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REACTIVATION OF ACETYLCHOLINESTERASE INHIBITED BY 1,2,2′-TRIMETHYLPROPYL METHYLPHOSPHONOFLUORIDATE (SOMAN) WITH HI-6 AND RELATED OXIMES

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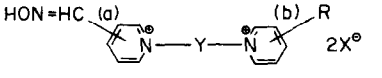
Bovine erythrocyte acetylcholinesterase inhibited by 1,2,2′-trimethylpropyl methylphosphonofluoridate (soman) is reactivated rapidly but incompletely by 1 mM of the bispyridinium mono-oximes HI-6, HS-6, HGG-12 and HGG-42 (pH 7.5, 25°). These oximes, especially HI-6 and HS-6, show a higher reactivating potency than conventional reactivators, like P2S, obidoxime or TMB4. The incomplete reactivation is studied in more detail with HI-6, which is found to be the most potent reactivator. Rapid reactivation and aging take place in the presence of the oxime (10–70 μM). The reactions are not consistent with a minimum scheme involving simultaneous aging of the inhibited enzyme and reactivation and aging of an inhibited enzyme–oxime complex. The formation of aged enzyme can be described as a first-order process. The formation of reactivated enzyme proceeds in a more complicated manner. HI-6 appears to have no effect on aging of the soman-inhibited enzyme. It is concluded that HI-6, being able to compete with the rapid aging of soman-inhibited acetylcholinesterase, has an extremely high reactivating potency, on which the therapeutic effect of the oxime is based.

Intoxication by a number of organophosphates can effectively be treated by certain oximes. The therapeutic activity of these compounds is based on their ability to reactivate acetylcholinesterase which has been inhibited by the organophosphate. The reactivation may be hampered by a transformation of the inhibited acetylcholinesterase into a form which cannot be reactivated by any known means, the so-called aging. Acetylcholinesterase inhibited by 1,2,2′-trimethylpropyl methylphosphonofluoridate (soman) exhibits a relatively very rapid aging *in vitro* as well as *in vivo* [1–4]. As a consequence, the conventional oximes, such as the pyridinium-oxime P2S and the bispyridinium di-oximes obidoxime (Toxogonin) and TMB4 (see Fig. 1 for structural formulae), do not show a satisfactory therapeutic effect [5–8].

During the last decade some bispyridinium mono-oximes became available as a result of the synthetic work in the laboratory of Prof. I. Hagedorn (Freiburg, F.R.G.). Representatives of these compounds, HS-6, HI-6, HGG-12 and HGG-42 (see Fig. 1), were reported to be effective antidotes in rodents [6–11] and in dogs [12, 13]. The reactivating potency *in vitro* of these oximes has hardly been investigated. Only HS-6 was described as a more potent reactivator of soman-inhibited acetylcholinesterase than TMB4 or obidoxime [14].

In the present work the reactivating potency *in vitro* of some bispyridinium mono-oximes was tested and compared with that of some well-known conventional oximes. After addition of an oxime to soman-inhibited acetylcholinesterase, two parallel reactions may proceed: reactivation and aging. Consequently, if the enzyme is reactivated to a certain extent, the oxime should have such a reactivating potency that the rate of the reactivation reaction is of the same order of magnitude as the rate of aging, or the oxime, in addition to being a reactivator, is able at the same time to retard or even stop the aging. In this paper also experiments are described to investigate the mechanism according to which the reactivation by the most potent reactivator, HI-6, is achieved.



Code name	a	Y	b	R	X
HS-6	2	CH_2OCH_2	3	$C(O)NH_2$	Cl
HI-6	2	CH_2OCH_2	4	$C(O)NH_2$	Cl
HGG-12	2	CH_2OCH_2	3	$C(O)C_6H_5$	Cl
HGG-42	2	CH_2OCH_2	3	$C(O)C_6H_{11}$	I
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 oximes studied.

MATERIALS AND METHODS

Materials. Bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was obtained from Sigma Chemical Co., St. Louis, MO, and had a specific activity 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, F.R.G., and obidoxime from E. Merck, Darmstadt, F.R.G. Soman, TMB4 and the bispyridinium monooximes were prepared in this laboratory. HS-6 and HI-6 were synthesized according to Schoene [15], and HGG-12 and HGG-42 according to Hagedorn [16]. 3-Cyclohexyl-pyridine, one of the starting compounds for the synthesis of HGG-42, was obtained according to Schölkopf *et al.* [17] and Bregovec *et al.* [18]. All other reagents were commercial products of an analytical grade.

General outline of reactivation and aging experiments. A general outline of the reactivation and aging experiments is given in Fig. 2. Soman-inhibited acetylcholinesterase was formed by incubation of the enzyme with soman at pH 10.2 (step I) in order to prevent as much as possible premature aging of the inhibited enzyme formed. At this pH the rate of aging of phosphonylated acetylcholinesterases is very slow. At the same time the excess of inhibitor will be removed by a rapid hydrolysis of the organophosphate. The procedure is essentially equal to that described previously for the formation of cyclopentyl methylphosphonylated acetylcholinesterase, the aging of which also proceeds rapidly at pH 7.5 and 25° ($t_{\frac{1}{2}}$ = 10 min) [19]. The cyclopentyl methylphosphonylated enzyme formed in this way could be reactivated up to 98 per cent. In a control experiment incubation of the enzyme for 1 hr at pH 10.2 and 25° did not influence the enzyme activity.

