er–Burk plot. Only in three instances were the correlation coefficients less than 0.99; the worst was 0.97.

Acknowledgment. We thank Saverio Sciacovelli for computing K_m and V_{max} and Vincenzo Carta for assistance in the experimental determination of kinetic parameters.

Registry No. 1, 2979-54-6; 2, 66895-70-3; 3, 81592-10-1; 4, 57461-26-4; 5, 91631-74-2; 6, 81592-17-8; 7, 81592-20-3; 8, 91631-98-0; 9, 91631-99-1; 10, 2979-53-5; 11, 81592-12-3; 12, 81592-08-7; 13, 81592-09-8; 14, 81592-11-2; 15, 91631-75-3; 16, 29736-20-7; 17, 91632-00-7; 18, 29736-22-9; 19, 2979-52-4; 20, 91632-01-8; 21, 29736-21-8; 22, 29736-18-3; 23, 3101-11-9; 24, 81592-05-4; 25, 81592-07-6; 26, 81592-15-6; 27, 81592-19-0; 28, 81592-18-9; 29, 81592-13-4; 30, 81592-16-7; ficin, 9001-33-6.

Structure–Activity Relationships for Reactivators of Organophosphorus-Inhibited Acetylcholinesterase: Quaternary Salts of 2-[(Hydroxyimino)methyl]imidazole

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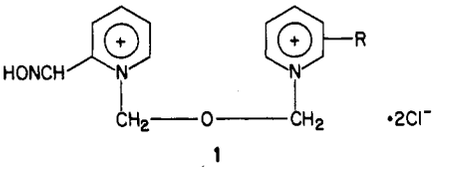
A series of 1,3-disubstituted-2-[(hydroxyimino)methyl]imidazolium halides were prepared and evaluated in vitro with respect to their ability to reactivate acetylcholinesterase inhibited by ethyl *p*-nitrophenyl methylphosphonate (EPMP) and 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD). The compounds conform to the general formula $\overline{N(CH_3)C(CHNOH)N(CH_2OR)CHCH}^{+}\cdot Cl^-$, where R = CH_3, $(CH_2)_3CH_3$, $(CH_2)_7CH_3$, $CH_2C_6H_5$, $CH_2C_{10}H_7$, $(CH_2)_3C_6H_5$, $CH(CH_3)_2$, $CH_2C(CH_3)_3$, and $CH(CH_3)C(CH_3)_3$. For comparison we also evaluated three known pyridinium reactivators, 2-PAM, HI-6, and toxogonin. The imidazolium aldoximes exhibit oxime acid dissociation constants (pK_a) in the range 7.9–8.1, bracketing the value of 8.0, believed to be optimal for acetylcholinesterase reactivation. With imidazolium compound in excess over inhibited enzyme, the kinetics of reactivation are well behaved for EPMP-inhibited AChE and depend on the nature of the alkyl ether group R. For GD-inhibited AChE, maximal reactivation was used to compare compounds because rapid phosphonyl enzyme dealkylation and enzyme reinhibition complicate interpretation of kinetic constants.

Various organophosphorus (OP) compounds are powerful inhibitors of synaptic acetylcholinesterase (AChE).[1] Standard therapy for intoxication by anti-AChE agents consists of coadministration of atropine and an AChE "reactivator".[2-4] Reactivators function as nucleophiles to displace OP moieties from inhibited AChE and thereby restore activity to the enzyme. Although research efforts over the past 25 years have produced dozens of experimental AChE reactivators, only three compounds have found extensive clinical application in managing anti-AChE agent poisoning. These three compounds, 2-PAM [2-[(hydroxyimino)methyl]-1-methylpyridinium halide], TMB4 [1,3-bis[4-[(hydroxyimino)methyl]-1-pyridinio]-propane dichloride], and toxogonin [1,3-bis[4-[(hydroxyimino)methyl]-1-pyridinio]-2-oxapropane dichloride], effectively reverse intoxication symptoms in cases of accidental pesticide or nerve agent poisoning by many OP agents. However, in animals these three pyridinium oximes are ineffective in preventing or treating intoxication by 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD) when GD is administered in quantities exceeding approximately 1.2 times the LD_{50}.[5-7]

In 1970, Oldiges and Schoene[5] reported that certain unsymmetrically bis(substituted pyridinium) dimethyl ether derivatives constitute effective therapy for GD poisoning in mice under conditions where 2-PAM, toxogonin, and TMB4 have insignificant therapeutic efficacy. The findings of Oldiges and Schoene evoked considerable interest[5-19] in the synthesis and evaluation of bis(pyridinium) dimethyl ether derivatives. Several structurally related AChE reactivators have been discovered that are useful in treating GD-intoxicated animals. The reactivators that are effective against GD conform to the general structure 1, where R = $C(O)NH_2$, $C(O)C_6H_5$, or $C(O)C_6H_{11}$



1

in the 3- or 4-position of the indicated pyridinium ring.[20]

† Organic Chemistry Department, SRI International.
‡ Biomedical Research Laboratory, SRI International.
§ Syntex Research Division.

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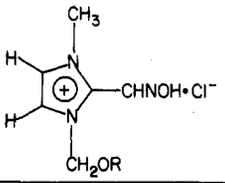
Table I. Selected Data for Imidazolium Compounds



compd[a]	R	yield[b], %	mp, °C	pK_a[c]	log P[d]	formula[e]
2a	CH_3	79	168–172 dec	7.96	−3.0	$C_7H_{12}N_3O_2Cl$
2b	$CH(CH_3)_2$	94	128–130 dec	7.93	−2.17	$C_9H_{16}N_3O_2Cl$
2c	$CH_2C(CH_3)_3$	50	167–168 dec	8.04	−0.91	$C_{11}H_{20}N_3O_2Cl$
2d	$CH(CH_3)C(CH_3)_3$	64	169–170 dec	8.01	−0.68	$C_{12}H_{22}N_3O_2Cl$
2e	$(CH_2)_3CH_3$	38	102–105 dec	8.04	−1.39	$C_{10}H_{18}N_3O_2Cl$
2f	$(CH_2)_7CH_3$	77	132–134 dec	8.01	+0.88	$C_{14}H_{26}N_3O_2Cl$
2g	$CH_2C_6H_5$	79	124–127 dec	7.94	−1.30	$C_{13}H_{16}N_3O_2Cl$
2h	$(CH_2)_3C_6H_5$	82	130–131 dec	8.05	−0.45	$C_{15}H_{20}N_3O_2Cl$
2i	$CH_2C_{10}H_7$	60	125–128 dec	8.11	−0.36	$C_{17}H_{18}N_3O_2Cl$

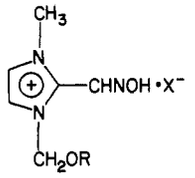
[a] See text for description of general route. [b] Yield for production of target compounds from immediate precursor. [c] Determined spectrometrically in 0.1 M phosphate buffer. [d] Log P is the octanol:buffer partition coefficient for 0.1 M, pH 7.6 phosphate buffer. [e] All compounds were analyzed for C, H, N, and Cl; analytical results were within ±0.4% of the theoretical values.

