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SHORT COMMUNICATIONS

New reactivators of phosphonylated acetylcholinesterase*

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Many oximes when used together with atropine provide treatment for poisoning by organophosphorus anticholinesterase compounds [1–3]. The mechanism of therapeusis involves reactivation of the vital enzyme acetylcholinesterase (acetylcholine acetyl hydrolase, EC 3.1.1.7, AChE) which is selectively inhibited by the organophosphorus poisons, equations 1 and 2,

$$AChE + PF \rightarrow AChE\text{-}P + F^- \quad (1)$$

$$AChE\text{-}P + oxime \rightarrow AChE + P\text{-}oxime \quad (2)$$

where *PF* represents an organophosphorus anticholinesterase such as sarin (i-propyl methylphosphonofluoridate), and *AChE-P* represents the inhibited, phosphonylated enzyme. In the latter, the phosphorus atom (with attached CH_3 and O-iPr groups) is covalently linked to the serine oxygen atom at the enzyme active site [4]. Reactivation involves transfer of the phosphonyl group to the oxime (*P-oxime*) with release of free (uninhibited) enzyme [1].

The rate of reactivation, equation 2, varies greatly with oxime structure [1–3, 5–7]. Of the large number of oximes tested, 2-PAM and toxigonin (Fig. 1) are therapeutic compounds of choice [2]. Both fall into a relatively limited family of comparatively rapid reactivators. The desirability of developing appreciably more rapid reactivators for improved therapy is generally recognized. Improvement would not only be valuable for the treatment of poisoning by sarin or insecticidal organophosphorus anticholinesterases, but it might be of particular value for poisoning by soman (pinacolyl methylphosphonofluoridate) which is refractory to currently known oximes [2, 8].

The property of rapid reactivation appears to be limited to quaternary pyridinium compounds containing the aldoximino group [1–3, 5–7]. Large numbers of such compounds have been and continue to be reported, without major improvement over the earliest oximes [4–7] typified by 2-PAM and TMB-4 (Fig. 1). In this paper, we report several additional compounds. These, too, are rapid reactivators. But, again, they are not better than existing compounds. All of the new oximes are related to 2-PAM. Several have 3-benzyloxy substituents on the pyridine ring, I–V, and two are 5-linked bispyridinium oximes, VII and VIII (Fig. 2). Choice of the latter was based upon the following rationale.

Among the monoquaternary pyridinium oximes, 2-, 3- and 4-PAM (the prefix numerals refer to the position on the pyridine ring to which the aldoximino group is attached), the reactivation rate varies sharply with the position of the aldoximino group (Table 1). 2-PAM is a far more rapid reactivator than either 3- or 4-PAM. Addition of a second charged group onto the 3- or 4-PAM molecules (X and TMB-4 are typical) increases the reactivation rate considerably. The ratios of these rates (diquaternary/corresponding monoquaternary) generally lies in the range of 20–50 [3]. With 2-PAM, the reverse occurs (2-PAM vs IX). Here the ratio is far below one. This unexpected decrease with the 2-PAM "diquarternary" suggested the possible influence of adverse steric effects. The decrease,

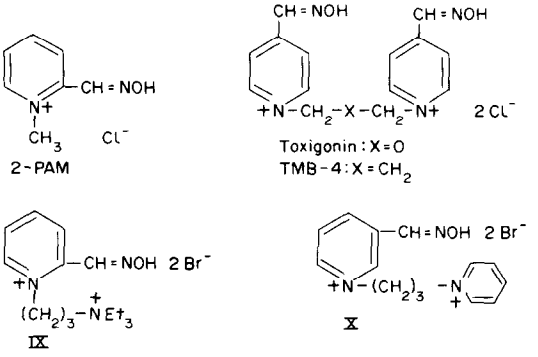
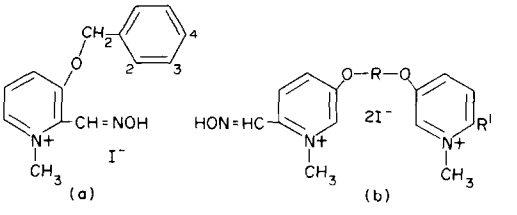


Fig. 1. Reference compounds.

we supposed, might be due to the presence of the bulky chain close to the aldoximino group which could interfere with its reaction (nucleophilic) on the phosphorus atom. By linking "2-PAMs" more distantly from the aldoximino group, at the 5-position, we hoped to overcome this disadvantage.