Aging and reactivation of the inhibited enzyme were started by addition of an oxime solution in phosphate buffer, pH 7.4 (step II). In reactivation experiments samples of this mixture were taken after various times (t) and added to an acetic acid/NaOH buffer, pH 5.0 (step III). At the final pH of this mixture, being 6.0, the reactivation reaction is stopped. A further incubation for at least 1 hr ensures the aging of all reactivatable inhibited enzyme. By this step it was possible to store samples taken at short time intervals until their activities (AIR'_t) could be determined in a rather time-consuming titrimetrical assay (step IV). Blanks were treated in the same manner, except that for the determination of the activity of the enzyme (A) [soman] = 0 (step I) and [oxime] = 0 (step II), for the determination of the activity of the enzyme incubated with oxime (AR) [soman] = 0 (step I), and for the determination of the activity of the inhibited enzyme (AI) [oxime] = 0 (step II). Blanks were determined at the start of an experiment. For the determination of the reactivation after 24 hr, values of A and AR measured after approximately 24 hr (t = approx. 24 hr in step II) were used.

The percentage of reactivation obtained at time t in a reactivation experiment (% $react'_t$) was calculated according to

$$\%\ react'_t = \frac{AIR'_t \frac{A}{AR} - AI}{A - AI} 100. \qquad (1)$$

In aging experiments steps I and II are identical to those of a reactivation experiment. Aging was followed from the decrease of the ability of the inhibited enzyme to be reactivated. Samples taken at various times (t) were mixed with a HS-6 solution and incubated for 3–10 hr (step V). The non-aged, inhibited enzyme is rapidly, but partially (see Results), reactivated by the relatively high concentration of HS-6. Maximum reactivation will be attained within 0.5 hr (see Results). For practical purposes, however, an incubation period of 3–10 hr was chosen. In the subsequent activity assay (step VI) the sum of the activity of the inhibited enzyme reactivated in step V and of the activity of the inhibited enzyme reactivated already at time t (step II) is determined (AIR'^a_t). For the determination of aging in the absence of an oxime an aging experiment was carried out in which only the buffer was used in step II instead of an oxime solution in buffer.

Blanks were determined at the start of an experiment and were carried out in the same manner, except that for the determination of the activity of the enzyme (A) [soman] = 0 (step I), [oxime] = 0 (step II) and [HS-6] = 0 (step V), for the determination of the activity of the enzyme incubated with oxime and subsequently with HS-6 (ARR')

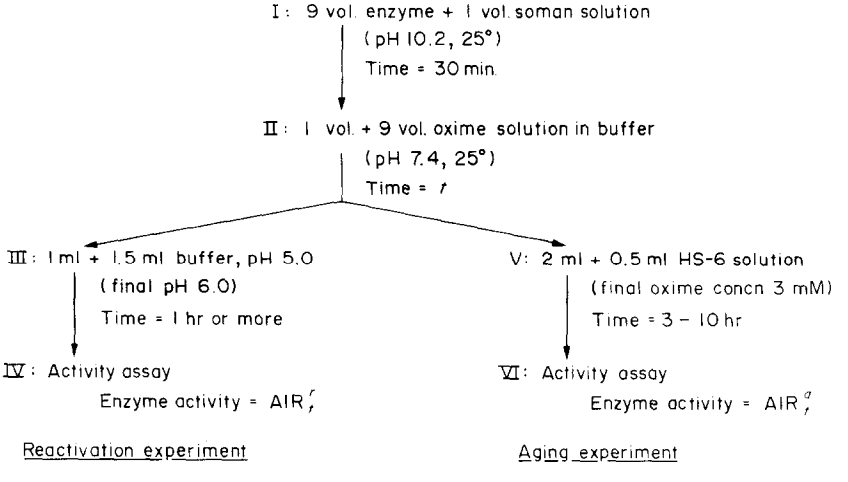


Fig. 2. General outline of a reactivation and of an aging experiment.

[soman] = 0 (step I), and for the determination of the activity of the inhibited enzyme (AI) [oxime] = 0 (step II) and [HS-6] = 0 (step V).

The percentage of reactivation obtained at time t in an aging experiment (% react$_t^a$) was calculated according to

$$\%\ \text{react}_t^a = \frac{\text{AIR}_t^a \dfrac{A}{ARR'} - AI}{A - AI} 100. \qquad (2)$$

