procedure for alkaline hydrolysis, and the product was obtained as a white solid: yield 1.34 g (80%); TLC R_f (D) 0.45. Anal. Calcd for $C_{38}H_{55}N_5O_{10}$: C, 61.52; H, 7.47; N, 9.44. Found: C, 61.36; H, 7.38; N, 9.51.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine (1.33 g, 1.8 mmol) in THF (15 mL), cooled in a ice–water bath, were added successively a solution of *O*-*tert*-butyl-L-threonine methyl ester hydrochloride (0.40 g, 1.8 mmol) and triethylamine (0.25 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.27 g, 1.8 mmol) in THF (10 mL), and a solution of DCC (0.37 g, 1.8 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred for 20 h. The reaction was treated following the standard procedure, and a white solid (1.52 g, 90%) was obtained, which showed a single spot on TLC R_f (C) 0.67; mp 158–160 °C. Anal. Calcd for $C_{47}H_{72}N_6O_{12}$: C, 61.82; H, 7.95; N, 9.20. Found: C, 61.65; H, 8.02; N, 9.33.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine.** To a solution of the preceding compound (1.51 g, 1.66 mmol) in MeOH cooled at 0 °C was added 4 mL of 1 N NaOH. The mixture was stirred at 0 °C for 30 min and then at room temperature for 36 h. Then an excess of 1 N NaOH (1.2 mL) was added and stirring continued for 24 h. The reaction mixture was treated following the standard procedure for alkaline hydrolysis and yielded the N,O-protected hexapeptide: yield 1.26 g (85%); mp 178–180 °C; TLC R_f (B) 0.8. Anal. Calcd for $C_{46}H_{70}N_6O_{12}$: C, 61.31; H, 8.05; N, 9.33. Found: C, 61.52; H, 8.11; N, 9.17.

Tyrosyl-D-serylglycyl-L-phenylalanyl-L-leucyl-L-threonine (10). *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine (0.230 g, 0.29 mmol) was dissolved at 0 °C in TFA saturated with HCl (1 mL). The reaction mixture was stirred at 0 °C during 30 min and then at room temperature for 30 min. Ether (50 mL) was added to the well-stirred mixture. The precipitate was collected and washed with ether (5 × 50 mL). The crude product was purified by gel filtration on LH20 (Pharmacia) with MeOH as eluent. Fractions containing the pure hexapeptide were evaporated in vacuo and lyophilized to yield 0.18 g (90%). The purity of the hexapeptide was checked both by TLC [single spot (R_f (D) 0.49)], and by HPLC on a Waters apparatus [(reversed phase μBondapak C18); solvent CH_3CN/NH_4AcO, buffer pH 4.2, 20:80, flow rate 1.2 mL/min, retention time of the single peak 612 s]. Anal. Calcd for $C_{33}H_{46}N_6O_{10}$: C, 57.71; H, 6.75; N, 12.24. Found: C, 57.88; H, 6.91; N, 12.12. NMR (Me_2SO-d_6) δ 3.77 (Tyr α H), 2.86 and 2.64 (β H), 6.98 and 6.61 (Ar H), 4.14 (Ser α H), 3.52 and 3.45 (β H), 3.65 (Gly α H), 4.39 (Phe α H), 2.99 and 2.76 (β H), 7.19 (Ar H), 4.14 (Leu α H), 1.48 (β H and γ H), 0.79 (CH_3), 3.91 (Thr α H), 3.91 (β H), 0.89 (CH_3).

These chemical shifts correspond to the spectrum of **10** performed on the Zwitterionic form. The slightly different chemical shifts reported for **10** in a preliminary communication[23] correspond to the trifluoroacetate form.

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Nonquaternary Cholinesterase Reactivators. Dialkylaminoalkyl Thioesters of α-Ketothiohydroximic Acids as Reactivators of Diisopropyl Phosphorofluoridate Inhibited Acetylcholinesterase

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We have prepared a series of α-ketothiohydroximic acid thioesters and evaluated them in vitro with respect to their ability to reactivate (diisopropylphosphoryl)acetylcholinesterase. The compounds conform to the general formula $RC(=O)C(=NOH)S(CH_2)_nNR_2'\cdot HCl$, where R = CH_3, C_6H_5, 4-$CH_3OC_6H_4$, 4-$NO_2C_6H_4$; n = 2, 3; and R′ = CH_3, C_2H_5, or i-C_3H_7. We also prepared 4-$BrC_6H_4C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot HCl$ and 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot CH_3I$ for comparison. The α-ketothiohydroximates exhibit oxime acid dissociation constants (pK_a) in the range 6.9 to 8.4, bracketing the value of pK_a = 7.9, believed to be optimal for acetylcholinesterase reactivation. The compounds are also good nucleophiles; bimolecular rate constants (k_n) for reaction with *p*-nitrophenyl acetate follow the expression $\log (k_n) = 6.7 - 0.69(14 - pK_a)$. The reactivation of (diisopropylphosphoryl)acetylcholinesterase is highly dependent on the α-ketothiohydroximate structure: 4-h incubation of inhibited enzyme at pH 7.6, 25 °C, with 1×10^{-3} M 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_3N(CH_3)_2\cdot HCl$ gives no detectable restoration of activity, whereas 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot HCl$ restores inhibited enzyme activity to 58% of control under identical conditions. With α-ketothiohydroximate in excess over inhibited enzyme, the kinetics of reactivation are governed by an equilibrium constant (K_r) for binding α-ketothiohydroximate to the inhibited enzyme and a nucleophilic displacement rate constant (k_r) for attack on phosphorus.

A variety of toxic organophosphorus (OP) esters owe their biological activity to phosphylation[1] of a serine hydroxyl at the active site of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), AChE.[2,3] Therapy for anti-AChE agent intoxication is based on coadministration of anticholinergics (e.g., atropine) to antagonize the effects of accumulated acetylcholine (ACh) and of AChE "reactivators" that displace the phosphyl residue from the active site and restore enzymatic activity.[4,5]

The last 30 years have witnessed considerable interest in elucidating the mechanism of AChE reactivation, and it is recognized[6,7] that the reaction proceeds as shown in

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eq 1, where EOH is active enzyme, EOP is phosphylated

$$EOP + R \overset{K_r}{\rightleftharpoons} [EOP{\cdot}R] \underset{H_2O}{\overset{k_r}{\rightarrow}} EOH + ROP \qquad (1)$$

enzyme, R is a reactivator, ROP is phosphylated reactivator, [EOP·R] is a complex between reactivator and inhibited enzyme, K_r (= [EOP][R]/[EOP·R]) is an equilibrium constant describing the affinity of the reactivator for the inhibited enzyme, and k_r is a rate constant for nucleophilic displacement of the phosphyl residue from the enzyme.

The development of useful reactivators has focused on compounds that combine a high affinity for the inhibited enzyme and strong nucleophilicity. In the latter regard, compounds featuring the oximino (=NOH) functionality have received the most attention, since the oximate anion is a particularly strong nucleophile toward OP esters. The dual requirements for nucleophilicity and for dissociation of oxime into oximate anion at physiological pH combine to give an optimal value for the oxime acid dissociation constant (pK_a), known empirically[5] to be $pK_a = 7.9$. Affinity for the inhibited enzyme has been approached by incorporating into reactivators cationic centers that provide for strong coulombic interaction with the anionic region(s) of AChE. Although various approaches to the development of AChE reactivators have been taken, pyridinium oximes, such as 2-[(hydroxyimino)methyl]-1-methylpyridinium halide (2-PAM), have been the compounds most studied.[8-11]

The proven therapeutic utility of pyridinium oximes notwithstanding, it is apparent that they have in common an important disadvantage in terms of limited tissue distribution. Hydrophobic cell membranes represent biological barriers to the transport of large ions from aqueous media into various tissues. This is especially true for passage of molecules from the serum into the central nervous system (CNS).[12,13] Nonionic OP esters are relatively lipophilic and differ significantly from the hydrophilic pyridinium oximes in terms of tissue penetration. The OP esters pass rapidly into various tissues, including the CNS,[14-16] but the pyridinium oximes penetrate at a much slower rate. As a result, the pyridinium oximes exhibit a high degree of selectivity for activity at peripheral vs. central sites.[17-27] Moreover, the disproportionately high concentration of pyridinium oximes in the serum results in rapid renal excretion and short biological half-lives.[11,28,29]

