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REACTIVATION AND AGING OF CYCLOPENTYL METHYLPHOSPHONYLATED ACETYLCHOLINESTERASE IN THE PRESENCE OF SOME 1-ALKYL-2-HYDROXYIMINOMETHYL-PYRIDINIUM SALTS

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Abstract—The effect of 2-hydroxyiminomethyl-1-methyl-pyridinium methanesulphonate (P2S) and its 1-pentyl and 1-heptyl analogues on the reactivation and aging of acetylcholinesterase inhibited by cyclopentyl methylphosphonofluoridate was studied at pH 7.5 and 25°. The reactions in the presence of the 1-pentyl- and 1-heptyl-pyridinium-oxime involve simultaneous aging of the inhibited enzyme, and reactivation and aging of the inhibited enzyme–oxime complex. The reactions in the presence of P2S are more complicated. Reinhibition of the reactivated enzyme by the phosphonylated P2S probably plays a substantial role in the reactivation process. The dissociation constants of the complexes of inhibited enzyme with the 1-pentyl- and 1-heptyl-pyridinium-oxime and the rate constants of reactivation and aging of these complexes were evaluated. From these results the maximum percentages of reactivation, which can be achieved with high concentrations of the oximes, are calculated as 30 and 48 for the 1-pentyl- and 1-heptyl-pyridinium-oxime, respectively. These oximes, which were found previously to accelerate the aging of acetylcholinesterase inhibited by ethyl dimethylphosphoramidocyanidate (tabun), retard the aging of the cyclopentyl methylphosphonylated enzyme. Different mechanisms for the aging of the two inhibited enzymes are suggested.

Certain organophosphates inhibit acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by phosphorylating the active site of the enzyme. The inhibited enzyme can be reactivated by nucleophilic substances, which displace the phosphorus moiety from the enzyme, such as 2-hydroxyiminomethyl-1-methyl-pyridinium methanesulphonate (P2S) and 1,1'-(1,3-propanediyl)bis(4-hydroxyiminomethyl-pyridinium) dibromide (trimedoxime). On storage, the inhibited enzyme gradually loses its ability to be reactivated. This conversion of the inhibited enzyme into a non-reactivatable form is called aging.

In reactivation studies of acetylcholinesterase inhibited by ethyl dimethylphosphoramidocyanidate (tabun) [1] it was observed that reactivation and aging of the inhibited enzyme occur simultaneously in the presence of P2S and some 1-(ar)alkyl analogues. Some of these oximes enhanced the rate of aging contrary to the general finding that quaternary ammonium ions, including P2S, have a retarding effect on aging [2–6].

The aging of the tabun-inhibited acetylcholinesterase proceeds very slowly; the half-life time is 46 hr at pH 7.5 and 25°. It is interesting to examine whether the above mentioned oximes are able to accelerate the aging of other inhibited acetylcholinesterases too, especially of those inhibited enzymes which show a rapid aging. Therefore, the influence of P2S and its 1-pentyl and 1-heptyl analogues on the reactivation and aging of acetylcholinesterase inhibited by cyclopentyl methylphosphonofluoridate was investigated. The time for 50 per cent of aging of this inhibited enzyme has been reported to be 21 min (pH 7.5, 25°) [7]. A kinetic analysis of the reactivation and aging reactions is described.

MATERIALS AND METHODS

Materials. Bovine erythrocyte acetylcholinesterase was obtained from Sigma Chemical Comp., St. Louis, Mo., U.S.A., and had a spec. act. of 27 nkat/mg protein at 25° in 0.6 mM phosphate buffer, pH 7.5, containing 3.2 mM acetylcholine perchlorate and 0.1 M potassium chloride. P2S was purchased from Dr. F. Raschig GmbH, W. Germany. The P2S analogues, 1-(3-aminocarbonylpyridinio)methoxymethyl-2-hydroxyiminomethyl-pyridinium dichloride (HS-6) and cyclopentyl methylphosphonofluoridate were prepared in this laboratory according to methods known from literature and had satisfactory elemental analysis. The structure of the oximes was confirmed by infrared and NMR spectroscopy. All other reagents were commercial products of an analytical grade.

