Biochemical Pharmacology, Vol. 33, No. 22, pp. 3573–3577, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

AGING AND REACTIVATABILITY OF PLAICE CHOLINESTERASE INHIBITED BY SOMAN AND ITS STEREOISOMERS

GÖRAN BUCHT and GERTRUD PUU*
Division of Experimental Medicine, National Defence Research Institute, Dept. 4, S-901 82 UMEÅ, Sweden

(*Received* 16 *February* 1984; *accepted* 9 *May* 1984)

Abstract—A simple and rapid method to study aging of soman-inhibited cholinesterases was developed. The method was applied to study the aging characteristics of soman-inhibited cholinesterase from the muscles of the plaice (*Pleuronectes platessa*). The orientation of the soman molecule in the active site is decisive both for the rate of aging and the degree of reactivation of unaged enzyme, a conclusion reached by using soman stereoisomers. Fluoride ions were found to affect reactivatability as well as aging rate.

Cholinesterases (EC 3.1.1.7 and EC 3.1.1.8) inhibited by organophosphates, binding covalently to a serine residue in the active site of the enzyme, can be reactivated by nucleophiles such as pyridinium aldoximes. Some inhibitor–enzyme complexes are, however, not reactivatable by oximes after some time. The explanation for this phenomenon first reported by Hobbiger in 1955 [1] is probably an enzyme-mediated dealkylation of the bound organophosphate [2, 3]. The process is called "aging" of the inhibitor–enzyme complex. The rate of the aging process is dependent on, for example, structure of the inhibitor, enzyme species, pH, temperature and ionic strength [4–7].

Soman-(1,2,2-trimethyl propyl methyl fluoro phosphonate) inhibited cholinesterases age very rapidly, with half-lives of a couple of minutes [3, 6]. The failure to counteract soman poisoning with oxime therapy in experimental animals is usually attributed to the fast formation of aged, unreactivatable inhibitor–enzyme complexes.

It has, however, been suggested by Bošković [8] and Heilbronn and Tolagen [9], that not only aging but also steric hindrance could be of importance for the inability of oximes to regenerate active enzyme.

We now report aging and reactivatability of cholinesterase from muscles of the plaice (*Pleuronectes platessa*), inhibited by soman. A very simple and rapid method to study aging is described. It is shown, by using stereoisomers of soman, that the orientation of bound soman is of importance both for aging rate and reactivatability. Both parameters are affected by fluoride ions.

MATERIALS AND METHODS

Soman (purity not less than 99%) was synthesized at the Chemistry Department of this institute.

Dr. Bengt Stridsberg also synthesized C_-- and C_+-soman: pinacolyl alcohol was resolved via its half-ester of phthalic acid using brucine (in acetone) and dehydroabiethyl amine (in ethanol) as resolving agents. Basic hydrolysis gave the *S* (+)- and *R* (−)-enantiomers, respectively, of pinacolyl alcohol with the observed optical rotations $\alpha_D^{25} = 6.390$ (neat), 99% optical purity, and $\alpha_D^{25} = -6.321$ (neat), 98% optical purity. The optical purity was determined with ^{1}H-NMR and a chiral lanthanide shift reagent.

The C_-- and C_+-isomers of soman were prepared in the usual manner from the alcohol and a mixture of methylphosphonic dichloride and difluoride. The observed optical rotations were C_--soman $\alpha_D^{25} = -12.967$ (neat) and C_+-soman $\alpha_D^{25} = 13.094$ (neat). ^{1}H- and ^{31}P-NMR analysis showed no change in optical purity.

Cholinesterase from the plaice (*Pleuronectes platessa*) was partially purified using a slight modification of the method described by Lundin *et al.* [10].

All chemicals used were of analytical grade. Filter paper (Munktell No. 3) was from Grycksbo Pappersbruk, Sweden.

Percentage reactivation and the rate constants of aging were calculated with the method suggested by Keijer *et al.* [7]:

$$\% \text{ reactivation} = \frac{\text{EIR} \times \frac{\text{E}}{\text{ER}} - \text{EI}}{\text{E} - \text{EI}} \times 100$$

where E is enzyme activity in the absence of inhibitor and reactivator, ER enzyme activity in the presence of reactivator, EI enzyme activity after inhibition and EIR enzyme activity after inhibition and reactivation.

Enzyme (5 mg/ml) was dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Four samples, with a volume of 25 μl, were withdrawn, put on filter papers and dried.

