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EFFECTS OF SOME MONO- AND BISQUATERNARY AMMONIUM COMPOUNDS ON THE REACTIVATABILITY OF SOMAN-INHIBITED HUMAN ACETYLCHOLINESTERASE *IN VITRO*

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Abstract—Acetylcholinesterase (AChE) inhibited by the organophosphate soman (1,2,2-trimethylpropylmethylphosphonofluoridate) rapidly becomes resistant to reactivation by oximes due to dealkylation of the soman–enzyme complex. This reaction is called aging. The effect of the four mono- and bisquaternary ammonium compounds tetramethylammonium (TMA), hexamethonium, decamethonium and suxamethonium on the reactivatability of soman-inhibited, solubilized AChE from human erythrocytes was investigated *in vitro*. All compounds were reversible inhibitors of AChE; the respective dissociation constants and the type of inhibition exhibited considerable differences. The affinities to both the active and the allosteric site were considerably higher for suxamethonium (K_{ii} 81.3 μM; K_i 15.9 μM) and decamethonium (K_{ii} 15.4 μM; K_i 4.4 μM) than for TMA (K_{ii} 1 mM; K_i 289.6 μM) and hexamethonium (K_{ii} 4.5 mM; K_i 331.8 μM).

The reactivation experiments were performed in a four-step procedure (soman-inhibition at 0° and pH 10, aging at 37° and pH 7.3, reactivation by the oxime HI 6 at 37° and pH 7.3 followed by AChE assay). After these four steps (total duration 55 min), AChE was inhibited by soman to 95–100%. HI 6 could reactivate about 20% of the inhibited enzyme. All effectors increased the AChE reactivatability by HI 6 when added *before* aging was started. The maximal increase in reactivatability was higher in the presence of 1.6 mM suxamethonium (+35.8%) and 150 μM decamethonium (+40%) than of 22 mM TMA (+22.5%) and 8.3 mM hexamethonium (+19.2%). If the effectors were added *after* 5 min of aging they increased the activity of soman-inhibited AChE, but to a considerably smaller extent than HI 6. A good correlation of the respective K_{ii} values and the effective concentrations of these drugs was observed, indicating that an allosteric binding site of AChE might be involved in the protective effect of these drugs.

The principal toxic mechanism of certain organophosphates, such as organophosphorus pesticides and the so-called nerve agents, is the inhibition of the acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by phosphylation of its catalytic site. The causal therapy of this intoxication consists of the reactivation of the inhibited enzyme by removing the phosphyl moiety. For that purpose, a large number of pyridinium oximes, like P2S, TMB4, obidoxime and the so called Hagedorn oximes, have been synthesized [1].

Acetylcholinesterase (AChE) inhibited by the organophosphate soman (1,2,2-trimethylpropylmethylphosphonofluoridate) undergoes a rapid dealkylation of the soman–enzyme complex. After the pinacolyl moiety is removed from the enzyme-bound soman, the phosphonylated enzyme cannot longer be reactivated by oximes [2–4]. This reaction is called *aging*. The search for drugs preventing aging remains an urgent task, because, in contrast to the enzyme of other species [5–8], human AChE inhibited by soman ages very fast (half time about 1.3 min) [9].

Bosković [10] suggested that the protection of the anionic site of AChE by quarternary ammonium compounds might prevent the rapid dealkylation (aging) of soman bound to the esteratic site of the catalytic unit. Up to now, nearly all beneficial effects of quaternary ammonium compounds (like tubocurarine, gallamine, methylpyridinium, decamethonium, hexamethonium and others) on aging had been demonstrated for non-human AChE [11–13] or for organophosphates other than soman (e.g. sarin [14]). The only positive effects on aging of human enzyme have been reported by Harris *et al.* [9] for SAD-128 and for atropine by Kuhnen and Schoene [15].

A somewhat different mechanism of action for bisquaternary ammonium compounds which might also result in an increased reactivatability by oximes has been suggested by Ohnesorge [16] and Lüllmann *et al.* [17]. The authors inhibited AChE with ^{3}H-diisopropylphosphorofluoridate (DFP) and found that these compounds reduced the phosphorylation rate of AChE.