Table I. Selected Data for Hydroximoyl Chlorides

	RC(=NOH)Cl			
R	yield, %	recrystn solvent	mp, °C	NMR (-NOH), δ
$C_6H_5C(=O)$	52	C_6H_6-CCl_4	123-126	13.68
$4\text{-}NO_2C_6H_4C(=O)$	18	C_6H_6	136-140	13.86
$4\text{-}CH_3OC_6H_4C(=O)$	51	C_6H_6	131-133	13.40
$CH_3C(=O)$	50	CCl_4	88-93	13.44
$4\text{-}BrC_6H_4$	65	CCl_4		

The possibility that therapy of OP ester intoxication could be significantly improved through the use of nonquaternary reactivators has been appreciated by several investigators, and there have been various attempts to design reactivators that better penetrate biological membranes. Examples include butanedione oxime (DAM),[30] 4-(dimethylamino)butanedione 2-oxime (isonitrosine),[18,31,32] the 3-(dimethylamino)propyl ester of oximinoacetic acid (OA3) and analogous amides,[29,33,34] the 2-(diethylamino)-ethyl thioester of 4-bromobenzothiohydroximic acid (LA54),[35-38] and 1,2,3-thiadiazole 5-aldoxime (TDA-5).[39] These nonquaternary reactivators have demonstrated some limited advantages over the pyridinium oximes, but to date none have been proven to be clearly superior in terms of overall therapeutic effectiveness.

Although different factors limit the utility of the individual nonquaternary compounds described above, it is

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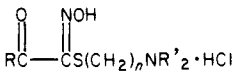
possible to rationalize their relative lack of antidotal efficacy primarily in terms of their poor activity as reactivators of inhibited AChE. In considering the molecular parameters that might affect the ability of the nonquaternary oximes to function as AChE reactivators, it is surprising to discover that structure–activity relationships for these materials have not been reported. Schoene[40] has used the determination of reactivation kinetics (the constants K_r and k_r) to good advantage in establishing structure–activity relationships for pyridinium oximes. It seems that application of this technique to nonquaternary reactivators would be straightforward and highly useful with respect to design of novel compounds with enhanced reactivity.

In view of the foregoing, we have undertaken an investigation of a series of α-ketothiohydroximic acid thioesters, given by the formula 1. Our choice of this general

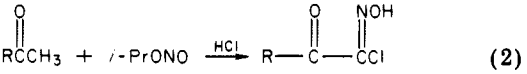


1

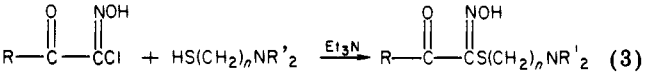
framework was based on considerations of synthetic flexibility, structural similarity to AChE substrates (e.g., ACh and acetylthiocholine), a functionality (protonated tertiary amine) providing for coulombic attraction to the anionic site of AChE, and the recognized requirement for compounds with oxime pK_a near 7.9. In the following we report the synthesis of the α-thiohydroximates and their characterization with respect to pK_a, nucleophilicity, and relative ability to reactivate AChE inhibited by diisopropyl phosphorofluoridate (DFP). We also investigated the kinetics of reactivation of the inhibited enzyme with the objective of identifying structure–activity relationships for reactivation. For comparison, we examined LA54 [4-Br-$C_6H_4C(=NOH)SCH_2CH_2N(C_2H_5)_2$·HCl], **2**, a thiohydroximate that has been touted[36] as a particularly effective reactivator, and 2-PAM (2-[(hydroxyimino)methyl]-1-methylpyridinium iodide), **3**.

Results and Discussion

Synthesis, Structure, and Acidity. We prepared the hydroximoyl chlorides and α-ketothiohydroximates described in Table I and II by reactions 2 and 3. Unop-



(2)



(3)

timized yields of reaction 3 ranged from 18 to 70%, based on the hydroximoyl chloride used.

Oxime acid dissociation constants (pK_a) were measured by spectrophotometric determination of oximate concentration in buffers of various pH.[41] From the data in Table II for **1a–i**, a plot (not shown) of pK_a vs. oxime proton NMR chemical shift is linear and conforms to eq 4. For

$$pK_a = 25.3\ (\pm 2.1) - 1.36\ (\pm 0.16)\ \delta \qquad (4)$$

the aroylthiohydroximates (**1a–c**), a plot (not shown) of pK_a vs. Hammet substituent constant (σ_p)[42] is also linear and conforms to eq 5. These correlations provide an

$$pK_a = 7.63\ (\pm 0.02) - 0.63\ (\pm 0.05)\ \sigma_p \qquad (5)$$

Table II. Selected Data for α-Ketothiohydroximates

RC(=NOH)S(CH$_2$)$_n$NR$_2$'·R''X

no.	R	n	R'	R''X	yield, %	TLC,[a] R_f	mp, °C	NMR (-NOH), δ	UV[b] λ_{max}, nm	UV[b] ϵ, M^{-1} cm^{-1} × 10^{-4}	pK_a	anal.
1a	$C_6H_5C(=O)$	2	C_2H_5	HCl	32	0.56	136–141	12.94	262	1.06	7.60	C, H, N, S, Cl
1b	4-$NO_2C_6H_4C(=O)$	2	C_2H_5	HCl	18	0.57	170–172.5	13.39	269	1.74	7.14	C, H, N, S, Cl
1c	4-$CH_3OC_6H_4C(=O)$	2	C_2H_5	HCl	65	0.65	139–140	12.69	230	1.12	7.82	C, H, N, S, Cl
									303	1.50		
1d	$CH_3C(=O)$	2	C_2H_5	HCl	24	0.45	156–161	13.12	273	0.87	7.40	C, H, N, S, Cl
1e	4-$CH_3OC_6H_4C(=O)$	2	CH_3	HCl	55	0.55	154–155.5	12.74	230	1.12	8.00	C, H, N, S, Cl
									308	1.68		
1f	4-$CH_3OC_6H_4C(=O)$	2	$(CH_3)_2CH$	HCl	70	0.64	179–184	12.70	230	1.13	8.01	C, H, N, S, Cl
									309	1.74		
1g	4-$CH_3OC_6H_4C(=O)$	3	CH_3	HCl	d	0.30	c	12.50	d	d	d	d
1h	4-$CH_3OC_6H_4C(=O)$	3	CH_3	$[HOC(=O)]_2$	29	0.42	151–153.5	e	d	d	8.42	C, H, N, S, Cl
1i	4-$CH_3OC_6H_4C(=O)$	2	C_2H_5	CH_3I	48	0.39	c	f	d	d	7.82	d
2	4-BrC_6H_4	2	C_2H_5	HCl	41	0.63	146–149	12.16	233	1.41	9.28	C, H, N, S, Cl, Br

[a] Silica gel, $CHCl_3$–MeOH (6:1). [b] In pH 7.6, 0.13 M phosphate buffer. [c] Hygroscopic syrup. [d] Not determined. [e] Broad singlet, apparently including oxalate protons. [f] In CD_3OD solution, OH exchanged with OD.

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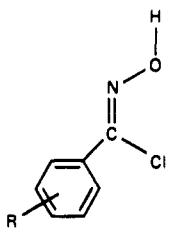
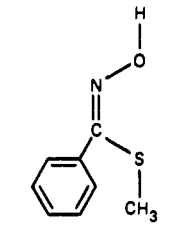
indication of the validity of the pK_a values, an important consideration because we have related α-ketothiohydroximate reactivity to the concentration of the dissociated form at a given pH and, hence, to the acidity of the oxime functionality. We determined $pK_a = 7.99$ for **3**, which is in good agreement with the value of 7.92 reported by Schoene and Strake.[40] Our value of $pK_a = 9.28$ for **2** is lower than that ($pK_a = 10.42$) determined potentiometrically by Krivenchuk et al.[36] We prefer our value because the spectrophotometric technique should be less sensitive than the potentiometric method to interference from the tertiary amine acid–base equilibrium.

