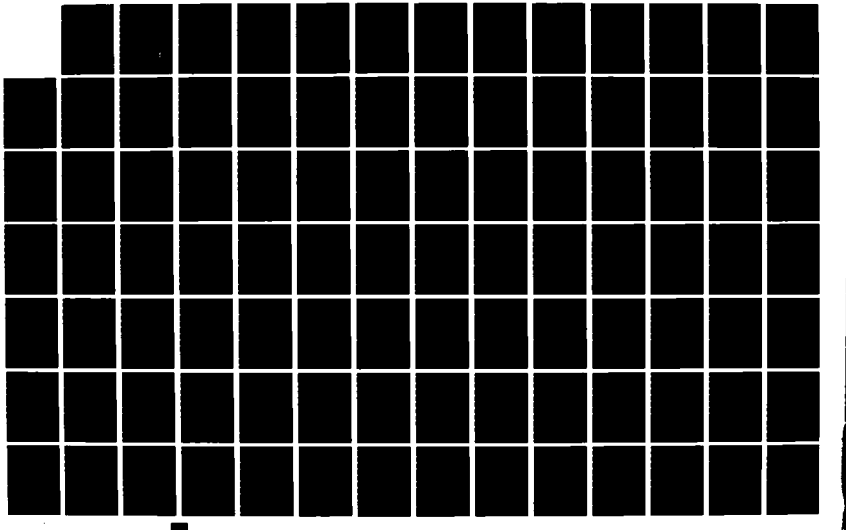
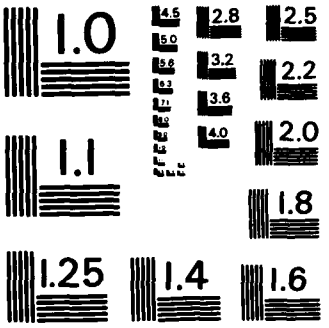
AD-A160 562 NONQUATERNARY CHOLINESTERASE REACTIVATORS(U) SRI INTERNATIONAL MENLO PARK CA R A KENLEY ET AL. 30 AUG 82 DAMD17-79-C-9178 1/2

UNCLASSIFIED F/G 6/1 NL





MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963 - A

AD-A160 562

AD _____

NONQUATERNARY CHOLINESTERASE REACTIVATORS

Annual Report

Richard Kenley, Robert Howd, Oliver Dailey,
Alexi Miller, Clifford Bedford, and John Winterle

30 August 1982

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-79-C-9178

SRI International
333 Ravenswood Avenue
Menlo Park, California 94025

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A160 562	3. RECIPIENT'S CATALOG NUMBER Annual Report
4. TITLE *(and Subtitle)* NONQUATERNARY CHOLINESTERASE REACTIVATORS		5. TYPE OF REPORT & PERIOD COVERED Oct. 1981 through Aug. 1982
		6. PERFORMING ORG. REPORT NUMBER SRI Project 1043
7. AUTHOR(s) Richard A. Kenley, Robert Howd, Oliver Dailey, Alexi Miller, Clifford Bedford, and John Winterle		8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9178
9. PERFORMING ORGANIZATION NAME AND ADDRESS SRI International 333 Ravenswood Avenue Menlo Park, CA 94025		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62734A.3M162734A875.AJ.421
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Ft. Detrick, Frederick, MD 21701-5012		12. REPORT DATE 30 August 1982
		13. NUMBER OF PAGES 179
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*		15. SECURITY CLASS *(of this report)* UNCLASSIFIED
		15a. DECLASSIFICATION DOWNGRADING SCHEDULE N/A

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

Chapter I published in Journal of Medicinal Chemistry, 1981, 1124. Chapters II and III submitted to Journal of Medicinal Chemistry for publication.

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Acetylcholinesterase
Ethyl p-nitropheynl methylphosphonate
α-Ketothiohydroximates
α-Heteroaromatic aldoximes
α-Heteroaromatic thiohydroximates in vitro reactivation

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

We prepared three general classes of compounds and evaluated them in vitro as reactivators of organophosphonate-inhibited acetylcholinesterase (AChE). The compounds conformed to the following general structures: $RC(:O)C(:NOH)S(CH_2)_n N(R')_2 \cdot HCl$, α-ketothiohydroximates, type 1; $R''-C(:NOH)H$, α-heteroaromatic aldoximes (R'' = oxadiazole, thiadiazole,

DD FORM 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

or triazole), type 2; and R''-C(:NOH)S $CH_2CH_2N(C_2H_5)_2 \cdot HCl$, α-hetero-aromatic thiohydroximates (R'' as above), type 3. In all cases, the objective was to develop nonquaternary compounds that can restore activity to phosphonylated-AChE and that can penetrate in vivo into the central nervous system.

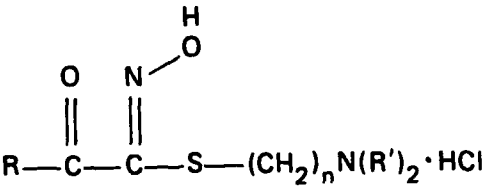
All the compounds reactivate inhibited AChE via pre-equilibrium binding to phosphonylated enzyme followed by nucleophilic attack on phosphorus to liberate active enzyme. We characterized each of the type 1, 2, and 3 compounds with respect to acidity, nucleophilicity, reversible anticholinesterase activity, and effective bimolecular rate constants (k_{eff}) for reactivation of ethyl methylphosphonyl-AChE. Within each class of compounds, the most active reactivators were: BrC_6H_4-C(:O)C(:NOH)$SCH_2CH_2N(C_2H_5)_2 \cdot HCl$, 1a; 3-(1-naphthyl)-5-hydroxyiminomethyl-1,2,4-oxadiazole, 2d; and 3-phenyl-1,2,4-oxadiazol-5-yl thiohydroximic acid 2-(diethylamino)ethyl S-ester, 3a. Compared with 2-PAM, relative k_{eff} values were 3a: 1a: 2d: 2PAM = 0.007:0.02:0.2:1. Compound 2d may be the most powerful nonquaternary reactivator ever reported; it is only 5 times less reactive than the standard pyridinium reactivator, 2-PAM. Compound 2d is a promising candidate for improved therapy against organophosphonate poisoning.

Compound 3a is a powerful reversible competitive inhibitor of AChE (K_I = 0.98 μM). Additionally, 3a protects AChE against inhibition by an organophosphonate and simultaneously reactivates any phosphonyl-AChE that forms. These characteristics recommend 3a as a pretreatment for organophosphonate poisoning.

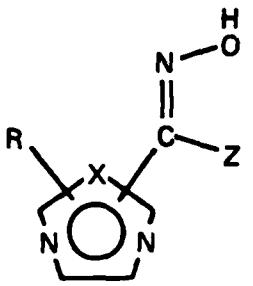
SUMMARY

The objective of the project is to develop improved therapeutic or pretreatment drugs against poisoning by anticholinesterase nerve agents. Our investigation focuses on nonquaternary oxime and hydroximic acid derivatives that will readily penetrate into hydrophobic tissue regions (e.g., the central nervous system) and at least equal the inherent reactivation potency of standard therapeutics such as 2-hydroxyimino-methyl-1-methylpyridinium iodide (2-PAM). The specific approach to the problem includes the following steps: preparing small quantities of test compounds, evaluating the compounds with respect to hydroxyimino-methyl group acidity and nucleophilicity, determining reversible anticholinesterase activity, determining kinetics for reactivation of organophosphorus ester-inhibited AChE, elucidating structure-activity relationships, designing new compounds with improved reactivity, and synthesizing 2- to 10-gram quantities of promising compounds for evaluation (at the U.S. Army Medical Research Institute of Chemical Defense) (USAMRICD) of antidotal efficacy in preventing or reversing nerve agent intoxication *in vivo*.

Our work to date has included three major class of compounds: α-ketothiohydroximic acid dialkylaminoalkyl S-esters (type 1); α-hetero-aromatic aldoximes (type 2); and α-heteroaromatic thiohydroximates (type 3). The general structures for these molecular types are as follows:



α-Ketothiohydroximate (1)



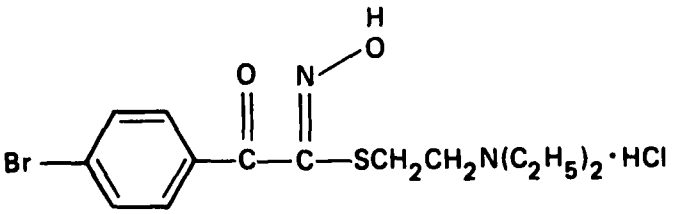
α-heteroaromatic aldoximes (2), Z = H

α-heteroaromatic thiohydroximates (3), $Z = SCH_2CH_2N(C_2H_5)_2 \cdot HCl$

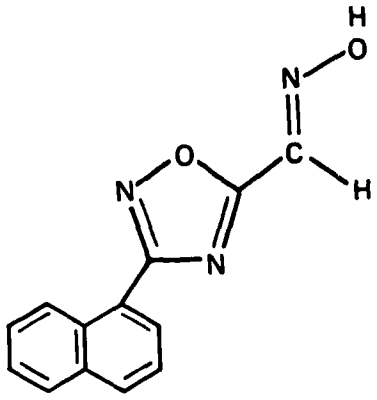
We have evaluated type 1, 2, and 3 compounds in vitro as reactivators of AChE inhibited by diisopropyl phosphorofluoridate (DFP) and by ethyl p-nitrophenyl methylphosphonate (EPMP).

Recently, we constructed an approved Dilute Chemical Surety Material (DCSM) Facility and began initial investigations of the *in vitro* reactivation of AChE inhibited by 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD).

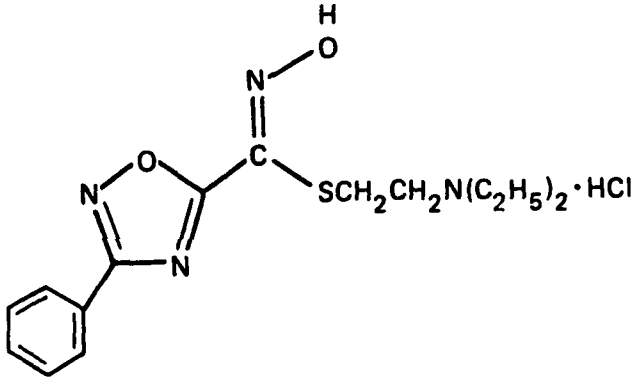
With respect to reactivation of EPMP-inhibited AChE, the most potent compounds (in each category of nonquaternary reactivators) are:



1a



2d

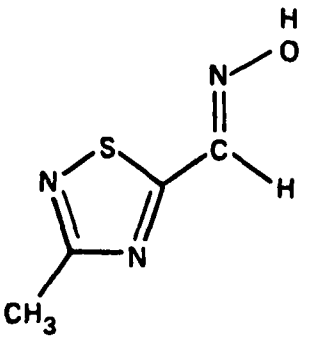


3a

where the specific compound numbers correspond to the abbrevations used in the body of this report.

As a measure of the reactivation potency of the test compounds, we determined effective bimolecular rate constants for reactivation (k_{eff} values). For the series 3a:1a:2d:2-PAM, observed k_{eff} values, respectively, were 1:2.7:27:135.

Compound 2d is only 5 times less reactive than the standard reactivator 2-PAM and is 40 times more reactive than 3-methyl-5-hydroxyiminomethyl-1,2,4-thiadiazole (4).



4

The literature reports that compound 4 is an effective antidote *in vivo* versus the anticholinesterase agent isopropyl methylphosphonofluoridate (GB). On the basis of our *in vitro* comparisons, we predict that 2d will also exhibit useful antidotal efficacy *in vivo*.

Compound 3a is unusual because it is both a powerful reversible AChE inhibitor and a modest reactivator of phosphonylated AChE. When micromolar concentrations of 3a are incubated with AChE before addition of excess EPMP, 3a simultaneously antagonizes irreversible inhibition by EPMP and reactivates any phosphonylated enzyme that does form. This interesting result suggests that 3a has potential as a pretreatment for organophosphorus ester poisoning.

In conclusion, we have demonstrated that it is indeed possible to design and prepare nonquaternary compounds that approach the inherent reactivation potency of the standard drug, 2-PAM. Coupled with the potential for improved tissue distribution by the nonquaternary compounds, this high reactivation potency promises to provide significant improvements in antidotal efficacy versus anticholinesterase agent poisoning. Whether the promise will be realized awaits direct experimental determination of the *in vivo* properties of our drugs. These *in vivo* experiments are planned for the very near future.