Kinetic studies [18] revealed that different bisquaternary compounds (gallamine, pancuronium, curare, succinyldicholine, decamethonium) bind to an "allosteric" site of AChE thus exerting an indirect influence on the active site. This allosteric site might

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account for the above-mentioned beneficial effects on organophosphate-inhibited AChE.

The purpose of this study was to investigate the influence of several mono- and bisquaternary ammonium compounds on the reactivatability of human erythrocyte AChE inhibited by soman. Particular attention was paid to perform most of the experiments at physiological temperature (37°) and pH (7.3).

MATERIALS AND METHODS

Materials. For all experiments we used solubilized AChE of human erythocytes (type XIII) purchased from Sigma (Munich, F.R.G.). Tetramethylammonium iodide (TMA), hexamethonium bromide and decamethonium bromide were purchased from Sigma (Munich, F.R.G.), suxameth onium chloride was a gift from Hormonchemie (Munich). All these drugs were of analytical purity. Soman was 92% pure. HI 6 was synthesized by Prof. Hagedorn (Freiburg, F.R.G.), recrystallized before use and its purity checked by HPLC. All other compounds used in the titrimetric and photometric assays were purchased from Sigma (Munich) or Merck (Darmstadt, F.R.G.).

Inhibition of AChE by quarternary ammonium compounds. Based on the assumptions of Kitz *et al.* [18], we started the experiments by evaluating the type of AChE inhibition of the quaternary compounds. The inhibition kinetics and calculations were performed according to Bisswanger [19]. K_i is the dissociation constant of the enzyme–inhibitor complex (inhibition at the active site) and K_{ii} the dissociation constant of the enzyme–substrate–inhibitor complex (inhibition at an allosteric site):

$$K_i = [E]\cdot[I]/[EI];\ K_{ii} = [ES]\cdot[I]/[ESI];$$

where E is the enzyme, I the inhibitor and S the substrate.

Five mg AChE (0.4–1.0 U/mg protein) was dissolved in 5 ml 52 mM phosphate buffer pH 7.3. Aliquots of 100 µl were diluted in 3 ml of the same buffer and mixed with 0.25 mM dithiobisnitrobenzoic acid (Ellman's reagent [20]). 0, 10, 50 or 100 µl of a 3 mM effector solution or 50 µl of a 30 mM effector solution (final concentrations 10, 50, 100 or 500 µM) in phosphate buffer pH 7.3 was added and incubated at 37°. After 10 min, 100 µl 156 mM acetylthiocholine (substrate, final concentration 5 mM) was added and ΔE/min was determined on a spectrophotometer at 405 nm. This assay was repeated at different substrate concentrations of 0.5, 0.25, 0.20, 0.15 and 0.1 mM.

The K_i and K_{ii} values of the different inhibitors were calculated on the base of the respective Lineweaver–Burk plots which were constructed by eye, using the following formulae according to Bisswanger [19]:

$$K_{ii} = \frac{[I]}{y\cdot V - 1};\ K_i = -\frac{K_m\cdot x\cdot[I]}{1 + ([I]:K_{ii}) + K_m\cdot x};$$

where x and y are the intersections of the graph with the x- and y-axis, $V = V_{max}$, K_m = Michaelis–Menten constant and $[I]$ the concentration of the inhibitor.