Specific examples of 1 include the following: HS-6 [R = 3-$C(O)NH_2$],[5] HI-6 [R = 4-$C(O)NH_2$],[5] HGG-12 [R = 3-$C(O)C_6H_5$],[18] HGG-42 [R = 3-$C(O)C_6H_{11}$],[5] BDB-26a [R = 4-$C(O)C_6H_5$],[7] and BDB-27a [R = 4-$C(O)C_6H_{11}$].[7]

In spite of the demonstrated therapeutic activity of type 1 compounds, a significant factor has hindered recommendation of these oximes for human use. Although their antidotal effectiveness is generally accepted, considerable controversy remains over the protective mechanisms of the oximes 1 in cases of GD poisoning. Various oximes that have structures similar to 1 are demonstrably poor antidotes for GD intoxication, thereby raising legitimate questions regarding the molecular parameters controlling the activity of oximes 1 and related compounds.

In view of the foregoing, we investigated compounds related to 1. Specifically, we prepared monoquaternary analogues of 1 that differ not in the substitution pattern or group (such substitutions having been widely investigated[21]) but rather in the nature of the alkyl ether moiety and in the nature of the quaternized heteroaromatic ring system.

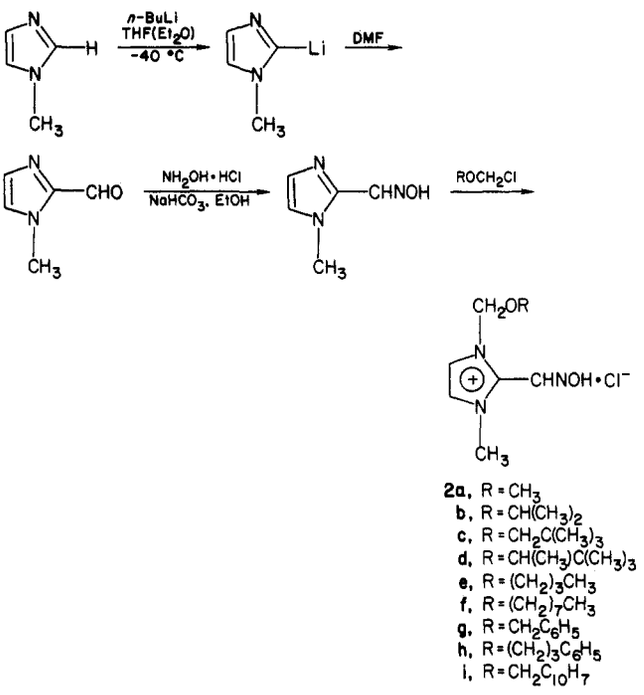
For this study we selected the imidazole ring system given by the general formula 2 on the basis of synthetic flexibility, reasonable isosteric similarity with pyridine, ease of quaternization[22] (providing for Coulombic attraction to anionic sites of AChE), and the recognized requirement for oximes with pK_a near 8.[1]



2

In the following we report the synthesis of nine type 2 compounds and their characterization with respect to pK_a, hydrolytic stability, reversible inhibition of AChE, and relative ability to reactivate electric eel AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate and by 3,3-dimethyl-2-butyl methylphosphonofluoridate. We also investigated the kinetics of reactivation for EPMP-inhibited AChE with the objective of identifying structure–activity relationships for reactivation. For comparison, we examined 2-PAM, toxogonin, and HI-6.

Scheme I



Results and Discussion

Synthesis, Structure, and Acidity. The imidazolium compounds 2 were prepared by the general synthesis route shown in Scheme I. Methylimidazole was converted to 1-methylimidazole-2-carboxaldehyde by the method of Iversen and Lund.[23] The aldehyde was converted to the oxime derivative in standard fashion. Reaction of oxime with the appropriate chloromethyl ethers, which were prepared from the respective alcohols,[24,25] provided the desired quaternary salts 2. The salts 2 were readily recrystallized from 2-propanol or 2-propanol/ethyl acetate mixtures to give analytically pure material.

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Type II. Kinetic Constants for Reactivation of Ethyl Methylphosphonyl-AChE by Various Test Compounds

compd[a]	[HOX],[b] μM	$[OX]^{-1}$,[c] μM^{-1}	$(k_{obsd})_c^{-1}$,[d] min	IC_{50},[e] μM	k_r, $min^{-1} \times 10^3$	K_r, $M \times 10^6$	k_{OX},[f] $M^{-1}\ min^{-1} \times 10^{-3}$	k_{HOX},[g] $M^{-1}\ min^{-1} \times 10^{-3}$
2a	30.0	0.115	22.5	290.0	59.4	7.86	7.6	2.2
	10.0	0.346	51.2					
	3.00	1.50	197					
	1.00	3.460						
2b	10.0	0.313	238	80.0	12.1	7.46	1.6	0.52
	3.00	1.050	778					
	1.00	3.130	2000					
2c	30.0	0.125	92.8	12.0	16.8	8.32	2.0	0.54
	10.0	0.376	229					
	3.00	1.250	743					
	1.00	3.760	1900					
2d	30.0	0.119	263	16.0	4.72	5.66	0.83	0.23
	10.0	0.357	565					
	3.00	1.190	1875					
	1.00	3.570	4430					
2e	30.0	0.125	162	20.0	7.58	1.99	3.8	1.0
	10.0	0.376	219					
	3.00	1.250	481					
	1.00	3.760	1110					
2f	10.0	0.357	336	7.0	3.29	0.336	9.8	2.8
	3.00	1.190	421					
	1.00	3.570	671					
2g	10.0	0.318	77.5	50.0	29.0	3.51	8.2	2.6
	3.00	1.060	162					
	1.00	3.180	417					
	0.300	10.600	417					
2h	10.0	0.382	350	15.0	3.29	0.391	8.4	2.2
	5.00	0.763	394					
	2.50	1.530	486					
	1.30	3.060	668					
2i	30.0	0.141	183	7.0	6.10	0.835	7.3	1.7
	10.0	0.424	225					
	3.0	1.412	354					
	1.0	4.240	745					
2-PAM	10.0	0.345	43.6	400	36.0	3.74	9.6	2.7
	4.00	0.804	109					
	1.50	2.300	307					
	0.500	6.900	735					
HI-6	30.0	0.049	120	400	20.6	33.8	0.61	0.41
	10.0	0.148	342					
	3.0	0.493	806					
	1.00	1.480	2490					

[a] See Table I for structure. [b] [HOX] is concentration of added test compound. [c] Calculated according to eq 14. [d] Calculated according to eq 11. [e] IC_{50} is the concentration of HOX that inhibits 50% of AChE activity. [f] From eq 15. [g] From eq 16.