We have considered the possibility of *E* and *Z* isomerization in the α-ketothiohydroximates insofar as the configuration of the oxime functionality can have a profound influence on the reactivation of inhibited AChE.[10] Exner and co-workers find that benzohydroxamoyl chlorides[43]

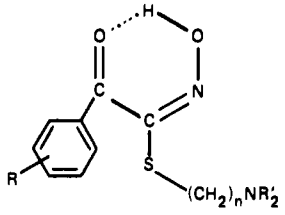


E-benzohydroxamoyl chlorides



E-methyl benzothiohydroximate

and methyl benzothiohydroximate[44] adopt the *E* configuration in solution. These workers also report IR O–H stretching frequencies near 3560 cm^{-1} for these compounds in $CHCl_3$. We find that **2** and the hydroximoyl chlorides listed in Table I exhibit O–H stretching frequencies at 3200 to 3300 cm^{-1} for spectra taken in Nujol mulls. By analogy, an *E* configuration seems likely for these compounds. Contrasting this, the Nujol mull spectra of the α-ketothiohydroximates show no sharp O–H bands in the 3000 to 4000 cm^{-1} region. An IR spectrum of the free base of **1a** in $CHCl_3$ solution reveals a very broad band centered at 2564 cm^{-1} that is typical of hydrogen-bonded O–H.[45] These observations suggest the *Z* configuration for the α-ketothiohydroximates, with six-center intramolecular hydrogen bonding of the oximino proton to the α-carbonyl group.



Z-aroylthiohydroximate

Nucleophilicity. As a measure of the inherent reactivities of the α-ketothiohydroximates and also as a control in reactivation experiments, we determined values for the bimolecular rate constant, k_n, for reaction of the α-ketothiohydroximates with *p*-nitrophenyl acetate (pNPA). We measured initial rates of production of *p*-nitrophenolate (pNP) from the reaction of 1.0×10^{-3} M pNPA with 10.0 to 100×10^{-6} M α-ketothiohydroximate at pH 7.6 and 25 °C. Under these conditions the rate of pNP formation is zero order, consistent with the rate law given in eq 6, where [OX] is the concentration of the anionic form of the α-ketothiohydroximate and k_0 is the pseudo-first-order rate constant for spontaneous hydrolysis of [pNPA]. At low conversions, both [pNPA] and [OX] remain constant, and eq 6 reduces to eq 7, where k_{eQ} is the equivalent first-order rate constant for pNP production and [OX] is calculated from the concentration of added test compound [HOX] and eq 8.

$$+d[pNP]/dt = k_n[pNPA][OX] + k_0[pNPA] \quad (6)$$

$$k_{eQ} = +d[pNP]/dt \cdot [pNPA]^{-1} = k_n[OX] + k_0 \quad (7)$$

$$[OX] = [HOX] \cdot [1 + \text{antilog}\,(pK_a - 7.6)]^{-1} \quad (8)$$

In accordance with eq 7, plots (not shown) of k_{eQ} vs. [OX] for the α-ketothiohydroximates, **2**, and **3** are linear with slope = k_n and intercept = k_0. Table III gives values of k_n and k_0 so determined, along with values of pK_B (= $14 - pK_a$), the conjugate base ionization constant for the test compounds.

As required by eq 7, the k_0 values are essentially independent of the reactivator used. The average for all values of k_0 from Table III is 2.3 (±0.4) $\times 10^{-3}$ min^{-1}, which is in good agreement with the value of $k_0 = 2.1 \times 10^{-3}$ min^{-1} reported by Jencks and Gilchrist.[46] For **3**, the value of $k_n = 9.6$ (±0.8) $\times 10^2$ M^{-1} min^{-1} compares to the value $k_n = 4.0 \times 10^2$ M^{-1} min^{-1} determined by Bergmann and Govrin[47] at pH 8.0 and 25 °C.

Figure 1 is a Bronsted plot (log k_n vs. pK_B) of the data of Table III. The data for the α-ketothiohydroximates conform to eq 9, where the β value (slope) of 0.69 ± 0.08 compares to the value of $\beta = 0.8$ commonly observed for reaction of pNPA with oxygen nucleophiles.[46,48]

$$\log k_n = 6.9\ (\pm 0.5) - 0.69\ (\pm 0.08)\ pK_B \quad (9)$$

Thus, the α-ketothiohydroximates behave as nucleophiles in the anticipated manner. Because the nucleo-

Table III. Rate Constants for Hydrolysis of *p*-Nitrophenyl Acetate (pNPA) in the Presence of α-Ketothiohydroximates[a]

no.[b]	k_n,[c] M^{-1} $min^{-1} \times 10^{-2}$	k_0,[d] $min^{-1} \times 10^3$	pK_B[e]
1a	3.9 ± 0.2	2.0 ± 0.4	6.40
1b	1.4 ± 0.1	2.8 ± 0.4	6.86
1c	6.1 ± 0.02	2.0 ± 0.4	6.18
1d	2.7 ± 0.1	2.8 ± 0.6	6.60
1e	5.1 ± 0.6	1.7 ± 0.4	6.00
1f	5.6 ± 0.4	2.4 ± 0.3	5.99
1h	13.4 ± 0.05	2.2 ± 0.2	5.58
1i	3.9 ± 0.03	1.8 ± 0.3	6.18
2	2.4 ± 0.2	2.6 ± 0.3	4.72
3	9.6 ± 0.8	2.9 ± 1.2	6.01

[a] Determined from equivalent first-order rates, k_{eQ}, for *p*-nitrophenolate production from 1.0×10^{-3} M pNPA in the presence of 10 to 100×10^{-6} M test compound at pH 7.6 and 25 °C in 0.1 M Mops buffer; see eq 7. [b] See Table II. [c] Nucleophilic displacement rate constant from slope of plot of [OX] vs. k_{eQ}; see eq 7 and 8. [d] Spontaneous hydrolysis rate constants, from intercept of plot of [OX] vs. k_{eQ}; see eq 7 and 8. [e] $pK_B = 14 - pK_A$.

(43) J. Smolikov, O. Exner, G. Barbara, D. Macciantelli, and A. Dondoni, *J. Chem. Soc., Perkin Trans. 2*, 1051 (1980).
(44) O. Exner, M. H. Benn, and F. Willis, *Can. J. Chem.*, **46**, 1873 (1968).
(45) L. J. Bellamy, "The Infrared Spectra of Complex Molecules", Wiley, New York, 1958.
(46) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, **90**, 2622 (1968).
(47) F. Bergmann and H. Govrin, *Biochimie*, **55**, 515 (1973).
(48) J. D. Aubort, R. F. Hudson, and R. C. Woodcock, *Tetrahedron Lett.*, **24**, 2229 (1973).

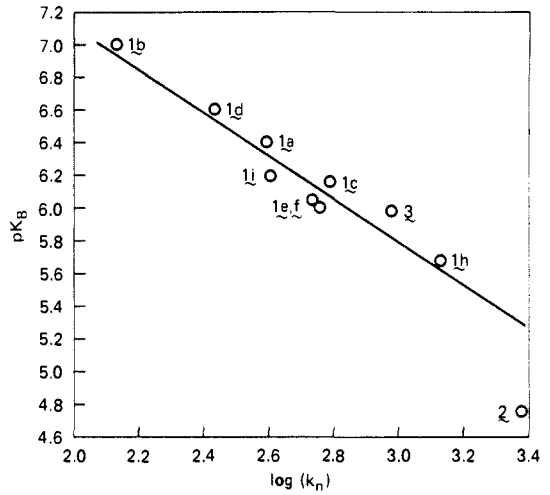


Figure 1. Conjugate base ionization constants (pK_B) vs. logarithm of bimolecular nucleophilic displacement rate constant (k_n) for hydrolysis of *p*-nitrophenyl acetate in the presence of various reactivators at pH 7.6, 25 °C.

philicity is proportional to the acidity of the oxime functionality and because the acidities of the aroylthiohydroximates obey a Hammet linear free-energy relationship, it is possible to "fine tune" both acidity and nucleophilicity by appropriate selection of aromatic substituent groups.