Table 1. Procedure for the assessment of the reactivatability of soman-inhibited AChE*

	Titrimetric	Photometric
1. Inhibition	300 µl AChE (*ca.* 0.4 U/ml) in veronal (3.3 mM, pH 10) +20 µl soman (15 nM) t = 30 min T = 0°	100 µl AChE (*ca.* 0.4 U/ml) in veronal (3.3 mM, pH 10) +7 µl soman (15 nM) t = 30 min T = 0°
2. Aging	+300 µl veronal (25 mM, pH 7.0) +10 µl *effector* in veronal (25 mM, pH 7.3) t = 5 min T = 37°	+100 µl veronal (25 mM, pH 7.0) +10 µl *effector* in veronal (25 mM, pH 7.3) t = 5 min T = 37°
3. Reactivation	+60 µl HI 6 (320 µM) in veronal (25 mM, pH 7.3) t = 20 min T = 37°	+20 µl HI 6 (320 µM) in veronal (25 mM, pH 7.3) t = 20 min T = 37°
4. Assay	Whole specimen diluted in 20 ml 3 mM Acetylcholinperchlorate and 0.1 M KCl, pHstat titration with 2 mM NaOH; registration of AChE activity in µl volume NaOH per 60 sec.	Whole specimen diluted in 3.0 ml Ellman's reagent (52 mM phosphate buffer, pH 7.3, 0.25 mM DTNB) and 0.1 ml acetylthiocholine (5 mM); registration of AChE activity in ΔE per 30 sec.

* The two methods were checked against each other. The results obtained by these different procedures were essentially identical and will therefore not be presented separately.

The K_{ii}/K_i ratio was calculated to get an estimate of the type of inhibition. Small values should indicate AChE inhibition at the allosteric site, high values inhibition at the active site.

Aging experiments. The reactivatability of AChE after a 5-min aging period in the presence and absence of an effector was measured by two methods, a titrimetric (pHstat method) and a photometric assay. All effects were checked by both methods. The procedure which is summarized in Table 1, consisted of four successive steps: inhibition—aging—reactivation—assay. The concentrations given were final concentrations referred to the third (reactivation) step. The concentration of HI 6 (320 μM) was evaluated as most effective in previous experiments using different concentrations of HI 6. Essentially identical results were obtained by the two different methods. Control runs were performed by replacing both the effector and the reactivator HI 6 or only the effector by distilled water. The results were corrected for the spontaneous hydrolysis of acetyl(thio)choline in the presence or absence of HI 6 respectively.

All steps except the first, i.e. aging, reactivation and assay, were performed at pH 7.3 and 37°. For the first step (inhibition) AChE was incubated together with soman in 3.3 mM veronal buffer pH 10 at 0° to prevent aging [4]. After 30 min, the enzyme was inhibited to about 60% as found in separate experiments. At this moment, aging was started by mixing the sample with 25 mM veronal buffer pH 7.0 to obtain pH 7.3 (see Table 1). At the same time, the respective effectors were added. Five minutes later, reactivation was started by addition of HI 6. At this moment (after 35 min total reaction time) the enzyme activity in the absence of any effector was still 6–12%.

The effectors were investigated at concentrations of their respective K_{ii} values and then diluted or concentrated to investigate the dose–response relationship. All effects were checked for statistical significance by a *t*-test for independent samples.

Effect of the quaternary compounds added after 5 min of aging. To get more insight into the mechanism of action we investigated the reactivation capacity of the four compounds. The different effectors were added at concentrations of their maximal effectiveness (based on the results of the forementioned experiments) together with, or without, HI 6 after the 5-min period of *in vitro*-aging (in step 3). This