The oxime was obtained only as the *E* isomer as previously reported.[26] The quaternary salts **2** were also all obtained configurationally pure as evidenced by proton NMR spectra. They all exhibited chemical shift values for the oxime hydroxyl proton between δ 13.30 and 13.70. By analogy with previously reported results[26] for the 1-methylimidazole oxime, the *E* configuration has been assigned to type **2** compounds.

All of the type **2** oximes exhibited acid dissociation constants (pK_a) near 8.0 in contrast to the value of 8.3 previously reported[26] for 1,3-dialkyl-substituted derivatives of **2**. The lower pK_a for derivatives **2** leads to an increase in effective oximate anion concentration at physiological pH. The higher anion concentration for compounds **2** at physiological pH should lead to more effective reactivation, since it is the oximate that actually serves as the attacking species on phosphorus.[27-29]

Reversible Acetylcholinesterase Inhibition. To correct for enzyme inhibition in our AChE assay and to probe possible correlations between test compound affinity for the enzyme active site and reactivity toward phosphonylated AChE, direct inhibition of AChE activity by type **2** compounds was determined. Eel AChE was incubated with three or more concentrations of each test compound and assayed for activity at intervals between 30 min and 4 h. Observed activities were invariant with time.

From the observed activities, the percentage enzyme inhibition (*I*) was calculated according to eq 1 where A_0

$$I = 100(A_0 - A_I)/A_0 \quad (1)$$

and A_I, respectively, are AChE activities in the absence and presence of added test compounds. The inhibitor concentration giving 50% enzyme inhibition (IC_{50} value) was calculated by linear least-squares regression of log $(I/100 - I)$ vs. log [HOX]. Table II summarizes the inhibition data.

From Table II it is clear that the quaternary imidazolium compounds differ substantially in their ability to reversibly inhibit the enzyme. Additionally, the inhibitory potency (IC_{50}) correlates with measured lipophilicity (log *P*). This relationship is shown in Figure 1 and conforms to eq 2 with a correlation coefficient of 0.92.

$$\log IC_{50} = (-0.44 \pm 0.07) \log P + \log (-2.07 \pm 0.11) \quad (2)$$

Reactivation of Eel AChE Phosphonylated by EPMP. Potential complicating side reactions, such as

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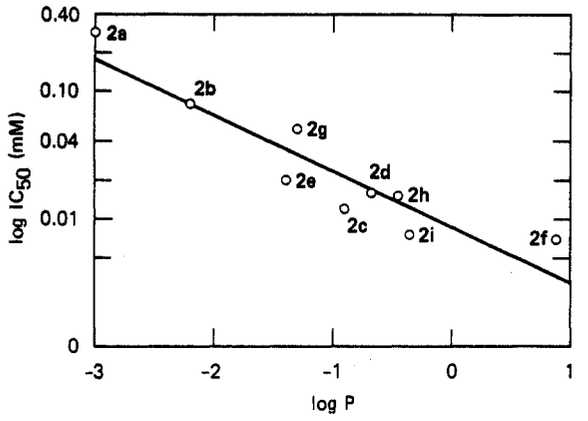

Figure 1. AChE inhibition potency (log IC_{50}) vs. octanol:water partition coefficient (log P) for type **2** compounds.

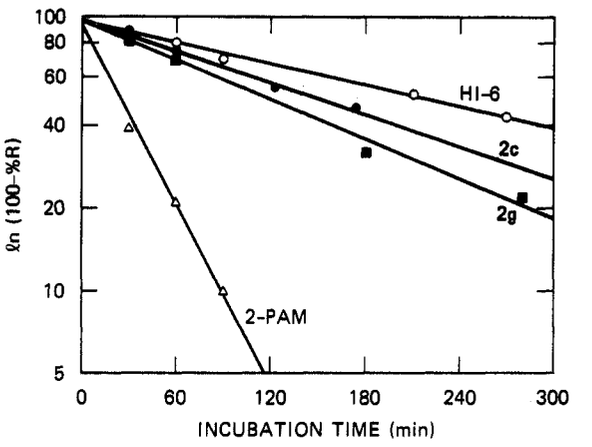

Figure 2. Semilogarithmic plot of ln (100 − % reactivation) vs. incubation time for reaction of ethyl methylphosphonyl-AChE with 10 μM of compounds **2c**, **2g**, 2-PAM, and HI-6.

AChE denaturation, dealkylation ("aging") of ethyl methylphosphonylated AChE, and enzyme inhibition by phosphonyl oxime, were shown to proceed at negligibly slow rates compared with the rate of oximate-induced reactivation of EPMP-inhibited AChE.[30]

Thus, the chemistry of the AChE/ethyl p-nitrophenyl methylphosphonate/oximate system used in the current investigations is satisfactorily described by the reaction set 3–8.

$$(C_2H_5O)CH_3P(O)OC_6H_4NO_2 + EOH \xrightarrow{k_i} EOP(O)CH_3(OC_2H_5) \quad (3)$$

$$EOP(O)CH_3(OC_2H_5) + OX \underset{}{\overset{K_r}{\rightleftharpoons}} [EOP(O)CH_3(OC_2H_5)\cdot OX] \quad (4)$$

$$[EOP(O)CH_3(OC_2H_5)\cdot OX] \xrightarrow{k_r} EOH \quad (5)$$

$$EOH + HOX \overset{K_i}{\rightleftharpoons} [EOH\cdot HOX] \quad (6)$$

$$EOP(O)CH_3(OC_2H_5) + H_2O \xrightarrow{k_{sp}} EOH \quad (7)$$

$$HOX \overset{K_a}{\rightleftharpoons} OX + H^+ \quad (8)$$

To determine reactivation kinetics, AChE was inhibited to approximately 90% of control activity and then incubated with various concentrations of test compounds. At timed intervals, aliquots were withdrawn and assayed for AChE activity. Results were corrected for spontaneous and oximate-induced hydrolysis of the substrate acetylthiocholine. With reactivators present in large excess over ethyl methylphosphonyl–AChE, restoration of enzyme activity followed pseudo-first-order kinetics according to eq 9, where k_{obsd} is the observed rate constant for oxi-

$$\ln (100 - \%\ R_t) = k_{obsd}\cdot t \quad (9)$$

mate-induced reactivation. In equation 9, % R_t is the observed percent reactivation at time t given by eq 10,

$$\%\ R_t = 100\frac{\{A_t[100/(100 - I)] - A_i\}}{A_c - A_i} \quad (10)$$

where A_c, A_i, and A_t, respectively, are observed activities for uninhibited (control) enzyme, enzyme after reaction with EPMP, and enzyme after incubation with reactivator for time t. Observed A_t values are multiplied by the factor $100/(100 - I)$ to correct for inhibition of AChE by added test compound.