Aging experiments. Inhibited acetylcholinesterase was obtained by incubating a mixture of 19 volumes of a solution of 1.16 mg enzyme/ml (approx. 5.3 nM of active sites) in 6.6 mM veronal buffer, pH 10, with one volume of 2 μM cyclopentyl methylphosphonofluoridate in water for 1 hr at pH 10.0 and 25°. Under these conditions over 96 per cent of the enzyme was phosphonylated and at the same time the excess of inhibitor was hydrolyzed. Incubation of acetylcholinesterase (1.1 mg/ml) in a sample of the inhibited enzyme solution for 2 hr at pH 7.5 and 25° gave no

inhibition of the enzyme, indicating a complete hydrolysis of the excess of inhibitor. In a control experiment incubation of the enzyme for 1 hr at pH 10 and 25° did not influence the enzyme activity. The aging, or aging and reactivation, was started by addition of four volumes of the inhibited enzyme solution to one volume of 40 mM phosphate buffer, without or with added 1-alkyl-pyridinium-oxime, respectively. The pH of the phosphate buffer was adjusted such that the final pH of the mixture was within the range 7.50 to 7.55. Aging of the inhibited enzyme was determined from the decrease of attainable reactivation. During the first 2 hr of the aging reaction 23 samples of 2.5 ml were taken at suitable time intervals and added to 0.13 ml of a 61 mM HS-6 solution in water containing 0.2 M disodium hydrogen phosphate. The final pH of the samples was adjusted to 8.1 by addition of 16–18 μl 0.5 N NaOH and the samples were allowed to reactivate for at least 20 hr at 25°. To determine the initial reactivatability of the inhibited enzyme 2 ml of the inhibited enzyme solution were directly added to a mixture of 0.5 ml of 40 mM phosphate buffer, without or with added 1-alkyl-pyridinium-oxime, and 0.13 ml of a 61 mM HS-6 solution in water containing 0.2 M disodium hydrogen phosphate. The final pH of this sample was adjusted to 8.1 by addition of 16–18 μl 0.5 N NaOH and allowed to reactivate for at least 20 hr at 25°. Then, the restored enzyme activity was measured in duplicate by using an apparatus developed by Keijer [8] for the automatic performance of pH-stat titrations. In the Radiometer titration equipment of the apparatus the burette unit SBU la was exchanged for an ABU 13 with a 0.25 ml-burette assembly. Samples of 1 ml were assayed for enzyme activity (AIR_t) at pH 7.8 and 25° with 23 ml of a 3.2 mM acetylcholine perchlorate solution in 0.1 M potassium chloride. The titrant was 0.05 N NaOH. Enzyme activities were corrected for spontaneous hydrolysis of the substrate. Blancs for the activity of the enzyme (A), the enzyme, without or with added 1-alkyl-pyridinium-oxime, incubated with HS-6 (AR) and the inhibited enzyme (AI) were run simultaneously.

The percentage of reactivation (% $react_t$) was calculated according to

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

Reactivation experiments. The inhibition of the enzyme and the removal of the excess of inhibitor were carried out as described for the aging experiments. Four to six reactivation experiments were performed with each oxime concentration used. The reactivation was started by addition of four volumes of the inhibited enzyme solution to one volume of the oxime solution in 40 mM phosphate buffer. The pH of the phosphate buffer was adjusted such that the final pH of the mixture was within the range 7.50 to 7.55. The enzyme was allowed to reactivate at 25°. The restored enzyme activity was determined with time intervals of 15 min during the first 2 hr by using the apparatus described for the aging experiments. In addition to this experiment, another three to five solutions of inhibited enzyme were prepared, immediately mixed with the oxime solution and incubated for various time periods of up to 15 min. Next, the restored enzyme activity was determined in the reactivation mixtures. Samples of 1 ml were assayed for enzyme activity as described for the aging experiments, except that the assay was carried out at pH 7.5 and that 0.17 ml of a 40 mM phosphate buffer, pH 7.5, was added to the assay mixture from a Braun perfusor as described previously [1]. Blancs were determined for the activity of the enzyme (A), the enzyme incubated with the oxime (AR) and the inhibited enzyme solution (AI).

Percentages of reactivation were calculated according to equation 1, where AIR_t is the activity of the inhibited enzyme after incubation with the oxime for time t.