Soman was added to the remaining enzyme solution to give a final concentration of 2×10^{-7} M. Two samples of 25 μl were withdrawn in a time series, the first pair at 0.5 min after soman and the

* To whom all correspondence should be addressed.

following ones at every third minute. The samples were put on strips of filter paper and immediately dried with a hair-drier in a fume-hood. The temperature at the papers was about 32° during drying. By using this procedure, aging was stopped and excess of soman removed.

When the time series was finished, the papers were transferred to tubes for elution. To two of the control samples and to one sample from each of the points in the time series, 1 ml 0.1 M sodium phosphate buffer pH 7.4 was added (to give E in duplicate and EI). To the other two control samples and samples from the time series 1.0 ml 0.1 mM Toxogonin in sodium phosphate buffer was added (to give ER in duplicate and EIR). Elution and reactivation took place during four hours. Enzyme activity was measured by Ellman's procedure [11], using acetylthiocholine iodide as substrate. Corrections were made for blanks, including the reaction between Toxogonin and DTNB.

When the effect of various compounds was investigated, the compound under study was added to the enzyme solution before withdrawal of enzyme for E and ER determinations and before addition of soman. Thus, both inhibition and aging took place in the presence of effector. Usually, a concentration corresponding to the I_{50}-value of the compound was chosen. The concentration was reduced 40-fold during reactivation.

For experiments with sodium fluoride, a higher concentration, 25 mM, was chosen, almost $100 \times I_{50}$ (3×10^{-4} M).

In order to reduce the protective effect of fluoride on inhibition by soman, higher soman concentrations were used in some experiments (4×10^{-7} or 6×10^{-7} M).

RESULTS

There are two advantages with the method to put the soman–enzyme solution on filter papers and dry the paper. Firstly, no aging can proceed in the absence of water. No decrease in reactivation degree was observed after prolonged storage of the papers. Secondly, excess soman evaporates and is thus easily removed. This was checked by adding extra acetylcholinesterase to the buffer used to eluate the inhibited enzyme from the paper. No inhibition of this extra cholinesterase could be noticed.

In Fig. 1 the aging curve for plaice cholinesterase, inhibited by soman, in 0.1 M sodium phosphate buffer pH 7.4 at 22° and reactivated by 0.1 mM Toxogonin is shown. The reproducibility of the method is satisfactory. The rate constant of aging, k_{obs}, was found to be 0.071 (S.D. 0.008)/min, corresponding to a half-life of 9.9 min. The initial reactivatability, i.e. percentage reactivatable enzyme extrapolated to time zero, was 35.3 (S.D. 0.8)%, when 0.1 mM Toxogonin was used as reactivator.

pH- and ionic strength-dependence

The rate of the aging reaction is known to be highly dependent on pH and ionic strength [6, 7]. As shown in Tables 1 and 2, this is the case also for soman-inhibited plaice cholinesterase.

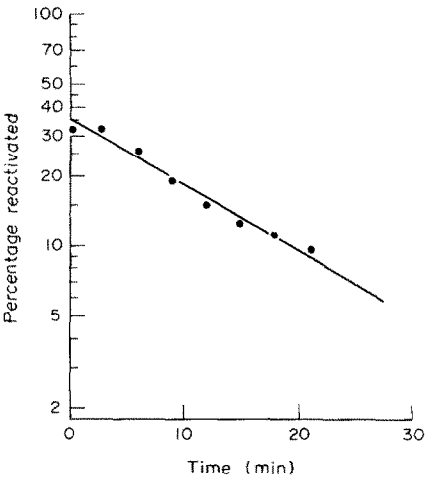

Fig. 1. Aging curve for cholinesterase from the plaice, inhibited by racemic soman. Inhibition and aging in 0.1 M sodium phosphate buffer, pH 7.4, at 22°. For reactivation 0.1 mM Toxogonin was used. Percentage reactivatable enzyme and the rate constant of aging were calculated as detailed in Materials and Methods. Initial reactivatable enzyme is defined as the intercept on the ordinate.

Reactivatability by various oximes

The initial reactivatability of soman-inhibited plaice cholinesterase was 35% when 0.1 mM Toxogonin was used as reactivator. We found this degree of reactivatability appropriate in order to be able to note any positive or negative effects of a number of compound on initial reactivatability and/or aging rate. However, we tested two other oximes, the monopyridinium oxime pralidoxime chloride ((2 - hydroxyimino - methyl)pyridinium - 1 - methyl chloride) and the bispyridinium oxime HI-6 ((2-hydroxyimino - methyl) - pyridinium - 1 - methyl - 4'-carbamoylpyridinium-1'-methylether dichloride). These oximes were both inferior to Toxogonin in reactivating soman-inhibited plaice cholinesterase. 0.1 mM 2-PAMCl gave an initial reactivatability of 16%, while the same concentration of HI-6 gave even less—only 12%.