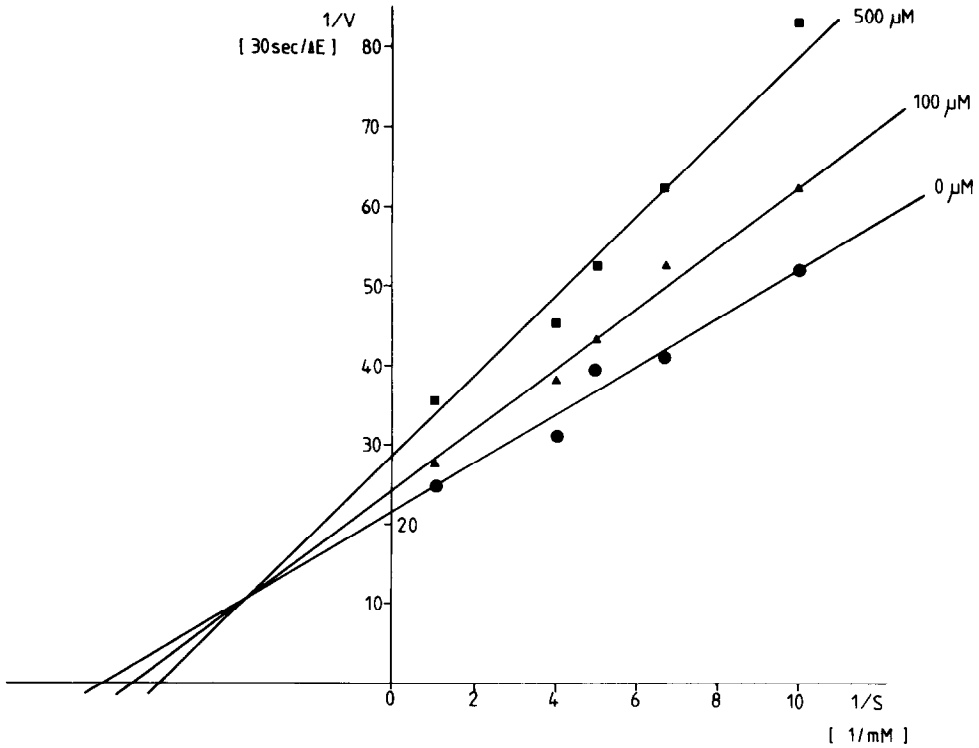

Fig. 1. Inhibition of the acetylcholinesterase (AChE) activity by tetramethylammonium iodide (TMA) at 37° and pH 7.3.

TMA was incubated at different concentrations (0, 100 and 500 μM) in a solution containing 3 ml 52 mM phosphate buffer pH 7.3 mixed with 0.25 mM dithiobisnitrobenzoic acid (Ellman's reagent) and 100 μl AChE (*ca.* 0.7 U/ml). After 10 min, 100 μl acetylthiocholine (substrate, final concentration 1 mM) was added and ΔE/min was determined on a spectrophotometer at 405 nm. The same measurements were repeated at different substrate concentrations of 0.5, 0.25, 0.2, 0.15 and 0.1 mM. The dissociation constants of TMA (K_i: 0.29 mM; K_{ii}: 1.00 mM) were calculated according to Bisswanger [19] on the base of the Lineweaver–Burk plot shown in this figure. For the other quaternary compounds the same procedure was used. The results are summarized in Table 2.

