

Acceleration of Oxime-Induced Reactivation of Organophosphate-Inhibited Fetal Bovine Serum Acetylcholinesterase by Monoquaternary and Bisquaternary Ligands

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ABSTRACT

Reactivation of organophosphate (OP)-inhibited acetylcholinesterase (AChE) by oximes is the primary reason for their effectiveness in the treatment of OP poisoning. Reactivation is reported to accelerate by quaternary ligands such as decamethonium, which is devoid of nucleophilicity. The mechanism of this enhancement is not known. To better understand the acceleration phenomenon, we examined ligand modulations of oxime-induced reactivation of methylphosphonylated AChE using 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide and fetal bovine serum AChE. Edrophonium, decamethonium, and propidium, three quaternary AChE ligands of different types, were tested as potential accelerators. Experiments were carried out with both soluble enzyme preparation and AChE conjugated to polyurethane. Kinetic measurements with oximes 2-[hydroxyiminomethyl]-1-methylpyridinium chloride, 1,1'-trimethylene bis-(4-hydroxyimino methyl)-pyridinium dibromide, and 1,1'-[oxybis-methylene]bis[4-(hydroxyimino)-methyl]pyridinium dichloride showed that in the presence of 50 μ M edrophonium, the reactivation rate constants increased 3.3–12.0-fold; 200 μ M decamethonium produced a 1.6–3.0-fold enhancement of reactivation rate constants by the same

oximes. Reactivation of the inhibited enzyme by 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxy-aminopyridinium)-dimethyl ether hydrochloride, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(3-carboxy-aminopyridinium)-dimethyl ether hydrochloride, and 1-[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2, 4-bis(hydroxyimino)methyl pyridinium dichloride was not affected by either ligand. Propidium slowed the reactivation of 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide-inhibited AChE by all oximes. Results suggest that the accelerator site may reside inside the catalytic gorge rather than at its entrance and acceleration may be due to the prevention of reinhibition of the regenerated enzyme by the putative product, the phosphonylated oxime. In addition to the nucleophilic property of the oximate anion, some of the reactivators may carry an accelerating determinant, as characterized with respect to edrophonium and decamethonium. Results offer possible explanations for the superiority of 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxy-aminopyridinium)-dimethyl ether hydrochloride over other oximes in the reactivation of specific AChE-OP conjugates.

AChE (EC 3.1.1.7), a serine hydrolase, catalyzes the hydrolysis of the neurotransmitter acetylcholine and terminates impulse transmission at cholinergic synapses. Acute

toxicity of OP compounds is attributed to irreversible inhibition of AChE, via covalent linkage to γ -oxygen of the active-site serine. Reactivation of inhibited AChE by nucleophiles such as oximes is an efficient way to attenuate toxicity; it plays a key role in the treatment of OP poisoning and has been the subject of investigation for decades to find more effective reactivators for the treatment of OP pesticide and

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ABBREVIATIONS: AChE, acetylcholinesterase; FBS, fetal bovine serum; OP, organophosphate; POX, phosphonylated oxime; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; EMP, O-ethyl methylphosphonyl; 7-HQ, 7-hydroxy-1-methyl quinolinium cation; ATC, acetylthiocholine; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); SAD-128, 1,1'-oxydimethylene bis-(4-*tert*-butylpyridinium chloride); 2-PAM, 2-[hydroxyiminomethyl]-1-methylpyridinium chloride; TMB₄, 1,1'-trimethylene bis-(4-hydroxyimino methyl)-pyridinium dibromide; LùH₆ (toxogonin), 1,1'-(oxybis-methylene)bis[4-(hydroxyimino)methyl]pyridinium dichloride; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxy-aminopyridinium)-dimethyl ether hydrochloride; HS-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(3-carboxy-aminopyridinium)-dimethyl ether hydrochloride; HLo7, 1-[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2, 4-bis(hydroxyimino)methyl pyridinium dichloride; MMB₄, 1,1'-methylene bis[4-(hydroxyiminomethyl)pyridinium dibromide].

chemical warfare agent toxicity. Reactivation of inhibited enzyme depends on the OP moiety, the nature and source of enzyme, and the structure of reactivator. In some cases, changes in one factor result in significant enhancement or retardation of the reactivation rate constants. One strategy to improve efficacy of reactivators is to characterize their structural features that accelerate displacement of OP-bound moiety and introduce them into newly designed oximes.

Many studies have attempted to delineate interactions between oximes and AChE-OP conjugates to find the structural features in oxime molecules and inhibited enzyme that determine reactivatability (Ashani *et al.*, 1995; Schwart *et al.*, 1995; Grosfeld *et al.*, 1996; Bencsura *et al.*, 1995; Saxena *et al.*, 1993; Masson *et al.*, 1997). Some investigators have also found that oxime-induced reactivation of AChE-OP conjugates can be accelerated by the quaternary ligands decamethonium and SAD-128 (Harris *et al.*, 1978; Luo *et al.*, 1995). This phenomenon was first observed in experiments with human erythrocyte AChE using soman and sarin to obtain inhibited enzyme (Harris *et al.*, 1978). These authors demonstrated that the bisquaternary compound SAD-128 slowed the aging of soman-inhibited AChE and thereby extended the period for oximes TMB₄ and LüH₆ to restore enzyme activity before aging took place. No data for the enhancement of reactivation rate constant were provided, but a relative increase in the fraction of the reactivated enzyme was noted. Slowing of the rate of aging in the presence of SAD-128 and other monoquaternary and bisquaternary ligands, such as tetramethylammonium, suxamethonium, and hexamethonium, was confirmed later (Štalc and Šentjerc, 1990; Grubič and Tomazič, 1989; Szinicz and Hallek, 1988). For sarin-inhibited AChE, it was suggested that the enhancement of reactivation could not be attributed to the same mechanism proposed for soman-inhibited enzyme because of the slow aging of AChE-sarin conjugate. Explanations such as decreasing the rate of inhibition by parent OP or conformational changes due to tight binding of these ligands at the peripheral site of the enzyme were suggested (Harris *et al.*, 1978). Because in none of these experiments were residual OPs completely removed before the addition of the oxime and the accelerating ligand, it is difficult to provide a straightforward explanation for the results, and the mechanism of acceleration phenomenon remains unclear. Interpretation of reactivation data is complicated by several parallel reactions: reactivation of inhibited enzyme, reinhibition of regenerated enzyme by putative POX (Fig. 1) (Hackley *et al.*, 1959; Schoene, 1972; Harvey *et al.*, 1986a, 1986b; Ashani *et al.*, 1995), and the possibility of different allosteric effects exhibited by the ligands used.