Soman stereoisomers

Soman has two chiral centres in the molecule, one at the phosphorus and one at the α-carbon in the pinacolyl moiety. There are thus four stereoisomers of soman, which can be designed C-P-, C-P+,

Table 1. Influence of pH on the aging rate of soman-inhibited plaice cholinesterase

pH	k_{obs}/min
6.1	0.335
7.0	0.141
7.4	0.071
8.1	0.028

To obtain constant ionic strength (I = 0.262), sodium phosphate buffers of different concentrations were used. Temperature, 22°.

Table 2. Influence of ionic strength on the aging rate of soman-inhibited plaice cholinesterase

Ionic strength	k_{obs}/min
2.62×10^{-3}	0.414
2.62×10^{-2}	0.219
0.262	0.071
1.31	0.041

The experiments were performed at 22° at pH 7.4, using sodium phosphate buffer of different concentrations (1, 10, 100 and 500 mM, respectively).

C_+P_- and C_+P_+. We used soman, resolved at the α-carbon, to study stereospecific aging of soman-inhibited cholinesterase. The C_-- and C_+- soman each thus contains two stereoisomers, but only one (P_-) is fast-reacting with acetylcholinesterases [12, 13].

In Fig. 2 the aging profiles for plaice cholinesterase, inhibited by C_-- and C_+-soman, are shown. C_--soman gave an inhibitor–enzyme complex, which aged slowly, with a half-life of 17 min. The initial reactivatability was low—only 17%. The reversed patter was found for C_+-soman, with a $t_{1/2}$ of 6 min and an initial reactivatability of 55%.

A comparison of the aging rate and reactivation degree for enzyme inhibited by racemic soman and by the isomeric forms, respectively, suggests that the cholinesterase in the former case is inhibited by about equal amounts of C_+P_-- and C_-P_--soman (Table 3).

Aging in the presence of compounds containing quarternary nitrogen

Bošković has suggested [8] that a protection of the anionic binding site in the active site by quarternary ammonium compounds during soman inhibition might result in a phosphonylated enzyme with an orientation of the soman residue such that reactivation is facilitated.

We tested this hypothesis by using a series of

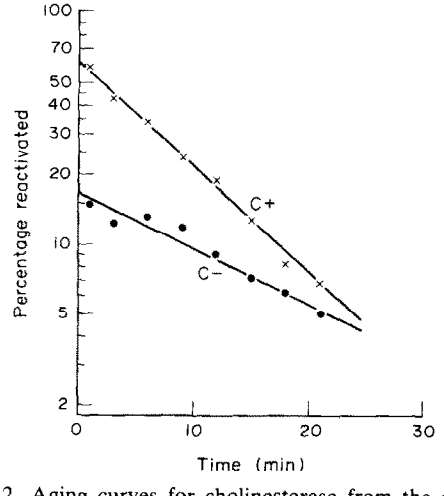

Fig. 2. Aging curves for cholinesterase from the plaice, inhibited by C_+- and C_--soman, respectively. Same experimental procedure as for Fig. 1.

Table 3. The effect of fluoride ions on initial reactivatability and rate constants of aging of plaice cholinesterase inhibited by C_+-, C_-- and racemic soman

	k_{obs} (min^{-1})	Initial reactivation (%)
Racemate		
Control	0.071 (0.008)†	35.3 (0.8)
25 mM NaF	0.094* (0.023)	42.1* (2.2)
C_+-soman		
Control	0.117 (0.023)	55.1 (6.8)
25 mM NaF	0.116 (0.013)	47.1* (3.3)
C_--soman		
Control	0.043 (0.010)	16.6 (3.4)
25 mM NaF	0.102* (0.031)	28.7* (9.6)

Inhibition and aging took place in 0.1 M sodium phosphate buffer, pH 7.4 (I = 0.262) at 22°.

* Significantly different ($P < 0.05$) from corresponding control.

† Standard deviations in parenthesis.

quarternary compounds. The ions studied were ammonium, choline, phenyltrimethylammonium, decamethonium, edrophonium, gallamine, tubocurarine and the oxime HI-6. The substances were added, usually in a concentration corresponding to the I_{50}-value, to the enzyme solution before soman. Thus, both inhibition and aging took place in the presence of effector compound. During reactivation, the concentration was decreased with a factor of 40, due to dilution.