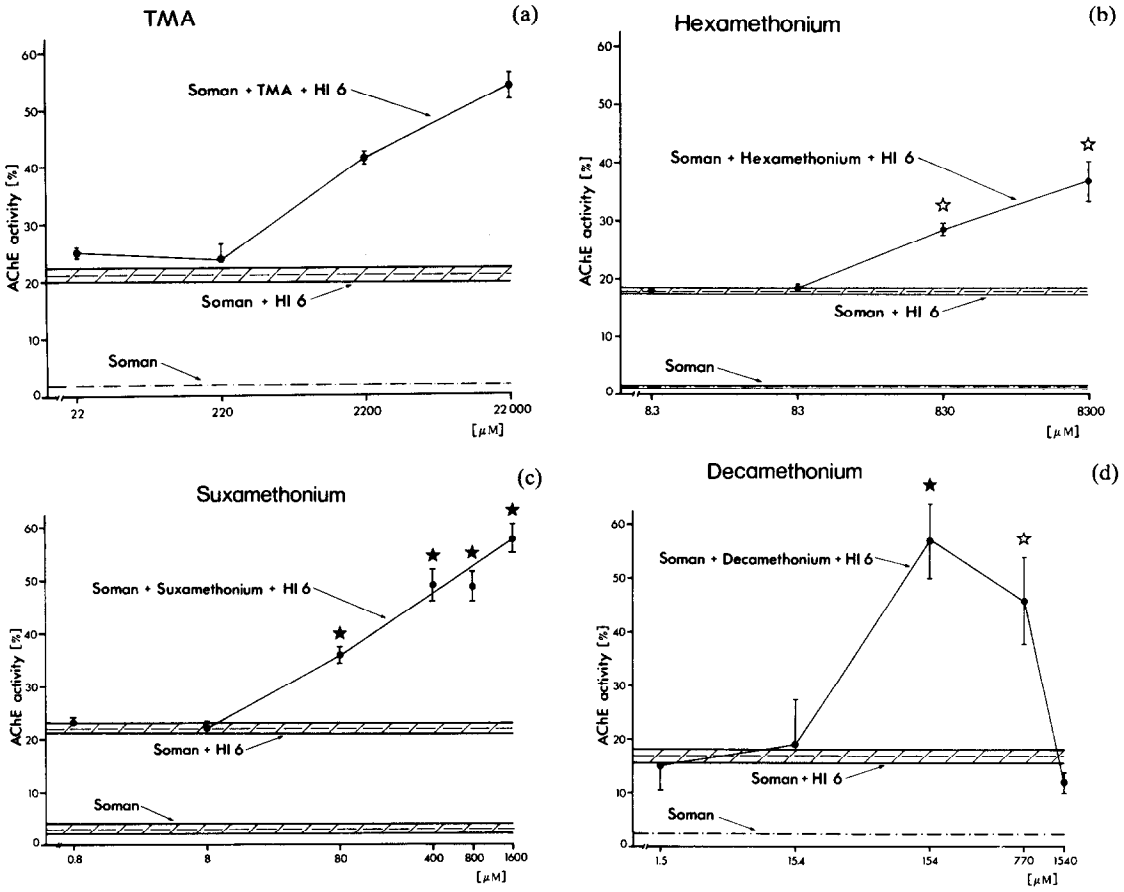

Fig. 2(a–d). Effect of four quaternary ammonium compounds (TMA, hexamethonium, suxamethonium and decamethonium) on the reactivation of soman-inhibited AChE by HI 6. AChE was incubated for 30 min in a solution containing soman at conditions preventing aging (0°, pH 10) [4]. Before starting aging by changing the temperature (37°) and pH (7.3), the respective compounds were added. After 5 min, AChE was reactivated by HI 6 for 20 min. Control runs were performed by replacing the effector (soman + HI 6) or both the effector and HI 6 (soman) by distilled water. All results are expressed as mean values ± SE of at least four experiments. Difference from control experiments was *t*-tested ($\star P < 0.05$; $\bigstar P < 0.01$).

mixture was then incubated at pH 7.3 and 37° for 20 min as described above.

RESULTS

AChE inhibition

All compounds caused an inhibition of AChE activity. As an example, the Lineweaver–Burk plot for tetramethylammonium (TMA) is shown in Fig. 1. The point of intersection of the different straight lines of the Lineweaver–Burk plot lied neither on the *x*- nor on the *y*-axis, indicating a mixed (competitive and non-competitive) type of inhibition. The dissociation constants of the four quaternary ammonium compounds investigated are summarized in Table 2. Although the type of inhibition appeared to be similar for all compounds, there was a marked difference in affinity: the dissociation constants (K_{ii} and K_i) for TMA and hexamethonium were about two orders of magnitude higher than for suxamethonium and decamethonium. This indicated a much higher affinity of the two latter compounds to both the peripheral and the active site. For all compounds, the K_{ii}/K_i ratio was markedly higher than 1. This was probably due to a higher affinity of these compounds to the active site than to a peripheral site. The K_{ii}/K_i ratio was particularly high for hexamethonium suggesting that this substance inhibits the AChE competitively rather than non-competitively.

Effectors added at the start of aging

Figures 2(a–d) show the effects of the bis- or mono-quaternary effectors on the reactivatability by HI 6 of soman-inhibited AChE after an aging period of 5 min. The respective control runs without effector and without or with HI 6 are shown on the bottom of the graphs as hatched bars (mean ± SE). When soman was added alone without effector or HI 6, AChE was found to be inhibited by 95–100% after the whole experimental procedure. When HI 6 was added after 5 min of aging, it reactivated about 17–22% of the enzyme. This reactivatability could be further increased by different effectors added at the start of aging (at step 2, see Table 1).