RESULTS

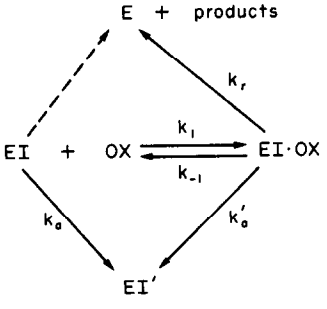
Reactivatability of the inhibited enzyme. Incubation of cyclopentyl methylphosphonylated acetylcholinesterase with 3 mM trimedoxime, obidoxime (Toxogonin®, 1,1'-(oxybis(methylene))bis(4-hydroxyiminomethyl-pyridinium) dichloride) or P2S at pH 8.1 and 25° restored 85–90 per cent of the enzyme activity. Almost complete reactivation, 96–100 per cent, was achieved by incubation with 3 mM HS-6 under the same conditions. This observation shows that under the conditions of the inhibition (pH 10) no aging occurs and that all the inhibited enzyme which has not aged after a certain time period of incubation, can be reactivated with HS-6. So, the percentage of aging of the inhibited enzyme after time t of incubation without or with added 1-alkyl-pyridinium-oxime (% ag_t) can be calculated as

$$\% \text{ ag}_t = 100 - \% \text{ react}_t \qquad (2)$$

where % $react_t$ is the percentage of reactivation obtained after time t of aging and a subsequent reactivation with 3 mM HS-6 (see *Aging experiments*).

The ability of the inhibited enzyme to be reactivated decreased in the presence of the 1-alkyl-pyridinium-oximes to a final value, which was achieved within two hr. An example is given in Fig. 1. The inhibited enzyme was reactivated with the oximes up to a final value (Fig. 1). The differences between these final values were three per cent or less. So, after two hours of incubation with one of the three oximes studied, a part of the inhibited enzyme has been reactivated and the rest of it has aged.

Kinetic analysis. We adopted the following scheme to describe the reactivation and aging of cyclopentyl methylphosphonylated acetylcholinesterase (EI) in the presence of the oxime (OX):



(3)

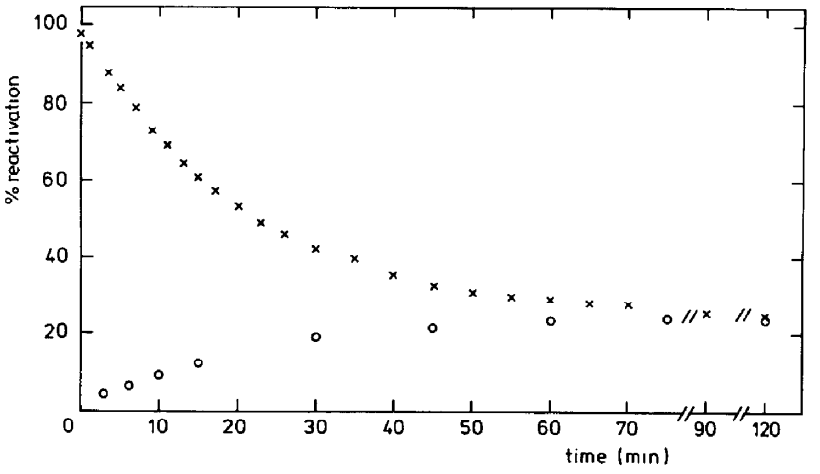


Fig. 1. Reactivation (O) and decrease of reactivatability (×) of cyclopentyl methylphosphonylated acetylcholinesterase in the presence of 0.3 mM 2-hydroxyiminomethyl-1-pentyl-pyridinium iodide at pH 7.5 and 25°.

where E, EI·OX and EI′ are the reactivated enzyme, the complex of inhibited enzyme with oxime, and the aged enzyme, respectively. Spontaneous reactivation was not observed. The oxime was used in a very large molar excess compared with the enzyme and hence, its concentration was assumed to be constant.