No significant increase in initial reactivatability was found for any of the substances investigated. Many of the compounds were of course protective against soman inhibition (EI values higher than in the absence of effector) and some had an influence of aging rate. The most pronounced effect was obtained by 1 mM (+) tubocurarine, which retarded aging ($t_{1/2} \approx 15$ min). Such an effect was also observed by Crone [14], using acetylcholinesterase from red blood cells and sarin (isopropyl methyl fluoro phosphonate) as inhibitor.

As the compounds were present not only during aging but also during inhibition, a change in reactivatability and aging rate could reflect that the ratio between C_- and C_+-soman inhibited enzyme was changed. Thus the slow aging in the presence of tubocurarine, for example, could be interpreted as a preference for C_--soman inhibition. However, the initial reactivatability was the same as for racemic soman.

Aging in the presence of fluoride ions

Fluoride, at high concentrations, was the only substance found, which did have a significant, positive effect on initial reactivatability. The percentage reactivatable enzyme, inhibited by soman racemate, increased 7% in the presence of 25 mM sodium fluoride (Table 3). Fluoride also had an accelerating effect on aging.

Fluoride ions diminished the great differences in reactivatability and aging rate between soman isomers. While k_{obs} for C_+-soman was unchanged in the presence of fluoride, maximal reactivation was

decreased 8%. The ions had much more pronounced effect on C_--soman inhibited enzyme, and increased aging rate as well as initial reactivatability.

In the studied system, fluoride ions could interact in several of the reactions, as the ions were present during inhibition, aging and reactivation. Firstly, fluoride protects against inhibition by irreversible inhibitors. If this protection is more pronounced for one of the soman isomers, a changed ratio of C_+- and C_--inhibited enzyme could be the consequence in the experiment with racemic soman. The increase in initial reactivatability and in aging rate observed for racemic soman inhibited enzyme could thus be interpreted as if C_+-soman had inhibited the enzyme to a greater extent. This possibility is, however, less likely, as we found that fluoride ions did show the same protective effect in inhibition experiments with the isolated isomers. Furthermore, fluoride had effects on aging rate and reactivation degree also of enzyme, inhibited by C_+- and C_--soman, respectively.

Secondly, fluoride might act as a reactivator. The increase in maximal reactivation found in the experiments with racemic and C_--soman might thus be explained by an enhancement of the oxime effect. However, when fluoride was added only to the oxime solution for reactivation of racemic soman inhibited enzyme, the same degree of reactivation was found as when only oxime was present. As fluoride obviously did not contribute to reactivation of enzyme, inhibited by racemic soman, containing equal amounts of C_-- and C_+-soman, it is also less likely that it contributed when only C_--soman inhibited enzyme was present. Furthermore, the observation of decreased reactivatability of C_+-soman inhibited enzyme does not support a hypothesis of fluoride acting as a reactivator.

DISCUSSION

The main purpose of the present work was to investigate if steric hindrance is an important factor for the inability of conventional oximes to reactivate soman-inhibited cholinesterase. We also wanted to find out if it is possible to overcome this steric hindrance—if there is any—by any other means, e.g. by quarternary ammonium compounds [8], than simply raising the concentration of oxime. By using soman isomers it was shown that the orientation of the soman residue in the active site of the enzyme is of great importance for the ability of an oxime to reactivate the enzyme. Furthermore, we found that the most favourable orientation for oxime attack also favoured aging, i.e. the splitting of the pinacolyl moiety from the soman residue. Histidine seems to be involved in the dealkylation [15]. We thus suggest that for C_+-soman, the C—O-bond is in closer proximity to this amino acid in the active site than is the case for C_--soman.

We did not find any evidence in favour of Bosković's hypothesis [8] that simple protection of the anionic binding site by quarternary ammonium compounds, which are reversible inhibitors, during soman inhibition could give a soman–enzyme complex more susceptible for oxime attack.

Such an effect was on the other hand obtained in great excess of fluoride ions. The interaction between fluoride and acetylcholinesterase and its substrates and inhibitors is complex. As an inhibitor, fluoride seems to bind preferentially to the enzyme–substrate complex but also to free and acetylated enzyme [16]. Fluoride protects against inhibition by organophosphates [17], carbamates [18] and methanesulfonates [19]. We also found that 2 mM sodium fluoride decreased the bimolecular rate constant, k_i, 10-fold for the reaction between electric eel acetylcholinesterase and soman (Forsberg and Puu, unpublished observation). This protection was entirely due to a change in the dissociation constant, K_d, i.e. the affinity of soman for the active site was greatly diminished.