Since spontaneous reactivation (reaction 7) proceeded at a nonnegligible rate, the observed reactivation rate constants were corrected according to eq 11, where k_{sp} was

$$(k_{obsd})_c = k_{obsd} - k_{sp} \quad (11)$$

calculated according to eq 12.

$$\ln [(A_c - A_i)/A_c] = k_{sp}\cdot t \quad (12)$$

All type **2** compounds restored enzyme activity at a significant rate. Linear least-squares regression of the data showed good adherance to eq 10. Semilogarithmic plots of (100 − % R) vs. time, shown in Figure 2, were linear to high conversions, and AChE reactivation was complete on long incubation with high reactivator concentrations. For clarity, not all the kinetic runs are plotted in Figure 2.

In an attempt to elucidate structure–activity relationships among the various reactivators, we examined the reactivation rate constant dependence on oxime concentration. For the reaction set given by eq 3–8, it can be shown[31-36] that eq 13 follows:

$$(k_{obsd})_c^{-1} = (1/k_r) + [OX]^{-1}(K_r/k_r) \quad (13)$$

where [OX] was calculated at pH 7.6 by eq 14. The

$$[OX] = [HOX]\{1 + \text{antilog } [pK_a - 7.6]\}^{-1} \quad (14)$$

constants K_r and k_r define the reactivation process with respect to [inhibited enzyme/oximate] complex dissociation and transformation of the complex to active enzyme. The derivation of eq 14 is straightforward and has been described elsewhere.[32,33,37]

According to eq 13, a plot of $(k_{obsd})_c^{-1}$ vs. $[OX]^{-1}$ is linear with slope = K_r/k_r. In the limit of low reactivator con-

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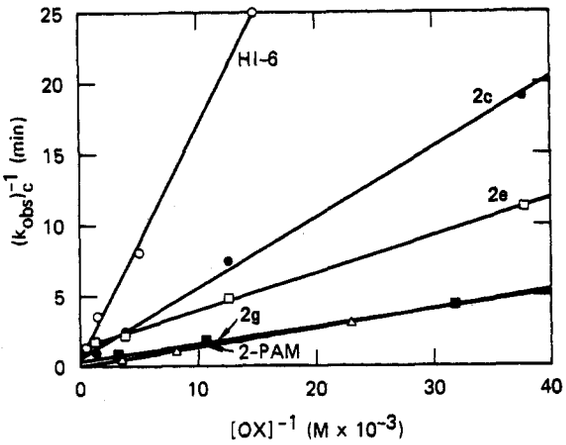


Figure 3. Reciprocal corrected observed rate constant for reactivation $(k_{obsd})_c^{-1}$ vs. reciprocal oximate concentration for reactivation of ethyl methylphosphonyl-AChE by compounds **2c**, **2e**, **2g**, 2-PAM, and HI-6.

centration ([OX] << K_r) the ratio of k_r to K_r is equivalent to k_{OX}, an apparent bimolecular rate constant for reactivation; that is,

$$k_r/K_r = k_{OX} \quad (15)$$

The bimolecular reactivation rate constant k_{OX} parallels the inherent activity of the oximate as a reactivator of the inhibited enzyme. Because various imidazolium aldoximes ionize to different extents at pH 7.6, the effective rate constant for reactivating inhibited AChE, k_{HOX}, is defined as the product of k_{OX} and the fraction of added test compound present as oximate at pH 7.6; that is,

$$k_{HOX} = k_{OX}\,[1 + \text{antilog}\,(pK_a - 7.6)]^{-1} \quad (16)$$

Table II summarizes values for reactivation rate constants calculated by using eq 13–16. Figure 3 is a double-reciprocal plot according to eq 13 for several type 2 compounds.

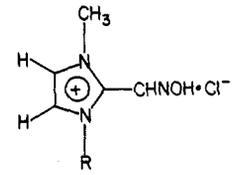
The table demonstrates the range of activaties for type 2 compounds investigated: k_{HOX} values varied from a low of 233 M^{-1} min^{-1} (**2d**) to a high of 2750 M^{-1} min^{-1} (**2f**). Relative k_{HOX} values for **2d**:**2f**:2-PAM were 1:11.4:11.5; thus type 2 compounds have activity comparable to 2-PAM. Several of the type 2 compounds exceed the activity of HI-6 in this model system.

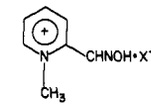
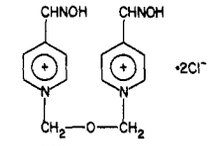
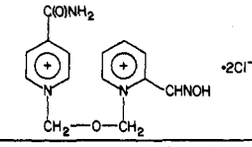
Reactivation of GD-Inhibited Eel AChE. GD-inhibited AChE differs from EPMP-inhibited enzyme in that inhibited enzyme dealkylation is much faster for GD than for EPMP. Determination of kinetic parameters was attempted by using a modification of De Jong and Wolring's procedure.[38] At timed intervals, aliquots were withdrawn from the pH 7.6 solutions and diluted 40-fold directly into a pH 8.0 buffer containing Ellman assay reagents.[39] The 40-fold dilution effectively stopped the reactivation process so that we could accurately calculate the percent reactivation after each incubation time period. The compounds clearly showed the expected time-dependent increase in R_t for each reactivator at all concentrations. In each case, R_t values approached a maximum, R_{max}, at about 30 min, but never approached the theoretical limit of R_{max} = 100. This behavior is consistent with concurrent rapid dealkylation coupled with slow reactivation of phosphonylated enzyme.

(38) De Jong, L. P. A.; Wolring, G. Z. *Biochem. Pharmacol.* **1980**, *29*, 2379.

(39) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.