CONTENTS

FIGURES

TABLES

OVERVIEW

Background

This report is a comprehensive summary of the technical effort undertaken on U.S. Army Medical Research and Development Command Contract DAMD17-79-C-9178, "Nonquaternary Cholinesterase Reactivators." The report covers progress from the initial award date of September 30, 1979 to August 1982. To reflect the major approaches of our investigation, we have subdivided this report into four chapters, each of which covers a distinct segment of our effort. The major distinction in each chapter is the general category of compound(s) investigated.

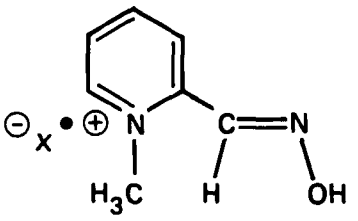
This overview summarizes the historical development of the program, citing major milestones achieved and any important changes taken in the general approach to the investigation. We also review the overall objectives and technical approach of the effort, highlight significant findings, identify critical trends, and forecast future directions in our research plan.

Objectives and Approach

The ultimate objective of this contract effort is the development of improved antidotes against intoxication by chemical warfare nerve agents. The critical imbalance in the relative chemical warfare capabilities of the United States and its potential adversaries is increasingly evident and dictates the need for an upgraded chemical warfare defensive posture. This defensive capability includes a requirement for safe and effective drugs to be used by combat troops for emergency first-aid or self-aid following tactical use of chemical warfare nerve agents.

Chemical warfare nerve agents inhibit the enzyme, acetylcholinesterase (AChE). Therapy of nerve agent intoxication relies on co-administration of anticholinergics (e.g., atropine) and so-called cholinesterase "reactivators." These reactivators interact with phosphonylated AChE to effect nucleophilic displacement at the phosphorus atom

and restore enzymatic activity. Currently available cholinesterase reactivators belong to a single class of organic compounds: hydroxyiminomethyl-substituted n-alkyl-pyridinium salts. The best known example of conventional cholinesterase reactivators is α-hydroxyiminomethyl-1-methylpyridinium halide, 2-PAM.



2-PAM and related pyridinium oximes share a fundamental disadvantage: as quaternary ammonium ions, they penetrate poorly from the serum through hydrophobic cell membranes. The hydrophilic nature of the pyridinium oximes seriously limits tissue distribution (especially within the central nervous system) and presumably also limits their antidotal efficacy.

Our approach to the development of improved nerve agent antidotes focuses on nonquaternary cholinesterase reactivators. In principle, it should be possible to find nonquaternary hydroximic acid derivatives that at least equal the inherent reactivation potency of the pyridinium oximes, but that significantly surpass the pyridinium salts with respect to penetration into tissues where reactivation of inhibited AChE is vitally needed.

Considerable evidence identifies the following requirements for high activity of cholinesterase reactivators:

- A nucleophilic hydroxyiminomethyl moiety.
- A hydroxyiminomethyl acid dissociation constant (pK_a) near 8.
- structural elements that contribute to strong binding of reactivator to the inhibited enzyme and permit the nucleophilic moiety to rapidly attack the inhibitor residue on the enzyme active surface.

In designing nonquaternary reactivators, we have attempted to incorporate all the above elements into molecules that do not feature a quaternary ammonium functionality.

To characterize and evaluate the candidate reactivators prepared during the project, we have relied on several kinetic probes. To reflect the inherent nucleophilicities of the test compounds, we have measured bimolecular rate constants, k_n, for reaction with acetylthiocholine. To ensure that the candidate compounds behave as nucleophiles in the anticipated fashion, we have related oxime pKa values to $\log(k_n)$ values in accordance with the usual Bronsted relationship. To identify the structural elements that contribute to high affinity for AChE, we have determined the degree to which the candidates themselves reversibly bind to AChE (i.e., I50 values for AChE inhibition). Finally, as a direct measure of reactivation potency, we have examined, <u>in vitro</u>, the kinetics of reactivation of phosphonylated AChE.

The reactivation process involves the following reactions:

$$HOX \overset{K_a}{\rightleftharpoons} OX + H^+ \qquad (1)$$

$$EOH + OP \xrightarrow{k_i} EOP \qquad (2)$$

$$EOP + OX \overset{K_r}{\rightleftharpoons} [EOP \cdot OX] \qquad (3)$$

$$[EOP \cdot OX] \xrightarrow{k_r} EOH + \text{products} \qquad (4)$$

where HOX is the protonated form of an oxime reactivator, OX is the active nucleophile (the oximate form), EOH is active enzyme, EOP is phosphonylated enzyme, and [EOP•OX] is a reversibly formed complex between reactivator and phosphonylated enzyme. The constants K_r and k_r characterize the binding of reactivator to inhibited enzyme and the transformation rate of [reactivator/inhibited enzyme] complex to active enzyme. It can be shown that

$$k_b = k_r/K_r \quad (5)$$

where k_b is equivalent to a bimolecular rate constant for reactivation of inhibited enzyme by the oximate form. Because at any pH only a fraction of added test compound dissociates to the active oximate form, we have defined an effective bimolecular reactivation rate constant as in equation (6)

$$k_{eff} = k_b\,[1 + \text{antilog}(pK_a - pH)]^{-1} \quad (6)$$

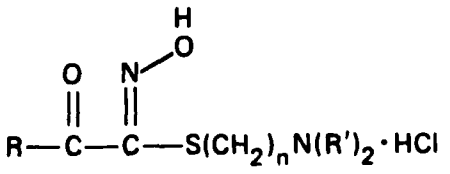
Our general approach to the problem involves the following iterative sequence:

- Synthesizing new candidates on the 100- to 500-mg scale.
- Determining pKa, k_n, and I50 values for the candidates.
- Determining k_r, K_r, k_b, and k_{eff} values for reactivation of phosphonylated enzyme by test compounds.
- Elucidating structure-activity relationships (SARs).
- Designing improved candidates on the basis of SARs.

The following section outlines specific elements of our programs.

Chronological Development of the Program

We began in 1979 with an investigation of α-ketothiohydroximates (1) as nonquaternary reactivators.

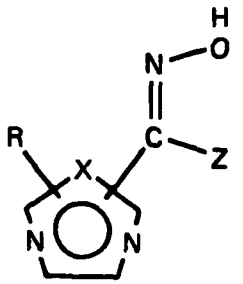


1

We used diisopropyl phosphorofluoridate (DFP) as an AChE inhibitor to test the type 1 compounds for activity in vitro. Chapter I below describes this phase of the program.

The DFP-inhibited enzyme proved to be a poor choice for evaluating our compounds: the diisopropyl phosphorylated AChE undergoes a complicating side reaction ("dealkylation") in parallel with oximate-induced reactivation. Dealkylation seriously interferes with accurate determination of reactivation kinetics, and in October 1980 we selected another inhibitor for use in our in vitro assay: ethyl p-nitrophenyl methylphosphonate (EPMP). As summarized in Chapter II, the EPMP-inhibited enzyme does not suffer significant side reactions and permits reliable determination of reactivation kinetics.

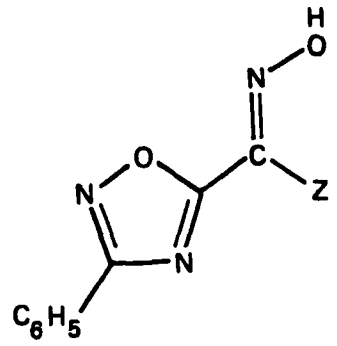
We found that the type 1 compounds, as a class, were not very potent reactivators of ethyl methylphosphonyl-AChE. For this reason, in March, 1981, we began to investigate two additional categories of reactivators: the α-heteroaromatic aldoximes (type 2 compounds) and α-heteroaromatic thiohydroximates (type 3 compounds). Chapter III describes our initial work on the heteroaromatic systems.



2, (Z = H)

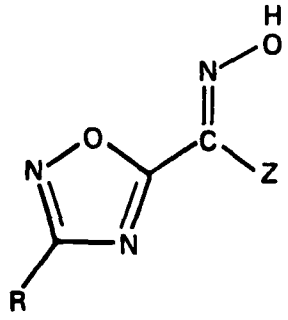
3, (Z = $SCH_2CH_2N(C_2H_5)_2 \cdot HCl$)

Of the 16 compounds reported in Chapter III, compound 2a was by far the most potent reactivator, and the analogous 3a exhibited modest reactivation potency plus a very pronounced reversible anticholinesterase activity.



2a (Z = H)
3a ($Z = SCH_2CH_2NEt_2 \cdot HCl$)

The unusual properties of 2a and 3a suggested the 3-substituted-1,2,4-thiadiazol-5-yl ring system as a likely avenue for more detailed investigations. In February, 1982, we initiated a study of this ring system whereby we varied the substituent, R, in the 3-position while maintaining the other elements of the basic 1,2,4-oxadiazol-5-yl aldoxime or thiohydroximate structure.



3-Substituted-1,2,4-oxadiazol-5-yl System

Chapter IV summarizes this segment of the investigation.

In October, 1981, we began constructing a facility for storage and use of the Dilute Chemical Surety Material (DCSM) 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD). Our goal was to evaluate our candidate reactivators in vitro versus a bona fide chemical warfare agent (i.e., GD). In April, 1982, we received our first shipment of DCSM: we were the first Medical R&D Command contractor to have an approved, operational DCSM facility. In vitro investigations with GD are under way and will constitute a significant fraction of our future effort.

Finally, in August 1982 we modified our original contractual effort to include the scale-up of particularly promising candidates to the 2- to 10-gram level. The objective of this additional effort was to prepare test compounds in the quantities necessary to support in vivo determinations of antidotal efficacy versus the nerve agent GD. The in vivo work (which will be performed at the U.S. Army Medical Research Institute for Chemical Defense) promises to provide the biological data that will allow us to reliably estimate the potential practical utility of compounds prepared under this contract.

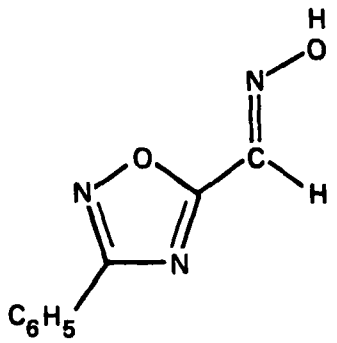
Major Findings

Our original work with DFP-inhibited AChE demonstrated the diisopropyl phosphorylated enzyme to be ill-suited to kinetic investigations. As an alternative, the EPMP-inhibited enzyme is an excellent choice. The ethyl methylphosphonylated-enzyme does not undergo complicating side reactions (e.g., enzyme denaturation, "dealkylation", or reinhibition by phosphonyl oxime) to a significant degree in the time required to effect essentially complete oximate-induced restoration of enzymatic activity. Additionally, we have developed a spectrophotometric enzyme assay with computer-assisted data analysis that is efficient, highly accurate, and reproducible. With this combination of inhibited enzyme and assay technique, we can rapidly evaluate the inherent reactivating potency of our test compounds.

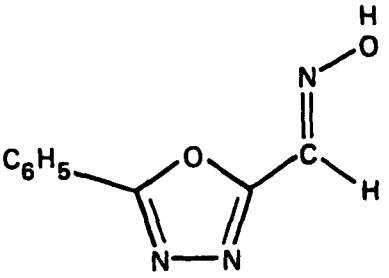
With respect to the three classes of reactivators investigated to date, we have shown that the type 1 compounds generally exhibit low activity as reactivators. In terms of k_{eff} values [see equation (6)] for reactivation of ethyl methylphosphonyl-AChE, the best type 1 compound is less reactive than 2-PAM by a factor of 50. We have argued (Chapter II) that entropic factors primarily limit the reactivation potencies of type 1 compounds. Relative to 2-PAM, the type 1 compounds exhibit a high degree of structural flexibility. Many low-energy conformations are available to the reactivator-inhibited enzyme complex for the type 1 compounds, and it seems likely that only a small fraction of these conformations position

the reactivator oximate functionality near the phosphorus atom of the inhibited enzyme. By comparison, 2-PAM binds to the inhibited enzyme with a limited number of orientations, a large fraction of which contribute to rapid attack on phosphorus. We conclude that structural rigidity is an important (previously undervalued) requirement for reactivator potency.

Within the general type 2 structure, we observed low reactivation potencies for most of the eight ring systems investigated: only the 3-substituted-1,2,4-oxadiazol-5-yl aldoximes show high reactivity toward phosphonylated AChE. For example the pair of analogous phenyl-substituted oxadiazoles shown below



1,2,4-oxadiazole



1,3,4-oxadiazole

differ in reactivity (k_{eff} values) by a factor of 15, the 1,2,4-isomer being more reactive. This fact plus the dependence of reactivator potency on the 3-substituent in the 1,2,4-oxadiazolyl system indicates that the heteroaromatic reactivators bind to a hydrophobic region on the enzyme active surface. Additionally, it appears that the heteroaromatic aldoximes bind quite rigidly and that therefore strict geometric requirements exist for reactivation potency.