The four compounds were effective at different

Table 2. Inhibition of human erythrocyte AChE by different mono- or bis-quaternary ammonium compounds

Substance	K_{ii} (10^{-6} M)	K_i (10^{-6} M)	K_{ii}/K_i
Tetramethylammonium	1000	290	3.5
Hexamethonium	4500	330	13.6
Decamethonium	15	4.4	3.5
Suxamethonium	81	16	5.1

The dissociation constants of the different compounds were evaluated according to [19] (see methods). All results represent mean values of at least two experiments.

concentrations. Decamethonium and suxamethonium exhibited a statistically significant ($P < 0.01$), beneficial effect at considerably lower concentrations (154 and 80 μM) than TMA and hexamethonium (2.2 mM and 830 μM). The effectiveness of TMA, hexamethonium and suxamethonium exhibited a dose-dependent increase, whereas decamethonium had a clear maximum of effectiveness at 150 μM. Its maximal effect was found at the 10-fold concentration of the K_{ii} value (see Table 2). A further increase resulted in a decreased efficacy (770 and 1500 μM).

To summarize the results, the reactivation of soman-inhibited AChE by HI 6 could be improved by the different effectors as follows (maximal effects in the range of concentrations investigated):

TMA (22 mM):	+ 22.5%;
Hexamethonium (8.3 mM):	+ 19.2%;
Suxamethonium (1.6 mM):	+ 35.8%;
Decamethonium (0.15 mM):	+ 40.0%;

The total reactivation obtained by these combinations reached maximal levels of about 60% of the original AChE activity (for decamethonium and suxamethonium).

Effectors added after 5 min of aging

If the quaternary ammonium compounds were added 5 min after the start of aging without HI 6, the AChE activity was slightly increased in comparison to control experiments (Fig. 3a). This effect depended on the effector concentration and was significantly different ($P < 0.01$) from control values for suxamethonium (at 810 μM) and hexamethonium (830 μM, 8.3 mM). No significant effect was found with TMA and decamethonium. The reactivation obtained by HI 6 could not be further augmented by any of these compounds (Fig. 3b).

DISCUSSION

The data presented demonstrate that quaternary ammonium compounds can increase the reactivatability by HI 6 of soman-inhibited human AChE *in vitro* at nearly physiological temperature and pH (Fig. 2a–d and Fig. 3a,b). All drugs investigated were inhibitors of the AChE (Table 2). Their respective dissociation constants, however, varied considerably, in agreement with the results of previous investigations [17, 21–23].

The affinities to both the active and an allosteric binding site of the AChE were about two orders of magnitude lower for TMA and hexamethonium than for suxamethonium and decamethonium (Table 2). In addition, the type of inhibition was not uniform for all quaternary compounds. According to the literature [18, 22], the kinetic analysis revealed a mixed, competitive and non-competitive type of inhibition for TMA, decamethonium and suxamethonium (Fig. 1 and Table 2). The K_{ii}/K_i ratios of these three compounds were quite similar (3.5–5.1) and relatively low compared to hexamethonium (13.6). As suggested by Schuh [23] and Lüllmann *et al.* [17], the affinity of hexamethonium to the active site is probably much higher than to any allosteric site, and it can therefore be considered a competitive inhibitor in contrast to the other quaternary ligands investigated in this study.

Moreover, the affinity to the allosteric site (K_{ii}) of these compounds appeared to be a reliable indicator of the effective concentration, because quaternary compounds with a low affinity (TMA, hexamethonium; see Table 2) exhibited their protective effect on AChE at much higher concentrations than those with a relatively high affinity (decamethonium, suxamethonium). In contrast, almost no effect was observed at K_i concentrations. The correlation of the K_{ii} values and the effective concentrations supports the hypothesis [10] that a peripheral modulatory site might be involved in the protective action of these quaternary ammonium ligands.