Reactivation experiments. Inhibited acetylcholinesterase was obtained by incubating a mixture of 9 vol. of a solution of 9.3 mg enzyme/ml (approx. 43 nM of active sites) in 6.6 mM veronal buffer, with 1 vol. of 3 μM soman in water for 30 min at pH 10.2 and 25° in a Radiometer pH-stat equipment. Under these conditions over 95 per cent of the enzyme was phosphonylated. After incubation of a mixture of 0.2 ml of the inhibited enzyme solution and 1.8 ml of 0.9 mg acetylcholinesterase/ml of 40 mM phosphate buffer, pH 7.4, for 5.5 hr at 25°, less than 5 per cent inhibition was observed, indicating an almost complete hydrolysis of the excess of inhibitor. Reactivation and aging was started by addition of 1 vol. of the inhibited enzyme solution to 9 vol. of an oxime solution in 40 mM phosphate buffer, pH 7.4. In the reactivation experiments with 1 mM of the various oximes the final pH was 7.35–7.4. Samples of 1 ml were taken after 0.5, 1, 1.5 and 24 hr of reactivation at 25°. In the reactivation experiments with various concentration of HI-6 the final pH was 7.40–7.45. The enzyme was allowed to reactivate at 25° for 40–45 min. Samples of 1 ml were taken every 0.5 min or, in the experiment with 10 μM HI-6, every 1 min, during the first 5 or 10 min, respectively. During the subsequent 35–40 min another 5–10 samples were taken. The reactivation reaction was stopped by adding the samples to 1.5 ml acetic acid/NaOH buffer, pH 5.0, adjusting the pH to 6.0. Next, this mixture was incubated at room temperature for at least 1 hr to allow all reactivatable inhibited enzyme to age. After addition of 25 μl 1 N NaOH the enzyme activity of 2 ml was measured by using an apparatus developed by Keijer [20] for the automatic performance of pH-stat titrations. In the Radiometer titration equipment of the apparatus the burette unit SBU 1a was exchanged for an ABU 13 with a 0.25 ml-burette assembly. The activity assay was performed at pH 7.5 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. In a control experiment the enzyme activity was measured after certain times of reactivation, both directly and after treatment with the acetic acid/NaOH buffer. It was found that the treatment with the buffer stops the reactivation completely and has no effect on the restored enzyme activity.

Aging experiments. The inhibition of the enzyme and the removal of the excess of inhibitor were carried out as described for the reactivation experiments. The aging, or aging and reactivation, was started by addition of 1 vol. of the inhibited enzyme solution to 9 vol. of 40 mM phosphate buffer, pH 7.4, without or with added HI-6, respectively. The mixture had a pH within the range 7.41–7.48. Samples of 2 ml were taken every 0.5 min during the first 5 min, or every 1 min during the first 10 min in the experiments without HI-6 and with 10 μM HI-6. During the subsequent 35 or 30 min another 10–12 samples were taken. The samples were added to 0.5 ml of a 15 mM HS-6 solution in 50 mM disodium hydrogen phosphate, the pH of which was adjusted to 7.8. The final pH was adjusted to 7.9 by addition of 10 μl 1.2 N NaOH and the samples were allowed to reactivate for 3–10 hr at 25°. Samples of 1 ml were assayed for enzyme activity as described for the reactivation experiments, except that the assay was carried out at pH 7.8. In addition, the enzyme activity of a sample of the mixture of the inhibited enzyme and phosphate buffer with HI-6 incubated for 24 hr at 25° was measured.

RESULTS

Reactivating potency of various oximes

More than 95 per cent of the enzyme was already inhibited within 5 min of incubation of the enzyme with soman at pH 10.2 and 25°. The degradation of the excess of soman, however, required an incubation period of 30 min. After this treatment the inhibited enzyme was reactivated to 71 per cent by 3 mM HS-6 (pH 7.9, 25°). After incubation of the inhibited enzyme at pH 10.2 for longer time periods up to 120 min, 3 mM HS-6 restored the enzyme activity to a lower percentage, viz. 1–1.5 per cent less per incubation period of 30 min. From this it can be extrapolated that 2 per cent

$$\left[= \frac{100}{71 + (1 \text{ or } 1.5)} \times (1 \text{ or } 1.5) \right]$$

of the total enzyme activity is not reactivatable at the start of the reactivation and aging reactions, i.e. after incubation of the enzyme with soman for 30 min at pH 10.2 and 25°.

Maximum reactivation was obtained with 1 mM of the oximes studied within 30 min of reactivation. The averages of the percentages of reactivation found after 0.5, 1, 1.5 and 24 hr of reactivation are given in Table 1. The incomplete reactivation may be due to the simultaneous occurrence of reactivation by the oxime and of aging of the soman-inhibited enzyme even in the presence of an oxime. The reactions were further investigated for the most potent reactivator, HI-6.

Table 1. Percentage of maximum reactivation (% react$'_\infty$) of soman-inhibited acetylcholinesterase on incubation with 1 mM oxime at pH 7.4 and 25°

Oxime	% React$'_\infty$
P2S	18
TMB4	22
Obidoxime	23
HS-6	67
HI-6	77
HGG-12	31
HGG42	51

In the activity assay the oximes present at a concentration of 0.03 mM showed some inhibiting effect on the rate of hydrolysis of acetylcholine. The inhibition was independent of the time of incubation of the enzyme with the oxime, except in the case of HGG-12. This oxime initially caused 39 per cent of inhibition, but after 24 hr of incubation 80 per cent of the enzyme activity was inhibited.

Reactivation and aging in the presence of HI-6

Reactivation and aging experiments of soman-inhibited acetylcholinesterase with HI-6 were carried out with oxime concentrations ranging from 10 to 70 μM. At the lowest HI-6 concentration a reasonably measurable increase of activity with time was obtained in the reactivation experiment (final reactivation: 14 per cent) and at the highest concentration a decrease of reactivation with time which was still reasonably measurable, was obtained in the aging experiment (total decrease: 22 per cent of reactivation). An example of the results of a reactivation and of an aging experiment is given in Fig. 3. A final percentage of reactivation seems to be obtained within 30 min. These values (% react$'_\infty$) are 3–7 per cent less than the final value obtained in the corresponding aging experiments (% react$^a_\infty$). After 24 hr of incubation, however, the percentage of reactivation is approximately equal to the value of % react$^a_\infty$. Apparently, a small part of the inhibited enzyme is slowly reactivated and does not age in the presence of HI-6. In the absence of the oxime the aging proceeded according to first-order kinetics up to 90 per cent conversion of the inhibited enzyme, after which the rate of aging slowed down.