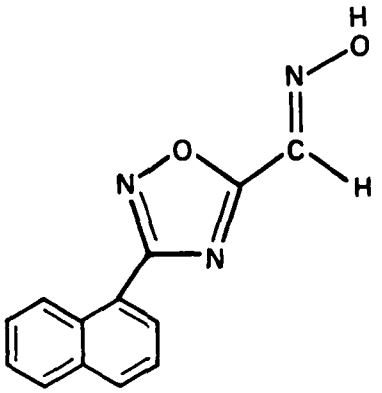
By comparison, none of the type 3 compounds are potent reactivators. The generally low pK_a values ($\approx$ 6.5) fall well below the optimal range of pK_a = 7.5 to 8.5, and largely limit the potency of the type three compounds investigated to date.

Interestingly, several of the type 3 compounds are reversible AChE inhibitors. Compound 3a, in particular, is a potent inhibitor (I50 = 0.98 μM) indicating strong binding of the compound to the enzyme active

site. We suspected that 3a might also antagonize irreversible enzyme inhibition by EPMP. In fact, when micromolar concentrations of 3a are preincubated with AChE, subsequent addition of excess EPMP does not lead to rapid complete loss of activity as is observed when 3a is not present initially. Rather, enzyme activity decreases initially, and then reaches a steady-state value that is proportional to the concentration of 3a present. At 3.0 μM, 3a protects 45% of the AChE from phosphonylation. This behavior is consistent with a dual mode of action for 3a: masking AChE against reaction with EPMP and reactivating any phosphonylated enzyme that does form.

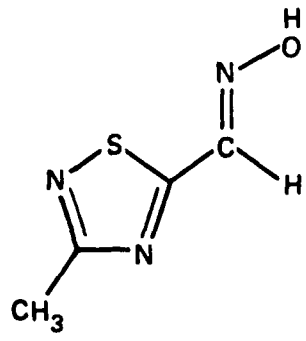
This discovery raises the very intriguing possibility that preincubation of AChE with 3a might be a useful way to prevent AChE phosphonylation by organophosphonates such as GD that are normally refractory to reactivation. It is also interesting to consider possible modifications to the basic structure of 3a that would increase the hydroxyiminomethyl pK_a from 6.5 to approximately 8 while leaving intact the elements that contribute to strong reversible binding of 3a to the AChE active site. In our future work we will search for such modified structures.

Finally, we note the phenomenal activity of compound 2d,



2d

the most potent reactivator that we have prepared. In terms of k_{eff} values, 2d is less reactive than 2-PAM toward ethyl methylphosphonyl-AChE by a factor of 5. On the basis of comparable literature data (Chapter IV), we conclude that, in vitro, 2d is the most powerful nonquaternary reactivator ever reported and that it is the only known nonquaternary reactivator that equals the reactivation potency of 2PAM within a factor of 10. In our in vitro assay, 2d is 40 times more potent than the thiadiazole, 4,



4

a reactivator with known antidotal properties versus the nerve agent GB. The relative reactivities of 2d and 4a suggest that 2d will have particularly pronounced activity in an in vivo evaluation. We are currently undertaking the preparation of 2d on a large enough scale sufficient to support in vivo testing of its activity.

Chapter I

DIALKYLAMINOALKYL S-ESTERS OF α-KETOTHIOHYDROXIMIC ACIDS AS REACTIVATORS OF DIISOPROPYL PHOSPHORO-FLUORIDATE-INHIBITED ACETYLCHOLINESTERASE

INTRODUCTION

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 thirty 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 equation (1)

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

where EOH is active enzyme, EOP is phosphylated 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 (pKa), 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-hydroxyiminomethyl-1-methyl-pyridinium halide (2-PAM), have been the compounds most studied.[8-11]

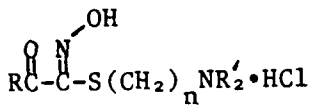
The proven therapeutic utility of pyridinium oximes notwithstanding, it is apparent that they have in common an important disadvantage in terms of limited tissue distribution. Hydrophobic cell membranes represent biological barriers to the transport of large ions from aqueous media into various tissues. 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 pyridinum oximes exhibit a high degree of selectivity for activity at peripheral versus 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]

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 butanedioneoxime (DAM),[30] 4-dimethylaminobutanedione 2-oxime (isonitrosine),[18,31,32] the 3-(diethylamino)propyl ester of oximinoacetic acid (OA3) and analogous amides,[29,33,34] the 2-(diethylamino)-ethyl S-ester 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 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 S-esters, given by the formula 1;



1

Our choice of this general 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 ($4\text{-}BrC_6H_4C(:NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$), 2, a thiohydroximate that has been touted[36] as a particularly effective reactivator, and 2-PAM (2-hydroxyiminomethyl-1-methyl-pyridinium iodide), 3.

RESULTS AND DISCUSSION

Synthesis, Structure, and Acidity

We prepared the hydroximoyl chlorides and α-ketothiohydroximates described in Tables 1 and 2 by reactions (2) and (3)

Table 1

SELECTED DATA FOR HYDROXIMOYL CHLORIDES
RC(:NOH)Cl

R	Yield, %	Recryst. Solvent	mp, °C	NMR (-NOH), δ
$C_6H_5C(:O)-$	52	C_6H_6-CCl_4	123-126	13.68
$4-NO_2C_6H_4C(:O)-$	18	C_6H_6	136-140	13.86
$4-CH_3OC_6H_4C(:O)-$	51	C_6H_6	131-133	13.40
$CH_3C(:O)-$	50	CCl_4	88-93	13.44
$4-BrC_6H_4-$	65	CCl_4	--	--

Table 2

SELECTED DATA FOR α-KETOTHIOHYDROXIMATES
$RC(:NOH)S(CH_2)_nNR'_2 \cdot R''X$

Compound	R	n	R′	R″X	Yield, %	TLC,[a] R_f	mp, °C	NMR (-NOH), δ	UV[b] λ_{max}, nm	UV[b] ε, $M^{-1}cm^{-1} \times 10^{-4}$	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	$4\text{-}NO_2C_6H_4C(:O)$	2	C_2H_5	HCl	18	0.57	170-172.5	13.39	269	1.74	7.14	C,H,N,S,Cl
1c	$4\text{-}CH_3OC_6H_4C(:O)$	2	C_2H_5	HCl	65	0.65	139-140	12.69	230 303	1.12 1.50	7.82	C,H,N,S,Cl
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	$4\text{-}CH_3OC_6H_4C(:O)$	2	CH_3	HCl	55	0.55	154-155.5	12.74	230 308	1.12 1.68	8.00	C,H,N,S,Cl
1f	$4\text{-}CH_3OC_6H_4C(:O)$	2	$(CH_3)_2CH$	HCl	70	0.64	179-184	12.70	230 309	1.13 1.74	8.01	C,H,N,S,Cl
1g	$4\text{-}CH_3OC_6H_4C(:O)$	3	CH_3	HCl	d	0.30	c	12.50	d	d	d	d
1h	$4\text{-}CH_3OC_6H_4C(:O)$	3	CH_3	$[HOC(:O)]_2$	29	0.42	151-153.5	e	d	d	8.42	C,H,N,S,Cl
1i	$4\text{-}CH_3OC_6H_4C(:O)$	2	C_2H_5	CH_3I	48	0.39	c	f	d	d	7.82	d
2	$4\text{-}BrC_6H_4$	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 proton .

[f] In CD_3OD solution, OH exchanged with OD.

$$RCCH_3 + iPrONO \xrightarrow[HCl]{} R-C(=O)-C(=NOH)-Cl \quad (2)$$

$$R-C(=O)-C(=NOH)-Cl + HS(CH_2)_nNR'_2 \xrightarrow{Et_3N} R-C(=O)-C(=NOH)-S(CH_2)_nNR'_2 \quad (3)$$

Unoptimized yields of reaction (3) ranged from 18% to 70%, based on hydroximoyl chloride used.

Oxime acid dissociation constants (pKa) were measured by spectrophotometric determination of oximate concentration in buffers of various pH.[41] From the data in Table 2 for 1a-1i, a plot (not shown) of pKa versus oxime proton NMR chemical shift is linear and conforms to equation (4)

$$pKa = (25.3 \pm 2.1) - (1.36 \pm 0.16)\ \delta \quad (4)$$

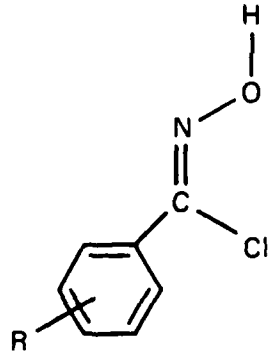
For the aroylthiohydroximates (1a, 1b, and 1c), a plot (not shown) of pKa versus Hammet substituent constant (σ_p)[42] is also linear and conforms to equation (5)

$$pKa = (7.63 \pm 0.02) - (0.63 \pm 0.05)\ \sigma_p \quad (5)$$

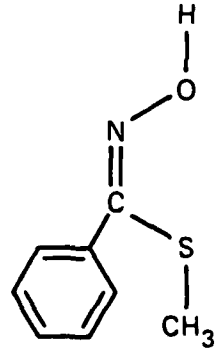
These correlations provide an indication of the validity of the pKa values, an important consideration because we have related α-ketothiohydroximate reactivity to the concentration of the dissociated form at a given pH and hence to the acidity of the oxime functionality. We determined pKa = 7.99 for 3, which is in good agreement with the value of 7.92 reported by Schoene and Strake.[40] Our value of pKa = 9.28 for 2 is lower than that

(pKa = 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 coworkers find that benzohydroxamoyl chlorides[43] and methyl benzothiohydroximate[44] adopt the E-configuration in solution.

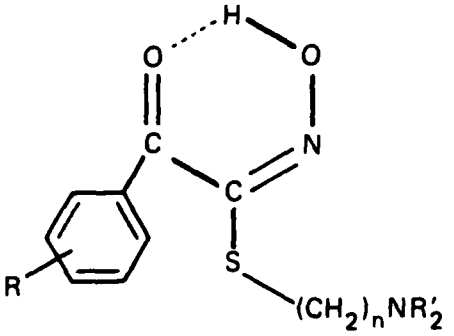


E-benzohydroxamoyl chlorides



E-methyl benzothiohydroximate

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 1 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 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 x 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 equation (6):

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

where [OX] is the concentration of the anionic form of the α-ketothiohydroximate, and k_o is the pseudo-first-order rate constant for spontaneous hydrolysis of [pNPA]. At low conversions both [pNPA] and [OX] remain constant and equation (6) reduces to:

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

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 equation (8).

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

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

As required by equation (7), the k_o values are essentially independent of the reactivator used. The average for all values of k_o from Table 3 is $(2.3 \pm 0.4) \times 10^{-3}$ min^{-1}, which is in good agreement with the value of $k_o = 2.1 \times 10^{-3}$ min^{-1} reported by Jencks and Gilchrist.[46] For 3, the value of $k_n = (9.6 \pm 0.8) \times 10^2$ M^{-1} compares to the value $k_n = 4.0 \times 10^2$ M^{-1} min^{-1}

Table 3

RATE CONSTANTS FOR HYDROLYSIS OF p-NITROPHENYL ACETATE (pNPA) IN THE PRESENCE OF α-KETOTHIOHYDROXIMATES[a]

Compound[b]	k_n[c], M^{-1} min^{-1} x 10^{-2}	k_o[d], min^{-1} x 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 x 10^{-3} M pNPA in the presence of 10 to 100 x 10^{-6} M test compound at pH 7.6 and 25°C in 0.1 M MOPS buffer; see equation (7).

[b] See Table 2.

[c] Nucleophilic displacement rate constant from slope of plot of (OX) vs k_{eQ}; see equations (7) and (8).

[d] Spontaneous hydrolysis rate constant, from intercept of plot of [OX] vs k_{eQ}; see equations (7) and (8).

[e] $pK_B = 14 - pK_A$.

determined by Bergmann and Govrin[47] at pH = 8.0 and 25°C.