To clarify the mechanism of ligand-induced acceleration of oxime reactivation, we examined the effect of several quaternary ligands on the reactivation of MEPQ-inhibited FBS AChE (Fig. 1). MEPQ (Levy and Ashani, 1986) was used to inhibit the enzyme for the following reasons. (1) The high bimolecular inhibition rate constant permits a near-stoichiometric inhibition protocol using relatively low concentrations of reactants. (2) Inhibited AChE does not readily age. (3) The OP/enzyme conjugate EMP-AChE represents a series of methylphosphonyl-AChE conjugates obtained with methylphosphonofluorides such as soman and sarin. FBS AChE is available in relatively large quantities as purified enzyme, and much experimental data have been accumulated on its

inhibition and reactivation characteristics (Ashani *et al.*, 1990; Wolfe *et al.*, 1994). Sequence alignment suggests that the amino acid residues lining the active-site gorge of FBS and human AChEs are identical (Cygler *et al.*, 1993). Three reversible ligands, edrophonium, decamethonium, and propidium, which interact with AChE by different mechanisms (Taylor and Lappi, 1975; Radić *et al.*, 1991; Harel *et al.*, 1993; Barak *et al.*, 1994), were studied for their effects on the reactivation of MEPQ-inhibited FBS AChE by various oxime reactivators. Time courses of oxime-induced reactivation were monitored, and the second-order reactivation rate constants were determined with and without accelerating ligands. In an attempt to remove the putative reactivation byproduct POX from the microenvironment of the enzyme during reactivation, FBS AChE immobilized on polyurethane sponge was used. Results shed light on the effects of added ligands on oxime-induced reactivation and provide clues for explaining the differences observed in the reactivation potency of various oximes.

Experimental Procedures

Materials. MEPQ was prepared according to a previously reported procedure (Levy and Ashani, 1986). 2-PAM, TMB₄, LüH₆ (toxogonin), HI-6, HS-6, HLo7, and MMB₄ (Fig. 1) were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC). Decamethonium and propidium were purchased from Sigma Chemical (St. Louis, MO). Electrophoretically pure FBS AChE (5000 units/mg; 1 unit of enzyme will hydrolyze 1.0 μ mol/min acetylcholine at pH 8.0 at 25°) was prepared in our laboratory according to a method reported previously (De La Hoz *et al.*, 1986). Bio-Spin 6 chromatography columns were purchased from BioRad (Hercules, California); FBS AChE sponge was prepared by covalent linking of the tetrameric form of the enzyme to the polyurethane foam (LeJeune *et al.*, 1996).

AChE assay. FBS AChE activity was determined spectrophotometrically according to the method of Ellman *et al.* (1961). One nanomole of FBS AChE has 400 units of activity. The assay mixture (3.2 ml) contained 1 mM ATC as substrate and 1 mM DTNB in 50 mM phosphate buffer, pH 8.0. All measurements were performed at 25°.

Determination of dissociation constants of the complex between native FBS AChE and oxime. Dissociation constants for complexes involved in the reversible inhibition of native FBS AChE by oximes were determined as described previously (Ashani *et al.*, 1995). Briefly, increasing concentrations of ATC were added to FBS AChE that was preincubated with various concentrations of oxime and 1 mM DTNB in 50 mM phosphate buffer, pH 8.0, at 25°. Activities were corrected for oxime-catalyzed hydrolysis of ATC. Plot of 1/V versus 1/S (Lineweaver-Burk plot) yielded a series of straight lines with different slopes. The slopes of these lines were replotted against the oxime concentration. The K_{ox} value was determined from the x intercept of the straight lines obtained from the secondary plot.

Dose-response profiles of acceleration by quaternary ligands. To 0.75 unit of FBS AChE in 50 mM phosphate buffer, pH 8.0, containing 0.05% BSA, a stoichiometric amount of MEPQ was added to ensure ~98% inhibition of enzyme activity. The mixture, in a final volume of 50 μ l, was incubated at 25° for 1 hr. To reactivate the enzyme, 1 mM oxime was added with or without the quaternary ligand to be tested and further incubated for 15 min at 25° in a total volume of 100 μ l (final concentration of the enzyme, 18.75 nM). The incubation mixture was applied to a prewashed Bio-Spin 6 chromatography column and immediately centrifuged at 1000 $\times g$ for 2 min. Control experiments based on inhibition of fresh FBS AChE solution by the column effluent clearly showed that all the three reversible ligands, at concentrations as high as 1 mM, could be effectively removed by this technique. Control enzyme samples were treated in

the same manner except that no MEPQ was added to the enzyme solution. Enzyme activity of the sample was determined as described above. Percent reactivation was calculated as described previously (Ashani *et al.*, 1995).

Determination of reactivation rate constants in the presence and absence of accelerating ligands. A substoichiometric amount of MEPQ (sufficient to inhibit ~90% of enzyme activity) was added to 2.5 units of FBS AChE in a final volume of 50 μ l of 50 mM phosphate buffer, pH 8.0, containing 0.05% BSA and incubated for 1 hr at 25°. The incubation mixture was diluted 10-fold with the same buffer to produce a final concentration of 12.5 nM enzyme. This minimized the effects of residual MEPQ on the reactivation rate. The final oxime concentration in the reactivation mixture ranged from 10

μ M to 3 mM. At specified time intervals, 10 μ l of reactivation mixture was withdrawn and diluted into 3.2 ml of assay mixture to monitor the change in enzyme activity. The reactivation rate constants in the presence of accelerators were determined using 50 μ M edrophonium or 200 μ M decamethonium. These concentrations caused only marginal inhibition of the enzyme activity (<5%) after dilution in the assay buffer.

