolation to the therapeutic effect of a series of oximes, ultimately in man.

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