All four compounds augmented the reactivatability by HI 6 of soman-inhibited human erythrocyte AChE when added at the start of aging. However, considerable differences between the quaternary ammonium compounds could be observed with respect to the shape of the concentration–effect curves of these drugs and their efficacy. Decamethonium was the only compound to exhibit a bell-shaped concentration–effect curve with a maximum at 154 μM. At higher concentrations, its beneficial effect decreased (770 μM) or even disappeared (1.5 mM). The other three compounds (TMA, hexamethonium, suxamethonium) showed a steady concentration-dependent increase of efficacy in the range investigated, even if we used higher concentrations than with decamethonium.

The comparison of the ability of the four quaternary ammonium compounds to improve the reactivatability of soman-inhibited AChE revealed further important differences. Decamethonium and suxamethonium showed higher effects (+40 and

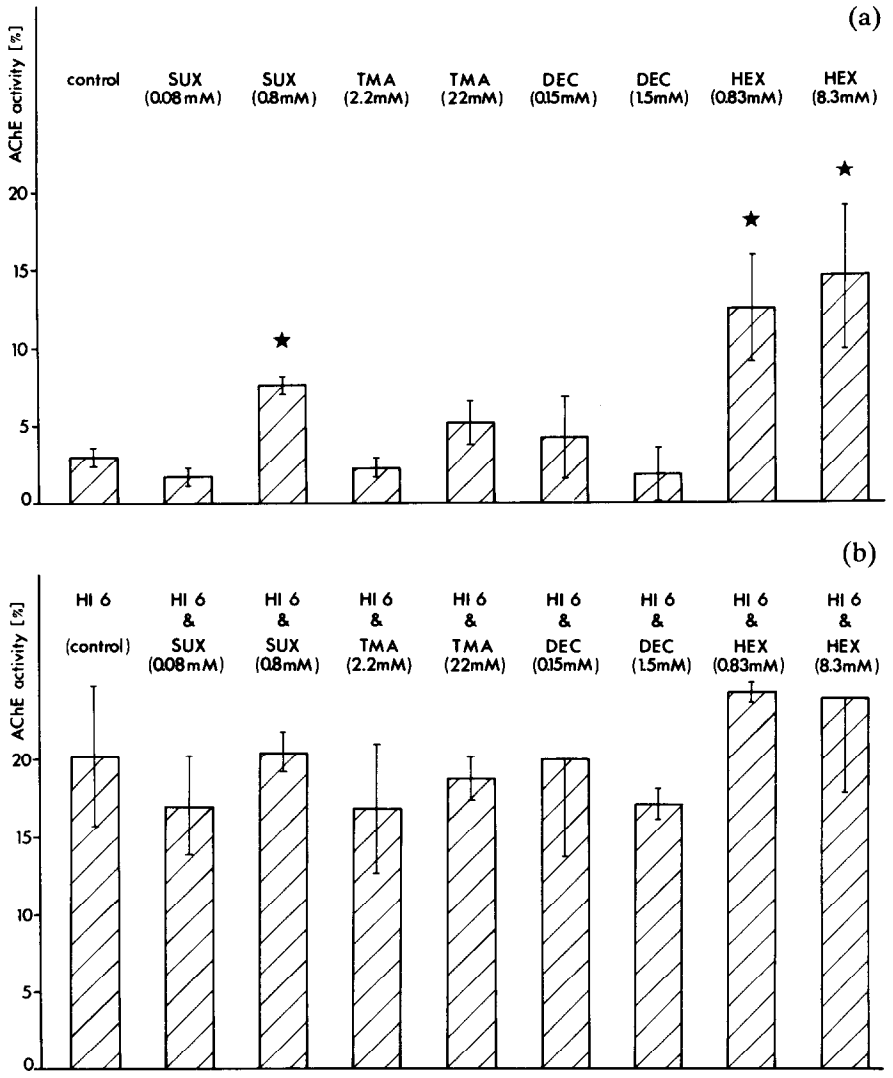

Fig. 3(a, b). Effect of quaternary ammonium compounds on soman-inhibited AChE after a 5-min period of aging. The same procedure as indicated in the legend of Fig. 2(a–d) (see Table 1) was used with the only exception that the different effectors were added (alone or together with HI 6) 5 min *after* aging was started. All results are expressed as mean values ± SD of at least four experiments. Difference from control experiments was *t*-tested (★$P < 0.01$).