Due to the chirality of the phosphorus in MEPQ, the phosphonyl conjugate EMP-AChE consists of two stereoisomers that are reactivated at different rates. The reactivation rates were calculated assuming an equal distribution of the two components of conjugated enzyme under the conditions used for the inhibition. Regression and

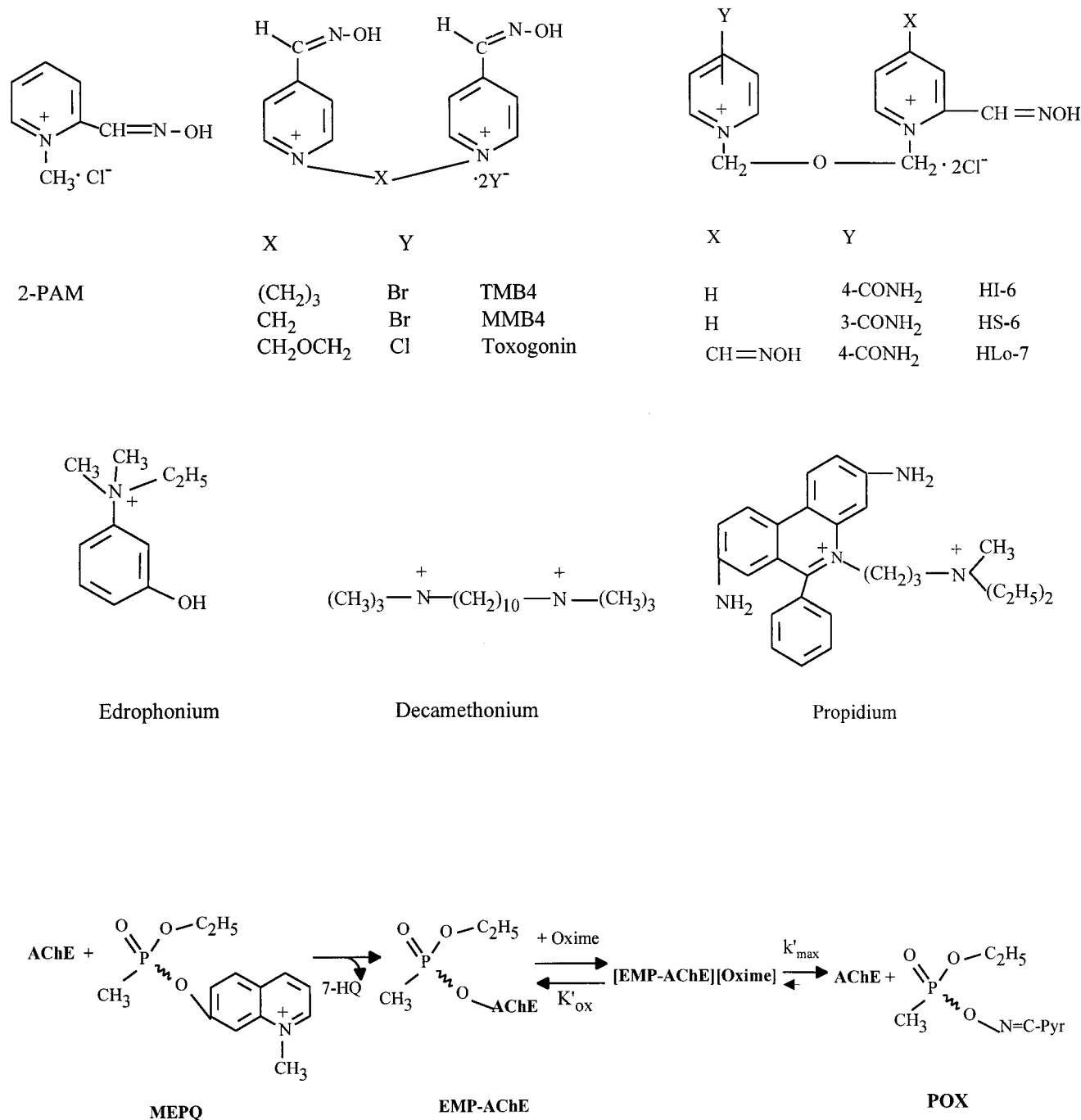
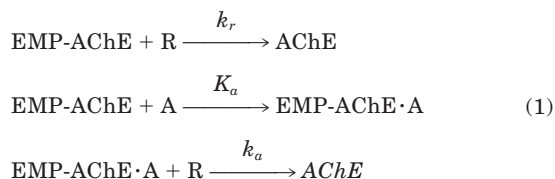


Fig. 1. Structure of oximes and accelerating ligands and the kinetic scheme for inhibition and reactivation of FBS AChE by MEPQ. 7-HQ, hydroxyquinulium leaving group, EMP-AChE, inhibited enzyme. POX, phosphonylated oxime. Pyr, pyridinium moiety of POX.

statistical analyses were performed using Prism Version 2.0, 1995 (GraphPAD Software, San Diego, CA).

Determination of the dissociation constant of the complex between edrophonium and AChE-OP conjugate. To estimate the dissociation constant of the complex between the accelerator and the inhibited enzyme, the following kinetic scheme was used in accordance with the assumptions made by Kitz and Wilson for the acceleration of the methanesulfonylation of AChE by substituted ammonium cations (Kitz and Wilson, 1963).



where EMP-AChE and AChE are the inhibited and reactivated enzymes, R and A are oxime and accelerator, EMP-AChE·A is the complex of accelerator with the phosphorylated enzyme (with the dissociation constant K_a , and k_r and k_a are the rate constants of the reactivation in the absence and presence of the accelerator. The mathematical solution of the kinetic scheme is approximated by:

$$k_{\text{obs}}/k_r = (K_a + \beta A)/(K_a + A) \quad (2)$$

where k_{obs} is the observed rate constant in presence of accelerator, and $\beta = k_a/k_r$.

Assay of enzyme activity of FBS AChE immobilized on polyurethane foam (sponge). We cut 10 mg of sponge (containing ~0.1 unit of enzyme activity) into small pieces and placed them into 10 ml of 50 mM phosphate buffer, pH 8.0. To the sponge suspension, 0.33 ml of 30 mM ATC was added and mixed thoroughly for 5 min. An aliquot of 1.033-ml supernatant was transferred to a cuvette with 33 μ l of 30 mM DTNB, and the absorbance at 412 nm was measured immediately. Enzyme activity is expressed as units of activity/g of sponge.

Reactivation of MEPQ-inhibited FBS AChE immobilized on sponge. We placed 10 mg of sponge into 1 ml of 0.375 μ M MEPQ solution for 30 min to inhibit >98% of AChE activity. The sponge was washed twice with 10 ml of 50 mM phosphate buffer, pH 8.0, and packed into a 3-mm internal diameter column. The column was washed with either reactivator/buffer solution or reactivator/ligand/buffer solution at a flow rate of 2 ml/min. After 15 min, the column was washed with 50 mM phosphate buffer, pH 8.0. The sponge was extruded from the column and assayed for enzyme activity as described above. Controls were treated in the same way except that no OP was added.