Expression of values of % react^r and % react^a as enzyme concentrations. To analyse kinetically the results of the reactivation and aging experiments the values obtained of % react^r and of % react^a are expressed as enzyme concentrations in the following manner. Let [E], [EI'] and [EI] be the concentrations of all forms of reactivated, aged and inhibited, but still reactivatable, enzyme, respectively. At the start of reactivation and aging $[E]_o = [EI']_o = 0$ and, since it was found that 2 per cent of the total inhibited enzyme concentration ($[E_{tot}]$) was not reactivatable

$$[EI]_o = \frac{98}{100}[E_{tot}]. \tag{3}$$

At time t of the reactions

$$[E]_t + [EI']_t + [EI]_t = [EI]_o. \tag{4}$$

For the results of the reactivation experiments it follows that

$$\frac{\%\ \text{react}^r_t}{100} = \frac{[E]_t}{[E_{tot}]} \tag{5}$$

and after complete reaction

$$\frac{\%\ \text{react}^r_\infty}{100} = \frac{[E]_\infty}{[E_{tot}]}. \tag{6}$$

To transform values of % react^a into an expression of enzyme concentrations it is assumed that after different times of incubation of the inhibited enzyme with HI-6 the same fraction (α) of the reactivatable inhibited enzyme is reactivated by the subsequent incubation with 3 mM HS-6. Then, it follows for the results of the aging experiments that

$$\frac{\%\ \text{react}^a_o}{100} = \frac{\alpha[EI]_o}{[E_{tot}]} \tag{7}$$

$$\frac{\%\ \text{react}^a_t}{100} = \frac{[E]_t + \alpha[EI]_t}{[E_{tot}]} \tag{8}$$

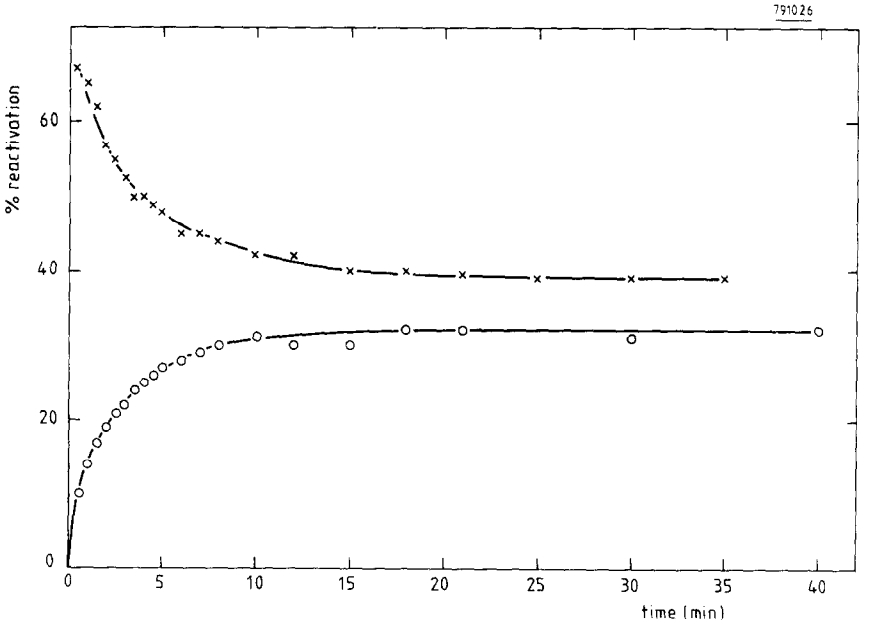


Fig. 3. Percentage of reactivation obtained in a reactivation experiment (○, % react$'_t$, see Eqn. 1) and in an aging experiment (×, % react^a_t, see Eqn. 2) carried out with soman-inhibited acetylcholinesterase in the presence of 40 μM HI-6 at pH 7.4 and 25°.

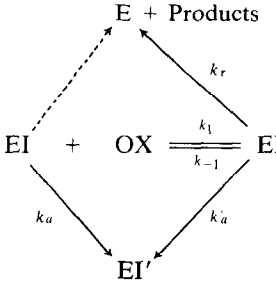
and

$$\frac{\%\ \text{react}^a_\infty}{100} = \frac{[E]_\infty}{[E_{tot}]}. \tag{9}$$

From Eqns. (3) and (7) it follows that

$$\alpha = \frac{\%\ \text{react}^a_0}{98}. \tag{10}$$

Kinetic analysis according to a minimum reaction scheme. The minimum reaction scheme to describe the simultaneous reactivation and aging of phosphonylated acetylcholinesterase in the presence of an oxime (OX) is



(11)

Spontaneous reactivation of the soman-inhibited enzyme was not observed. For this scheme it was derived [21] that

$$[EI]_t = [EI]_0\, e^{-k_{obs}t} \tag{12}$$

and

$$[E]_t = [E]_\infty(1 - e^{-k_{obs}t}), \tag{13}$$

where

$$k_{obs} = \frac{k_a\frac{k_{-1}}{k_1} + k'_a[OX] + k_r[OX]}{\frac{k_{-1}}{k_1} + [OX]}. \tag{14}$$