Sarin was obtained from the Process Development Group, Edgewood Arsenal. The AChE was isolated from electric eel (Sigma); the concentration in active sites was estimated to be 2×10^{-5} M. The monoquaternary compounds, I–V, [9], were kindly provided by Dr. L. Berkowitz of these laboratories. Compounds VI-VIII were prepared at the Ash–Stevens Laboratories. Their preparation will be reported elsewhere. The compounds satisfied normal criteria for purity; elemental analysis (CHI) in each case was within acceptable limits. Unaged sarin-inhibited AChE was prepared by reaction between the two at pH 10.2. To 300 μl glycine buffer, 10^{-4} M, pH 10.2, there was added 100 μl of suitably diluted AChE and then 10 μl of an aqueous solution of sarin [prepared from a stock solution of 1% (v/v) sarin in carefully dried benzene]; final



(a) Phenyl ring substitution:
I, unsubst.; II, 2-Cl; III, 4-Cl; IV, 2-CH_3; V, 3-CH_3

(b) Diquaternary oximes:

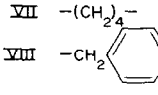
	R	R'
VI	$-(CH_2)_2-$	$-CH_3$
VII	$-(CH_2)_4-$	$-CH=NOH$
VIII		$-CH=NOH$

Fig. 2. Test compounds.

* The views of the authors do not purport to reflect the position of the Department of Defense.

Table 1. Reactivation of sarin-inhibited eel AChE*

	$k_{2(app)}$ (M^{-1} min^{-1})	Diquaternary/ Monoquaternary oxime, rate ratio
Monoquaternary		
2-PAM	2200	<0.014
3-PAM	2	33
4-PAM	140	43
Diquaternary		
IX	<30	
X	67	
TMB-4	6000	

* The pH value is 7.4. Data of H. Michel. See Ref. 3.

concentrations were approximately 2×10^{-8} M AChE (in active sites) and 10^{-7} M sarin. After room temperature incubation for 30 min, the enzyme was fully inhibited and the excess sarin had been hydrolyzed (to innocuous products). Complete destruction of excess sarin was confirmed in each run by assay of an aliquot for further inhibition of fresh enzyme. Aging of the phosphonylated enzyme was minimized by performance of the phosphonylation reaction at elevated pH [10]. However, zero aging was not assumed. The extent of reactivatability (reactivation end point) was determined in each run.

Reactivation was performed at constant pH with a Radiometer autotitrator (TTT1, ABU1, SBR2, PHA 630, fitted with 0.25 ml burette) using the method of Kitz *et al.* [11]. Unless otherwise stated, the reactivation medium (3–5 ml) contained 0.225 M KCl, 0.025% gelatin and 0.0048 M acetylcholine, pH 7.4, 25°. Titrant was 0.0008 M carbonate-free sodium hydroxide. Near the end of each run, TMB-4 was added to a concentration of 10^{-4} M. Reactivation to maximum activity took place within 2–3 min. The reactivation end point could be computed after correction for inhibition of the enzyme by the added TMB-4. Since many of the reactivators are themselves inhibitors [3] (reversible competitive), it was necessary to separately determine the effect of the added TMB-4 to each oxime (at its test concentration). In the absence of other oximes, 10^{-4} M TMB-4 inhibits AChE 30 per cent under the cited test conditions.

In previously reported studies of AChE reactivation, it has always been assumed that the product of oxime reactivation was identical with the original enzyme. We have confirmed this assumption. Two aliquots (3 ml) of sarin-inhibited AChE were individually incubated for 30 min at room temperature in 0.2 μ aqueous phosphate, pH 7.0, containing 10^{-3} M TMB-4 and each of the enzymatically active solutions was subjected to Lineweaver and Burk analysis [12]. Two samples of uninhibited enzyme were treated in an identical fashion. Enzymatic activity was measured spectrophotometrically (Zeiss PMQ, Beckman Log-Linear recorder) at 272 nm using phenyl acetate of varying concentrations as substrate. For each assay, 3 μl of reactivation solution was added to a cuvette containing 3.0 ml of a solution of phenyl acetate in 0.2 μ phosphate, pH 7.0, which had been pre-equilibrated for 10 min at 25° in a thermostatted cell holder. It had previously been established that, at a concentration of 10^{-6} M, TMB-4 has no effect on the kinetic properties of AChE. For the uninhibited enzyme, $K_m = 1.19 \pm 0.06 \times 10^{-3}$ M, $V_{max} = 0.13$; reactivated enzyme, $K_m = 1.18 \pm 0.18 \times 10^{-3}$ M, $V_{max} = 0.07$. Incomplete reactivation, suggested by the reduced value of V_{max} has been reported by several investigators [13, 14]. This becomes particularly pronounced at low oxime concentrations [13], under which conditions reactivation kinetics become unexpectedly complex.*

* G. M. Steinberg, unpublished observations.

Table 2. Reactivation of sarin-inhibited eel AChE by oximes, pH 7.4, 25°, in the presence of 0.0048 M acetylcholine*

Oxime	Conc ($M \times 10^6$)	$k_{2(app)}$ (M^{-1} min^{-1})	Inhibition*† (%)
TMB-4	10	7.0×10^3	2
I	50	280	54
II	50	170	91
III	50	176	81
IV	50	70	70
V	50	224	68
VI	3	4.7×10^3	53
VII	2	4.8×10^3	
VIII	10	2.8×10^3	80

* Apparent second-order reactivation rate constants were computed from equation 3.

† Reduction in activity of the native enzyme by oxime under the reactivation test conditions.