Results

Dissociation constants of FBS AChE-oxime complexes. Lineweaver-Burk plots for most of the oximes revealed competitive inhibition. K_{ox} values (mM) determined from the slopes of Lineweaver-Burk plot versus oxime concentrations were as follows (standard error <25%): 2-PAM, 0.16; TMB₄, 0.037; LüH₆, 0.4; MMB₄, 0.90; HI-6, 0.09; HS-6, 0.22; and HL07, 0.042.

Spontaneous reactivation of EMP-AChE in the presence of different quaternary ligands. MEPQ-free phosphorylated enzyme was obtained by gel filtration using Bio-Spin column chromatography. Control experiments showed that residual OP could be completely removed from the inhibited enzyme by this procedure. No significant acceleration of the spontaneous reactivation of EMP-AChE could be observed in the first 2 hr of incubation in presence of 20 μ M propidium, 50 μ M edrophonium, or 200 μ M decamethonium

(Fig. 2). However, on prolonged incubation (22 hr), it seemed that the three ligands produced different effects on spontaneous reactivation. Edrophonium more than doubled the spontaneous reactivation rate of the AChE-OP conjugate; decamethonium did not change the rate; and propidium decreased the spontaneous reactivation rate by <50%. However, these changes were essentially negligible compared with changes observed in oxime-induced reactivation with the same ligands.

Effect of increasing concentration of quaternary ligands on oxime-induced reactivation of EMP-AChE. A dose-response acceleration of reactivation of EMP-AChE was observed with edrophonium and decamethonium. Edrophonium displayed a remarkable enhancement of reactivation by 2-PAM, TMB₄ and LüH₆ (Fig. 3, A–C) in the concentration range of 10 μ M to 1 mM and produced a greater acceleration than decamethonium. The peripheral-site ligand propidium slowed the reactivation process with all oximes tested, also in a concentration-dependent manner. None of the ligands could promote HI-6-induced reactivation of the phosphorylated enzyme (Fig. 3D). In these experiments, the concentration of the leaving group product of MEPQ, 7-HQ, was present at ~18 nM. In control experiments with a 7-HQ-free inhibited enzyme obtained by gel filtration, the reactivation measured was similar to that observed in the presence of 7-HQ. Therefore, at the specified concentration, it did not significantly affect the results.

Kinetic rate constants for the reactivation of EMP-AChE in the presence of accelerators. Although minor deviations occurred at low concentrations of TMB₄ and LüH₆, in most cases the data fitted well to an exponential association equation for the two-component model in the determination of first-order reactivation rate constant, k_{obs} . The magnitudes of k_{obs} values for the fast reactivation component of the inhibited enzyme were usually several-fold larger than those of the slow reactivation component.

Secondary plots of k_{obs} versus oxime concentration were used to obtain the second-order reactivation rate constants. Plot of k_{obs} versus oxime concentration is expected to yield a curve that asymptotically approaches a constant value when

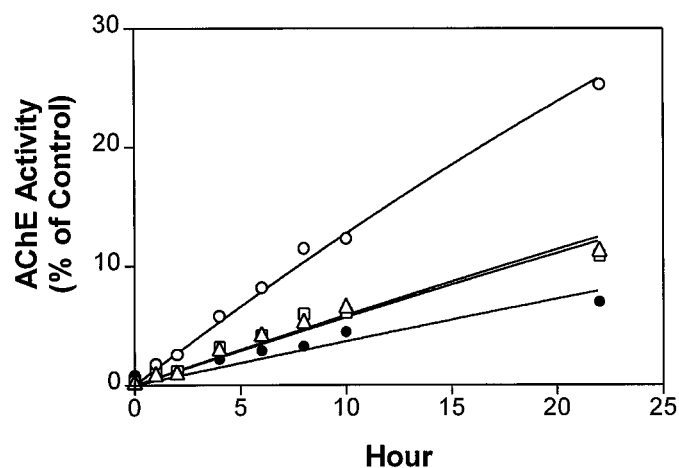


Fig. 2. Spontaneous reactivation of MEPQ-inhibited FBS AChE with and without quaternary ligands. EMP-AChE free of excess inhibitor was incubated in 50 mM phosphate buffer, pH 8.0, with or without ligands at 25°, and enzyme activity was monitored at specified time intervals. Δ , Control. \square , 200 μ M decamethonium. \circ , 50 μ M edrophonium. \bullet , 20 μ M propidium.

the concentration of oxime is sufficiently high. This was the case only for 2-PAM and HI-6, and the bimolecular rate constants (k_r) were obtained by dividing the unimolecular rate constant (k'_{\max}) by the dissociation constant of the phosphorylated enzyme/oxime complex, K'_{ox} (Fig. 1). Because the plots for TMB₄ and LüH₆ resulted in straight lines, the bimolecular rate constants of the reactivation by these oximes were calculated from the slope of the line for the concentration range used. Reactivation rate constants of EMP-AChE by 2-PAM, HI-6, TMB₄, and LüH₆ in the absence and presence of edrophonium and decamethonium are summarized in Table 1. In the presence of 50 μM edrophonium, the bimolecular rate constants for the fast reactivation component of the EMP/AChE conjugate by 2-PAM, TMB₄, and LüH₆ increased 3.3-, 5.3-, and 4.5-fold, respectively. A greater increase in the reactivation constant of the slow component was observed with TMB₄ and LüH₆ (8.7- and 12.0-fold, respectively). No changes in k_r could be detected with HI-6. In the presence of 200 μM decamethonium, similar acceleration effects were observed as for edrophonium, but to a lesser degree; the bimolecular rate constants of both the fast and slow components were changed 1.6–3-fold. Again, no

distinct changes were observed in the reactivation rate constant of EMP-AChE by HI-6 in the presence of 200 μM decamethonium.

Dissociation constant of the complex between edrophonium and EMP-AChE. The plot of the ratio of the edrophonium-accelerated rate constant over the normal rate constant (i.e., k_{obs}/k_r) versus edrophonium concentration is shown in Fig. 4. The saturation curves imply that the acceleration phenomenon may be peculiar to an enzyme-ligand interaction. The dissociation constants (K_a) of the complex between edrophonium and the inhibited enzyme were $20.6 \pm 4.8 \mu\text{M}$ ($\beta = 10.7$) and $19.5 \pm 3.5 \mu\text{M}$ ($\beta = 14.3$) for the reactivation of the fast component of EMP-AChE with TMB₄ and LüH₆, respectively.