Table III. Percentage of Maximum Reactivation (% R_{max}) for GD-Inhibited Eel AChE by Oximes[a]



compd	R	% R_{max}[b]
2a	CH_2OCH_3	15.0
2b	$CH_2OCH(CH_3)_2$	9.8
2c	$CH_2OCH_2C(CH_3)_3$	5.4
2d	$CH_2OCH(CH_3)C(CH_3)_3$	0.8
2e	$CH_2O(CH_2)_3CH_3$	17.4
2f	$CH_2O(CH_2)_7CH_3$	9.3
2g	$CH_2OCH_2C_6H_5$	25.5
2h	$CH_2O(CH_2)_3C_6H_5$	19.3
2i	$CH_2OCH_2C_{10}H_7$	20.4
2-PAM		23.3
toxogonin		36.5
HI-6		48.7

[a] A 1 mM concentration was used for all test oximes; incubation with oxime was conducted in buffer at pH 7.6 and 25 °C. [b] Maximum reactivation for all test oximes was achieved by 30-min incubation.

For each of the compounds we calculated observed pseudo-first-order reactivation rate constant values, k_{obsd}, according to eq 17, where eq 17 is identical with that de-

$$\ln\left(\frac{R_{max} - R_t}{R_{max}}\right) = k_{obsd} \cdot t \quad (17)$$

rived by De Jong and Wolring[38] for reactivation of GD-inhibited bovine erythrocyte AChE. In all cases, the calculated k_{obsd} values decreased with increasing oximate concentration, contrary to the expected behavior. Thus we conclude, as did De Jong and Wolring[38] in their investigations of GD-inhibited AChE reactivation, that enzyme reinhibition along with fast phosphonyl enzyme dealkylation preclude accurate determination of kinetic constants. Instead, we rely on observed R_{max} values to reflect relative reactivator potencies vs. GD-inhibited AChE, as shown in Table III.

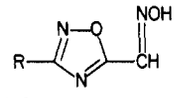
As with the EPMP-inhibited AChE described above, a broad range of activities were observed for the compounds investigated: % R_{max} values varied from a low of 0.80 (**2d**) to a high of 25.5 (**2h**). Unlike EPMP-inhibited AChE, the R_{max} for HI-6 exceeded that of **2h** by a factor of 2.

Structure–Activity Relationships. For most type 2 compounds the k_{HOX} determined for EPMP-inhibited eel AChE paralleled the R_{max} measured for GD-inhibited eel AChE. Alkyl branching on the alkoxyalkyl moiety, compounds **2b–2d**, significantly decreased the in vitro reactivation potency toward both EPMP- and GD-inhibited AChE. The *n*-octoxymethyl compound **2f** displayed anomalous behavior. While being the most potent in vitro reactivator of EPMP-inhibited AChE, it was only moderately reactive toward GD-inhibited AChE. For this se-

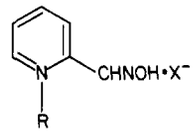
ries, incorporation of a second aryl substituent favors in vitro reactivation of GD-inhibited AChE (e.g., compounds **2g–2i**).

By limiting this work to a series of 1-(alkoxyalkyl)-3-methylimidazolium oximes, we hoped to minimize the effects of oximate nucleophilicity (pK_a) and geometry on reactivation while maximizing steric and hydrophobic binding effects. Although other as yet undefined binding forces may be involved, it appears that both hydrophobic forces and steric interactions contribute strongly to reactivation potency in this series of compounds. Thus the search for new imidazolium compounds with increased activity should reasonably focus on substituents that feature high octanol:buffer partitioning and low steric bulk. We identify R = octoxymethyl, (benzyloxy)methyl, and (3-phenylpropoxy)methyl as substituent groups satisfying these criteria, since **2f**, **2g**, and **2h** are among the most reactive quaternary imidazolium compounds investigated in vitro to date.

Interestingly, the benzyl and octyl substituent groups confer high activity to other classes of reactivators. For example, in the 3-substituted-5-[(hydroxyimino)-methyl]-1,2,4-oxadiazoles, the compound with R = n-C_8H_{17}



was the most powerful nonquaternary reactivator tested against EPMP-inhibited AChE (k_{OX} = 2120 M^{-1} min^{-1}).[40] Similarly, in the series of N-alkyl- and N-aralkylpyridinium 2-aldoximes, De Jong and co-workers[41–44] found 1-



benzyl-2-[(hydroxyimino)methyl]pyridinium halide to be the most active reactivator of AChE inhibited by several different organophosphorus compounds.

Conclusions

Nine imidazolium aldoximes were prepared and evaluated as reactivators of ethyl methylphosphonylated and 3,3-dimethyl-2-butyl methylphosphonylated eel AChE. On the basis of this work and earlier investigations by ourselves[30,31,40] and others,[21,45–48] we can now identify some of the molecular parameters that govern the activity of quaternary imidazolium cholinesterase reactivators.

For EPMP-inhibited AChE, steric interactions between oxime reactivators and enzyme dictate the inherent in vitro reactivity of imidazolium reactivators. Substituents (alkoxy/aralkoxymethyl) can be extensively varied and still provide reactivators with pK_a values in the useful range. Of the oxime-substituted imidazoles, only the 2-[(hydroxyimino)methyl]imidazolium compounds provide such effective oxime pK_a values. Furthermore, incorporating the alkoxymethyl groups on the imidazole ring lowers the (hydroxyimino)methyl pK_a values by approximately 0.2–0.3 unit relative to alkyl groups.

While the lipophilic effects of the substituents are directly correlated with potency for direct inhibition of eel AChE, they do not correlate with reactivation potency. Alkyl branching on the alkoxymethyl group, as found in compounds **2b–2d**, greatly reduces in vitro reactivation potency (toward both EPMP- and GD-inhibited AChE) while having little or no effect on IC_{50}. Incorporation of a relatively flat aryl substituent favors in vitro reactivation. It is not clear whether the variable effects of these substituents are due to optimization of the orientation of oximate toward the phosphonylated AChE or to other as yet undefined geometric constraints.

The imidazolium aldoximes **2a**, **2f**, **2g**, and **2h** were found to possess excellent properties for reactivation of organophosphorus-inhibited eel AChE in vitro. The compounds were approximately equal to 2-PAM in their ability to reactivate EPMP- and GD-inhibited AChE and were superior to HI-6 with respect to reactivation of EPMP-inhibited AChE. The ease and versatility of synthesis makes the imidazolium family of reactivators an attractive alternative to existing therapeutic oximes. The application of structure–activity relationships to reactivation of inhibited AChE has led to important advances in organophosphorus agent therapy with respect to development of pyridinium oximes. It is our belief that this approach can be applied to the imidazolium oximes and can ultimately lead to the design of improved therapeutics for organophosphorus intoxication. Further investigations will be directed toward optimization of the molecular parameters important for in vivo efficacy, such as a favorable tissue distribution, a moderate rate of metabolism, and a high therapeutic index. We are currently exploring these possibilities.