Let

$$K_d = \frac{k_{-1}}{k_1} = \frac{[EI][OX]}{[EI \cdot OX]} \tag{4}$$

and

$$[EI_{tot}] = [EI] + [EI \cdot OX]. \tag{5}$$

It is derived [1] for scheme (3) that

$$[EI_{tot}]_t = [EI_{tot}]_0 \, e^{-k_{obs}t} \tag{6}$$

where

$$k_{obs} = \frac{k_a K_d + k'_a[OX] + k_r[OX]}{K_d + [OX]} \tag{7}$$

$$[EI']_t = \frac{k_a K_d + k'_a[OX]}{k_{obs}(K_d + [OX])}[EI_{tot}]_0(1 - e^{-k_{obs}t}) \tag{8}$$

and

$$[E]_t = \frac{k_r[OX]}{k_{obs}(K_d + [OX])}[EI_{tot}]_0(1 - e^{-k_{obs}t}). \tag{9}$$

When $t = \infty$, $[EI']_\infty = (k_a K_d + k'_a[OX])[EI_{tot}]_0/k_{obs} \times (K_d + [OX])$ and $[E]_\infty = k_r[OX][EI_{tot}]_0/k_{obs}(K_d + [OX])$. Hence, equations 8 and 9 can be rewritten to

$$\ln([EI']_\infty - [EI']_t) = \ln[EI']_\infty - k_{obs}t \tag{10}$$

and

$$\ln([E]_\infty - [E]_t) = \ln[E]_\infty - k_{obs}t. \tag{11}$$

from equations 8 and 9 it follows that

$$\frac{[E]_t}{[EI']_t} = \frac{[E]_\infty}{[EI']_\infty} = \frac{k_r[OX]}{k_a K_d + k'_a[OX]}. \tag{12}$$

By expressing the relative concentrations of aged enzyme and of reactivated enzyme as %ag and %react$_t$, respectively, equations 10 and 11 can be represented by

$$\ln(\%\,ag_\infty - \%\,ag_t) = \ln(\%\,ag_\infty - \%\,ag_0) - k_{obs}t \tag{13}$$

and

$$\ln(\%\,react_\infty - \%\,react_t) = \ln(\%\,react_\infty) - k_{obs}t \tag{14}$$

where $\%\,ag_\infty$, $\%\,react_\infty$ and $\%\,ag_0$ are the final percentage of aging, of reactivation and the percentage of aging at time zero, respectively. Equations 7 and 12 are used for the evaluation of the kinetic parameters K_d, k_r and k'_a from the data obtained in the aging and reactivation experiments after rearranging the equations as follows. Equation 7 can be written as

$$k_a - k_{obs} = \frac{k_{max}[OX]}{k_d + [OX]} \tag{15}$$

where

$$k_{max} = k_a - k'_a - k_r \tag{16}$$

and from equations 12 and 16 it is derived that

$$k'_a = \frac{k_a - k_{max} - (k_a K_d/[OX])([E]_\infty/[EI']_\infty)}{1 + ([E]_\infty/[EI']_\infty)}. \tag{17}$$

Kinetic parameters. The rate of aging of cyclopentyl methylphosphonylated acetylcholinesterase was found as 7.1×10^{-2} min^{-1}.

Aging and reactivation of the inhibited enzyme in the presence of the 1-pentyl- and 1-heptyl-pyridinium-oxime can be described by scheme (3). Plots of $\ln(\%ag_\infty - \%ag_t)$ vs t (equation 13) for aging and plots of $\ln(\%react_\infty - \%react_t)$ vs t(Equation 14) for reactivation gave straight lines with intercept $\ln(\%\,ag_\infty - \%\,ag_0)$ and $\ln(\%\,react_\infty)$, respectively. Two examples are given in Fig. 2. The slopes of the lines, k_{obs}, obtained for the aging experiments were approximately equal to those of the corresponding reactivation experiments in agreement with the proposed reaction scheme (equations 13 and 14). The more extensive number of data of the aging experiments were used for the evaluation of values of k_{obs} according to least-squares analysis.

The results of the aging experiments carried out in the presence of P2S (0.1–1.0 mM) also obeyed equation 13. However, plots of the data of the corresponding reactivation experiments according to equation 14