AChE Reactivation Control Experiments. All observed enzyme activities were corrected for spontaneous and oximate-catalyzed hydrolysis of substrate. For pNPA as substrate (see Experimental Section), the initial rates of oximate-catalyzed hydrolysis were actually greater than the enzyme-catalyzed rates for high reactivator concentrations. However, for the high concentration (1.0×10^{-3} M) of pNPA used in the assay, the test compounds ($10.0–100 \times 10^{-6}$ M in the assay) were rapidly consumed. Thus, after an initially high rate of pNP production, the rate leveled off within 2 to 4 min to a value dependent only on the enzyme-catalyzed reaction plus a contribution due to spontaneous hydrolysis of pNPA. The latter could be accounted for by the value of k_0 determined above. In the case of reactions assayed by the Ellman technique, corrections for nonenzymatic substrate hydrolysis were smaller and could be made by running blanks with reactivator in an appropriate concentration but with no enzyme added.

Spontaneous reactivation of (diisopropylphosphoryl)acetylcholinesterase was negligible over 24 h. However, the inhibited enzyme (EOP) rapidly converted ("aged") into a nonreactivatable species (EOP′) as shown in reaction 10, where EOP is (diisopropylphosphoryl)acetylcholinesterase and EOP′ is a dealkylated, inhibited enzyme.[5] We determined k_a under our experimental conditions by incubating the inhibited enzyme for timed intervals before reactivating for 60 min at 25 °C with 1.0×10^{-3} M **3**. Semilog plots of A_t/A_0 vs. time (where A_t is AChE activity after incubation for t min before reactivation with **3** and A_0 is the activity of inhibited enzyme reactivated immediately) were linear for at least three half-lives. For three determinations the average value of k_a was 1.61 (±0.08) $\times 10^{-3}$ min^{-1}, corresponding to a half-life for aging of 7.1 h.

$$\text{EOP} \xrightarrow{k_a} \text{EOP}' \qquad (10)$$

We incubated AChE with the various test compounds to check for reversible or irreversible inhibition of the enzyme under the conditions of the reactivation experiments. Thus, we incubated AChE with each test compound (1.00×10^{-3} M) and withdrew aliquots at 0, 3, and 24 h for determination of enzyme activity. A sample of AChE without added test compound served as control. Except for **1f**, none of the compounds gave any evidence of inhibition of the enzyme using this procedure. For **1f** the average value of enzyme activity at 0, 3, and 24 h was 92 ± 12% of control. For all the other compounds at all three time points, the average value of the enzyme activity was 102 ± 3% of control. Thus, the inhibition of AChE due to the reactivators themselves can be neglected in our assays of enzyme activity.

Table IV. Percent reactivation (% r_t) of (Diisopropylphosphoryl)acetylcholinesterase after Incubation with 1.00×10^{-3} M Reactivator at 25 °C, pH 7.6

no.[a]	% r_t[b] after incubation for t h		
	$t = 2$	$t = 4$	$t = 24$
1a[c]	63	83	80
1b	18	20	25
1c	31	58	55
1d	13	28	23
1e	56	65	71
1f	4.3	2.8	21
1h	0.0	0.0	16
1i	5.6	9.5	18
2[c]	8	8	9
3	109	105	85

[a] See Table II. [b] % r_t calculated from eq 11. [c] Reactivator concentration = 2.00×10^{-3} M.

Finally, we checked the α-ketothiohydroximates for hydrolytic stability at pH 7.6 and 25 °C. As evidenced by UV–visible spectra and by rate constants, k_n, for reaction with pNPA, hydrolysis of the compounds over 24 h was negligible.

Percent AChE Reactivation. To determine the relative activities of the α-ketothiohydroximates as reactivators of (diisopropylphosphoryl)acetylcholinesterase we added the DFP-inhibited enzyme to 1.00×10^{-3} M solutions of the test compounds and determined enzyme activity after 2-, 4-, and 24-h incubation periods. Percent reactivation was calculated according to eq 11, where % r_t is the percent

$$\%\ r_t = 100(E_t/E_0) \qquad (11)$$

reactivation after t minutes of incubation, E_t is the enzyme activity at time t, and E_0 is the control enzyme activity. Table IV summarizes the data for the α-ketothiohydroximates, plus **2** and **3**.

From the table, it is seen that the α-ketothiohydroximates do function as reactivators and that their activity is highly dependent on structure. In the series of dimethyl-, diethyl-, and diisopropylaminoethyl thioesters of *p*-methoxybenzoylthiohydroximic acid (**1e,c,f**), activity decreases with increasing size of the alkyl substituent, indicating a steric effect on the reactivation. It is also significant that the quaternary α-ketothiohydroximate **1i** is actually less reactive than the tertiary analogue **1c**. We anticipated that the tertiary alkylamine functionality would be completely protonated at pH 7.6 and that the protonated species would provide the coulombic interaction with the anionic subsite necessary for enhanced affinity of the reactivator for the inhibited enzyme. If this were not the case, the quaternary ammonium analogue would predictably exhibit considerably higher activity than the nonquaternary reactivator. The fact that **1c** is actually more reactive than the methylated **1i** can be rationalized on the basis of strong coulombic interactions for both compounds plus a steric effect that lowers the affinity of

Table V. Formulas for Calculating Kinetic Constants for Reactivation of Inhibited AChE

plot	abscissa	ordinate	slope$_{(n)}$	intercept$_{(n)}$	equation	eq no.
1	t	$\ln(\%\, r_\infty - \%\, r_t)$	$S_1 = -k_{obsd}$	$I_1 = \ln(\%\, r_\infty)$	$[EOP']_\infty/[EOH]_\infty = (100 - \exp[I_1])/\exp[I_1]$	13, 17
2	$[OX]^{-1}$	$[EOP']_\infty/[EOH]_\infty$	$S_2 = k_a K_r/k_r$	$I_2 = k_a'/k_r$	$k_a' = I_2 k_r$	16
3	$[OX]^{-1}$	$(k_{obsd} - k_a)^{-1}$	$S_3 = K_r/k_{max}$	$I_3 = 1/k_{max}$	$K_r = S_3/I_3$	15, 18
					$k_r = [(I_3)^{-1} + k_a]\cdot[1 + I_2]^{-1}$	18, 16

the *N*-methyl derivative for the inhibited enzyme.

Comparison of the 2-(diethylamino)ethyl and 3-(dimethylamino)propyl thioesters of *p*-methoxybenzoylthiohydroximic acid (**1c**, and **1h**, respectively) shows lower activity for the 3-(dimethylamino)propyl analogue. While this could be interpreted as an effect of reactivator structure on activity, it must be noted that the pK_a of **1h** (Table II) is 0.6 log unit higher than that of **1c**. Therefore, at pH 7.6, **1c** is 40% ionized (eq 8), where **1h** is only 14% ionized to the nucleophilic oximate anion. This example demonstrates that the simple screening for activity by determining percent reactivation under standard conditions suffices to rate similar compounds in terms of relative potency but can fail to permit an elucidation of the molecular parameters that govern reactivity.

The shortcomings of the simple screening method are also apparent when **3** is compared with the α-ketothiohydroximates. Because reactivation by **3** is complete within 2 h, it is impossible to use the data of Table IV to quantitatively compare it with the α-ketothiohydroximates. To do so requires a careful study of reactivation as a function of time and reactivator concentration. Results of such a study are presented in the section that follows.

Finally, the data for 2-(diethylamino)ethyl *p*-bromobenzothiohydroximate, **2**, deserve comment. We find **2** to be considerably inferior to **3** as a reactivator of (diisopropylphosphoryl)acetylcholinesterase and attribute the lack of activity of **2** primarily to its high pK_a (= 9.28) and the correspondingly low fraction (1.4% at pH 7.6) of the ionized species available to reactivate the enzyme. These results are in marked contrast to those of Krivenchuk et al.[36] who obtained 35 and 41% reactivation of (ethylmethylphosphonyl)butyrylcholinesterase after incubation with **3** and **2**, respectively.

Even considering the differences in inhibited enzymes used, it is difficult to rationalize the large differences in the relative activities of **2** and **3** reported by us and by Krivenchuk and co-workers. In the absence of additional details regarding the experimental method employed by Krivenchuk et al., we cannot speculate about other reasons for this apparent discrepancy. In any case, **2** proves to be a very poor reactivator in our experiments, and we believe that published reports of its antidotal effectiveness[35-38] should be interpreted with some caution.