Figure 1 is a Bronsted plot (log k_n versus pK_B) of the data of Table 3. The data for the α-ketothiohydroximates conform to equation (9):

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

where the β-value (slope) of 0.69 ±0.08 compares to the value of β = 0.8 commonly observed for reaction of pNPA with oxygen nucleophiles.[46,48]

Thus the α-ketothiohydroximates behave as nucleophiles in the anticipated manner. Because the nucleophilicity is proportional to 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 Details 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 x 10^{-3} M) of pNPA used in the assay, the test compounds (10.0 to 100 x 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_o determined above. In the case of reactions assayed by the Ellman technique, corrections for nonenzymatic

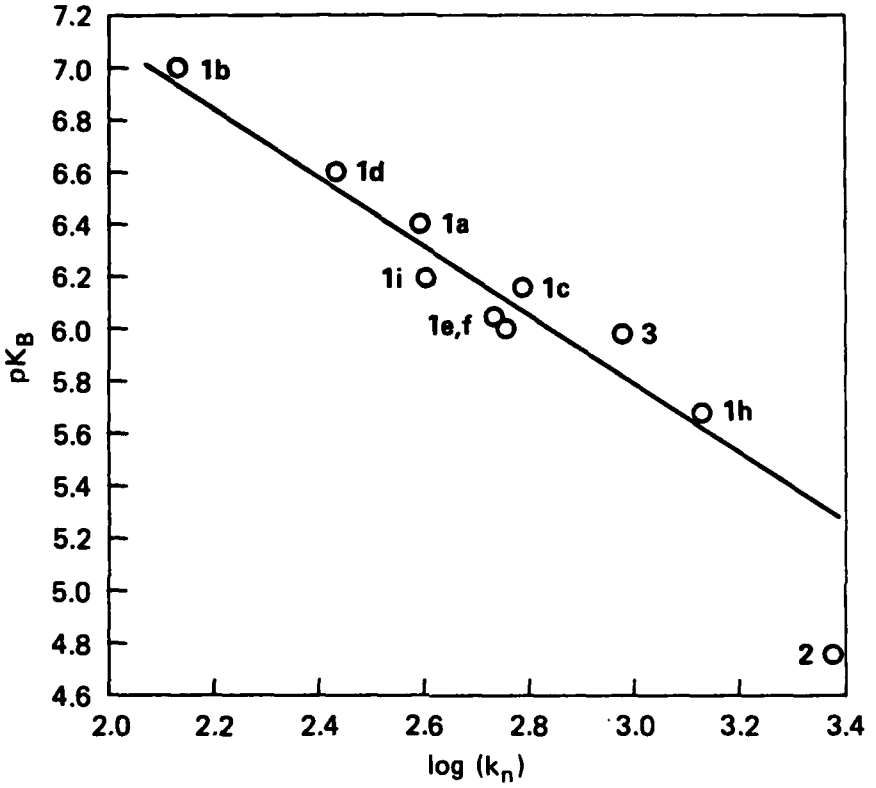


FIGURE 1 CONJUGATE BASE IONIZATION CONSTANTS (pK_B) VERSUS LOGARITHM OF BIMOLECULAR NUCLEOPHILIC DISPLACEMENT RATE CONSTANT (k_n) FOR HYDROLYSIS OF p-NITROPHENYL ACETATE IN THE PRESENCE OF VARIOUS REACTIVATORS AT pH = 7.6, 25°C

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 diisopropyl phosphoryl-AChE was negligible over 24 h. However, the inhibited enzyme (EOP) rapidly converted ("aged") into a nonreactivatable species (EOP′) as shown in reaction (10):

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

where EOP is diisopropyl phosphoryl-AChE 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_c versus time (where A_t is AChE activity after incubation for t min before reactivation with 3 and A_o is activity of inhibited enzyme reactivated immediately) were linear for at least three half-lives. For three determinations the average value of k_a was $(1.61 \pm 0.08) \times 10^{-3}$ min^{-1}, corresponding to a half-life for aging of 7.1 h.

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.

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 diisopropyl phosphoryl-AChE, 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 is calculated according to equation (11):

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

where $\%r_t$ is the percent reactivation after t min incubation, E_t is the enzyme activity at time t, and E_o is the control enzyme activity. Table 4 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 S-esters of p-methoxybenzoylthiohydroximic acid (1e, 1c, and 1f), 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 analog 1c. We anticipated that the tertiary alkylamine functionality would be completely protonated at pH = 7.6 and that the protonated species would

Table 4

PERCENT REACTIVATION ($\%r_t$) OF DIISOPROPYL PHOSPHORYL-AChE AFTER INCUBATION WITH 1×10^{-3} M REACTIVATOR AT 25°C, pH = 7.6

Compound[a]	$\%r_t$[b] After Incubation for t hr		
	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 2.

[b] $\%r_t$ calculated from equation (11).

[c] Reactivator concentration = 2.00×10^{-3} M.

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 analog would predictably exhibit considerably higher activity than the nonquaternary reactivator. The fact that 1c is actually more reactive than the methylated 1f can be rationalized on the basis of strong coulombic interactions for both compounds plus a steric effect that lowers the affinity of the N-methyl derivative for the inhibited enzyme.

Comparison of the 2-(diethylamino)ethyl and 3-(dimethylamino)propyl S-esters of p-methoxybenzoylthiohydroximic acid (1c, and 1h , respectively) shows lower activity for the 3-(dimethylamino)propyl analog. While this could be interpreted as an effect of reactivator structure on activity, it must be noted that the pKa of 1h (Table 2) is 0.6 log units higher than that of 1c . Therefore at pH 7.6, 1c is 40% ionized [equation (8)], whereas 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 4 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, $\underset{\sim}{2}$, deserve comment. We find $\underset{\sim}{2}$ to be considerably inferior to $\underset{\sim}{3}$ as a reactivator of diisopropyl phosphoryl-AChE and attribute the lack of activity of $\underset{\sim}{2}$ 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 ethyl methylphosphonyl-butyrylcholinesterase after incubation with $\underset{\sim}{3}$ and $\underset{\sim}{2}$, respectively.

Even considering the differences in inhibited enzymes used, it is difficult to rationalize the large differences in the relative activities of $\underset{\sim}{2}$ and $\underset{\sim}{3}$ reported by us and by Krivenchuk and coworkers. 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, $\underset{\sim}{2}$ is 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 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):

$$EOP + OX \underset{}{\overset{Kr}{\rightleftharpoons}} [EOP \cdot OX] \xrightarrow{k_r} EOH + POX \qquad (1)$$

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

$$[EOP \cdot OX] \xrightarrow{k'_a} EOP' + OX \qquad (12)$$

where the reactivator is represented by the 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:

$$\ln (\%r_{\infty} - \%r_t) = \ln (\%r_{\infty}) - k_{obs} \cdot t \qquad (13)$$

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

$$k_{max} = k'_a - k_a + k_r \qquad (15)$$

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

where k_{obs} 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 equation (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 equation (10). The ratio $[EOP']_{\infty}/[EOH]_{\infty}$ is calculated from observed values of $\%r_{\infty}$ according to equation (17):

$$[EOP']_{\infty}/[EOH]_{\infty} = (100 - \%r_{\infty})/\%r_{\infty} \qquad (17)$$

Rearranging equation (14) gives

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

Thus a plot [equation (13)] of ℓn ($\%r_\infty - \%r_t$) versus time gives slope = $-k_{obs}$ and intercept = ℓn ($\%r_\infty$); a plot [equation (16)] of $[EOP']_\infty/[EOH]_\infty$ versus $[OX]^{-1}$ gives slope = k_aK_r/k_r and intercept = k_a'/k_r; and a plot [equation (18)] of $(k_{obs} - k_a)^{-1}$ versus $[OX^{-1}]$ 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 5 summarizes the necessary derivations. At low reactivator concentrations, equation (19) holds:

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

where k_b is a 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 in the dissociated form at pH = 7.6. Thus from equations (8) and (19), k_{eff} is given by equation (20):

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

To probe these relationships, 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 equation (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

Table 5

FORMULAS FOR CALCULATING KINETIC CONSTANTS FOR REACTIVATION OF INHIBITED AChE

Plot	Abscissa	Ordinate	$Slope_{(n)}$	$Intercept_{(n)}$	Calculate	Equation
1	t	$\ln(\%r_\infty - \%r_t)$	$S_1 = -k_{obs}$	$I_1 = \ln(\%r_{oo})$	$[EOP']_\infty/[EOH]_\infty = (100 - \exp[I_1])/\exp[I_1]$	(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	$[OX]^{-1}$	$(k_{obs}-k_a)^{-1}$	$S_3 = K_r/k_{max}$	$I_3 = 1/k_{max}$	$K_r = S_3/I_3$	(15), (18)
					$k_r = [(I_3)^{-1} + k_a] \cdot [1 + I_2]^{-1}$	(18), (16)

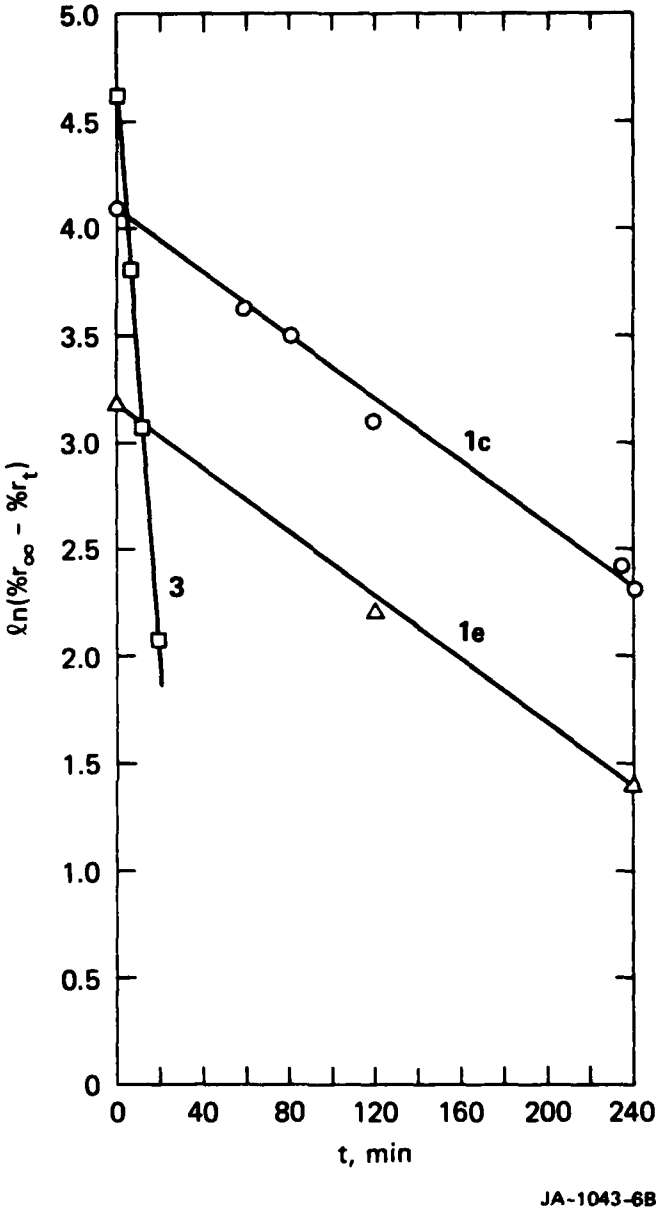


FIGURE 2 NATURAL LOGARITHM OF PERCENT MAXIMAL REACTIVATION MINUS REACTIVATION AT TIME t, $\ell n(\%r_\infty - \%r)$ VERSUS TIME AT 25°C, pH = 7.6 FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE WITH 1.0×10^{-3} M 3, 1.0×10^{-3} M 1c, AND 0.20×10^{-3} M 1e

unimportant under the experimental conditions.[50] Table 6 summarizes the values of k_{obs} and $\ell n(\%r_\infty)$ obtained for a range of concentrations of all four reactivators. Figure 3 is a double-reciprocal plot of $(k_{obs} - k_a)^{-1}$ versus $[OX]^{-1}$ 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 equation (18). A similar plot (not shown) of $[OX]^{-1}$ versus $[EOP']_\infty/[EOH]_\infty$ conforms to equation (16) for the reactivators investigated. Data from Table 6 and from least squares treatment of the data according to equations (16) and (18) are summarized in Table 7 along with kinetic constants calculated as in Table 5 and equations (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_{obs} 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 equation (16). These intercepts are used to calculate k_a', and we view the values for k_a' in Table 7 as tentative. The intercepts calculated from equation (18) are also subject to a degree of uncertainty, and this must be taken into consideration when interpreting values for k_r.