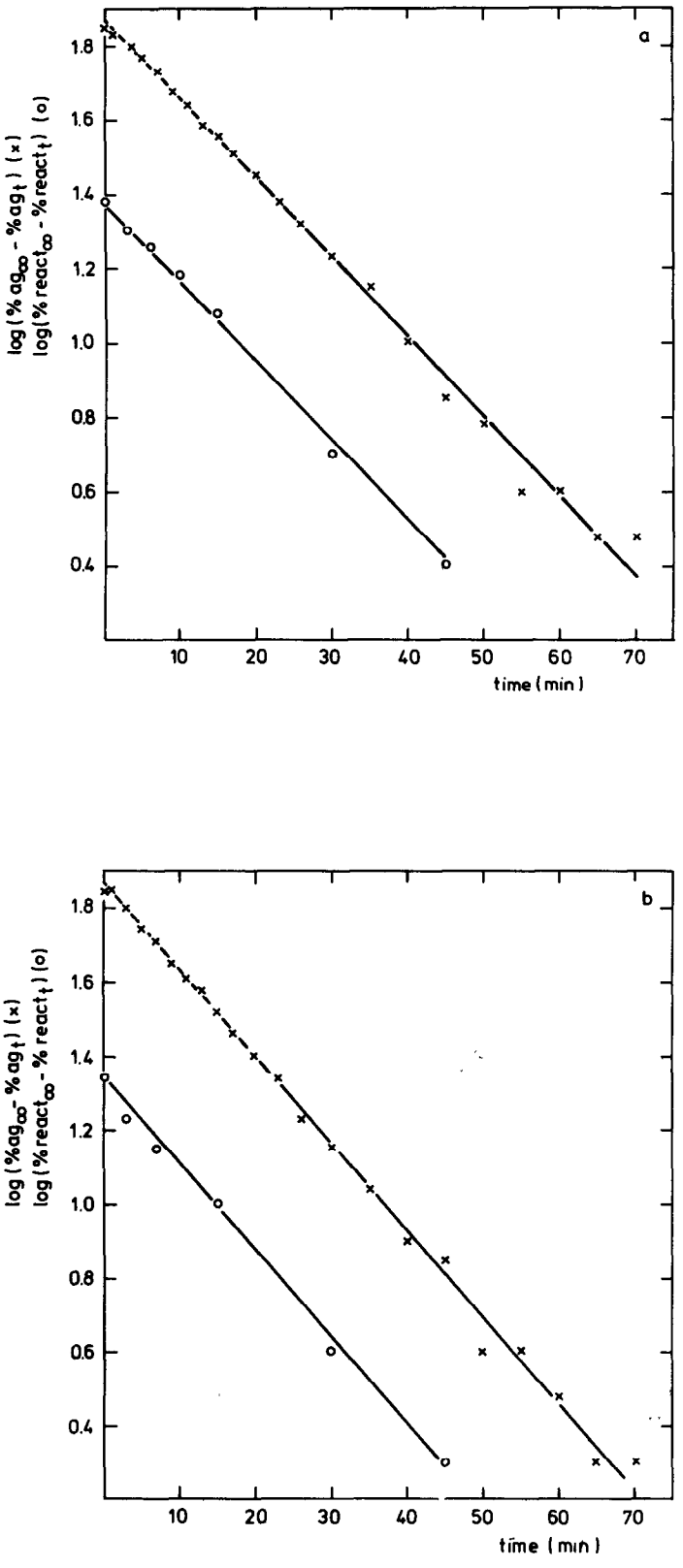


Fig. 2. Plots of log ($\%\ ag_\infty - \%\ ag_t$) vs t (×) for the decrease of reactivatability and plots of log ($\%\ react_\infty - \%\ react_t$) vs t (O) for the reactivation of cyclopentyl methylphosphonylated acetylcholinesterase at pH 7.5 and 25° in the presence of

a. 0.3 mM 2-hydroxyiminomethyl-1-pentyl-pyridinium iodide,
b. 0.3 mM 1-heptyl-2-hydroxyiminomethyl-pyridinium iodide.