AChE Reactivation Kinetics. The work of several authors[7,40] makes it clear that the kinetics of reactivation of phosphylated AChE cannot be adequately described by a simple bimolecular substitution reaction mechanism.

De Jong and Wolring[49,50] have devised a kinetic scheme that accounts for the reversible formation of the complex between reactivator and inhibited enzyme, as well as the parallel processes of reactivation and dealkylation of phosphylated AChE. The scheme consists of reactions 1, 10, and 12, where the reactivator is represented by the

$$EOP + OX \overset{K_r}{\rightleftarrows} [EOP\cdot OX] \overset{k_r}{\rightarrow} EOH + POX \quad (1)$$

$$EOP \overset{k_a}{\rightarrow} EOP' \quad (10)$$

$$[EOP\cdot OX] \overset{k_a'}{\rightarrow} EOP' + OX \quad (12)$$

oximate anion (OX), and K_r, k_r, and k_a are as previously defined. Provision is made in reaction 12 for dealkylation of the equilibrium complex, [EOP·OX], to the nonreactivatable form of the inhibited enzyme, EOP′, with a rate constant, k_a', that may or may not differ from k_a.

Following the derivation of De Jong and Wolring, it can be shown that

$$\ln(\%\, r_\infty - \%\, r_t) = \ln(\%\, r_\infty) - k_{obsd}\cdot t \quad (13)$$

$$k_{obsd} - k_a = \frac{k_{max}[OX]}{K_r + [OX]} \quad (14)$$

$$k_{max} = k_a' - k_a + k_r \quad (15)$$

$$\frac{[EOP']_\infty}{[EOH]_\infty} = \frac{k_a K_r [OX]^{-1}}{k_r} + \frac{k_a'}{k_r} \quad (16)$$

where k_{obsd} is the pseudo-first-order (oximate in excess) rate constant for reactivation, $\%\, r_\infty$ is the maximum attainable percent reactivation, k_{max} is defined as in eq 15, $[EOP']_\infty$ is the amount of nonreactivatable enzyme at infinite time, $[EOH]_\infty$ is the concentration of active enzyme at infinite time, and k_a is determined in an independent experiment according to eq 10. The ratio $[EOP']_\infty/[EOH]_\infty$ is calculated from observed values of $\%\, r_\infty$ according to eq 17. Rearranging eq 14 gives eq 18. Thus, a plot (eq

$$[EOP']_\infty/[EOH]_\infty = (100 - \%\, r_\infty)/\%\, r_\infty \quad (17)$$

$$(k_{obsd} - k_a)^{-1} = \frac{K_r[OX]^{-1}}{k_{max}} + \frac{1}{k_{max}} \quad (18)$$

13) of $\ln(\%\, r_\infty - \%\, r_t)$ vs. time gives slope = $-k_{obsd}$ and intercept = $\ln(\%\, r_\infty)$, a plot (eq 16) of $[EOP']_\infty/[EOH]_\infty$ vs. $[OX]^{-1}$ gives slope = $k_a K_r/k_r$ and intercept = k_a'/k_r, and a plot (eq 18) of $(k_{obsd} - k_a)^{-1}$ vs. $[OX]^{-1}$ gives slope = K_r/k_{max} and intercept = $1/k_{max}$. Because k_a is independently known (vide supra), the determination of $\%\, r_\infty$ and $\%\, r_t$ as a function of time and reactivator concentration permits calculation of the important kinetic constants K_r and k_r. Table V summarizes the necessary derivations. At low reactivator concentrations, eq 19 holds, where k_b is a

$$k_b = k_r/K_r \quad (19)$$

bimolecular rate constant for reactivation in the limit of low reactivator concentration. Finally, we define k_{eff} as a quantitative measure of the effectiveness of reactivation. This term is governed by the intrinsic activity of an oximate anion (k_b) and the fraction of reactivator that exists

(49) L. P. A. De Jong and G. Z. Wolring, *Biochem. Pharmacol.*, **27**, 2911 (1978).
(50) L. P. A. De Jong and G. Z. Wolring, *Biochem. Pharmacol.*, **27**, 2229 (1978).
(51) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism", 2nd ed, Wiley, New York, 1961, p 49.

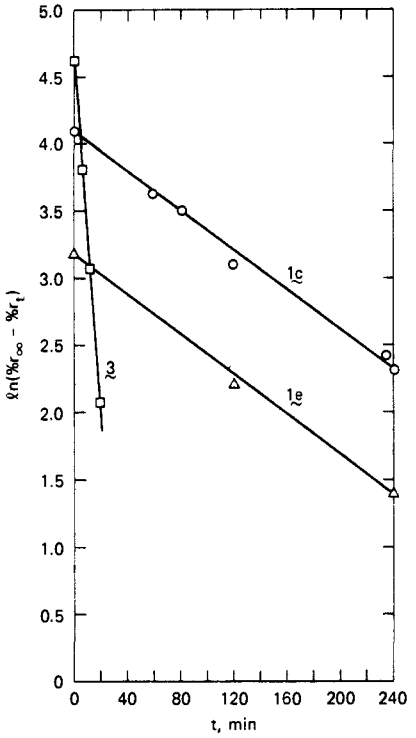


Figure 2. Natural logarithm of percent maximal reactivation minus reactivation at time t, ln ($\% r_\infty - \% r_t$) vs. time at 25 °C, pH 7.6, for reactivation of (diisopropylphosphoryl)acetylcholinesterase with 1.0×10^{-3} M **3** (□–□), 1.0×10^{-3} M **1c** (○–○), and 0.20×10^{-3} M **1e** (△–△).

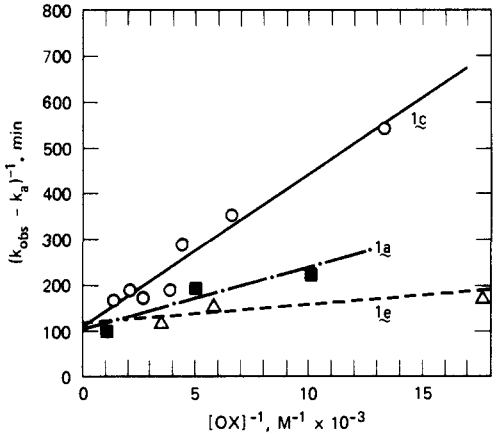


Figure 3. Double-reciprocal plot of observed first-order reactivation rate constant minus dealkylation rate constant $[(k_{obsd} - k_a)^{-1}]$ vs. oximate concentration ($[OX]^{-1}$) for reactivation of (diisopropylphosphoryl)acetylcholinesterase by **1a** (■–■), **1c** (○–○), and **1e** (△---△).

in the dissociated form at pH 7.6. Thus, from eq 8 and 19, k_{eff} is given by eq 20. To probe these relationships,

$$k_{eff} = k_b[1 + \text{antilog}\ (pK_a - 7.6)]^{-1} \qquad (20)$$

we determined $\% r_\infty$ and $\% r_t$ as a function of time for various concentrations of **3**, **1a**, **1c**, and **1e**. Figure 2 is a sample plot of eq 13 for 1.00×10^{-3} M **3** and **1c** and 0.200×10^{-3} M **1e**. The linearity of the plots indicates that complicating factors, such as reinhibition of the enzyme by phosphorylated reactivator, are unimportant under the experimental conditions.[50] Table VI summarizes the values of k_{obsd} and ln ($\% r_\infty$) obtained for a range of concentrations of all four reactivators. Figure 3 is a double-reciprocal plot of $(k_{obsd} - k_a)^{-1}$ vs. $[OX]^{-1}$ for **1a**, **1c**, and **1e**. The plot for **3** is not shown because of the difference in scale for $[OX]^{-1}$, but the data for **3** conform reasonably well to eq