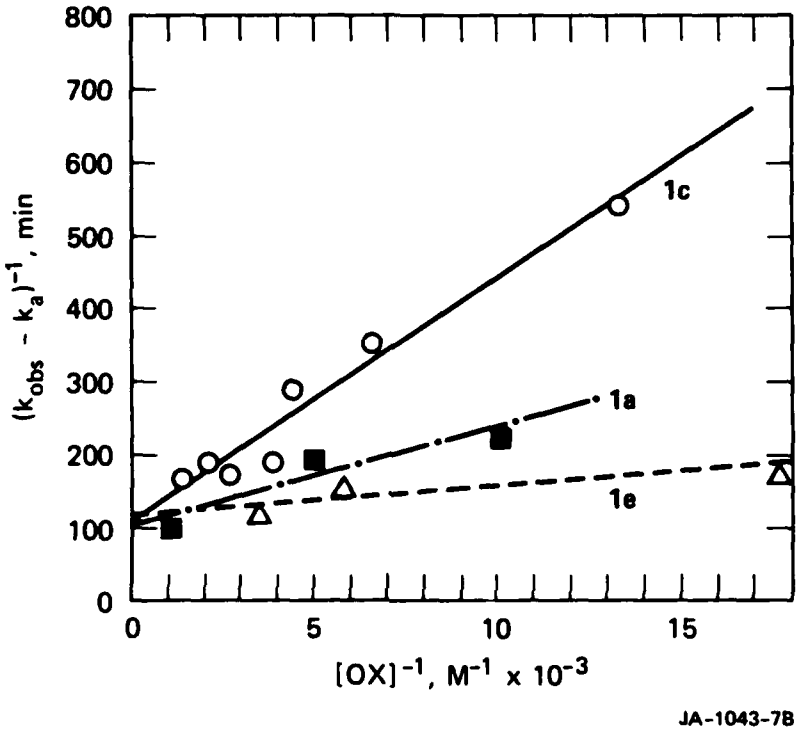


FIGURE 3 DOUBLE-RECIPROCAL PLOT OF OBSERVED FIRST-ORDER REACTIVATION RATE CONSTANT MINUS DEALKYLATION RATE CONSTANT, $(k_{obs}-k_a)^{-1}$, VERSUS OXIMATE CONCENTRATION ($[OX]^{-1}$ FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE BY 1a, 1c, AND 1e

Table 6

PSEUDO-FIRST-ORDER OBSERVED RATE CONSTANTS, k_{obs}, FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE AS A FUNCTION OF REACTIVATOR CONCENTRATION AT 25°C, pH = 7.6

Compound[a]	[HOX], M x 10^4	[OX][b], M x 10^4	k_{obs}[c], min^{-1} x 10^3	$\ln(\%r_\infty)$[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 2.

[b] Calculated from [HOX] and equation (8).

[c] Calculated from slope of least squares plot of data according to equation (13).

[d] Calculated from intercept of least squares plot of $\ln(\%r_\infty - \%r_t)$ according to equation (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 (ref. 51).

Table 7

KINETIC CONSTANTS FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE AT 25°C, pH = 7.6

Compound[a]	[HOX] M x 10^4	$[OX]^{-1}$[b] M^{-1} x 10^{-3}	$(k_{obs}-k_g)^{-1}$[c] min	$\frac{[EOP']_\infty}{[EOH]_\infty}$	k'_a/k_r[e]	$\frac{k_a K_r}{k_r}$[f] M x 10^4	$K_r/k_{r\,max}$[g] M-min x 10^2	$1/k_{max}$[h] min	k'_a[i] min^{-1} x 10^3	k_r[j] min^{-1} x 10^3	k_r[k] M x 10^4	k_b[l] M^{-1} min^{-1}	k_{eff}[m] M^{-1} min^{-1}	k_{eff} (Relative)
1a	2.00	10.0	231	3.12	0.21 ± 0.7	3.2 ± 1.1	1.4 ± 0.36	103 ± 24	2.0	9.3	1.3	70	34	3.1
	4.00	5.00	190	2.37										
	20.0	1.00	106	0.203										
1c	2.0	13.3	538	2.69	0.17 ± 0.18	2.1 ± 0.30	3.30 ± 0.31	106 ± 19	2.4	8.6	2.9	30	11	1
	4.00	6.65	353	2.05										
	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	2.0	17.6	170	3.29	-0.018 ± 0.11	1.9 ± 0.10	0.32 ± 0.19	117 ± 21	0.19	10	0.27	180	108	9.6
	6.0	5.86	151	1.17										
	10.0	3.51	115.	0.576										
3	0.0300	1156	200	0.964	0.11 ± 0.050	0.0075 ± 0.0008	0.017 ± 0.003	15 ± 14	6.7	61	0.11	5500	1600	136
	0.0800	431	129	0.506										
	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 2.

[b] Calculated from [HOX] and equation (8).

[c] From k_{obs} values of Table 6; $k_g = 1.61 \times 10^{-3}$ min^{-1}, see text.

[d] $[EOP']_\infty = (100 - \%r_\infty) + \%r_\infty$.

[e] Intercept of least squares plot of data according to equation (16).

[f] Slope of least squares plot of data according to equation (16).

[g] Slope of least squares plot of data according to equation (18).

[h] Intercept of least squares plot of data according to equation (18).

[i] Calculated as shown in Table 5.

[j] Calculated as shown in Table 5.

[k] Calculated as shown in Table 5.

[l] Calculated from equation (19).

[m] Calculated from equation (20).

[n] See Table 6.

These caveats notwithstanding, the kinetic constants given in Table 7 do reflect some significant structure-activity relationships for the reactivation 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 = 1.61×10^{-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 $K_r = 0.11 \times 10^{-4}$ M and $k_r = 61 \times 10^{-3}$ min^{-1} for 3 combine with a pKa 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]

CONCLUSIONS

The α-ketothiohydroximates are moderately active reactivators of diisopropyl phosphoryl-AChE. In this respect they should be compared to reactivators such as TDA-5[39] and 1-(2-hydroxyiminomethylpyridinium)-3-(3-carboxamidopyridinium)dimethyl ether 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 dialkylaminoalkane thiols. 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 $\underset{\sim}{3}$ apparently accelerates the rate at which diisopropyl phosphoryl-AChE converts to a nonreactivatable form. Similar results[50] with ethyl dimethylphosphoramido-AChE suggest that this may be a general phenomenon 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 DETAILS

Melting points (uncorrected) were obtained with a Fischer-Johns apparatus, NMR spectra (in DMSO-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.

Materials

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 $i\text{-}C_3H_7ONO$ 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 1 gives selected data for the hydroximoyl chlorides.

S-(N,N-Dialkylamino)alkyl α-Ketothiohydroximates (1a-1i). 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 2 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 x 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, m.p. 139-140°C, TLC ($CHCl_3$-MeOH, 6:1) R_f 0.65; NMR δ 12.69 (S, 1H, =NOH), 10.55 (broad, 1H, NH^+), 7.93

(d, 2H, Ar), 7.1 (d, 2H, Ar), 3.87 (s, 3H, OCH_3), 2.7-3.4 (m, 8H, CH_2), 1.13 (t, 6H, CH_3); IR (nujol) ν 2560br,m, 1661s, 1600s, 989s cm^{-1}. Slightly impure fractions of free base, 109 mg, were similarly converted to the hydrochloride and recrystallized 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-diethylaminoethanethiol hydrochloride (510 mg; 3.0 mmol) and sodium methoxide (162 mg; 3.0 mmol) in 15 mL of isopropanol.[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, 1H, NOH), 10.42 (broad, 1H, NH^+), 7.59 (ϕ, 4H, Ar), 3.3-2.8 (m, 8H, CH_2), 1.05 (t, 6H, CH_3); IR (nujol) ν 3215s, 2632s, 1587m, and 922s cm^{-1}.

Methods

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 x 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 diisopropyl phosphoryl-AChE, 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 x 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 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 $\approx 10 \times 10^{-6}$ 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}.

p-Nitrophenyl Acetate Assay. A 100-µL aliquot of enzyme stock 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 3 min, during which period inhibition of the enzyme is complete. After the incubation period, the entire solution is added to a 1 cm x 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 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 200 μL final volume. These solutions are incubated at 25°C±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 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^{-8} to 2.00×10^{-5} M reactivator, and 1.0×10^{-3} M substrate. Observed enzyme activities are corrected (see Results Section) 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 $\overset{\sim}{3}$ 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 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.

LITERATURE CITED

1. We use the term "phosphylation" when we do not wish to distinguish between phosphorylation and phosphonylation.

2. Heath, D. F.; Organophosphorus Poisons--Anticholinesterases and Related Compounds, Pergamon Press, New York, 1961.

3. Koelle, G. B.; "Neurohumoral Transmission and the Autonomic Nervous System in The Pharmacological Basis of Therapeutics," L. Goodman and L. Gilman, Eds., MacMillan, New York, 1965, pp. 404-444.

4. Wills, J. H.; International Encyclopedia of Pharmacological Therapy, Section 13, Vol. I, Pergamon Press, New York, 1970, pp. 357-469.

5. Usdin, E.; International Encyclopedia of Pharmacological Therapy, Section 13, Vol. I, Pergamon Press, New York, 1970, pp. 47-356.

6. (a) Hagedorn, I; Stark, I.; Lorenz, H. P.; Angew. Chem. Int. Ed., 1972, 11:307.
(b) Englehard, N.; Prchal, K.; Nenner, M.; Angew. Chem. Int. Ed., 1967, 6:615.

7. (a) Green, A. L.; Smith, H. J.; *Biochem. J.*, 1958, 68:28.
(b) Green, A. L.; Smith, H. J.; Biochem. J., 1958, 68:32.
(c) Wang, E.I.C.; and Braid, P. E.; J. Biol. Chem., 1967, 242:2683.

8. Namba, T.; Nolte, C. T.; Jackrel, J.; Grob, D.; Am. J. Med., 1971, 50:475.

9. Sim, V. M.; in Drill's Pharmacology in Medicine, 3rd ed., McGraw-Hill, New York, 1965, p. 971.

10. Ellin, R. I.; Wills, J. H.; J. Pharm. Sci., 1961, 53:955.

11. McNamara, B. P.; "Oximes as Antidotes in Poisoning by Anticholinesterase Compounds," Edgewood Arsenal Special Publication 5B-SP-76004, 1976, avail. NTIS (AD-A023 243/9ST).

12. Blasberg, R. G.; National Cancer Institute Monograph 46, pp. 19-27 (1977).

13. Hall, D. P.; Handb. Exp. Pharmakol., 1971, 27:1.

14. DeCandole, C. A.; Douglas, W. W.; Evans, C. L.; Holmes, R.; Spencer, K.E.U.; Torrance, R. W.; Wilson, K. M.; Brit. J. Pharmac. Chemother., 1968, 8:466.

15. Lipp, J. A.; Arch. Int. Pharmacol., 1968, 175:161.

16. Bajgar, J.; Biochem. Pharmacol., 1972, 21:687.

17. Kewitz, H.; Archiv. Biochem. Biophys., 1957, 66:263.

18. Kewitz, H.; Nachmansohn, D.; Archiv. Biochem. Biophys., 1957, 66:271.

19. Rutland, J. P.; Br. J. Pharmacol., 1958, 13:399.

20. Hobbiger, F.; Vojvodic, V.; Biochem. Pharmacol., 1967, 16:455.

21. Hobbiger, F.; and Vojvodic, V.; Biochem. Pharmacol., 1966, 15:1677.

22. Milosevic, M.P.; Andjelkovic, D.; Nature (Lond.), 210, 206 (1966).

23. Rosenberg, P.; Biochem. Pharmacol., 1960, 3:212.

24. Filbert, M. G.; Fleisher, J. H.; Lochner, M. A.; Biochim. Biophys. Acta, 1972, 284:164.

25. Bajgar, J.; Patocka, J.; Jakl, A.; Hrdina, V.; Acta Biol. Med. Germ., 1975, 34:1049.

26. Bajgar, J.; Jakl, A.; Hrdina, V.; Biochem. Pharmacol., 1971, 20:3230.

27. Bajgar, J.; Eur. J. Pharmacol., 1972, 19:199.

28. Jager, B. V.; Stagg, G. N.; Green, N.; Jager, L.; Bull. John Hopkins Hosp., 1958, 102:225.

29. Benschop, H. P.; DeJong, L.P.A.; Vink, J.A.J.; Kienhuis, H.; Berends, F.; Elskamp, D.M.W.; Kepner, L. A.; Meeter, E.; Visser, R.P.L.S.; "The Prophylactic Value of Oximes Against Organophosphate Poisoning," in Medical Protection Against Chemical Warfare Agents, Almquist and Wiksell, Uppsala, 1976, pp. 120-131.