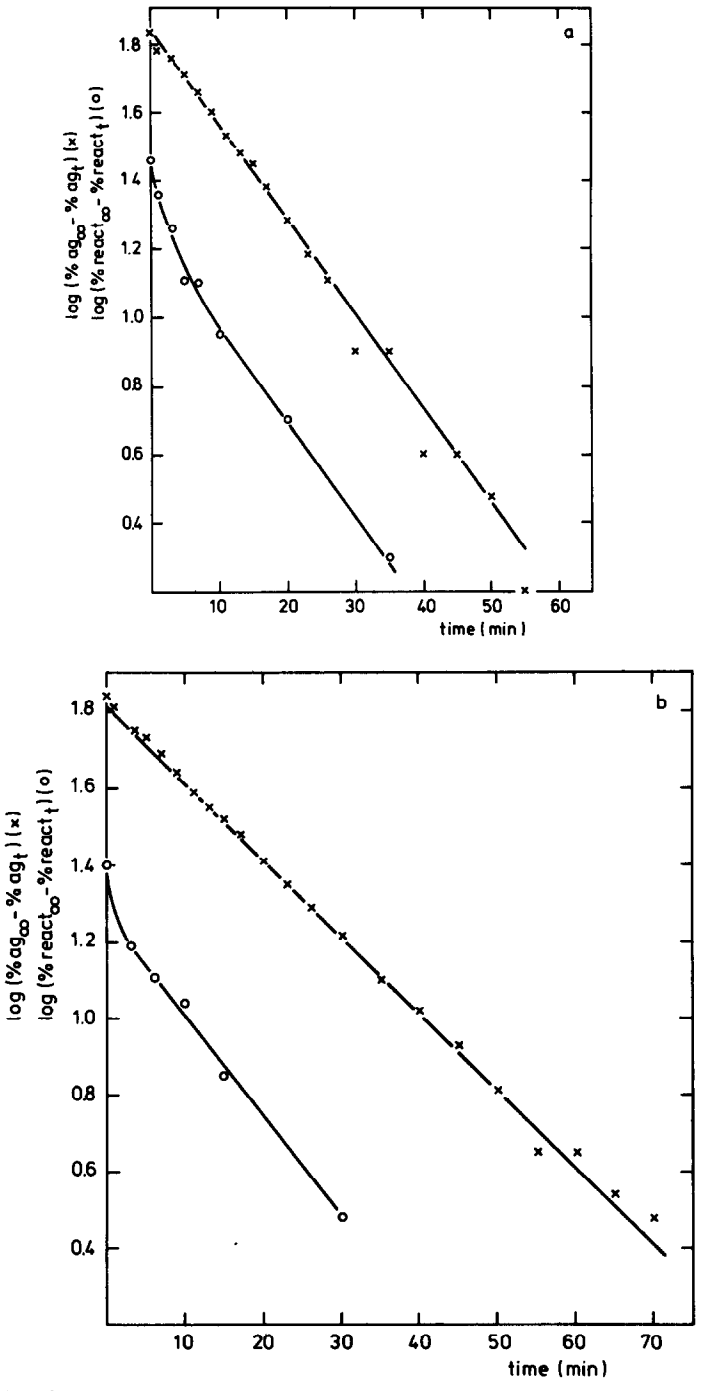


Fig. 3. Plots of log ($\%ag_\infty - \%ag_t$) vs $t(x)$ for the decrease of reactivatability and plots of log($\%react_\infty - \%react$:) vs $t(O)$ for the reactivation of cyclopentyl methylphosphonylated acetylcholinesterase in the presence of 0.3 mM P2S at pH 7.5 and 25°. Initial concentrations of inhibited enzyme:

a. 0.88 mg/ml,
b. 1.76 mg/ml.

curved.An example is given in Fig. 3. Results of aging and reactivation experiments with twice the inhibited enzyme concentration in the presence of 0.3 mM P2S showed more strongly marked deviations of the proposed kinetics, as illustrated in Fig. 3. This finding suggests that reinhibition of the reactivated enzyme by the phosphonylated P2S, which is formed on reactivation, is involved in the process. Deviations of the proposed kinetics were not noticed for the results of similar experiments with 0.3 mM 1-pentyl- or 1-heptyl-pyridinium-oxime.

Values of k_{obs} were determined with various concentrations of the 1-pentyl- and 1-heptyl-pyridinium-oxime (see Table 1). From these data the parameters

Table 1. Kinetic constants and their standard errors of aging and reactivation of cyclopentyl methylphosphonylated acetylcholinesterase in the presence of 2-hydroxyiminomethyl-1-pentyl-pyridinium iodide (I) and 1-heptyl-2-hydroxyiminomethyl-pyridinium iodide (II) at pH 7.5 and 25°

Oxime	Range of oxime concentrations used (mM)	k_{max} (10^{-2} min^{-1})	K_d (mM)	k'_a (10^{-2} min^{-1})	k_r (10^{-2} min^{-1})
I	0.1–0.6 (6)*	2.8 ± 0.2	0.029 ± 0.022	3.0 ± 0.2	1.3 ± 0.3
II	0.1–0.6 (5)	3.1 ± 0.6	0.17 ± 0.09	2.1 ± 0.2	1.9 ± 0.6

* Values in parentheses give the number of oxime concentrations used.