Table VI. Pseudo-first-order Observed Rate Constants, k_{obsd}, for Reactivation of (Diisopropylphosphoryl)acetylcholinesterase as a Function of Reactivator Concentration at 25 °C, pH 7.6

no.[a]	[HOX], M × 10⁴	[OX],[b] M × 10⁴	k_{obsd},[c] min⁻¹ × 10³	ln ($\% r_\infty$)[d]
1a	2.00	1.00	5.90 ± 0.8	3.19 ± 0.04
	4.00	2.00	6.82 ± 1.7	3.39 ± 0.10
	20.0	10.0	11.0[e]	4.42[e]
1c	2.00	0.752	3.02 ± 0.16	3.30 ± 0.01
	4.00	1.52	4.40 ± 0.62	3.49 ± 0.04
	6.00	2.26	5.13 ± 1.0	3.79 ± 0.16
	7.00	2.63	7.12 ± 0.27	4.10 ± 0.03
	10.0	3.76	7.24 ± 0.26	4.05 ± 0.04
	12.0	4.51	6.90 ± 0.17	4.22 ± 0.02
	20.0	7.52	7.65 ± 0.47	4.30 ± 0.06
1e	2.00	0.569	7.46 ± 0.41	3.15 ± 0.063
	6.00	1.71	8.17 ± 1.1	3.83 ± 0.16
	10.0	2.85	10.3 ± 1.5	4.15 ± 0.23
3	0.0300	0.00870	6.57 ± 0.38	3.93 ± 0.049
	0.0800	0.0232	9.31 ± 0.11	4.20 ± 0.014
	0.100	0.0289	19.6 ± 0.80	[f]
	0.160	0.0463	20.2 ± 1.1	4.42 ± 0.083
	0.200	0.0571	29.8 ± 1.4	[f]
	10.0	2.89	125 ± 10	4.49 ± 0.12

[a] See Table II. [b] Calculated from [HOX] and eq 8. [c] Calculated from slope of least-squares plot of data according to eq 13. [d] Calculated from intercept of least-squares plot of ln ($\% r_\infty - \% r_t$) according to eq 13, using experimentally determined values of $\% r_\infty$, except as noted. [e] Calculated from three points, including $\% r_\infty$, no valid statistical data. [f] No infinity point measured. Slopes calculated from initial points by the method of Guggenheim.[51]

18. A similar plot (not shown) of $[OX]^{-1}$ vs. $[EOP']_\infty/[EOH]_\infty$ conforms to eq 16 for the reactivators investigated. Data from Table VI and from least-squares treatment of the data according to eq 16 and 18 are summarized in Table VII along with kinetic constants calculated as in Table V eq 19 and 20.

At this point, some discussion of the precision of the measurements seems appropriate. Accurate determination of $\% r_\infty$ is essential for reliable calculation of values for k_{obsd} and especially for the ratio $[EOP']_\infty/[EOH]_\infty$. We did not make replicate determinations of $\% r_\infty$ values in our experiments, and this is a major source of uncertainty in calculating kinetic constants. The uncertainty is largest for least-squares determinations of intercepts calculated from eq 16. These intercepts are used to calculate k_a', and we view the values for k_a' in Table VII as tentative. The intercepts calculated from eq 18 are also subject to a degree of uncertainty, and this must be taken into consideration when interpreting values for k_r.

These caveats notwithstanding, the kinetic constants given in Table VII do reflect some significant structure–activity relationships for the reactivators in question. Concerning the rate of dealkylation of the inhibited enzyme–oximate complex, the values of k_a' range from 0.19 to 2.4×10^{-3} min⁻¹ for **1e**, **1c**, and **1a**. These compare to $k_a = 1.61 \times 10^{-3}$ min⁻¹ for the inhibited enzyme in the absence of reactivator and indicate no significant effect of the α-ketothiohydroximates on the rate of dealkylation. For **3**, however, $k_a' = 6.7 \times 10^{-3}$ min⁻¹, suggesting that **3** accelerates the aging of DFP-inhibited enzyme. Similar effects of pyridinium oximes on dealkylation of ethyl dimethylphosphoramidocyanidate inhibited AChE were reported by De Jong and Wolring.[50]

Insofar as reactivation is concerned, **3** is characterized both by a strong affinity for the inhibited enzyme and high nucleophilicity toward phosphorus. Our values of K_r =

Table VII. Kinetic Constants for Reactivation of (Diisopropylphosphoryl)acetylcholinesterase at 25 °C, pH 7.6

no.[a]	[HOX], M × 10^4	$[OX]^{-1}$,[b] M^{-1} × 10^{-3}	$(k_{obsd} - k_a)^{-1}$,[c] min	$[EOP']_\infty/[EOH]_\infty$[d]	k_a'/k_r[e]	k_aK_r/k_r,[f] M × 10^4	K_r/k_{max},[g] M·min × 10^2	$1/k_{max}$,[h] min	k_a',[i] min^{-1} × 10^3	k_r,[j] min^{-1} × 10^3	K_r,[k] M × 10^4	k_b,[l] M^{-1} min^{-1}	k_{eff},[m] M^{-1} min^{-1}	k_{eff} (rel)
1a	2.00	10.0	231	3.12	0.21 ±	3.2 ± 1.1	1.4 ±	103 ± 24	2.0	9.3	1.3	70	34	3.1
	4.00	5.00	190	2.37	0.7		0.36							
	20.0	1.00	106	0.203										
1c	2.00	13.3	538	2.69	0.17 ±	2.1 ±	3.30 ±	106 ± 19	2.4	8.6	2.9	30	11	1
	4.00	6.65	353	2.05	0.18	0.30	0.31							
	6.00	4.42	281	1.26										
	7.00	3.80	180	0.657										
	10.0	2.66	176	0.742										
	12.0	2.20	187	0.470										
	20.0	1.33	164	0.357										
1e	2.00	17.6	170	3.29	−0.018 ±	1.9 ±	0.32 ±	117 ± 21	0.19	10	0.27	380	108	9.8
	6.00	5.86	151	1.17	0.11	0.10	0.19							
	10.0	3.51	115	0.576										
3	0.0300	1156	200	0.964	0.11 ±	0.0075 ±	0.017 ±	15 ± 14	6.7	61	0.11	5500	1600	145
	0.0800	431	129	0.506	0.050	0.0008	0.003							
	0.100	346	55.5	*n*										
	0.160	216	53.6	0.203										
	0.200	175	35.4	*n*										
	10.0	3.50	8.13	0.122										

[a] See Table II. [b] Calculated from [HOX] and eq 8. [c] From k_{obsd} values of Table VI; k_a = 1.61 × 10^{-3} min^{-1}; see text. [d] $[EOP']_\infty = (100 - \% r_\infty)/\% r_\infty$. [e] Intercept of least-squares plot of data according to eq 16. [f] Slope of least-squares plot of data according to eq 16. [g] Slope of least-squares plot of data according to eq 18. [h] Intercept of least-squares plot of data according to eq 18. [i] Calculated as shown in Table V. [j] Calculated as shown in Table V. [k] Calculated as shown in Table V. [l] Calculated from eq 19. [m] Calculated from eq 20. [n] See Table VI.

0.11 × 10^{-4} M and k_r = 61 × 10^{-3} min^{-1} for **3** combine with a pK_a value of 7.99 to give an effective bimolecular rate constant, k_{eff}, for reactivation of 1600 M^{-1} min^{-1}. By this measure **3** is 140 times more effective than **1c** and approximately 15 times more active than **1e**, the most effective of the α-ketothiohydroximates. It is interesting to note that for the α-ketothiohydroximates the displacement rate constant (k_r) shows little dependence on reactivator structure, whereas values for K_r vary by a factor of 25 for the compounds examined. This observation can be used to rationalize the relative activities of the compounds. It also demonstrates how the precise determination of reactivation kinetics for a series of related thiohydroximates could be used to "map" the inhibited enzyme, thereby providing insight into the molecular requirements for reactivation. Unfortunately, DFP proved to be a poor choice of inhibitor for these determinations because of uncertainties imposed by the rapid rate of dealkylation. A reasonable alternative to DFP would be ethyl *p*-nitrophenyl methylphosphonate,[52] an inhibitor that yields a phosphonylated enzyme that undergoes neither dealkylation nor spontaneous reactivation to a significant degree at 25 °C.[53] Investigations along these lines are in progress and will be reported in a separate communication.