30. Childs, A. F.; Davies, D. R.; Green, A. L.; Rutland, J. P.; Br. J. Pharmacol., 1955, 10:462.

31. Steinberg, G. M.; Bolger, J.; J. Am. Pharm. Assoc., 1957, 46:188.

32. Shein, G. K.; Farmakol. Toksikol., 1967, 30:491.

33. Kuznetsov, S. G.; Somin, I. N.; Khim.-Farm. Zh., 1967, 1:30.

34. Balasova, E. K.; Kartaseva, J. J.; Rosengart, V. I.; Biokhim., 1968, 33:29.

35. Krivenchuk, V. E.; Petrunkin, V. E.; Khim.-Farm. Zh., 1973, 7:13.

36. Krivenchuk, V. E.; Brizgailo, L. I.; Petrunkin, V. E.; Biokhim., 1974, 39:1163.

37. Kagan, Yu. S.; Kokshareva, N. V.; Sasinovich, L. M.; Krivenchuk, V. E.; Farmakol Toksikol., 1975, 38:294.

38. Kokshareva, N. V.; Kovtun, S. D.; Kagan, Yu. S.; Mizyukova, I. G.; Medvedev, B. M.; Byull. Eksp. Biol. Med., 1977, 83:29.

39. Benschop, H. P.; VandenBerg, G. R.; Van Hooidonk, C.; De Jong, L.P.A.; Kientz, C. E.; Berends, F.; Kepner, L. A.; Meeter, R.; Visser, R.P.L.S.; J. Med. Chem., 1979, 22:1306.

40. Schoene, K.; Strake, E. M.; Biochem. Pharmacol., 1971, 20:2527.

41. Albert, A.; Sergeant, E. P.; Ionization Constants of Acids and Bases, J. Wiley and Sons, Inc., New York, 1962.

42. Kosower, E. M.; An Introduction to Physical Organic Chemistry, J. Wiley and Sons, Inc., New York, 1968, p. 49.

43. Smolikov, J.; Exner, O.; Barbara, G.; Macciantelli, D.; Dondoni, A.; J. Chem. Soc. Perkin Trans. II, 1051 (1980).

44. Exner, O.; Benn, M. H.; Willis, F.; Can. J. Chem., 1968, 46:1873.

45. Bellamy, L. J.; The Infrared Spectra of Complex Molecules, John Wiley and Sons, Inc., New York, 1958.

46. Jencks, W. P.; Gilchrist, M.; J. Am. Chem. Soc., 1968, 90:2622.

47. Bergmann, F.; Govrin, H.; Biochimie, 1973, 55:515.

48. Aubort, J. D.; Hudson, R. F.; Woodcock, R. C.; Tett. Lett., 1973, (24):2229.

49. De Jong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol., 1978, 27:2911.

50. De Jong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol., 1978, 27:2229.

51. Frost, A. A.; Pearson, R. G.; Kinetics and Mechanism, 2nd ed., John Wiley and Sons, Inc., New York, 1961, p. 49.

52. Fukuto, T. R.; Metcalf, R. L.; J. Am. Chem. Soc., 1959, 81:372.

53. Berry, W. K.; Davies, D. R.; Biochem. J., 1966, 100:572.

54. Kepner, L. A.; Wolthuis, O. L.; Eur. J. Pharmacol., 1978, 48:377.

55. Levin, N.; Hartung, W. H.; Org. Syn., Coll., 1955, 3:192.

56. Brachwitz, H.; Z. Chem., 1966, 6:313.

57. Hesse, G.; Krehbiel, G.; Ber., 1955, 88:130.

58. Chiang, Y. H.; J. Org. Chem., 1971, 36:2146.

59. Benn, M. H.; Can. J. Chem., 1964, 42:2393.

60. Still, W. C.; Kahn, M.; Mitra, H.; J. Org. Chem., 1978, 43:2923.

61. Douglass, M. L.; DeSalva, S. J.; U.S. 3,953,590 (1976).

62. Main, A. R.; Miles, K. E.; Braid, P. E.; Biochem. J., 1961, 78:769.

63. Mounter, L. A.; Whittaker, V. P.; Biochem. J., 1953, 54:551.

64. Briggs, A. I.; Trans. Far. Soc., 1954, 50:800.

65. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M.; Biochem. Pharmacol., 1961, 7:88.

Chapter II

α-KETOTHIOHYDROXIMATES AS REACTIVATORS OF ETHYL METHYLPHOSPHONYL-ACETYLCHOLINESTERASE IN VITRO

INTRODUCTION

Biologically active organophosphorus compounds are widely used in agriculture and medicine.[1-7] Certain highly toxic organophosphonates are also stockpiled for use in war.[8-10] The potential for deliberate or accidental poisoning by organophosphorus esters dictates a requirement for safe and effective therapeutics.

Most toxic organophosphorus esters are irreversible inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) AChE.[1,11-13] The inhibition proceeds via phosphylation* of a serine hydroxyl at the "esteratic" region of the enzyme active site. Conventional therapy of organophosphorus ester intoxication entails coadministration of atropine [to antagonize the effects of accumulated acetylcholine (ACh)] and AChE "reactivators" that restore activity to the enzyme.[14-17] Currently, pyridinium oximes are the only clinically used reactivators. Examples are 2-hydroxyiminomethyl-1-methylpyridinium iodide (2PAM) and 1,3-bis(4-hydroxyiminomethyl-1-pyridinium)propane dibromide (TMB4).

Pyridinium oximes can reactivate AChE because of two factors: an oxime acid dissociation constant (pKa) equal to approximately 8; and a cationic moiety (the n-alkyl pyridinium ion) at the appropriate distance from the oxime to give a structural similarity to ACh. The oxime acid dissociation constant contributes to a high proportion of the

*We use the term "phosphylation" when we do not distinguish between "phosphonylation" and "phosphorylation."

anionic (oximate) form of the reactivator at physiological pH. The oximate acts as a nucleophile to displace the organophosphorus moiety from the serine hydroxyl. The cationic moiety interacts electrostatically with an aspartic acid carboxylate at the "anionic" region of the enzyme active site and affords reversible binding of reactivator to the inhibited enzyme. The geometry of the pyridinium oximes places the oximate close to the phosphylated serine so that the nucleophilic displacement reaction proceeds readily.

Although the pyridinium oximes are useful therapeutics, the quaternary ammonium functional group common to this class of compounds limits their antidotal activity insofar as the hydrophilic pyridinium cations penetrate membranes poorly. This causes a disproportionately high serum concentration of the pyridinium reactivators, rapid renal elimination, and marginal antidotal activity in regions (such as the central nervous system) where organophosphorus esters elicit pronounced physiological responses.

In principle, it should be possible to develop nonquaternary AChE reactivators that would not only equal the inherent activity of the pyridinium oximes toward phosphylated AChE but would also exhibit a tissue distribution more like that of hydrophobic organophosphorus esters. We earlier[18] investigated series of α -ketothiohydroximic acid S-esters, given by the general formula, 1:

$$RC_6H_4\overset{O}{\overset{\|}{C}}-\overset{NOH}{\overset{\|}{C}}-S(CH_2)_nNR_2'\cdot HCl$$

1

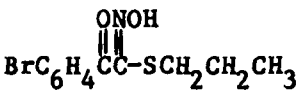
where R was chosen to "fine-tune" the oxime pK_a; the tertiary amine functional group was incorporated to facilitate penetration into hydrophobic tissues while also providing coulombic interaction (via the protonated ammonium salt) with the anionic region of the enzyme active site; and n was varied to optimally separate the cationic and nucleophilic moieties of the reactivator.

Previously[18] we reported the synthesis of nine structurally related

α-ketothiohydroximates and characterized them with respect to structure, acidity, nucleophilicity, and activity in vitro as reactivators of diisopropyl phosphoryl-AChE. These were moderately active reactivators, and we tried to quantitatively compare their activity by determining reactivation kinetics. Unfortunately, diisopropyl phosphoryl-AChE undergoes a side reaction (dealkylation, see below) with a rate comparable to the reactivation reaction. This introduced uncertainty into our determination of reactivation kinetics and thwarted further attempts to establish quantitative structure-activity relationships.

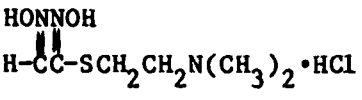
To resolve these issues we have now examined the activity of five thiohydroximates as reactivators of ethyl methylphosphonyl-AChE in vitro. Our objectives were to develop a useful in vitro model to accurately determine kinetics of reactivation and to establish structure-activity relationships for the thiohydroximates as reactivators.

This report describes the synthesis of: 2-(diethylamino)ethyl p-bromobenzoylthiohydroximate (Code SR 3018), 1a (R = Br, n = 2, R' = C_2H_5); 3-(dimethylamino)propyl p-bromobenzoylthiohydroximate, 1d (R = Br, n = 3, R' = CH_3); the related compounds n-propyl p-bromobenzoylthiohydroximate, 2:



2

and 2-(dimethylamino)ethyl thioglyoximate, 3:



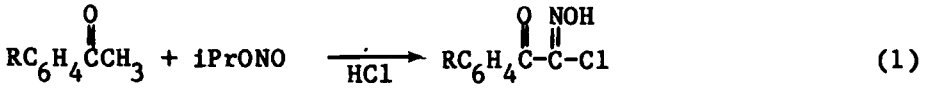
3

We also detail our evaluation of selected thiohydroximates as reactivators in vitro of AChE inhibited by ethyl p-nitrophenyl methylphosphonate (EPMP). For comparison, we examined the activity of 2-hydroxyiminomethyl-1-methylpyridinium iodide, 4 as a reactivator of EPMP-inhibited AChE.

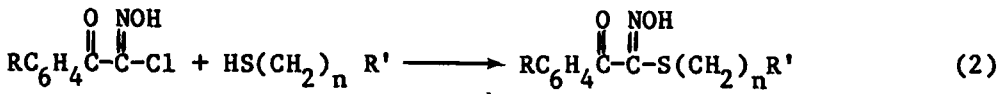
RESULTS AND DISCUSSION

Synthesis and Structure

Table 1 shows structures and pertinent data for compounds used in the current investigation. We prepared the new compounds 1a, 1d, and 2 as previously described[18] for 1b and 1c via reactions (1) and (2):



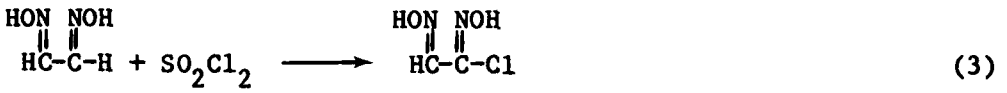
(1)



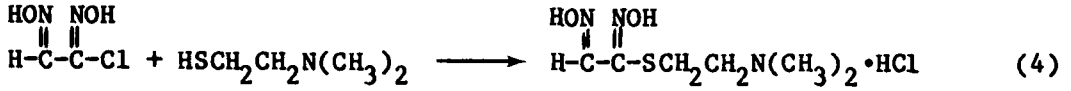
(2)

1 and 2

We obtained the thioglyoximate, 3, by chlorination of glyoxime followed by esterification with 2-(dimethylamino)ethane thiol, reactions (3) and (4):



(3)



(4)

3

Table 1

SELECTED DATA FOR THIOHYDROXIMATES

Compound	Structure	pK_a[a]	NMR[b] (NO-H) δ, ppm	Reference
1a	$4\text{-}BrC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$	7.44	13.01	this work
1b	$4\text{-}MeOC_6H_4C(O)C(NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$	7.82	12.69	18
1c	$4\text{-}MeOC_6H_4C(O)C(NOH)SCH_2CH_2CH_2N(CH_3)_2 \cdot HCl$	8.42	12.50	18
1d	$4\text{-}BrC_6H_4C(O)C(NOH)SCH_2CH_2CH_2N(CH_3)_2 \cdot H_2C_2O_4$	7.66	c	this work
2	$4\text{-}BrC_6H_4C(O)C(NOH)SCH_2CH_2CH_3$	8.40	12.60	this work
3	$(HON)CHC(NOH)SCH_2CH_2N(CH_3)_2 \cdot HCl$	8.37	12.47(br) 11.84(s)	this work

[a]Determined spectrophotometrically in 0.1 M phosphate buffer.
[b]Relative to TMS in DMSO-d_6.
[c]Broad singlet, apparently including oxalate protons.