K_d and k_{max} were estimated by fitting equation 15 to a set of $k_a - k_{obs}$, [OX] data by means of the non-linear regression method of Wilkinson [9]. The value of k'_a was calculated according to Equation 17 by using the values obtained for K_d and k_{max}. In these calculations $[E]_\infty/[EI']_\infty$ was expressed as the ratio of $\%\,react_\infty$ and $\%\,ag_\infty - \%\,ag_0$ which were obtained from the reactivation experiments and the corresponding aging experiments, respectively. The value of k'_a was obtained as the mean of values calculated for the individual oxime concentrations. Next, k_r was calculated according to equation 16. The results are given in Table 1. Standard errors of K_d, k_{max} and k_r were calculated by means of methods described by Wilkinson [9].

At high oxime concentrations equation 12 becomes equal to

$$\frac{[E]_\infty}{[EI']_\infty} = \frac{k_r}{k'_a}. \qquad (18)$$

So, the ratio $100\,k_r/(k'_a + k_r)$ represents the maximum percentage of reactivation which can be achieved with these oximes at pH 7.5 and 25°. The maximum percentages of reactivation derived from the k_r and k'_a values given in Table 1 are 30 and 48 for the 1-pentyl- and 1-heptyl-pyridinium-oxime, respectively. These values corresponding within the experimental error to the final percentage of reactivation found with a high concentration of the 1-pentyl compound (1 mM, $K_d/[OX] = 0.03$), and of the heptyl compound (3 mM, $K_d/[OX] = 0.06$), being 34 and 45, respectively. The final percentages of reactivation obtained with 0.1, 1 and 3 mM P2S were 11, 60 and 77%, respectively.

DISCUSSION

The oxime HS-6 is able to restore almost completely the enzyme activity of cyclopentyl methylphosphonylated acetylcholinesterase, although the inhibited enzyme exhibits a rapid aging. The percentages of reactivation achieved with the well-known reactivators trimedoxime, obidoxime or P2S are less. In reactivation experiments carried out with acetylcholinesterase inhibited by 1,2,2-trimethylpropyl methylphosphonofluoridate (soman), which ages very rapidly, HS-6 is also a better reactivator than these oximes [10]. Obviously, either the aging of these phosphonylated acetylcholinesterases proceeds more slowly in the presence of HS-6 than in the presence of the other oximes or HS-6 reactivates the inhibited enzyme faster or both.

The reactivation and aging of cyclopentyl methylphosphonylated acetylcholinesterase in the presence of the 1-pentyl- or 1-heptyl-pyridinium-oxime can be described with scheme (3). The results obtained with P2S, however, do not obey this scheme. From experiments carried out with twice the initial enzyme concentration it was concluded that reinhibition of the reactivated enzyme by the phosphonylated P2S formed plays a substantial role in the process. Acetylcholinesterase shows a five to seven times higher affinity to the 1-heptyl or 1-pentyl-pyridinium-oxime than to P2S [1, 11]. So, the different course of the reactivation process in the presence of P2S and of its 1-pentyl or 1-heptyl analogues may partly be due to a better protection offered by the two latter oximes of the reactivated enzyme against reinhibition by the phosphonylated oxime formed. Phosphonylated oximes may be very potent anticholinesterases, but usually degrade rapidly in aqueous solution [12]. It is reasonable to assume that substitution of the 1-methyl group of the phosphonylated P2S by pentyl or heptyl will hardly affect its stability. However, these substitutions might decrease the anticholinesterase activity of the phosphonylated oxime.

The affinity of tabun-inhibited acetylcholinesterase to 1-(ar)alkyl-2-hydroxyiminomethyl-pyridinium salts was found to increase with increasing lipophilicity of the oximes [1]. In contrast, cyclopentyl methylphosphonylated acetylcholinesterase shows a higher affinity to the 1-pentyl-pyridinium-oxime than to the more lipophilic 1-heptyl analogue. A similar result was obtained by Patočka [13] in his reactivation experiments with acetylcholinesterase inhibited by isopropyl methylphosphonofluoridate (sarin) which exhibited a higher affinity to 1-butyl-2-hydroxyiminomethyl-pyridinium iodide than to the more lipophilic 1-hexyl analogue. These results show that the contribution of the alkyl group to the binding of these oximes to phosphorylated or phosphonylated acetylcholinesterases also depends on the substituents at the phosphorus atom attached to the esteratic site of the enzyme, although the 1-alkyl group is probably bound near the anionic site of the enzyme [1, 11].