Conclusion

The α-ketothiohydroximates are moderately active reactivators of (diisopropylphosphoryl)acetylcholinesterase. In this respect they should be compared to reactivators, such as TDA-5[39] and 2-[(hydroxyimino)methyl]-3′-carboxamido-4,4′-(oxydimethyl)dipyridinium dichloride (HS6),[40,54] that are inferior to **3** as reactivators but that, nevertheless, are effective therapeutics for intoxication by isopropyl or 2,2-dimethyl-2-butyl methylphosphonofluoridate.

We regard the α-ketothiohydroximates as a significant advancement in the study of organophosphorus agent therapy not so much because they are clearly superior reactivators but because they represent a novel class of materials and offer the promise of an extremely useful research tool for probing the mechanisms of cholinesterase reactivation on the molecular level. We have shown that the synthesis of α-ketothiohydroximates is at once facile and highly flexible. Readily available starting materials can be used to prepare a variety of hydroximoyl chlorides, each of which can, in turn, be esterified with a series of dialkylaminoalkanethiols. This allows systematic variation not only of the oxime acid dissociation constant and nucleophilicity but also of structural features in the portion of the molecule that must interact with the anionic region of the AChE active site. We have demonstrated that there are pronounced structural effects on the activity of the α-ketothiohydroximates and that these can be quantitated through determination of reactivation kinetics.

The application of structure–activity relationships to reactivation of inhibited AChE has led to important advances in OP agent therapy with respect to development of pyridinium oximes. It is our hope that the α-ketothiohydroximates can be applied similarly to the design of improved therapeutics based on nonquaternary reactivators.

As a final point, we find that **3** apparently accelerates the rate at which (diisopropylphosphoryl)acetylcholin-

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esterase converts to a nonreactivatable form. Similar results[50] with (ethyldimethylphosphoramido)acetylcholinesterase suggest that this may be a general phenomenom for phosphorylated AChE. We conclude that this possibility should be probed in greater detail with the objective of developing a more complete understanding of molecular mechanisms of reactivation of inhibited AChE.

Experimental Section

Melting points (uncorrected) were obtained with a Fischer-Johns apparatus, NMR spectra (in Me_2SO-d_6 unless otherwise specified, Me_4Si internal reference, δ 0.0) were determined with a Varian Model EM 390 or Model XL-100-15 spectrometer; signals are designated as s (singlet), d (doublet), t (triplet), or m (multiplet). UV–visible spectra were measured with a Perkin-Elmer Model 575 spectrophotometer. Enzyme kinetics were followed in 1-cm path-length cuvettes using a Gilford Model 2000 spectrophotometer equipped with a four-position sample changer. The spectrophotometer output was coupled to a DEC MINC-11 computer programmed to give a continuously updated display of absorbance values and least-squares slope, intercept, and correlation coefficient for each cuvette. The system also provides for graphics display and permanent storage of the data in a disc file. A description of the program is available on request. We conducted all kinetic experiments at 25 °C and pH 7.6 in 0.1 M *N*-morpholinopropanesulfonic acid (Mops) buffer plus $MgCl_2$ (0.01 M), NaN_3 (0.002%), and bovine serum albumin (0.1%). Reported error limits for rate constants are standard deviations determined by least-squares linear regression analysis.

Lyophilized electric eel AChE (Worthington) was used, with a nominal activity of 1.4×10^3 ACh units per milligram. *p*-Nitrophenyl acetate (pNPA), 3-(*N*-morpholino)propanesulfonic acid (Mops), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and acetylthiocholine were used as supplied by the manufacturer (Sigma Chemical Co.). We prepared isopropyl nitrite[55] in small amounts and stored it at 5 °C until immediately before use. Where results of elemental analyses are given, only the symbols of the elements are indicated when experimental values agree within ±0.4% of theoretical.

Hydroximoyl Chlorides. The α-aroylhydroximoyl chlorides $4\text{-}RC_6H_4C(=O)C(=NOH)Cl$, where R = H, CH_3O, or NO_2, were prepared by treating the corresponding acetophenones with *i*-C_3H_7ONO and HCl as described by Brachwitz.[56] $CH_3C(=O)C(=NOH)Cl$ was similarly prepared from chloroacetone.[57] *p*-Bromobenzohydroximoyl chloride was prepared by chlorination of $p\text{-}BrC_6H_4CHNOH$.[58,59] Table I gives selected data for the hydroximoyl chlorides.

***S*-(*N*,*N*-Dialkylamino)alkyl α-Ketothiohydroximates (1a–i).** All compounds were prepared from the corresponding hydroximoyl chlorides by the same general procedure. **1i** and **1h** were isolated, respectively, as the iodide and oxalic acid salts. The remaining compounds were isolated as the hydrochloride salts. Table II presents selected data for the α-ketothiohydroximates. The preparation of *S*-[2-(diethylamino)ethyl] 4-methoxybenzoylthiohydroximate hydrochloride (**1c**) is described as a typical example.

Diethylaminoethanethiol hydrochloride (340 mg, 2 mmol) was added to a solution of 404 mg (4 mmol) of triethylamine in 4 mL of $CHCl_3$. Then 427 mg (2 mmol) of α-chloro-α-oximino-*p*-methoxyacetophenone was added with stirring. The light orange-brown solution was stirred at room temperature for 4 h. Washing the solution with water removed little color; the solution was dried and concentrated to leave 780 mg of yellow syrup. An ether solution of the syrup was washed several times with water (which removed most of the color), dried, and concentrated. The syrupy residue (650 mg, showing several impurities by TLC) was purified on a silica gel column (14 × 2.5 cm) by "flash" chromatography[60] using $CHCl_3$–MeOH (8:1) as eluting solvent. From the fractions that showed no impurities, we obtained 479 mg of a light yellow syrup: TLC, R_f 0.6 ($CHCl_3$–MeOH, 6:1).

The syrup was dissolved in 10 mL of MeOH–ether (1:1), then filtered, and treated with 0.5 mL of ether saturated with dry HCl. Ether was added to turbidity. Slight warming caused the separation of white crystals; after chilling, the product was collected, washed with ether, and dried at 25 °C (0.1 mm) to yield 374 mg of **1c**: mp 139–140 °C; TLC ($CHCl_3$–MeOH, 6:1) R_f 0.65; NMR δ 12.69 (s, 1 H, =NOH), 10.55 (br, 1 H, NH^+), 7.93 (d, 2 H, Ar), 7.1 (d, 2 H, Ar), 3.87 (s, 3 H, OCH_3), 2.7–3.4 (m, 8 H, CH_2), 1.13 (t, 6 H, CH_3); IR (Nujol) ν 2560 (br m), 1661 (s), 1600 (s), 989 (s) cm^{-1}. Slightly impure fractions of free base, 109 mg, were similarly converted to the hydrochloride and recrystalized from methanol–ether to yield 35 mg: mp 134–135 °C; total yield 60%.

***S*-[2-(Diethylamino)ethyl] 4-Bromobenzothiohydroximate (2).** 4-Bromobenzohydroximoyl chloride (705 mg, 3.0 mmol) was treated with 2-(diethylamino)ethanethiol hydrochloride (510 mg, 3.0 mmol) and sodium methoxide (162 mg, 3.0 mmol) in 15 mL of 2-propanol.[61] The crude product was purified by "flash" chromatography on silica gel ($CHCl_3$–MeOH, 6:1) and final recrystallization from methanol–ether to give 450 mg (41% yield) of white crystalline product: mp 146–149 °C; TLC ($CHCl_3$–MeOH, 6:1) R_f 0.63 (UV and I_2 detected); NMR δ 12.16 (s, 1 H, NOH), 10.42 (br, 1 H, NH^+), 7.59 (m, 4 H, Ar), 3.3–2.8 (m, 8 H, CH_2), 1.13 (t, 6 H, CH_3); IR (nujol) ν 3215 (s), 2632 (s), 1587 (m), 922 (s) cm^{-1}.