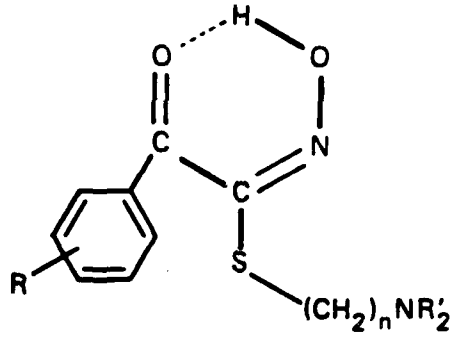
We previously showed that for various 1, oxime acidity correlated well with =NO-H proton NMR chemical shift. Combining data for 1a, 1d, 2, 3, and glyoxime (pK_a = 9.9, NMR = 11.62 δ) with data previously obtained[18] for α-ketothiohydroximates gives equation (5):

$$pK_a = (28.6 \pm 1.2) - (1.62 \pm 0.096)\ \delta \qquad (5)$$

Similarly for 1 with n = 2, R' = C_2H_5, and R = NO_2, H, MeO, and Br, the pK_a data of Table 1 and reference 18 correlate with the Hammett substituent constant σ_p[19] to give equation (6):

$$pK_a = (7.61 \pm 0.005) - (0.78 \pm 0.01)\ \sigma_p \qquad (6)$$

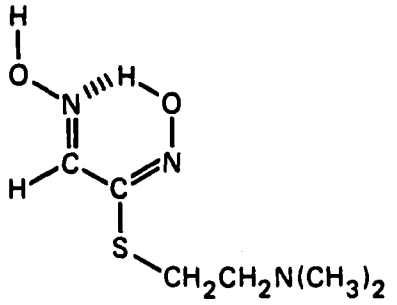
On the basis of IR spectral data, we previously proposed an E-configuration for α -ketothiohydroximates with six-center intramolecular hydrogen bonding of the hydroxyimino proton to the α-carbonyl group. Compounds (1a) and (2) should also exist in the E-configuration.



E-α-Ketothiohydroximate

The NMR spectrum of 3 exhibited two hydroxyimino proton signals: a broad resonance centered at 12.4 ppm and a sharp peak at 11.84 ppm. The methine =C-H proton resonance for 3 was centered at 7.80 ppm. By comparison, the NMR of glyoxime in DMSO-d_6[20] shows hydroxyimino protons at 11.62 and methine protons at 7.83 ppm. These data suggest that one of the hydroxyimino protons in 3 is internally hydrogen-bonded and that

the other retains the same configuration as in glyoxime. A structure consistent with these data is the E, E-thiohydroximate:



E,E-Configuration for 3

Control Experiments

The inhibition of AChE by EPMP and subsequent reactions of ethyl methylphosphonyl-AChE in the presence or absence of oximate reactivators are described by equations (7) through (15):

$$EOH + R_p\text{-}C_2H_5O(Me)P(O)OC_6H_4NO_2 \xrightarrow{k_7} EOP(O)(Me)OC_2H_5\text{-}Sp + HOC_6H_4NO_2 \quad (7)$$

$$EOH + S_p\text{-}C_2H_5O(Me)P(O)C_6H_4NO_2 \xrightarrow{k_8} EOP(O)(Me)OC_2H_5\text{-}R_P + HOC_6H_4NO_2 \quad (8)$$

$$C_2H_5O(Me)P(O)OC_6H_4NO_2 + OX + H^+ \xrightarrow{k_9} C_2H_5O(Me)P(O)OX + HOC_6H_4NO_2 \quad (9)$$

$$EOP(O)(Me)OC_2H_5 + H_2O \xrightarrow{k_{10}} EOH + HOP(O)(Me)OC_2H_5 \quad (10)$$

$$EOP(O)(Me)OC_2H_5 \text{ (or EOH)} + H_2O \xrightarrow{k_{11}} \text{denatured enzyme} \quad (11)$$

$$EOP(O)(Me)OC_2H_5 + H_2O \xrightarrow{k_{12}} EOP(O)(Me)OH + HOC_2H_5 \quad (12)$$

$$EOP(O)(Me)OC_2H_5 + OX + H^+ \underset{k_r}{\overset{K_r}{\rightleftharpoons}} [EOP(O)(Me)OC_2H_5 \cdot OX] \xrightarrow{k_r} EOH + C_2H_5O(Me)P(O)OX \quad (13)$$

$$C_2H_5O(Me)P(O)OX + EOH \xrightarrow{k_{14}} EOP(O)(Me)OC_2H_5 + OX + H^+ \qquad (14)$$

$$C_2H_5O(Me)P(O)OX + H_2O \xrightarrow{k_{15}} \text{products} \qquad (15)$$

$$HOX \underset{}{\overset{K_a}{\rightleftharpoons}} OX + H^+ \qquad (16)$$

Equations (7) and (8) show the reaction of enzyme (EOH) with EPMP to yield ethyl methylphosphonyl-AChE, $EOP(O)Me(OC_2H_5)$. The designations R_p- and S_p- in equations (7) and (8) refer to the stereochemical configuration about the chiral phosphorus atom in EPMP. By analogy with other chiral phosphonate esters[20-23], one isomer of EPMP (probably the R_p- enantiomer) should inhibit AChE more rapidly than the other isomer. For clarity, in reactions (9) through (15) we have omitted designations for the stereoisomers of EPMP and its derivatives.

Reaction (9) shows formation of phosphonyl oxime, $C_2H_5O(Me)P(O)OX$, by direct reaction of EPMP with the oximate form of a reactivator (OX).

Reactions (10) and (11) are unimolecular reactions of inhibited (or uninhibited) enzyme. Reaction (10) is the spontaneous reactivation process and reaction (11) includes any nonspecific reactions that lead to unrecoverable loss of enzyme activity. Reaction (12) shows dealkylation of ethyl methylphosphonyl-AChE to a species, EOP(O)(Me)OH, that cannot be reactivated by oximates.

As shown in reaction (13), the reaction of oximate with inhibited enzyme proceeds via reversible formation of an oximate/inhibited enzyme complex, $[EOP(O)(Me)OC_2H_5 \cdot OX]$, followed by nucleophilic attack on phosphorus to yield active enzyme and phosphonyl oxime. Phosphonyl oxime, in turn, partitions between two pathways: reinhibition of AChE [reaction (14)] or hydrolytic decomposition to noninhibitory products [reaction (15)].

Finally, reaction (16) is the acid-base equilibrium between oximate and oxime (HOX).

The scheme given by reactions (7) through (16) demonstrates the complexity of the AChE-inhibitor-reactivator system. Reactivation kinetics can be accurately determined and meaningfully interpreted only when reaction (13) greatly predominates reactions (9) through (12), (14), and (15).

We originally chose to investigate EPMP as an inhibitor because ethyl methylphosphonyl-AChE reportedly[24] undergoes reactions (10) and (12) very slowly. We planned to circumvent problems associated with reaction (7) and the presence of unreacted inhibitor by adding EPMP to a slight excess of the enzyme. We recognized that reactions (8), (9), and (14) could introduce complicating factors, but anticipated that reaction (13) would predominate under conditions of high reactivator concentration and low enzyme concentration. Under such conditions, reaction (13) is first-order in [AChE] and pseudo-zero-order in [OX]. Reactions (8) and (14), however, are second-order overall, i.e., first-order in [AChE] and also first-order in $[S_p\text{-}C_2H_5O(Me)P(O)C_6H_4NO_2]$ or in $[C_2H_5O(Me)P(O)OX]$, respectively. Thus low enzyme concentrations should favor reaction (13).

To determine the accuracy of these expectations, we first measured enzyme activities under different reaction conditions and at different time points. The values determined were:

A_o = uninhibited AChE activity

A_t = AChE activity at time t

A_{max}(t min) = maximal AChE activity observed (t = duration of experiment)

A_I = ACHE activity after addition of EPMP

The first step was to determine the concentration of EPMP that would inhibit various levels of purified eel AChE to approximately 5% of original activity. For a solution of AChE at a nominal concentration of 65 ACh units mL^{-1}, incubation with 0.40 μM of EPMP yielded maximal inhibition (12% of original activity) within 30 to 60 min. A rough estimate of the rate of the inhibition reaction gave $k_7 \approx 3 \times 10^5\ M^{-1}\ min^{-1}$ at 25°C.

We next examined spontaneous reactivation of inhibited enzyme, reaction (10), as a function of enzyme concentration at 37°C. We inhibited a stock solution of 62.5 ACh U.-mL^{-1} AChE to approximately 5% of original activity. The stock solution of inhibited enzyme was then diluted to three different concentrations (3.13, 0.625, and 0.125 ACh U.-mL^{-1}) of total AChE and incubated for timed intervals. We withdrew aliquots from each incubation solution and diluted the aliquots into substrate solution for assay of activity, choosing the final aliquot volumes and dilution factors to give identical concentrations of total enzyme in the assay solution for all three incubation solutions. In this experiment we determined rate constants for spontaneous reactivation according to equation (17):

$$\ell n(A_I) = k_{10} \cdot t \qquad (17)$$

The results in Table 2 show that both the rate of reaction (10) and the maximal activity observed after 1440 min incubation of inhibited enzyme varied inversely with the concentration of total enzyme in the incubation solution. This indicates that some EPMP remained in solution (probably the less reactive S_p- enantiomer) after the initial rapid inhibition of the stock enzyme solution and that at long reaction times additional enzyme inhibition [reaction (8)] competed with reaction (10).

To probe the effects of enzyme level on reactivation of ethyl methylphosphonyl-AChE by oximates, we repeated the above experiment, diluting the stock solution of inhibited enzyme to three concentrations into 0.010 mM 4. As before, we withdrew aliquots at timed intervals

Table 2

SPONTANEOUS REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT THREE CONCENTRATIONS OF TOTAL ENZYME

Total [AChE],[a] ACh-U.-mL^{-1}	A_o,[b,c] μM min^{-1}	A_I, μM min^{-1}	A_{max} (t = 1440 min), μM min^{-1}	k_{10},[d] min^{-1} x 10^3
0.125	12.5	0.767	3.31	0.85 ± 0.1
0.625	12.5	0.875	2.53	0.45 ± 0.69
3.13	12.5	0.548	1.43	0.35 ± 0.21

[a]Total [AChE] in incubation solution.
[b]AChE activity in assay solution. Not equal to AChE activity in incubation solution.
[c]For definition of symbols, see text.
[d]Calculated according to equation (17), k_{10} is the observed rate constant for spontaneous reactivation.

for assay, choosing aliquot volumes to give the same level of total enzyme in all three assay solutions.

Figure 1 shows the results of this experiment and illustrates two important points. First, the rates of oximate-induced reactivation varied inversely with the level of enzyme in the incubation solution. Second, at the lowest AChE concentration investigated, (4) completely restored AChE activity to control (uninhibited) values.

The effect of AChE level on reactivation rates indicates that reinhibition of the enzyme by phosphonyl oxime [reaction (14)] was significant only at the two higher AChE concentrations. At the 0.125 ACh U.-mL^{-1} level of AChE, enzyme reactivation [reactions (13) and (15)] predominated over the reinhibition reaction, and for this reason we performed all subsequent reactions at the 0.125-ACh U.-mL^{-1} level. The complete restoration of AChE activity by 4 also demonstrated that dealkylation [reaction (12)] was slow compared with reaction (13). We

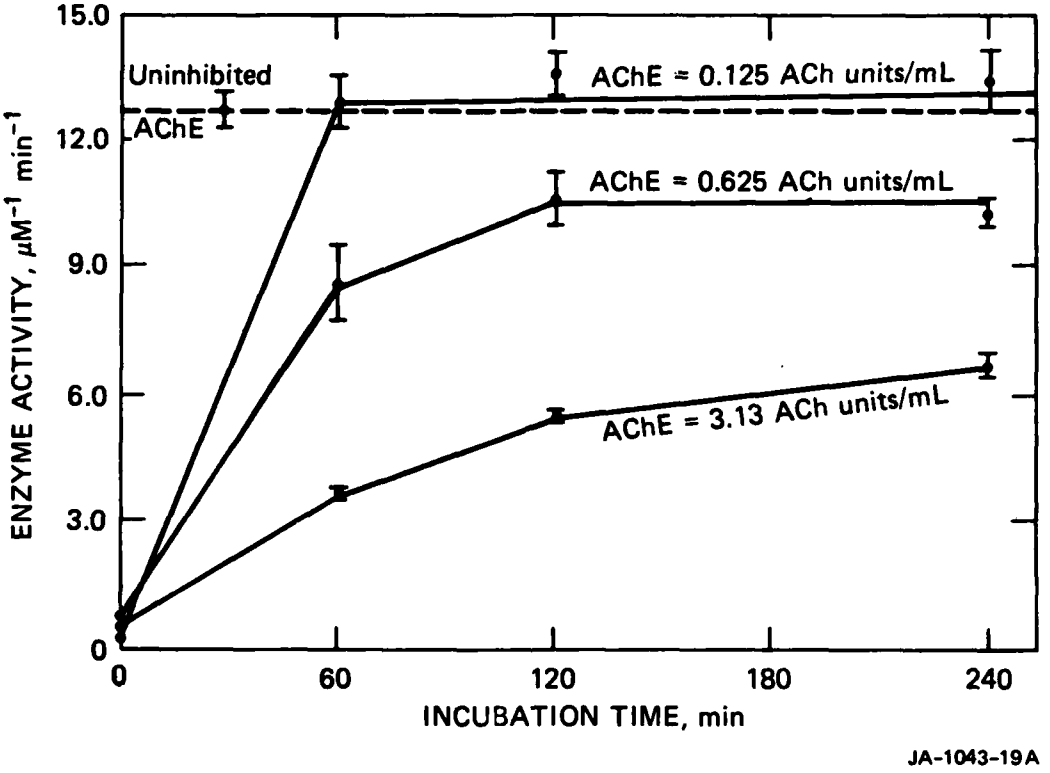


FIGURE 1 REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY 0.010 mM 4 AT 37°C AS A FUNCTION OF TOTAL ENZYME CONCENTRATION IN THE INCUBATION SOLUTION

(Error bars indicate means ± S.E. for n = 4 determinations.)

further proved the unimportance of reaction (12) in a separate experiment by incubating ethyl methylphosphonyl-AChE for timed invervals at 25°C before adding the inhibited enzyme to 1.0 mM 4 and assaying for activity after reactivating the enzyme. In this experiment, 4 reactivated AChE to 106% and 93% of uninhibited enzyme activity after pre-incubation periods of 6 and 28 h, respectively.