The rate constants of reactivation (k_r) and of aging (k'_a) of the inhibited enzyme from its complex with the 1-pentyl- and 1-heptyl-pyridinium-oxime differ slightly. As follows from Equation 12 the maximum percentage of reactivation which can be achieved with high concentrations of the oximes, is determined by the ratio of the values of k_r and k'_a (see also Equation 18). Consequently, this percentage does not only

depend on the reactivation potency of the oximes, but also on the extent to which the oximes affect the rate of aging of the inhibited enzyme.

The oximes retard the aging of the inhibited enzyme (k'_a). This observation is consistent with the finding that quaternary ammonium ions generally have a retarding effect on aging [2–6]. Binding of quaternary ammonium ions probably causes a conformational change of the inhibited enzyme in such a way that the catalysis of the aging reaction is disadvantageously affected. The 1-pentyl- and 1-heptyl-pyridinium-oxime were found to accelerate the aging of tabun-inhibited acetylcholinesterase. These results suggest that the aging of the two inhibited enzymes proceeds via different mechanisms. The aging of cyclopentyl methylphosphonylated acetylcholinesterase is believed to take place by the release of the cyclopentyl group probably as a carbonium ion after fission of the carbon–oxygen bond [2, 7, 14]. However, as the release of the ethyl group in the tabun-inhibited enzyme is less probable as a consequence of the instability of the ethyl carbonium ion, the aging of this inhibited enzyme might proceed via the release of the ethoxy group by cleavage of the phosphorus–oxygen bond. The accelerating effect brought about by some oximes might be due to a nucleophilic attack of the compounds at the phosphorus atom of the inhibited enzyme. Subsequently, two reactions can proceed, either the enzyme is the leaving group (reactivation) or the ethoxy group is the leaving group (aging). A similar mechanism was proposed by Erlanger *et al.* [15] for the interaction of hydroxylamine with diethyl phosphorylated chymotrypsin. However, an alternative mechanism for the aging of tabun-inhibited acetylcholinesterase, in which the dimethylamino group is split off, cannot be ruled out. A study of the effect of the alkyl-pyridinium-oximes on the aging of diethyl phosphorylated or ethyl methylphosphonylated acetylcholinesterase might contribute to a better understanding of the mechanism of aging.

REFERENCES

1. L. P. A. de Jong and G. Z. Wolring, *Biochem. Pharmac.* **27**, 2229 (1978).
2. F. Berends, C. H. Posthumus, I. v. d. Sluys and F. A. Deierkauf, *Biochim. biophys. Acta* **34**, 576 (1959).
3. W. K. Berry and D. R. Davies, *Biochem. J.* **100**, 572 (1966).
4. K. Schoene and R. Wulf, *Arzneimittel-Forsch.* **22**, 1802 (1972).
5. H. D. Crone, *Biochem. Pharmac.* **23**, 460 (1974).
6. S. H. Sterri, *Biochem. Pharmac.* **26**, 656 (1977).
7. H. P. Benschop and J. H. Keijer, *Biochim. biophys. Acta* **128**, 586 (1966).
8. J. H. Keijer, *Analyt. Biochem.* **37**, 439 (1970).
9. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
10. O. L. Wolthuis, H. J. Clason-van der Wiel and R. P. L. S. Visser, *Eur. J. Pharmacol.* **39**, 417 (1976).
11. L. P. A. de Jong and G. Z. Wolring, *Croat. chem. Acta* **47**, 383 (1975).
12. L. P. A. de Jong and D. I. Ceulen, *Biochem. Pharmac.* **27**, 857 (1978).
13. J. Patočka, *Sb. ved. Pracé lek. Fak. Hradci Králové*, **16**, 121 (1973).
14. H. O. Michel, B. E. Hackley, Jr., L. Berkowitz, G. List, E. B. Hackley, W. Gillilan and M. Pankau, *Archs Biochem. Biophys.* **121**, 29 (1967).
15. B. F. Erlanger, W. Cohen, S. M. Vratsanos, H. Castleman and A. G. Cooper, *Nature, Lond.* **205**, 868 (1965).