Acetylcholinesterase Assay. Two different spectrophotometric methods were used to determine AChE activity. The hydrolysis of pNPA to *p*-nitrophenolate[62,63] (λ_{max} = 402 nm, ϵ = 1.50×10^4 M^{-1} cm^{-1})[64] was used to assay enzyme activity in determinations of percent reactivation at 2, 4, and 24 h. For determination of the rate of dealkylation of diisopropyl phosphoryl-AChE, the inhibition of AChE by reactivators, and kinetics of reactivation of (diisopropylphosphoryl)acetylcholinesterase, we assayed enzyme activity by the method of Ellman,[65] monitoring production of 5-thio-2-nitrobenzoic acid (λ_{max} = 412 nm, ϵ = 1.36×10^4 M^{-1} cm^{-1}). The pNPA assay proved to be the more convenient technique, but it was also less precise due to the relatively rapid spontaneous hydrolysis of pNPA. Details of the methods follow.

***p*-Nitrophenyl Acetate Method.** The lyophilized enzyme is dissolved in 0.1 M, pH 7.6, Mops buffer at a nominal concentration of 5×10^2 ACh units per milliliter and maintained at −10 °C until immediately before use. *p*-NPA is dissolved in acetonitrile/ethanol (1:4) at 0.10 M. The enzyme stock solution is diluted by a factor of 1.0×10^3 with Mops buffer and 10 μL of substrate stock solution is added to 990 μL of diluted enzyme to give a final concentration of 1.0×10^{-3} M substrate and a nominal concentration of 2×10^{-9} M AChE. Under these conditions the rate of enzymatic hydrolysis of pNPA is $\sim 10 \times 10^{-6}$ M min^{-1}, and the rate for spontaneous hydrolysis of pNPA is 2.3×10^{-6} M min^{-1}.

Ellman Method. The 500 ACh units per mL stock solution is diluted by a factor of 2.0×10^3 in Mops buffer, DTNB is made to 1.0×10^{-2} M in pH 7, 0.1 M phosphate buffer, and acetylthiocholine is made to 7.5×10^{-2} M in 1:9 H_2O/EtOH. Combination of 20 μL of enzyme solution with 30 μL of DTNB, 20 μL acetylthiocholine, and 930 μL of 0.1 M phosphate buffer, pH 8, gives an analytical solution with final concentrations of 3.0×10^{-4} M DTNB, 7.5×10^{-4} M acetylthiocholine, and, nominally, 2×10^{-11} M AChE. Under these conditions the enzyme-catalyzed rate of thiocholine production is $8.75 (\pm 0.01) \times 10^{-6}$ M min^{-1}, and the rate of spontaneous hydrolysis is 7.6×10^{-8} M min^{-1}.

Acetylcholinesterase Inhibition and Reactivation. *p*-Nitrophenyl Acetate Assay. A 100-μL aliquot of enzyme stock

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solution is diluted to 0.6 mL with Mops buffer and 100 μL is removed and diluted 20-fold for noninhibited control assays. A 5-μL aliquot of 1.0×10^{-2} M DFP in ethanol is added to the remaining 0.5 mL of enzyme solution and incubated for 3 min, during which period inhibition of the enzyme is complete. After the incubation period, the entire solution is added to a 1 × 20 cm glass column packed to a bed height of 12 cm with Sephadex G-50, 50–100 mesh, the solution is eluted under suction with Mops buffer, and 1.0- to 2.0-mL fractions are collected. The enzyme elutes primarily in the 2- to 4-mL fraction, and DFP primarily in the 7- to 9-mL fraction. The elution of DFP in the AChE fraction was negligible as evidenced by incubating noninhibited AChE with the 2- to 4-mL fraction and assaying for enzymatic activity. For reactivation studies, candidate reactivators are dissolved to 1.0×10^{-2} M in water and diluted to 3.0×10^{-6} to 2.0×10^{-3} M in 100 μL of inhibited enzyme solution plus the quantity of buffer required to give a 200 μL final volume. These solutions are incubated at 25 ± 0.2 °C in a shaker bath, and 25-μL aliquots are removed at timed intervals. The 25-μL aliquots are added to 965 μL of Mops buffer plus 10 μL of 1.0×10^{-2} M pNPA, and the enzyme activity is assayed. Thus, the final concentrations in the assay solutions are as follows: 1×10^{-9} M inhibited AChE, 3.00×10^{-6} to 2.00×10^{-5} M reactivator, and 1.0×10^{-3} M substrate. Observed enzyme activities are corrected (see Results) for spontaneous and reactivator-catalyzed hydrolysis of substrate to give net activities: 100% enzyme activity is defined as the activity restored by incubation of inhibited enzyme with 1.0×10^{-3} M **3** for 60 min at 25 °C.

Ellman Procedure. The general procedure described above is followed except that lower enzyme concentrations are required. Typically, a 10-μL aliquot of stock AChE is diluted to 0.6 mL in Mops buffer and incubated with a 4-μL aliquot of 1.0×10^{-2} M DFP for 10 min. From the 1- to 4-mL column fraction of inhibited enzyme, 400-μL aliquots are withdrawn and diluted to 600 μL with Mops buffer and the volume of reactivator stock solution required to give a 3.00×10^{-6} to 2.00×10^{-3} M reactivator concentration. At timed intervals, 20-μL aliquots of the enzyme plus reactivator solution are withdrawn and assayed as described above. Thus, the final concentrations in the assay solution are as follows: 1.0×10^{-10} M AChE, 3.00×10^{-8} to 2.00×10^{-5} M reactivator, 3.0×10^{-4} M DTNB, and 7.5×10^{-4} M acetylthiocholine.

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Trapping of Metabolically Generated Electrophilic Species with Cyanide Ion: Metabolism of Methapyrilene

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The popular antihistamine methapyrilene [*N*,*N*-dimethyl-*N*′-(2-pyridyl)-*N*′-(2-thienylmethyl)-1,2-ethanediamine] recently has been shown to be a potent hepatocarcinogen. Metabolic studies with rabbit liver 100000*g* microsomal preparations have resulted in the partial characterization of the in vitro metabolic profile of methapyrilene. Evidence for the formation of the *N*-oxide and the three possible carbinolamines resulting from the NADPH-dependent oxidation of the (dimethylamino)ethyl side chain nitrogen and carbon atoms of methapyrilene is presented. Attempts to trap iminium ion intermediates with electrophilic alkylating potential by coincubating methapyrilene with sodium cyanide have led to the isolation of *N*-(cyanomethyl)normethapyrilene. The possibility of characterizing the iminium ion intermediate that would result from the oxidative deamination of the dimethylamino moiety was precluded by the chemical instability of the corresponding α-cyano amine, which undergoes a spontaneous retro-Michael reaction and hydrolysis to the corresponding amide. The results are discussed in terms of the metabolic activation of methapyrilene to potential alkylating species.

Recent studies have established that the widely used histamine H_1-receptor antagonist methapyrilene [*N*,*N*-dimethyl-*N*′-(2-pyridyl)-*N*′-(2-thienylmethyl)-1,2-ethanediamine (1)] is a potent hepatocarcinogen.[1] Although the carcinogenic properties of many xenobiotics are thought to be mediated by metabolically generated electrophilic intermediates that form covalent bonds with nucleophilic functionalities present on biomacromolecules,[2] it is not clear what bioactivation pathway(s) methapyrilene might undergo. The in vivo conversion of methapyrilene to *N*-nitroso metabolites, at one time considered a possible metabolic activation pathway for this compound,[3] does not appear to account for its carcinogenicity.[4]

We have been interested in the possible metabolic conversion of tertiary amines to electrophilic iminium ions. These reactive species, which theoretically may form by ionization of intermediary α-carbinolamine metabolites,[5] have been demonstrated indirectly by isolation of the corresponding α-cyano amines from liver microsomal coincubations of nicotine and 1-benzylpyrrolidine with the nucleophilic trapping agent sodium cyanide.[6] In the present paper we report the results of our studies on the in vitro metabolism of methapyrilene and of our attempts to characterize the metabolic conversion of this hepatocarcinogen to reactive iminium ions.

Results and Discussion

In Vitro Metabolism of Methapyrilene in the Absence of Sodium Cyanide. Our metabolic work employed NADPH-supplemented 100000*g* microsomal preparations obtained from phenobarbital-induced rats or from un-

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