Dependence of the edrophonium-induced acceleration on the initial concentration of the phosphorylated enzyme. Because reactivation can be slowed by reinhibition of the regenerated enzyme by POX, a product that may have accumulated during reactivation (Fig. 1), and because the stability of this intermediate depends on the oxime used (Hackley *et al.*, 1959; Schoene, 1972; Harvey *et al.*, 1986b), it was important to examine the dependence of the

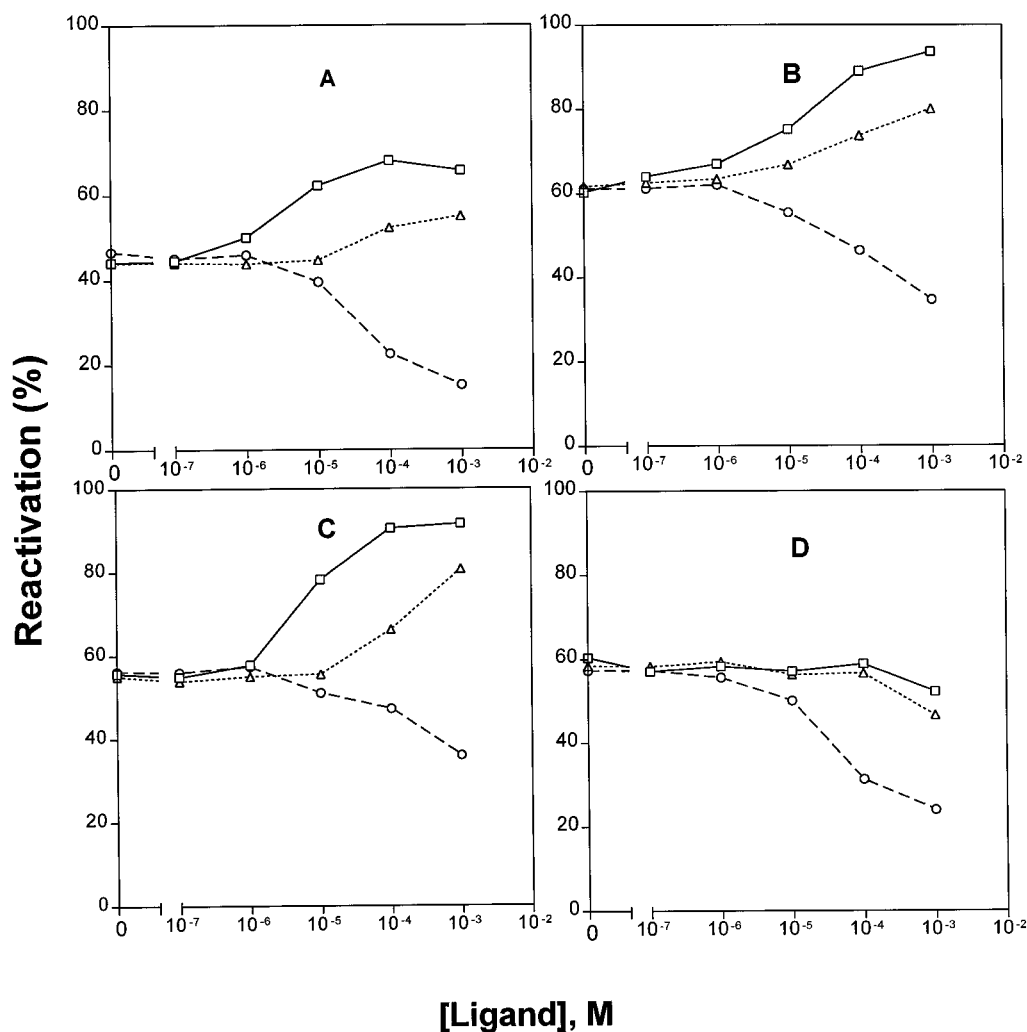


Fig. 3. Modulation profiles of oxime-induced reactivation of MEPQ-inhibited FBS AChE by quaternary ligands. AChE was inhibited with a stoichiometric amount of MEPQ at 25° for 60 min, followed by reactivation with oximes in presence or absence of ligands for 15 min at 25°. Ligands were removed by Bio-Spin column centrifugation before assaying for enzyme activity; A, 1 mM 2-PAM. B, 1 mM TMB₄. C, 1 mM LüH₆. D, 1 mM HI-6. □, Edrophonium. △, Decamethonium. ○, Propidium. The data are representative of three experiments.

acceleration on the initial concentration of the phosphonylated enzyme. It was assumed that an increase in the concentration of the inhibited enzyme at a fixed oxime concentration would increase the concentration of POX, which in turn could decrease the overall rate of reactivation. This allowed the examination of the relationship between the acceleration and POX reinhibition of various oximes. For this purpose, the time courses of reactivation with TMB₄, LüH₆, 2-PAM, MMB₄, HI-6, HS-6, and HLo7 were monitored at initial concentrations of 1.25 and 12.5 nM of EMP-AChE, with and without 50 μ M edrophonium. The results for TMB₄, LüH₆, 2-PAM, and HI-6 are shown in Fig. 5. Results of MMB₄ resemble those of 2-PAM, and those of HS-6 and HLo7 are almost identical with those of HI-6 (data not shown).