Experimental Section

Materials. Nuclear magnetic resonance spectra were recorded on a Varian Associates EM-360 or EM-390 spectrometer; chemical shifts are reported in parts per million (δ) from an internal tetramethylsilane standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Melting points were determined on a Fisher-Johns or Mel-Temp melting point apparatus and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tn, and are reported in Table I.

Tetrahydrofuran (THF) was distilled from benzophenone ketyl and stored under nitrogen over 4A molecular sieve. Metalation reactions were conducted in oven-dried apparatus under an inert atmosphere. Ethyl p-nitrophenyl methylphosphonate (EPMP) was prepared by conversion of diethyl methylphosphonate to ethyl methylphosphonochloridate followed by reaction with p-nitrophenol.[49] 3,3-Dimethyl-2-butyl methylphosphonofluoridate was supplied by the U.S. Army Medical Research and Development Command (20 mg in 10 mL of water) and stored at −70 °C until use. **CAUTION**: EPMP and GD are extremely toxic anticholinesterase agent. It must be handled with gloves and in a fume hood or at high dilutions at all times. Toxogonin and HI-6 were prepared by the method of Hagedorn.[8,12]

Chloromethyl Ethers. All chloromethyl ethers were prepared from the corresponding alcohols by using previously reported procedures.[24,25] Of the chloromethyl ethers prepared, only three

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(41) De Jong, L. P. A.; Benschop, H. P.; Van den Berg, G. R.; Wolring, G. Z.; De Korte, D. C. *Eur. J. Med. Chem.-Chim. Ther.* **1981**, *16*, 257.
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(46) Schoene, K.; Strake, E. M. *Biochem. Pharmacol.* **1971**, *20*, 1041.
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(49) Fukuto, T. R.; Metcalf, R. L. *J. Am. Chem. Soc.* **1959**, *81*, 372.

were previously unreported compounds. These exhibited the following properties.

Chloromethyl 1-methylnaphthyl ether: prepared from 1-(hydroxymethyl)naphthalene and isolated in 17% yield as an impure, colorless, viscous oil: bp 98–100 °C (0.12 torr); ^{1}H NMR ($CDCl_3$) δ 5.17 (s, 2 H, CH_2), 5.52 (s, 2 H, CH_2Cl), 7.37–8.35 (m, 7 H, aryl). The major contaminant, 1-(chloromethyl)naphthalene, did not interfere with subsequent reactions.

1-Chloro-3,4,4-trimethyl-2-oxapentane (chloromethyl pinacolyl ether): prepared from pinacolyl alcohol (Aldrich) and isolated in 33% yield as a colorless oil: bp 90–92 °C (95 torr); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 9 H, CH_3), 1.12 (d, 3 H, J = 6.0 Hz, CH_3), 3.50 (q, 1 H, J = 6.0 Hz, CH), 5.57 (s, 2 H, CH_2Cl). Anal. Calcd: C, 56.19; H, 9.43; Cl, 23.69. Found: C, 56.15; H, 9.49; Cl, 23.91.

Chloromethyl 3-phenylpropyl ether: prepared from 3-phenyl-1-propanol (Aldrich) and isolated in 48% yield as a colorless oil: bp 117–118 °C (7.0 torr); ^{1}H NMR ($CDCl_3$) δ 1.83 (d of quintets, 2 H, J = 6.0 Hz, J = 2.0 Hz, CH_2), 2.63 (t, 2 H, J = 6.0 Hz, CH_2), 3.60 (t, 2 H, J = 6.0 Hz, CH_2), 5.42 (s, 2 H, CH_2Cl), 7.20 (s, 5 H, phenyl). Anal. Calcd: C, 65.04; H, 7.10; Cl, 19.20. Found: C, 65.24; H, 7.29; Cl, 19.06.

2-[(Hydroxyimino)methyl]-1-methylimidazole. The 1-methyl-2-formylimidazole was prepared by the method of Iversen and Lund.[23] The water-soluble oxime derivative was most conveniently prepared by treating the formylimidazole with a slight excess of hydroxylamine hydrochloride and $NaHCO_3$ at reflux in EtOH for 1 h, filtering inorganics while still warm, concentrating the filtrate, and recrystallizing the residue from 2-propanol: mp 170–172 °C (lit.[26] mp 170 °C).

General Procedure for Imidazolium Salts 2. The quaternary salts were prepared by dissolving the imidazole oxime in THF–DMF (6:1) and adding 2 equiv of the appropriate chloromethyl ether. After stirring at room temperature for 24–48 h, the precipitates were filtered, washed with dry THF or diethyl ether, and vacuum dried. The following salts were prepared via this procedure.

2-[(Hydroxyimino)methyl]-1-(methoxymethyl)-3-methylimidazolium Chloride (2a). Recrystallization from EtOH yielded analytically pure **2a** as colorless prisms: mp 168–172 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 3.37 (s, 3 H, OCH_3), 4.03 (s, 3 H, NCH_3), 5.77 (s, 2 H, NCH_2O), 8.08 (d, 1 H, J = 1.5 Hz, aryl), 8.17 (d, 1 H, J = 1.5 Hz, aryl), 8.62 (s, 1 H, CHN), 13.45 (s, 1 H, NOH).

2-[(Hydroximino)methyl]-1-(isopropoxymethyl)-3-methylimidazolium Chloride (2b). Recrystallization from EtOH yielded analytically pure **2b** as a white powder: mp 128–130 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 1.07 (d, 6 H, J = 6.0 Hz, CH_3), 3.77 (m, 1 H, CH), 4.00 (s, 3 H, CH_3), 5.78 (s, 2 H, CH_2), 8.05 (d, 1 H, J = 2.0 Hz, aryl) 8.18 (d, 1 H, J = 2.0 Hz, aryl), 8.60 (s, 1 H, CH), 13.43 (s, 1 H, NOH).

1-[(2,2-Dimethylpropoxy)methyl]-2-[(hydroxyimino)-methyl]-3-methylimidazolium Chloride (2c). Recrystallization from 2-propanol gave analytically pure **2c** as white crystals: mp 167–168 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 0.83 (s, 9 H, CH_3), 3.20 (s, 2 H, CH_2), 4.02 (s, 3 H, CH_3), 5.83 (s, 2 H, CH_2), 8.10 (d, 1 H, J = 1.0 Hz, aryl), 8.18 (d, 1 H, J = 1.0 Hz, aryl), 8.60 (s, 1 H, CH), 13.50 (s, 1 H, NOH).