After substitution of $[E]_t$ and $[E]_\infty$ according to Eqns. (5) and (6), Eqn. (13) leads to

$$\ln(\%\ \text{react}^r_\infty - \%\ \text{react}^r_t) = \ln(\%\ \text{react}^r_\infty) - k_{obs}t. \tag{15}$$

From Eqns. (13), (8) and (9) it is derived after substitution of $\alpha[EI]_t$ in Eqn. (8) by using Eqns. (12) and (7), that

$$\ln(\%\ \text{react}^a_t - \%\ \text{react}^a_\infty) = \ln(\%\ \text{react}^a_0 - \%\ \text{react}^a_\infty) - k_{obs}t. \tag{16}$$

The data obtained in the reactivation and aging experiments were plotted according to Eqns. (15) and (16), respectively. An example is given in Fig. 4. Similar plots were obtained with the other HI-6 concentrations. Obviously, the results do not obey the minimum reaction scheme (11). The plots give straight lines except for the last part of the reactions, but the slopes of the plots for the reactivation experiments and for the corresponding aging experiments, which should be equal (k_{obs}), differ significantly ($P < 0.01$, Behrens–Fisher test) and the intercepts of the plots of log (% react$^r_\infty$ − % react^r_t) vs t for the data obtained in the experiments with 40–70 μM HI-6 largely deviate from the value of log (% react$^r_\infty$) (Eqn. 15).

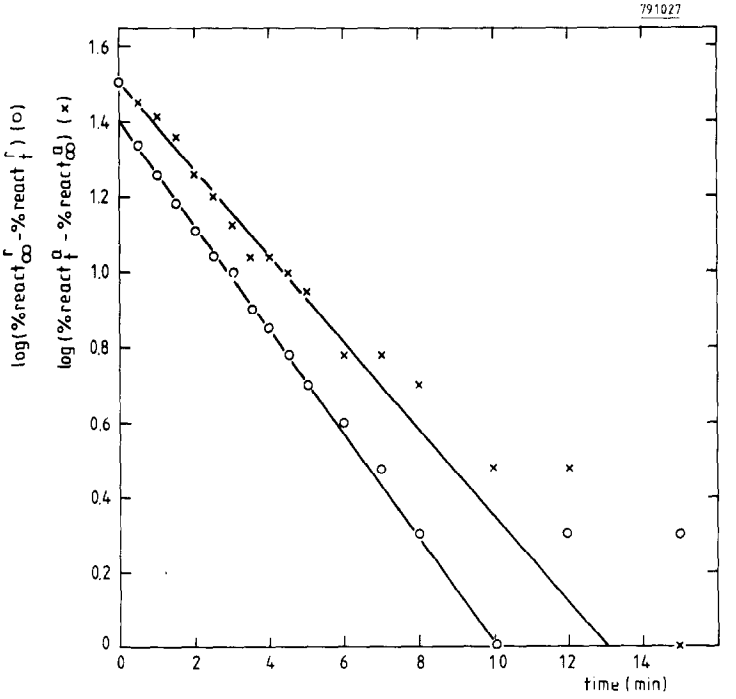


Fig. 4. Plot of log (% react$^r_\infty$ − % react^r_t) vs t (○) according to Eqn. (15) for data of a reactivation experiment and plot of log (% react^a_t − % react$^a_\infty$) vs t (×) according to Eqn. (16) for data of an aging experiment carried out with soman-inhibited acetylcholinesterase in the presence of 40 μM HI-6 at pH 7.4 and 25°.