Reactivation rate data are given in Tables 2 and 3. In Table 2, rates were calculated at a single concentration of oxime. The apparent second order reactivation rate constants, $k_{2(app)}$, were computed from equation 3,

$$k_{2(app)} = \frac{k_{(obs)}}{[oxime]} \tag{3}$$

where $k_{(obs)}$ is the observed first-order reactivation rate in the presence of oxime of concentration, [*oxime*]. No distinction is made between protonated and unprotonated forms of the oxime. Percentage inhibition represents the extent of inhibition of unphosphonylated enzyme by the stated concentration of oxime in the reactivation medium.

The $k_{2(app)}$ values give only a rough measure of the comparative reactivating effectiveness of the group of compounds, since the reaction is not truly second order. However, for screening purposes, this is quite adequate. Per equation 4, the reaction involves an intermediate complex, *AChE-P—oxime*. In Table 3, results of the

$$AChE\text{-}P + oxime \underset{}{\overset{K_R}{\rightleftharpoons}} AChE\text{-}P \ldots oxime$$

$$AChE\text{-}P \ldots oxime \xrightarrow{k_r} AChE + P\text{-}oxime \tag{4}$$

more detailed kinetic analysis are given for the faster reactivators, compounds VI–VIII, together with those of the reference compounds 2-PAM and TMB-4. Values for K_R, the dissociation constant of the phosphonylated enzyme-oxime complex, and for k_r, the first-order reaction rate constant, were calculated according to the method discussed by Wang and Braid [15], equation 5.

$$\frac{1}{k_{(obs)}} = \frac{1}{k_r} + \frac{K_R}{k_r[oxime]} \tag{5}$$

The plot of $1/k_{(obs)}$ vs $1/[oxime]$ yields a straight line with ordinate intercept $= 1/k_r$ and slope $= K_R/k_r$.

Table 3. Kinetic constants of reactivation of sarin-inhibited eel AChE by oximes, pH 7.4, 25°

Oxime	K_R (M)	k_r (min^{-1})
2-PAM	1.8×10^{-4}	0.38
TMB-4	6.1×10^{-5}	0.723
VI	5.6×10^{-5}	0.244
VII	5.4×10^{-6}	0.069
VIII	1.2×10^{-5}	0.0825

The results with the compounds reported here are somewhat disappointing. Simple addition of an aromatic side chain to 2-PAM, with the increased opportunity for hydrophobic or π bonding to the enzyme, as in the monoquaternary compounds, reduces the reactivation rate. The 2-PAM "diquaternaries" VII and VIII are markedly superior to IX, yet they have been returned only to the activity of the parent 2-PAM. Still, we feel that the rationale on which the design of these compounds is based is reasonable and bears additional exploration. Perhaps our choice of compounds was too narrow. AChE is known to have extensive hydrophobic binding regions near the anionic subsite [16, 17] which should be exploitable. And the combination of the intrinsic reactivity of 2-PAM with the diquarternary multiplication factor observed for 3- and 4-PAMs, if it could be achieved, would give compounds with second-order reactivation rate constants of the magnitude of 10^5–10^6 M^{-1} min^{-1}. Such an objective is well worth additional effort.

Biochemistry Group, Biomedical Laboratory, Edgewood Arsenal, Md. 21010
GEORGE M. STEINBERG*
JOYCE CRAMER†
Ash–Stevens, Inc. Detroit, MI 48202, U.S.A.
ARTHUR B. ASH

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* Present address: DCBD, NCI, Bldg. 10, Rm 4BO9, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20014.
† Present address: VA Hospital, New Haven, CT.

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Effect of chronic ethanol administration on liver alcohol dehydrogenase activity in mice

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It has been established that about 90 per cent of ingested ethanol is metabolized in the liver [1] and that the rate of ethanol metabolism is markedly increased in humans and experimental animals after chronic ethanol consumption [2–5]. The enzyme alcohol dehydrogenase (ADH), located in hepatic parenchymal cells, has a primary function in the metabolism of ethanol by degrading it to acetaldehyde [6]. Studies designed to investigate the possibility of increased ADH activity to explain increased ethanol metabolism after chronic administration have produced a variety of results. Some investigators found an increase in ADH activity after chronic administration [2,7–9] and others found no change or a slight decrease [10]. Strains of mice which have a genetically determined preference for ethanol in a two-bottle test showed heightened activity of both ADH and acetaldehyde dehydrogenase prior to alcohol adminstration [11]. However, the relationship between liver ADH activity and ethanol metabolism is poorly correlated in the intact animal regardless of strain [12]. Furthermore, an increase in the rate of ethanol metabolism can occur without changes in ADH activity [3,4,13,14], and thus the overall correlation between ADH activity and increased ethanol metabolism is not strong. More recent investigations have been directed toward explaining the enhanced rates of ethanol metabolism via a microsomal ethanol-oxidizing system [15] or by the increased rate of nicotinamide dinucleotide regeneration by the mitochondria in a hypermetabolic state induced in the liver by chronic ethanol consumption [16].

The present study rather than attempting to elucidate the relationship between ADH activity and ethanol metabolism was designed to examine the induction effect of