+35.8%) at lower concentrations (150 μM and 1.6 mM) than hexamethonium and TMA (+19.2 and +22.5%; 8.3 and 22 mM). These findings partially correspond to a recently published study of Berman and Decker [13] who found the highest efficacy for decamethonium in retarding aging of methylphosphono-AChE.

According to the literature [9–14, 16, 17] two mechanisms of action might be responsible for the beneficial effect of mono- or bisquarternary ammonium compounds on soman-inhibited AChE: (1) the prevention of enzyme phosphonylation and (2) the reduction of the dealkylation rate of the phosphonylated enzyme. A direct reactivation of the phosphonylated enzyme by these compounds is not probable because of the absence of an oxime group [24]. On the base of the results presented, both mechanisms are possible. The first mechanism would explain, why these compounds, especially hexamethonium and suxamethonium, increased the activity of AChE incubated with soman when added alone after a 5-min aging period. At that time, the AChE was not completely inhibited (residual activity 6–12%, see Methods); the quaternary compounds probably prevented the phosphonylation of the remaining, uninhibited active sites. This is in good agreement with previous studies using DFP-inhibited AChE [16, 17].

However, there was indirect evidence that this is not the only mechanism of action of quaternary ammonium compounds upon soman-inhibited AChE. Especially decamethonium is likely to retard

the dealkylation reaction (aging) of soman-inhibited AChE, as suggested by Berman and Decker [13] for methylphosphono-AChE. Our data support this hypothesis, as decamethonium exerted the highest effect of all compounds investigated on the reactivatability of soman-inhibited AChE by HI 6 (Fig. 2d), but the smallest effect when added alone (Fig. 3a). Thus, decamethonium might rather retard aging than prevent the phosphonylation of AChE by soman, in contrast to the other three drugs investigated which seem to slow the phosphonylation of AChE at a relatively higher degree.

Our data do certainly not allow exhaustive considerations on structure–activity relationships. Further polymethonium compounds should be investigated. However, our data are in agreement with the assumption of Berman and Decker [13] that bisquaternary polymethonium compounds are more effective in increasing the reactivatability than monoquaternary compounds as shown for TMA in this study and for decyltrimethylammonium and hexyltrimethylammonium by the forementioned authors [13]. Additionally, an interquarternary chain length of 10 methylene groups (decamethonium) might result in a higher efficacy than of 6 groups (hexamethonium). Perhaps the same interquaternary distance of 1.45 nm which is believed to be partially responsible for the depolarising activity of decamethonium and suxamethonium [25] is optimal to improve the reactivatability.

In conclusion, both the prevention of phosphonylation and the decrease of dealkylation of the phosphonylated AChE may contribute to the beneficial action of mono- or bisquaternary compounds on soman-inhibited enzyme. The majority of publications dealing with aging of organophosphate-inhibited AChE neglects the prevention of phosphylation [9, 13, 15]. As shown by this work and by previous investigations [16, 17], this effect might well contribute to an increased reactivatability and should therefore be considered in further studies.

Concerning the practical therapeutic aspects of soman-poisoning, the mechanism of the beneficial effects becomes less important, because both prevention of phosphonylation and retardation of aging are rather of prophylactic than of therapeutic interest. As shown by Ohnesorge [16] with DFP, these ammonium compounds might also exert protective effects *in vivo*. The toxicity of all compounds investigated in this study is certainly too high to take them into consideration for routine antidotal administration. In agreement with Crone [14] and Gray *et al.* [12] we therefore stress the need for less toxic agents.

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