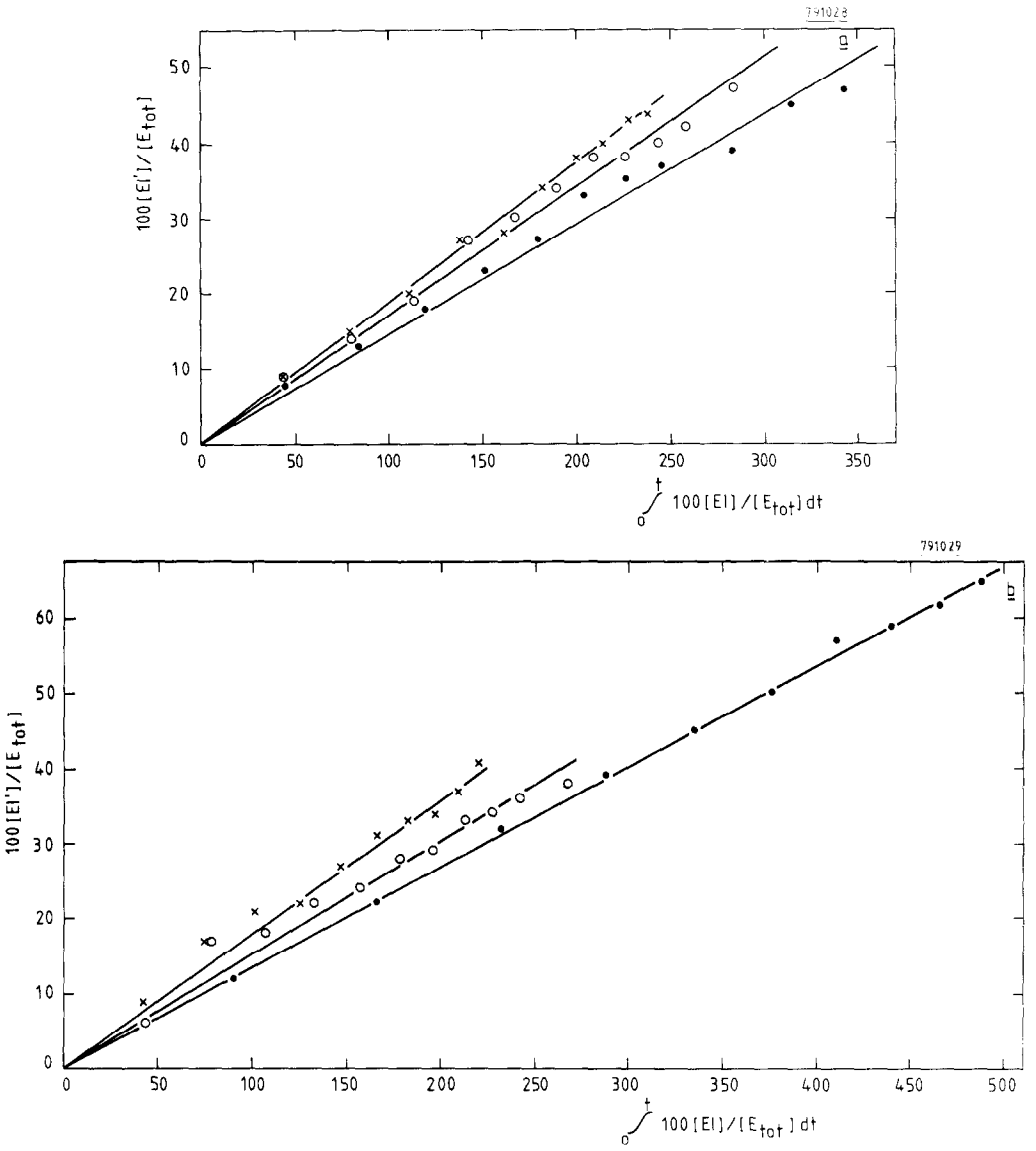


Fig. 5. Plots of 100 $[EI']_t/[E_{tot}]$ against $\int_0^t 100[EI]/[E_{tot}]\,dt$. The data were calculated (see Eqns. 19 and 20) from results obtained in reactivation and aging experiments with soman-inhibited acetylcholinesterase carried out at pH 7.4 and 25° in the presence of HI-6 at a concentration of (a) 30 μM (●), 40 μM (○) or 50 μM (×); (b) 10 μM (●), 60·μM (○) or 70 μM (×).

Evaluation of the overall rate constant of aging. Aging is a first-order reaction of a phosphorylated or phosphonylated acetylcholinesterase. In the presence of HI-6 the soman-inhibited enzyme may be present in more than one form, for instance as such and as a complex with the oxime, which may age at different rates. Nevertheless, the overall rate of formation of the aged enzyme might be described by

$$\frac{d[EI']}{dt} = k_{ag}[EI], \qquad (17)$$

where k_{ag} represents the overall rate constant of aging. Whether the obtained results obey this equation was examined as follows.

Equation (17) can be written as

$$\frac{100[EI']}{[E_{tot}]} = k_{ag}\int \frac{100[EI]}{[E_{tot}]}\,dt. \qquad (18)$$

From Eqns. (5), (8) and (10) it is obtained that

$$\frac{100[EI]_t}{[E_{tot}]} = \frac{98(\%\ \text{react}_t^a - \%\ \text{react}_t')}{\%\ \text{react}_0^a} \qquad (19)$$

and from Eqns. (4), (5), (7), (10) and (19) it is derived that

$$\frac{100[EI']_t}{[E_{tot}]} = 98 - \%\ \text{react}_t' - \frac{98(\%\ \text{react}_t^a - \%\ \text{react}_t')}{\%\ \text{react}_0^a}. \qquad (20)$$

The integral

$$\int_0^t \frac{98\ (\%\ \text{react}_t^a - \%\ \text{react}_t')}{\%\ \text{react}_0^a}\,dt$$

was calculated for each time t at which values of

Table 2. Rate constant of aging of soman-inhibited acetylcholinesterase and the overall rate constant of aging in the presence of HI-6 (k_{ag} of Eqn. 17) at pH 7.4 and 25°

HI-6 concentration (μM)	Rate constant of aging (min^{-1})
—	0.16
10	0.14
30	0.15
40	0.17
50	0.19
60	0.15
70	0.18

% react$_i^a$ and % react$_i'$ were determined, according to the trapezoidal rule, by means of a computer. Values of % react$_0^a$ were found by extrapolation of plots of log (% react$_i^a$ − % react$_\infty^a$) vs t as given in Fig. 4. The values obtained for the integral were plotted against the corresponding values of 100 $[EI']_i/[E_{tot}]$ calculated according to Eqn. (20). The plots obtained for the various concentrations of HI-6 are given in Figs. 5a and b. The data taken from the part of the reactions in which approximately 80 per cent of the final concentration of the aged enzyme is formed, reasonably fit Eqn. (18). The data from the last part of the reactions deviate from a single line. Values of k_{ag} were calculated as the slopes of the plots by means of the method of least-squares. The values are given in Table 2 together with the rate constant of aging determined in the absence of HI-6 which was calculated as the slope of a plot of log % react$_i^a$ vs t.