2-[(Hydroxyimino)methyl]-3-methyl-1-[(3,3-dimethyl-2-butoxy)methyl]imidazolium Chloride (2d). Recrystallization from 2-propanol yielded analytically pure **2d** as colorless crystals: mp 169–170 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 0.73 (s, 9 H, CH_3), 1.02 (d, 3 H, J = 6.0 Hz, CH_3), 3.33 (q, 1 H, J = 6.0 Hz, CH), 4.00 (s, 3 H, CH_3), 5.83 (s, 2 H, CH_2), 8.05 (d, 1 H, J = 2.0 Hz, aryl), 8.22 (d, 1 H, J = 2.0 Hz, aryl), 8.60 (s, 1 H, CH), 13.43 (s, 1 H, NOH).

1-(1-Butoxymethyl)-2-[(hydroxyimino)methyl]-3-methylimidazolium Chloride (2e). Two recrystallizations from ethyl acetate/2-propanol (10:3) gave analytically pure white crystals of **2e**: mp 102–105 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 0.70–1.70 (br m, 7 H, alkyl), 3.53 (t, 2 H, J = 6.0 Hz, CH_2), 4.05 (s, 3 H, CH_3), 5.87 (s, 2 H, CH_2), 8.18 (d, 1 H, J = 2.0 Hz, aryl), 8.30 (d, 1 H, J = 2.0 Hz, aryl), 8.63 (s, 1 H, CH), 13.53 (s, 1 H, NOH).

2-[(Hydroxyimino)methyl]-3-methyl-1-(1-octoxymethyl)-imidazolium Chloride (2f). Recrystallization from EtOH yielded analytically pure off-white crystals of **2f**: mp 132–134 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 0.87 (m, 3 H, CH_3), 1.18 (br s, 12 H, methylene), 3.50 (t, 2 H, OCH_2), 3.98 (s, 3 H, NCH_3), 5.75 (s, 2 H, NCH_2O), 7.97 (d, 1 H, J = 1.5 Hz, aryl), 8.05 (d, 1 H, J = 1.5 Hz, aryl), 8.57 (s, 1 H, CH), 13.30 (s, 1 H, NOH).

1-[(Benzyloxy)methyl]-2-[(hydroxyimino)methyl]-3-methylimidazolium Chloride (2g). Recrystallization from EtOH gave analytically pure **2g** as an off-white solid: mp 124–126 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 3.98 (s, 3 H, CH_3), 4.65 (s, 2 H, CH_2), 5.90 (s, 2 H, CH_2), 7.35 (s, 5 H, phenyl), 8.00 (d, 1 H, J = 2.0 Hz, aryl), 8.13 (d, 1 H, J = 2.0 Hz, aryl), 8.60 (s, 1 H, CH), 13.42 (s, 1 H, NOH).

2-[(Hydroxyimino)methyl]-3-methyl-1-[(3-phenylpropoxy)methyl]imidazolium Chloride (2h). Recrystallization from 2-propanol gave analytically pure **2h** as colorless crystals: mp 130–131 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 1.80 (m, 2 H, CH_2), 2.60 (t, 2 H, J = 6.0 Hz, CH_2), 3.57 (t, 2 H, J = 6.0 Hz, CH_2), 4.07 (s, 3 H, CH_3), 5.09 (s, 2 H, CH_2), 7.25 (s, 5 H, phenyl), 8.20 (d, 1 H, J = 2.0 Hz, aryl), 8.30 (d, 1 H, J = 2.0 Hz, aryl), 8.70 (s, 1 H, CH), 13.63 (s, 1 H, NOH).

2-[(Hydroxyimino)methyl]-3-methyl-1-[(1-naphthylmethoxy)methyl]imidazolium Chloride (2i). Recrystallization from 2-propanol/ethyl acetate (1:1) yielded analytically pure **2i** as a white solid: mp 125–128 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 3.97 (s, 3 H, CH_3), 5.18 (s, 2 H, CH_2), 6.03 (s, 2 H, CH_2), 7.58 (m, 4 H, aryl), 8.03 (m, 4 H, aryl), 8.25 (d, 1 H, J = 2.0 Hz, aryl), 8.67 (s, 1 H, CH), 13.61 (s, 1 H, NOH).

Physical Measurements. Reactivator pK_a values were determined spectrophotometrically in 0.1 M phosphate buffer by the method of Albert and Sargent.[50] Octanol:water partition coefficients were determined spectrophotometrically by the method of Fujita et al.[51] The aqueous phase for all log P determinations was pH 7.4, 0.1 M phosphate buffer.

Competitive Inhibition of Acetylcholinesterase. For analysis of AChE competitive inhibitory potency, AChE plus four to six different concentrations of the compounds are dissolved in 0.1 M phosphate buffer, and duplicates are assayed three times over a period of about 2 h. The results, invariant with time, are averaged for each concentration and the IC_{50} determined by the linear transformation equation discussed earlier. Only values between 10% and 90% inhibition are used for the calculation. The actual procedure is as follows.

Drug solutions are made up in water at appropriate concentrations between 10^{-2} and 10^{-6} M. Aliquots of 0–700 μL are added to 5935 μL of 0.1 M, pH 8.0 phosphate buffer. To this is added 365 μL of an AChE solution in 0.1 M, pH 7.6 Mops buffer at 0.42 U/mL, followed by addition of water to make the total volume 7000 μL. The solution is mixed well and then incubated at 25 °C. To 960-μL aliquots are added 30 μL of 0.10 M dithiobis-[nitrobenzoic acid] (DTNB) and 10 μL of 0.075 M acetylthiocholine (AcSCh), and the increasing absorbance is monitored by a UV–visible spectrometer at 412 nM (AChE = 0.022 U/mL, DTNB = 3×10^{-3} M, AcSCh = 7.5×10^{-4} M).

Acetylcholinesterase Reactivation after EPMP Inhibition. Unless otherwise noted, all experiments were conducted at 25.0 ± 0.1 °C in pH 7.6, 0.1 M morpholinopropanesulfonic acid (Mops) buffer plus NaN_3 (0.002%), $MgCl_2$ (0.01 M), and bovine serum albumin (0.01%). Enzyme activities were assayed by the Ellman method[39] on a Gilford-modified DU spectrophotometer coupled to a HP-85 laboratory computer for automatic rate determination. All rate constants were determined by least-squares linear regression analysis with error limits reported as standard deviation from the mean.