Based on the effect of the enzyme concentration on the time course of reactivation, the oximes were categorized into three groups. (1) For TMB₄ and LüH₆, reactivation by these oximes displayed the highest dependence on the initial concentration of the phosphonylated enzyme. The acceleration by edrophonium was notable for the two enzyme concentrations. (2) For

TABLE 1

Effect of monoquaternary and bisquaternary ligands on oxime-induced reactivation rate constant (k_r) of MEPQ-inhibited FBS AChE

In all the cases, data were analyzed assuming a 50:50 ratio between the two kinetic species of EMP-AChE; for the determination of k_r , five or six concentrations of oxime were used ranging from 10 μ M to 3 mM. Values are mean \pm standard error of three or four determinations.

| Oxime | k_r | | |
|-------------------------------|--------------------------------|-----------------------------|--------------------------------|
| | Control | Edrophonium (50 μ M) | Decamethonium (200 μ M) |
| | $M^{-1} \cdot \text{min}^{-1}$ | | |
| 2-PAM ^a | | | |
| Fast | 273 \pm 79 | 894 \pm 115 | 434 \pm 34 |
| Slow | 33 \pm 7 | 118 \pm 41 | 74 \pm 26 |
| TMB ₄ ^b | | | |
| Fast | 1176 \pm 77 | 6182 \pm 479 | 2281 \pm 397 |
| Slow | 37 \pm 7 | 325 \pm 18 | 88 \pm 15 |
| LüH ₆ ^b | | | |
| Fast | 1114 \pm 157 | 5003 \pm 443 | 2117 \pm 197 |
| Slow | 30 \pm 6 | 358 \pm 31 | 89 \pm 18 |
| HI-6 ^a | | | |
| Fast | 736 \pm 80 | 790 \pm 196 | 604 \pm 142 |
| Slow | 54 \pm 10 | 66 \pm 24 | 52 \pm 23 |

^a Obtained by dividing k_{max} by K_{ox} . In the case of 2-PAM, the changes of k_r in the presence of the two ligands were mainly contributed by the decrease of K_{ox} with no change in k_{max} , whereas for HI-6, no such changes was detectable in the presence of either ligand.

^b Calculated from the slope of k_{obs} versus [oxime] plot.

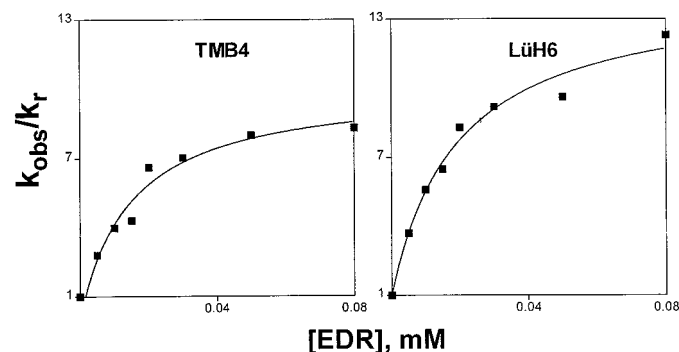


Fig. 4. Plots of k_{obs}/k_r versus edrophonium concentration in accordance with eq. 1. Increasing concentrations of edrophonium were added to MEPQ-free EMP-AChE (12.5 nM) followed by 0.05 mM TMB₄ or LüH₆, and incubated at 25°. The k_r value was determined without edrophonium, and k_{obs} is the reactivation rate constant at the specified edrophonium concentration. Data points were fitted to eq. 2.

2-PAM and MMB₄, the effect of enzyme dilution was definitely observed but significantly less pronounced compared with the first group. In addition, the acceleration phenomenon with edrophonium diminished when a low concentration of EMP-AChE was used. (3) For HI-6, HS-6, and HLo7, this third group was unaffected by either the initial concentration of enzyme or the presence of edrophonium.

Enhancement of oxime-induced reactivation by edrophonium and decamethonium in FBS AChE conjugated to polyurethane sponge. To examine further the hypothesis that the observed acceleration was due to a decrease in reinhibition of free enzyme by POX, immobilized FBS AChE was used to remove continuously low-molecular-weight ligand produced during the reactivation process. Under such conditions, reinhibition of the reactivated enzyme by POX was expected to attenuate. For this purpose, a modified Ellman procedure was developed for assaying enzyme activity in the sponge. Plot of absorbance at 412 nm versus time provided a straight line over the first 5 min (not shown).

Fig. 6 shows reactivation results obtained with FBS AChE sponge inhibited with MEPQ and eluted continuously for 15 min with 0.1 mM 2-PAM, LüH₆, TMB₄, or HI-6 in the presence or absence of the accelerating ligands. The reactivation of the immobilized enzyme was distinctively faster than with enzyme in homogenous solution. Nearly the same level of reactivation could be achieved with one tenth of the oxime concentration used in the experiments with EMP-AChE in solution. Although no attempt was made to obtain rate constants for the reactivation of AChE conjugated to sponge, the acceleration effect of edrophonium and decamethonium on oxime-induced reactivation was still discernible, and enhancement of reactivation followed the same rank order as that observed for enzyme in solution.

Discussion

An important finding of this study was that among the three types of ligands tested, the active-site ligand edrophonium is the most powerful accelerator of oxime-induced reactivation of EMP-AChE. This acceleration was quite substantial, and with 50 μ M edrophonium, the bimolecular rate constant of the reactivation by LüH₆ was increased 12-fold. Decamethonium, which spans the peripheral- and active-site region (Taylor and Jacobs, 1974; Harel *et al.*, 1993; Barak *et al.*, 1994), was less potent. The peripheral-site ligand propidium not only lacked acceleration properties but also slowed the reactivation of MEPQ-inhibited FBS AChE (Fig. 3). This is reasonable because binding of propidium to the peripheral site may sterically or allosterically prevent the oxime from entering into the active site of the enzyme, as occurs in substrate hydrolysis (Barak *et al.*, 1995). These observations suggest that the acceleration site may reside near the catalytic center inside the gorge rather than near the entrance. The fact that edrophonium lacks nucleophilicity, as judged from its negligible effect on spontaneous reactivation, rules out the possibility that the accelerator directly attacks the P atom of the enzyme-bound EMP moiety. The probability that the hydroxyl group of edrophonium is involved in the acceleration of the displacement of OP moiety by oximes is decreased in light of the structure of other accelerators, such as decamethonium and SAD-128 (Harris *et al.*, 1978; Luo *et al.*, 1995).