Analogously, the rate of formation of reactivated enzyme might be represented by

$$\frac{d[E]}{dt} = k_{react}[EI] \tag{21}$$

or

$$\frac{100[E]}{[E_{tot}]} = k_{react} \int \frac{100[EI]}{[E_{tot}]}\, dt, \tag{22}$$

where k_{react} is the overall rate constant of reactivation. Plots of values obtained for the integral against the corresponding values of % react$_i'$ (≈ 100 $[E]_i/[E_{tot}]$, see Eqn. 5) are given in Figs. 6a and b. The data cover that part of the process in which approximately 80 per cent of the final concentration

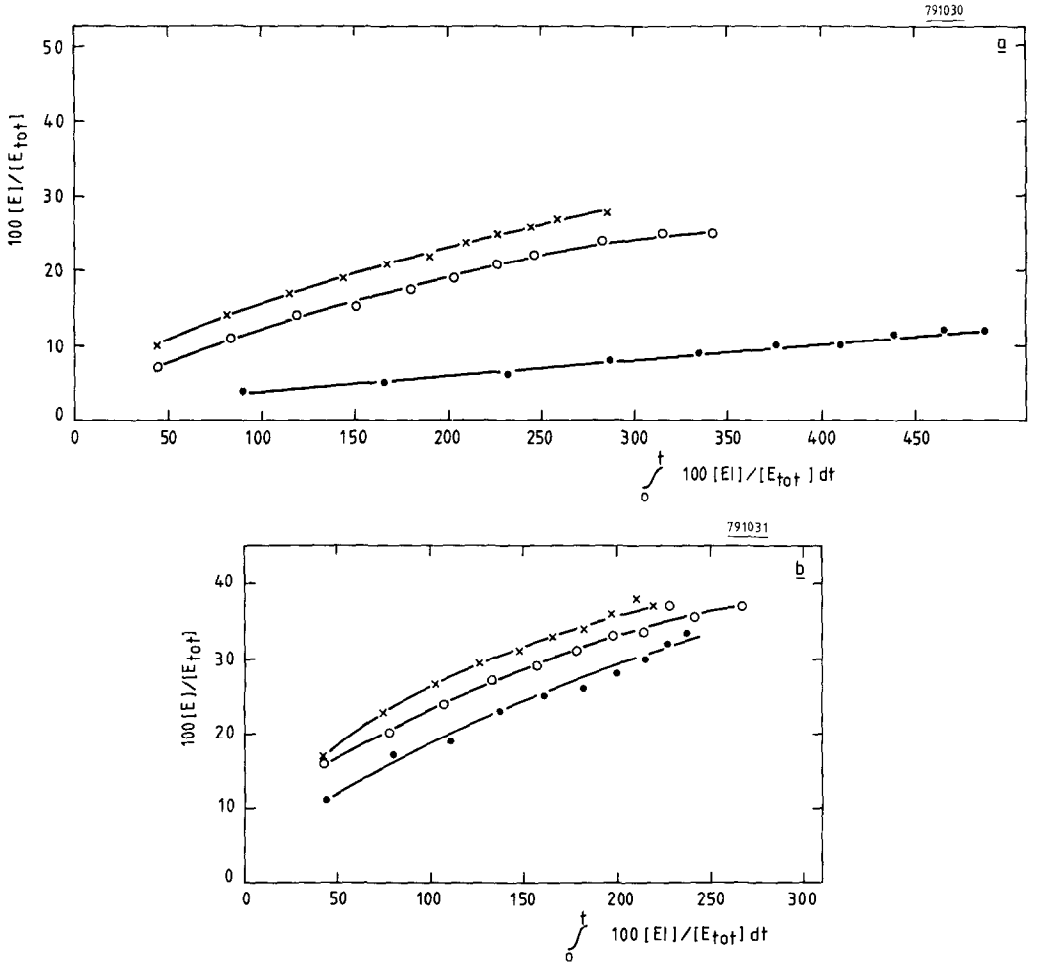


Fig. 6. Plots of 100 $[E]_i/[E_{tot}]$ against $\int_0^t$ 100 $[EI]/[E_{tot}]$ dt. The data were calculated (see Eqns. 5 and 20) from results obtained in reactivation and aging experiments with soman-inhibited acetylcholinesterase carried out at pH 7.4 and 25° in the presence of HI-6 at a concentration of (a) 10 μM (●), 30 μM (○) or 40 μM (×); (b) 50 μM (●), 60 μM (○) or 70 μM (×).

of the reactivated enzyme is formed. The results clearly show that the reactivation process cannot be described by Eqn. (22).

DISCUSSION

Incubation of acetylcholinesterase with soman at pH 10.2 and 25° for 30 min appears to be a suitable procedure for the formation of soman-inhibited enzyme which is almost completely reactivatable. For the assay of the reactivation of the inhibited enzyme at short time intervals the pH of the samples taken was lowered to 6.0 which stops the reactivation. At the same time reactivatable inhibited enzyme is rapidly transferred into aged enzyme at this pH. So, it was possible to store samples of the reactivation mixture until their activities could be measured in the time-consuming titrimetrical assay. Aging of inhibited acetylcholinesterase is mostly determined from the decrease of the ability of the inhibited enzyme to be reactivated by an oxime. None of the oximes studied, however, was able to reactivate completely soman-inhibited acetylcholinesterase. It was shown that the percentages of incomplete reactivation obtained can be used to calculate the rate constant of aging provided that the same fraction of the reactivatable enzyme in the samples taken at different times of aging is reactivated by the oxime added.