In general, eel AChE (Sigma) was reacted with the quantity of EPMP, giving approximately 90% inhibition of activity in 20 min. Aliquots of inhibited enzyme were then withdrawn and diluted in Mops buffer containing known concentrations of reactivators. The inhibited enzyme was incubated with reactivators for timed intervals and assayed (in duplicate) for activity. In parallel experiments, uninhibited AChE and inhibited AChE in the absence of added reactivator were assayed for activity to

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determine, respectively, rates of enzyme denaturation and spontaneous reactivation. As a control in our AChE assay and to check for hydrolytic stability of the type 2 compounds, acetylthiocholine hydrolysis by the compounds was determined as a function of time. For each compound, observed thiocholine production rates (d[thiocholine]/dt) were invariant to within SD = ±10% over the 3- to 4-h incubation period normally used in the in vitro assay of EPMP reactivation. Furthermore, UV spectra of the test compounds taken at pH 6.4, 7.6, and 8.5 showed little or no significant changes over a 24-h period. These results exclude the possibility of significant hydrolytic degradation of the test compounds under our experimental conditions. Observed enzyme activities were corrected for inhibition by the test compounds and for spontaneous and reactivator-induced substrate hydrolysis as discussed earlier.

Dilution factors and aliquot volumes were determined experimentally for the various transfers involved in the experiments. An exact procedure giving good precision in replicate assays is as follows.

Dilute 110 μL of (nominally) 500 acetylcholine units/mL of enzyme solution with 110 μL of Mops buffer to give enzyme "stock" solution, 250 U/mL. For determining uninhibited AChE activity, dilute 25 μL of stock solution to 20 mL with Mops buffer (AChE 0.31 U/mL) and withdraw 50 μL for assay (see below).

To inhibit the AChE, dilute 140 μL of stock solution in 132 μL of Mops plus 8 μL of EPMP (1×10^{-5} M in C_2H_5OH). To determine activity of the inhibited AChE, incubate 20 min, withdraw 10 μL, dilute to 4.0 mL in Mops (0.31 U/mL AChE, $<< 7 \times 10^{-10}$ M EPMP), and assay 50 μL.

For reactivation studies, dilute 100 μL of the inhibited AChE solution to 1.0 mL with Mops buffer, remove 25 μL (for each incubation), and dilute to 1.0 mL with Mops (0.31 U/mL AChE, $<< 7 \times 10^{-10}$ M EPMP) plus reactivator at several concentrations from 0 to 10^{-3} M. Incubate at 25 °C for 4 h, removing duplicate 50-μL aliquots for assay at several time points.

For assay of AChE activity, add 50-μL aliquots of solution to be assayed to 910 μL of pH 8.0, 0.1 M phosphate buffer, plus 30 μL of 0.10 M dithiobis[nitrobenzoic acid], plus 10 μL of 0.075 M acetylthiocholine, and monitor increased absorbance at 412 nm vs. time (AChE = 0.016 U/mL, DTNB = 3×10^{-3} M, AcSCh = 7.5×10^{-4} M, reactivators = 0 to 5×10^{-5} M, EPMP $<< 3.5 \times 10^{-11}$ M).

Acetylcholinesterase Reactivation after GD Inhibition. For GD reactivation studies, procedures had to be modified from those described above because of the rapid dealkylation of GD-inhibited AChE. AChE was inhibited with GD at pH 10.1 and then diluted into Mops buffer for reactivation at pH 7.6. The high pH during inhibition retards aging and destroys excess GD. Aliquots were removed for assay at very short intervals to evaluate initial rates of reactivation and at least two longer time points to determine maximum percent reactivation. The dilution into pH 8.0 phosphate buffer for assay ensured that reactivation (a second-order reaction) would proceed at a negligible rate compared to aging (a first-order reaction). Thus percent reactivation would be unchanged in the assay tubes. The exact procedure is as follows.

Dilute 120 μL of an enzyme solution containing (nominally) 500 acetylcholine units/mL (in 0.025 M Mops) with 680 μL of 0.9% saline to give enzyme "stock" solution (75 U/mL of AChE).

To inhibit the AChE, add 150 μL of stock enzyme to 975 U/mL of pH 10.2, 0.05 M borate buffer (with 0.01% in BSA), plus 75 μL of 1×10^{-6} M GD in H_2O; this initiates the start of the incubation. For an uninhibited enzyme control, another tube is started concurrently containing the above solutions, but 75 μL of H_2O is substituted for the GD. In a typical experiment, pairs of such tubes are started 10 min apart, and each set is incubated at 25 °C for 30 min (9.4 U/mL of AChE, 6.2×10^{-8} M GD).

After inhibition, add 200 μL of the inhibited and uninhibited enzyme solutions to separate tubes that contain 700 μL of pH 6.8, 0.025 M Mops plus 100 μL of reactivator in H_2O at several concentrations from 0 to 10^{-3} M. An aliquot of the appropriate enzyme solution is added first to the uninhibited enzyme tube, then to the zero-drug tube, then to three drug-containing tubes at 20-s intervals (1.9 U/mL of AChE, $<< 1.2 \times 10^{-8}$ M GD).

To dilute for assay of AChE activity, deliver 15-μL aliquots (in duplicate) of reactivation solutions into 945 μL of pH 8.0, 0.1 M phosphate buffer into spectrophotometer cuvettes at 10-s intervals. Each drug-containing tube is sampled four times in duplicate in 5 min, and then zero-drug and drug-containing tubes are sampled at 30, 60, and 90 min. All the cuvettes are assayed, four at a time, as quickly as possible after the dilution, where each assay run takes about 5 min. For the assay, 30 μL of 0.1 M dithiobis[nitrobenzoic acid] plus 10 μL of 0.075 M acetylthiocholine is added to the cuvettes, and absorbance is monitored at 412 nm vs. time (AChE = 0.028 U/mL, DTNB = 3×10^{-3} M, AcSCh = 7.5×10^{-4} M, reactivators = 0 to 1.5×10^{-5} M, GD $<< 1.8 \times 10^{-10}$ M).

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Registry No. **2a**, 91900-09-3; **2b**, 91900-10-6; **2c**, 91900-11-7; **2d**, 91900-12-8; **2e**, 91900-13-9; **2f**, 91900-14-0; **2g**, 91900-15-1; **2h**, 91900-16-2; **2i**, 91900-17-3; chloromethyl 1-methylnaphthyl ether, 88045-68-5; 1-(hydroxymethyl)naphthalene, 4780-79-4; chloromethyl pinacolyl ether, 91900-18-4; pinacolyl alcohol, 464-07-3; chloromethyl 3-phenylpropyl ether, 90875-79-9; 3-phenyl-1-propanol, 122-97-4; 2-[(hydroxyimino)methyl]-1-methylimidazole, 20062-62-8; 1-methyl-2-formylimidazole, 13750-81-7; hydroxylamine hydrochloride, 5470-11-1; acetylcholinesterase, 9000-81-1.