Fluoride can also act as a reactivator [20] and has some antidotal effect *in vivo* [21, 22]. Fluoride does not, however, contribute to reactivation in the presence of oxime [20], and we could also observe this lack of effect. We find it thus less probable that the increase in reactivatability observed can be explained by simple enhancement of reactivation. We rather believe that fluoride changes the conformation of the enzyme–inhibitor complex and thus acts as an allosteric modulator.

This modulation also comes to an expression in the rate of aging. It has been proposed previously that allosteric effectors can control the rate of aging of phosphonylated cholinesterase [14]. The remarkable effect of some bispyridinium compounds, e.g. SAD-128 (1,1′-oxydimethylene bis-(4-*tert*-butylpyridinium chloride)) to influence aging rate [23, 24] and also to augment reactivation if present simultaneously with oxime [23] can probably also be explained by allosteric properties of these compounds. All modulators reported so far, which have effects on aging rate, reactivation rate and/or reactivatability, are of no therapeutic value, as the concentrations needed are equivalent to more or less toxic doses. Provided that steric factors are of importance, it is of greater practical interest that some newer oximes, above all HI-6, are effective reactivators of unaged, soman-inhibited cholinesterase of mammalian origin [25]. This conclusion also makes us believe that plaice cholinesterase is not a good model for testing effects of oximes, as this enzyme responded very poorly on HI-6 treatment.

Acknowledgements—We want to thank Dr. B. Stridsberg and Dr. G. Lindberg for synthesis of soman and its isomers, Ms. E. Artursson and Ms. M. Andersson for performing some of the experiments and Ms. G. Karlsson for skilfully preparing the manuscript.

REFERENCES

1. F. Hobbiger, *Br. J. Pharmac.* **10**, 356 (1955).
2. F. Berends, C. H. Posthumus, I. V. D. Sluys and F. A. Deierkauf, *Biochim. biophys. Acta* **34**, 576 (1959).
3. J. H. Fleisher and L. W. Harris, *Biochem. Pharmac.* **14**, 641 (1965).
4. W. K. Berry and D. R. Davies, *Biochem. J.* **100**, 572 (1966).
5. M. Sun, Z. Chang, M. Shau, R. Huang and T. Chou, *Eur. J. Biochem.* **100**, 527 (1979).
6. H. O. Michel, B. E. Hackley, L. Berkowitz, G. List,

E. B. Hackley, W. Gillilan and M. Pankau, *Archs. Biochem. Biophys.* **121**, 129 (1967).
7. J. H. Keijer, G. Z. Wolring and L. P. A. de Jong, *Biochim. biophys. Acta* **334**, 146 (1974).
8. Bošković, *Fundam. Appl. Toxic.* **1**, 203 (1981).
9. E. Heilbronn and B. Tolagen, *Biochem. Pharmac.* **14**, 73 (1965).
10. S. J. Lundin, Å. Bovallius, L. Holmberg and G. Lindner, FOA 1 Report, C 1330–34(36) (1969).
11. G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
12. J. H. Keijer and G. Z. Wolring, *Biochim. biophys. Acta* **185**, 465 (1969).
13. I. Nordgren, G. Lundgren, G. Puu and B. Holmstedt, *Archs. Toxic.* **55**, 70 (1984).
14. H. D. Crone, *Biochem. Pharmac.* **23** 460 (1974).
15. G. Beauregard, J. Lum and B. D. Roufogalis, *Biochem. Pharmac.* **30**, 2915 (1981).
16. T. L. Rosenberry, in *Advances in Enzymology*, Vol. 43 (Ed. A. Meister), p. 103. John Wiley, New York (1975).
17. E. Heilbronn, *Acta chem. scand.* **19**, 1333 (1965).
18. C. M. Greenspan and I. B. Wilson, *Molec. Pharmac.* **6**, 266 (1970).
19. C. M. Greenspan and I. B. Wilson, *Molec. Pharmac.* **6**, 460 (1970).
20. E. Heilbronn, *Biochem. Pharmac.* **14**, 1363 (1965).
21. L. Albanus, E. Heilbronn and A. Sundwall, *Biochem. Pharmac.* **14**, 1375 (1965).
22. J. G. Clement and M. Filbert, *Life Sci.* **32**, 1803 (1983).
23. L. W. Harris, W. C. Heyl, D. L. Stitcher and C. A. Broomfield, *Biochem. Pharmac.* **27**, 757 (1978).
24. K. Schoene, *Biochim. biophys. Acta* **525**, 468 (1978).
25. L. P. A. de Jong and G. Z. Wolring, *Biochem. Pharmac.* **29**, 2379 (1980).