The bispyridinium mono-oximes clearly show a much higher reactivating potency *in vitro* of soman-inhibited acetylcholinesterase than the conventional oximes. This result corresponds with the therapeutic activities found for these oximes against soman intoxication. The most potent reactivators are HS-6 and HI-6. HS-6 was already found to be a more potent reactivator than the conventional oximes of soman-inhibited acetylcholinesterase [14] and of cyclopentyl methylphosphonylated acetylcholinesterase, which also exhibits a rapid aging [$t_{\frac{1}{2}}$ (pH 7.5, 25°) 10 min] [19]. A time-dependent inhibition of acetylcholinesterase by one of the oximes, HGG-12, was observed, indicating that the enzyme reacts with HGG-12 or possibly with a decomposition product of the oxime formed at pH 7.5.

Aging and reactivation of soman-inhibited acetylcholinesterase proceed simultaneously in the presence of the oximes studied. Similar observations made for acetylcholinesterase inhibited by ethyl dimethylphosphoramidocyanidate (tabun) with P2S and some l-alkyl analogues [21] and for the cyclopentyl methylphosphonylated enzyme with l-pentyl and l-heptyl analogues of P2S [19] could be described with the minimum scheme (11). The reactions of soman-inhibited acetylcholinesterase in the presence of HI-6 do not obey this scheme. Aging of the soman-inhibited enzyme is shown to proceed in the presence of the oxime as a first-order process. The reactivation process is more complicated. Previously, the minimum scheme was also found not applicable to the reactivation and aging of cyclopentyl methylphosphonylated acetylcholinesterase in the presence of P2S [19]. Results were obtained indicating that reinhibition by the phosphonylated oxime formed on reactivation plays a substantial role in the reactions. This may afford an explanation for the present results too.

Aging of soman-inhibited acetylcholinesterase proceeds initially according to first-order kinetics, but becomes slower after 90 per cent of the inhibited enzyme has been converted. A similar observation was reported by Michel *et al.* [3]. The kinetics of aging occurring in the presence of HI-6 also show deviation from first-order as the reaction nears completion. This phenomenon may be due to the chirality of soman. Four stereoisomers are present in racemic soman owing to the chiral phosphorus atom and a chiral carbon atom in the pinacolyl group. The relative concentrations of the inhibited acetylcholinesterases formed by incubation with a large excess of racemic soman are determined by the ratio of the rate constants of inhibition for the stereoisomers. Since acetylcholinesterase shows a large stereospecificity in its reaction with the stereoisomers of soman having a different configuration around the phosphorus atom [22], only phosphonylated enzymes with the same configuration around the phosphorus atom will be obtained on inhibition with excess of racemic soman. The rate of reactivation and of aging of these inhibited enzymes may differ. The small part of the soman-inhibited acetylcholinesterase which shows slower aging and a much slower reactivation by HI-6 might be the inhibited enzyme formed with the slower inhibiting one of the two rapidly inhibiting stereoisomers having a different configuration around the carbon atom.

An effective reactivation of soman-inhibited acetylcholinesterase has to compete with the very rapid aging occurring simultaneously. This difficulty is overcome, at least partially, if a reactivator is able to retard the aging. Quaternary ammonium compounds not having any reactivating properties have been reported to retard the aging of acetylcholinesterase inhibited by soman [4, 23], and by other organophosphates [21, 24–26]. In the study of Harris *et al.* [4] an enhanced reactivation of soman-inhibited acetylcholinesterase by TMB4 or obidoxime in the presence of 1,1'-[oxybis(methylene)]bis[4-(1,1-dimethylethyl)-pyridinium] dichloride (SAD-128), which retards the aging of the inhibited enzyme, was found. This retarding effect by SAD-128 should increase the ratio of the rate of reactivation to that of aging, resulting in a higher final percentage of reactivation. In addition, binding of SAD-128 to a regulatory site and production of a conformational change rendering the inhibited enzyme more susceptible to reactivation by oxime, as proposed by Harris *et al.* [4], may contribute to the augmentative effect by this compound. For a proper understanding of the influence of a reactivator on aging it is useful to distinguish between the effect of the compound directly on aging itself, resulting in a different rate constant for the reaction, and the effect of indirectly slowing down of aging by withdrawing inhibited enzyme from the aging reaction owing to reactivation. Reactivators have been reported to reduce the rate constant of aging of cyclopentyl methylphosphonylated acetylcholinesterase [19], but to enlarge that of the tabun-inhibited enzyme [21]. The retarding or termination of the aging of soman-inhibited acetylcholinesterase found by Fleisher *et al.* [2] by

using high concentrations of P2S is probably mainly due to withdrawal of inhibited enzyme from the aging reaction by reactivation. The present results indicate that HI-6 has no effect on aging of the soman-inhibited enzyme, whereas this oxime is the most powerful reactivator known. The concentration of enzyme reactivated in the presence of 1 mM of the oxime is over three times as high as that of aged enzyme formed. This implies an extremely high reactivating potency of HI-6 for soman-inhibited acetylcholinesterase on which the therapeutic effect of this oxime is based.

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