Studies of inhibition of FBS AChE-catalyzed hydrolysis of acetylcholine by edrophonium provided a dissociation constant (K_I) of 0.1–0.2 μM (Ashani *et al.*, 1990; Saxena *et al.*, 1997). If the accelerator site is the same as the binding site of edrophonium to the native enzyme, the dissociation constant obtained from the acceleration experiments, K_a , would be expected to be in the same range. Although the K_a values for the acceleration in experiments with TMB₄ and LüH₆ were in excellent agreement (20.6 ± 4.8 and 19.5 ± 3.5 μM , respectively), they indicate that the complex EMP-AChE·A is ≥ 100 –200-fold less stable than that formed between the native enzyme and edrophonium. This is consistent with eq. 1 that assumed the binding of the accelerator to EMP-AChE. The binding site of edrophonium was determined on the basis of X-ray crystallography of its complex with native *Torpedo* AChE and studies with AChE mutants (Harel *et al.*, 1993; Barak *et al.*, 1994; Ordentlich *et al.*, 1995). The amino acid residues shown to interact with edrophonium in the native enzyme may not be available to stabilize edrophonium in the EMP-AChE conjugate (i.e., the active-site serine, the oxyanion constituents, and possibly the catalytic histidine). Indeed, the complex of edrophonium with some aged and nonaged OP conjugates of AChE was found to be 100–1000-fold less stable than the complex with native enzyme (Berman and Decker, 1986a, 1986b). The dissociation constant, K_a , from our experiments indicates that the stability of EMP-AChE·A is still 30-fold greater than the complex formed between edrophonium and the peripheral anionic site of mouse AChE ($K_I = 590$ μM) (Radić *et al.*, 1991). This substantiates the conclusion that the accelerator site is close to the catalytic center of the

enzyme. Although the experimental evidence suggests that the accelerator binding may be inside the gorge, it is difficult to designate a specific attachment locus.

Assuming that acceleration arises from binding of edrophonium near the catalytic site region, the next questions to be addressed are: By what mechanism does the ligand enhance the reactivation by 2-PAM, MMB₄, TMB₄, and LüH₆? Why is reactivation by HI-6, HS-6, and HLo7 not affected? At first, eq. 1 seems to offer a straightforward answer that is peculiar to conformational changes in the phosphorylated enzyme after the binding of the accelerator to the inhibited enzyme. According to this explanation, it is likely then that HI-6, HS-6, and HLo7 already are projected inside the gorge in orientations that do not require assistance of an accelerating ligand. However, not all results are consistent with an allosteric mechanism, and some observations that offer other explanations for the acceleration phenomenon are discussed.

Relationship between EMP-AChE concentration and rate of reactivation. From the results of the seven tested oximes, it clearly was shown that the dependence of reactivation on initial enzyme concentration correlated very well with edrophonium acceleration of the reactivation. Reinhibition by POX has been implied to explain the decrease in the rate of reactivation of concentrated OP-AChE solutions compared with diluted conjugates (Harvey *et al.*, 1986b; Ashani *et al.*, 1995). Because the inability of edrophonium to accelerate reactivation by HI-6, HS-6, and HLo7 parallels the lack of dependence of reactivation by these oximes on the initial concentration of EMP-AChE, it is possible that in addition to the mechanism summarized in eq. 1, acceleration is mani-

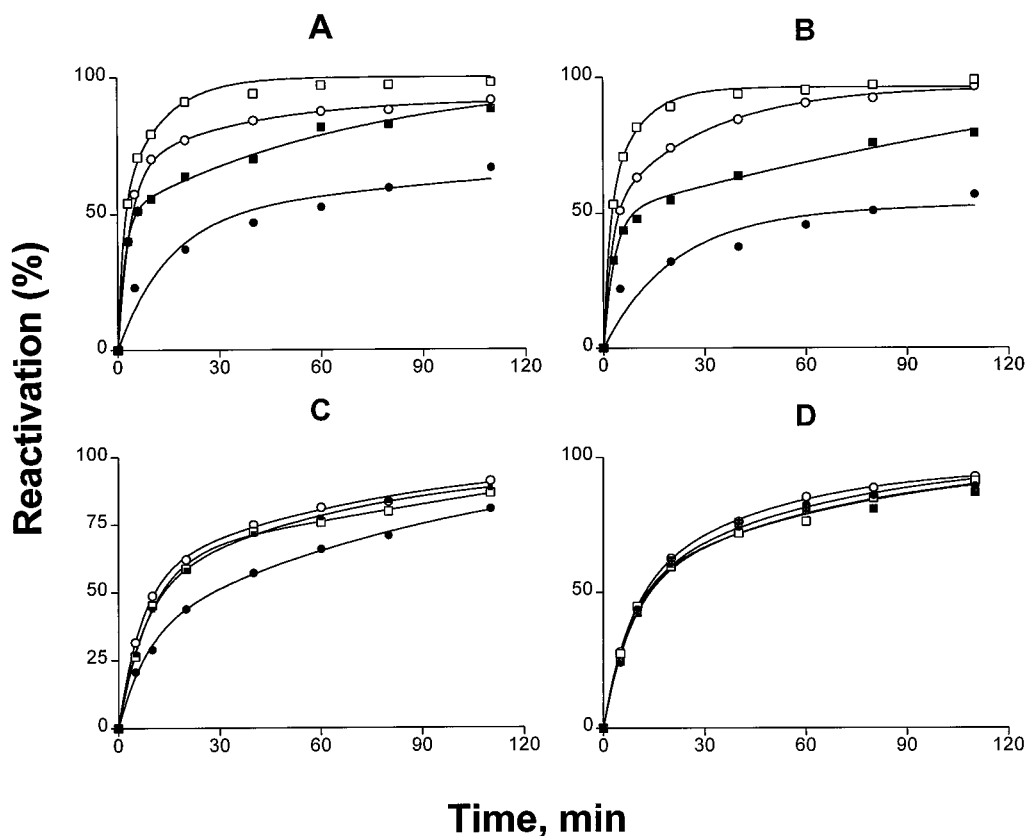


Fig. 5. Effect of initial concentration of EMP-AChE on reactivation time course. EMP-AChE conjugates, 1.25 nM (\square , \blacksquare) and 12.5 nM (\circ , \bullet), were incubated at 25° with fixed concentrations of oximes with (\square , \circ) and without (\blacksquare , \bullet) 50 μM edrophonium. A, 0.05 mM TMB₄. B, 0.05 mM LüH₆. C, 0.5 mM 2-PAM. D, 0.5 mM HI-6. The data are representative of three experiments.

fest by slowing the reinhibition by POX. Numerous reports have demonstrated protection of AChE against OP inhibition by preincubation of the enzyme with a reversible active-site ligand (Gray, 1984; Galli *et al.*, 1994; Grunwald *et al.*, 1994). The formation of a stable reversible complex between AChE and a ligand that arrests the catalytic machinery of AChE precludes phosphorylation of the active-site serine and increases the chances of hydrolytic inactivation of the OP inhibitor. However, for this hypothesis to hold, the 100–200-fold differences between K_a and K_I must be considered. This discrepancy may be reconciled by comparing the ratio [accelerator]/ K_a with [oxime]/ K_{ox} .

[Accelerator]/ K_a versus [oxime]/ K_{ox} . The results suggest that a ternary complex among EMP-AChE, oxime, and edrophonium may be formed and result in conformational changes that can underlie the observed acceleration. If the enhancement of reactivation is due to protection of the free enzyme from reinhibition, it also is possible for binding of the reactivator to free enzyme to destabilize the complex AChE·A. Therefore, K_a in eq. 1 is a macroscopic composite of more than one constant. Hence, the kinetic assumptions are more complex than those depicted in eq. 1. This can explain the relatively high K_a values compared with the K_I values of edrophonium for free enzyme. These considerations, together with the structural similarities between reactivators and accelerators (Fig. 1), raise the possibility that quaternary oximes are likely to carry their own accelerating determinant, and their binding site may overlap the accelerator site. Although oximes are poor inhibitors of the enzyme, they still can competitively inhibit the enzyme, with the dissociation constant, K_{ox} , ranging from 0.037 to 0.90 mM. The ratio of edrophonium concentration to its K_a value (2.5) is significantly more favorable in terms of occupying the accelerator site than the ratio [oxime]/ K_{ox} for TMB₄ and LüH₆ (<1.4; Fig. 5). For 2-PAM and HI-6, these ratios were 3.1 and 5.6, respectively. Thus, the rank order of the responsiveness of these four oximes to the acceleration phenomenon, TMB₄ ~ LüH₆ > 2-PAM >> HI-6, may be attributed to their partial

occupation of the accelerator site. However, these observations are not consistent with [oxime]/ K_{ox} ratios of HLo7 (1.19) and HS-6 (2.27), which were not affected at all, or [oxime]/ K_{ox} of MMB₄ (0.05), which was only moderately accelerated by 50 μ M edrophonium (not shown).

Stability of the putative POX. The possibility that differences in the stability of the reactivation product POX might explain the results also was considered. This hypothesis is supported two ways. First, as indicated, no unambiguous correlation was found between the [oxime]/ K_{ox} ratio and the acceleration produced by 50 μ M edrophonium, suggesting that the diverse response of the oximes to the accelerator was only partly influenced by the ability of edrophonium to compete with the oxime for an accelerator site. Second, POX derived from an oxime at position 2 of the pyridinium ring is significantly less stable than its 4 position homologue (Hackley *et al.*, 1959). Also, insertion of a second quaternary ammonium head, such as found in the 2-hydroximinomethyl containing reactivators HI-6, HS-6, and HLo7, is expected to further destabilize the 2-positioned phosphorylated oxime due to the increase in the positive charge density. The latter is known to facilitate nucleophilic displacements at the P atom. These arguments agree well with the observation that reactivation was not enhanced by accelerators, when HI-6, HS-6, and HLo7 were used, and support the contention that the stability of POX can determine the oxime responsiveness to the accelerating ligands.

Reactivation of sponge-bound AChE. The results with the immobilized EMP-AChE in sponge clearly showed that reactivation was faster than with EMP-AChE in solution, an observation that substantiates the proposed involvement of POX in the apparent rate of reactivation and presumably in the acceleration phenomenon. Enhancement of reactivation in the presence of edrophonium and decamethonium followed the same rank order that was observed for soluble enzyme. It seems that if POX indeed influences the reactivation time course, this inhibitor is only partly accessible to washout from the column, and edrophonium and decamethonium therefore can still exhibit partial acceleration on the reactivation. This is supported by a recent study of the reactivation of a diethylphosphoryl-butyrylcholinesterase conjugate by 2-PAM in the absence and presence of an OP hydrolase (Ashani *et al.*, 1997), which suggests that the putative POX inside the catalytic gorge may not be accessible to the OP hydrolase outside the gorge.

In conclusion, the results of this study indicate that the activity of oxime reactivators, which are likely to produce potent POX inhibitors during reactivation, can be enhanced by non-nucleophile accelerators such as edrophonium and decamethonium. Binding of the ligands near the active site of the enzyme seemed to prevent the reinhibition of the enzyme by POX. However, we cannot rule out the possibility that acceleration is promoted to some extent by conformational change or changes induced by the accelerator in the EMP/AChE conjugate. Finally, the reported superiority of HI-6 over 2-PAM, TMB₄, and LüH₆ as a reactivator of certain phosphorylated AChEs (De Jong and Wolring, 1980; Luo *et al.*, 1994) is proposed to stem from in part the poor stability of the corresponding POX intermediate or to be due to the ability of HI-6 to slow the reinhibition by the same mechanism attributed to the accelerator.

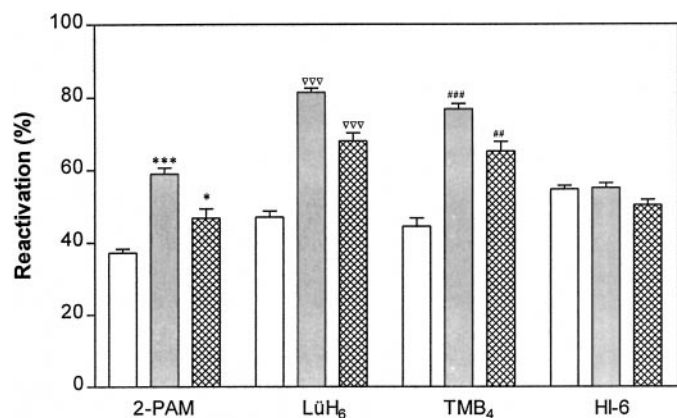


Fig. 6. Enhancement of oxime-induced reactivation of MEPQ-inhibited sponge FBS AChE by edrophonium and decamethonium. MEPQ-inhibited FBS AChE sponge was washed with phosphate buffer to remove excess OP. Reactivation was carried out for 15 min by passing oxime or oxime-ligand solutions through the sponge in a minicolumn. Oximes and ligand concentrations were 0.1 mM each. □, control; ▒, oxime with edrophonium; ▨, oxime with decamethonium. *, $p < 0.05$; and ***, $p < 0.001$ compared with 2-PAM control. ∇∇∇, $p < 0.001$ compared with LüH₆ control. ##, $p < 0.01$; and ###, $p < 0.001$ compared with TMB₄ control (Student's t test).

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