As a final control, we studied the oximate-induced reactivation of ethyl methylphosphonyl-AChE as a function of time and concentration of added 3. For comparison we also monitored spontaneous reactivation in the absence of added 3 and the loss of activity of uninhibited enzyme.

Figure 2 shows the results of this experiment and demonstrates that rates of uninhibited enzyme denaturation and spontaneous reactivation of inhibited AChE were slow compared with oximate-induced reactivation. As with 4, reactivation with high concentrations of 3 totally restored AChE activity to control values.

In summary, appropriate control experiments have demonstrated that under suitable reaction conditions the oximate-induced reactivation of ethyl methylphosphonyl-AChE proceeds to the virtual exclusion of complicating side reactions. The EPMP-inhibited eel AChE system appears to be ideally suited to determining reactivation kinetics. Accordingly, we examined rates of reactivation of ethyl methylphosphonyl-AChE as a function of concentration of added 1, 2, 3, and 4. The following section describes the results of this investigation.

Reactivation Kinetics

Incubation of ethyl methylphosphonyl-AChE with a large excess of reactivator restores enzyme activity according to pseudo-first-order kinetics, equation (18):

$$\ell n(100-R) = k_{obs} \cdot t \qquad (18)$$

where:

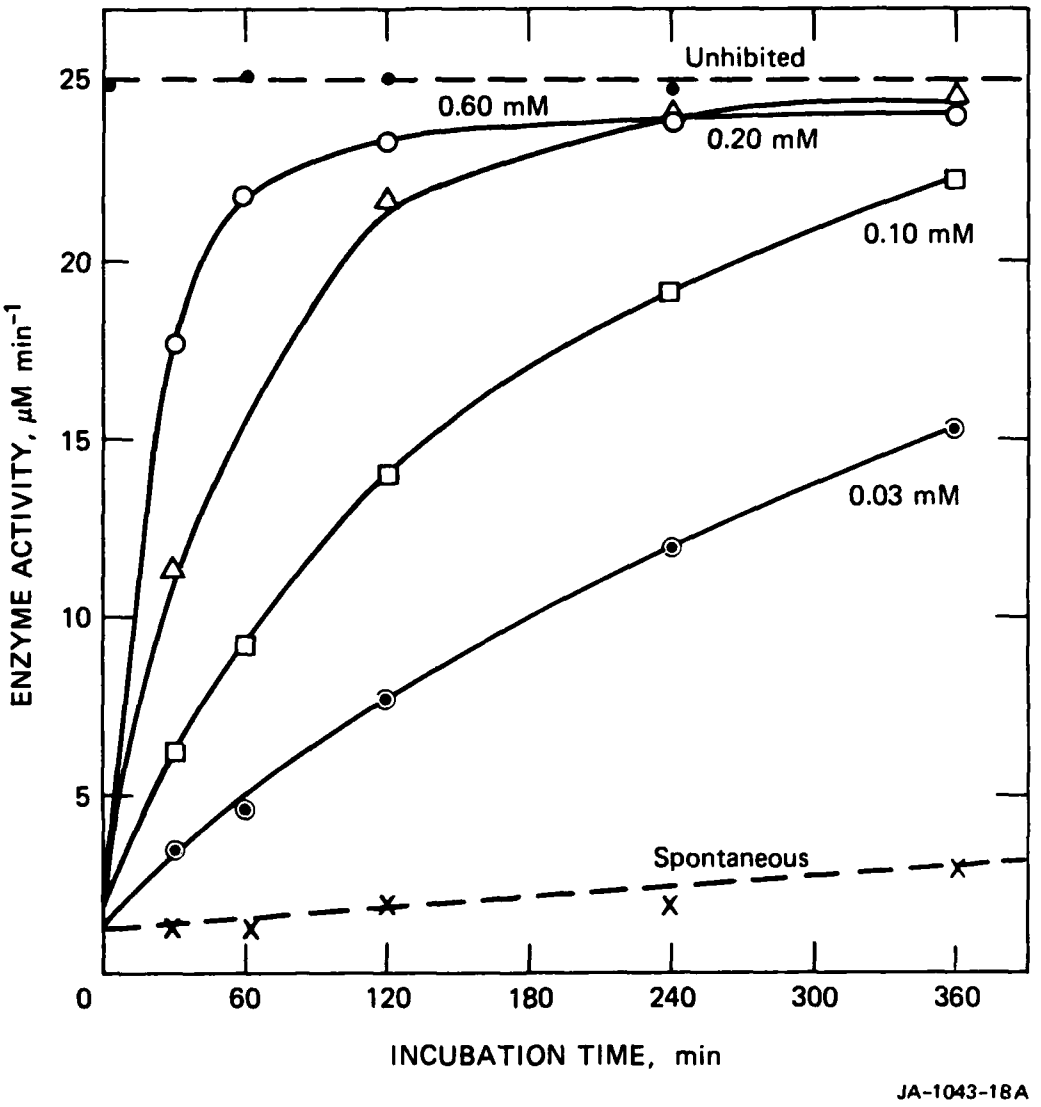


FIGURE 2 REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS CONCENTRATIONS OF 3

k_{obs} = observed first-order rate constant for reactivation

R = percent reactivation

$= 100(A_t - A_I)/(A_c - A_I)$

A_c = control AChE activity

$= A_o$ or $\frac{1}{2}$ $A_o + A_{max}$ [for 4] ,

and A_t and A_I are as defined in the preceding section.

We determined reactivation kinetics for 4 and for the compounds listed in Table 1. The exact procedure is described in the Experimental Details Section. Briefly, we incubated EPMP with a slight excess of AChE for 20 min to produce ethyl methylphosphonyl-AChE. The inhibited enzyme was then diluted into solutions containing various concentrations of reactivators, and aliquots were withdrawn at timed intervals for assay of activity. In each experiment involving a nonquaternary reactivator, we included at least one concentration of 4 to serve as a standard. In experiments where A_{max} for reactivation by 4 was nearly identical to A_o, we defined control activity, A_c, as the mean value of A_o and A_{max}. In cases where reactivation by 4 did not go to completion within the duration of the experiment, we set A_c equal to A_o. Spontaneous denaturation of AChE was negligible in all cases studied. In calculating values for R, we corrected for any contribution to observed activity due to spontaneous reactivation. To do this, we directly determined A_I values at each time point for the experiment and used these values to calculate R.

Table 3 summarizes k_{obs} values and other pertinent data for the kinetic experiments. For most runs, the reaction kinetics adhered well to equation (18); semilogarithmic plots of (100 - R) versus time were typically linear to 90% reactivation for experiments that were followed to high conversion. Figure 3 shows typical data plotted according to equation (18) for several of the test compounds. For clarity, not all of the kinetic runs are plotted in Figure 3. In most runs spontaneous reactivation was negligible compared with oximate-induced reactivation. For some relatively poor reactivators at low concentrations, spontaneous and induced reactivation proceeded at comparable rates, but the contribution due to spontaneous hydrolysis could be accurately subtracted.

Table 3

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) AND RELATED DATA[a] FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS

Experiment	Test Compound[b]	[HOX],[c] mM	A_o, $\mu M\ min^{-1}$	A_I, $\mu M\ min^{-1}$	$A_{max}(t)$[e] $\mu M\ min^{-1}$	A_c, $\mu M\ min^{-1}$	k_{10},[f] $min^{-1} \times 10^3$	k_{11},[g] $min^{-1} \times 10^3$	k_{obs},[h] $min^{-1} \times 10^3$
1	1a	0	9.19	1.99	--	9.19	0.34	0.064	--
		0.0300			4.31(20)				1.63 ± 0.039
		0.100			8.68				7.04 ± 0.081
		0.300			10.7				17.8 ± 0.81
		1.00			9.34				21.6 ± 1.8
	4	0.00150			6.16				3.58 ± 0.41
2	1a	0	11.9	0.397	--	11.7	1.2	< 0.05	--
		0.0600			6.63(300)				2.48 ± 0.11
		0.200			8.97				4.43 ± 0.29
		0.500			11.0				11.8 ± 1.0
	4	0.0100			11.5				18.2 ± 1.8
3	1b	0	12.3	1.94	--	12.3	0.39	0.11	--
		0.0300			1.44(300)				0.398 ± 0.026
		0.100			3.25				1.22 ± 0.046
		0.300			5.84				2.86 ± 0.18
		1.00			7.00				4.15 ± 0.45
	4	0.00150			6.75				3.43 ± 0.40
4	1c	0	10.5	0.243	--	10.7	2.2	< 0.02	--
		0.0300			2.13(360)				0.507 ± 0.024
		0.100			1.69				0.380 ± 0.0082
		0.300			4.04				1.23 ± 0.037
		1.00			5.66				2.07 ± 0.045
	4	0.0100			10.8				23.7 ± 4.3
5	1d	0	9.27	0.191	--	9.27	1.8	--	--
		0.00300			0.323(300)				0.0680 ± 0.0086
		0.0100			0.640				0.223 ± 0.018
		0.100			4.83				2.21 ± 0.018
6	2	0	12.4	0.265	--	12.4	2.9	0.16	--
		0.500			1.78(300)				0.362 ± 0.028
		1.00			2.43				0.581 ± 0.044
		2.00			2.74				0.664 ± 0.035
		3.00[i]			1.51				0.728 ± 0.082
	4	0.00150			7.71				2.98 ± 0.20
7[j]	3	0	25.3	1.21	--	25.0	2.3	0.046	--
		0.0300			15.4(360)				2.26 ± 0.0345
		0.100			22.2				5.60 ± 0.12
		0.200			24.5				15.9 ± 0.41
		0.600			23.4				33.4 ± 3.3
	4	0.0100			24.7				18.3 ± 4.4
8	4	0	11.5	0.195	--	9.56	0.77	--	--
		0.00050			3.67(360)				1.36 ± 0.14
		0.00150			6.60				3.06 ± 0.25
		0.00400			8.53				9.15 ± 0.91
		0.0100			8.68				23.7 ± 3.4

[a] k_{obs} = observed first-order rate constant for reactivation

R = percent reaction

$= 100(A_t - A_I)/A_c - A_I)$

A_c = control AChE activity

$= A_o$ or $1/2 \{ A_o + A_{max}$ [for 4] $\}$.

[b] See Table 1 for structures.

[c] [HOX] = concentration of added test compound.

[d] Reported value of A_I calculated at t = 0 from intercept of least-squares linear regression of A_I data according to equation (17).

[e] All A_{max} values determined at time, t, in minutes, given parenthetically for first A_{max} value in an individual experiment.

[f] Calculated according to equation (17).

[g] Calculated according to the expression $\ln(A_o) = k_{11} \cdot t$.

[h] Calculated according to equation (18).

[i] Control experiment showed that 2 at 3 mM inhibits AChE to 92% control activity. Corrected A_t values for 3m M 2 were calculated by dividing observed A_t values by 0.92.

[j] Data plotted in Figure 2.

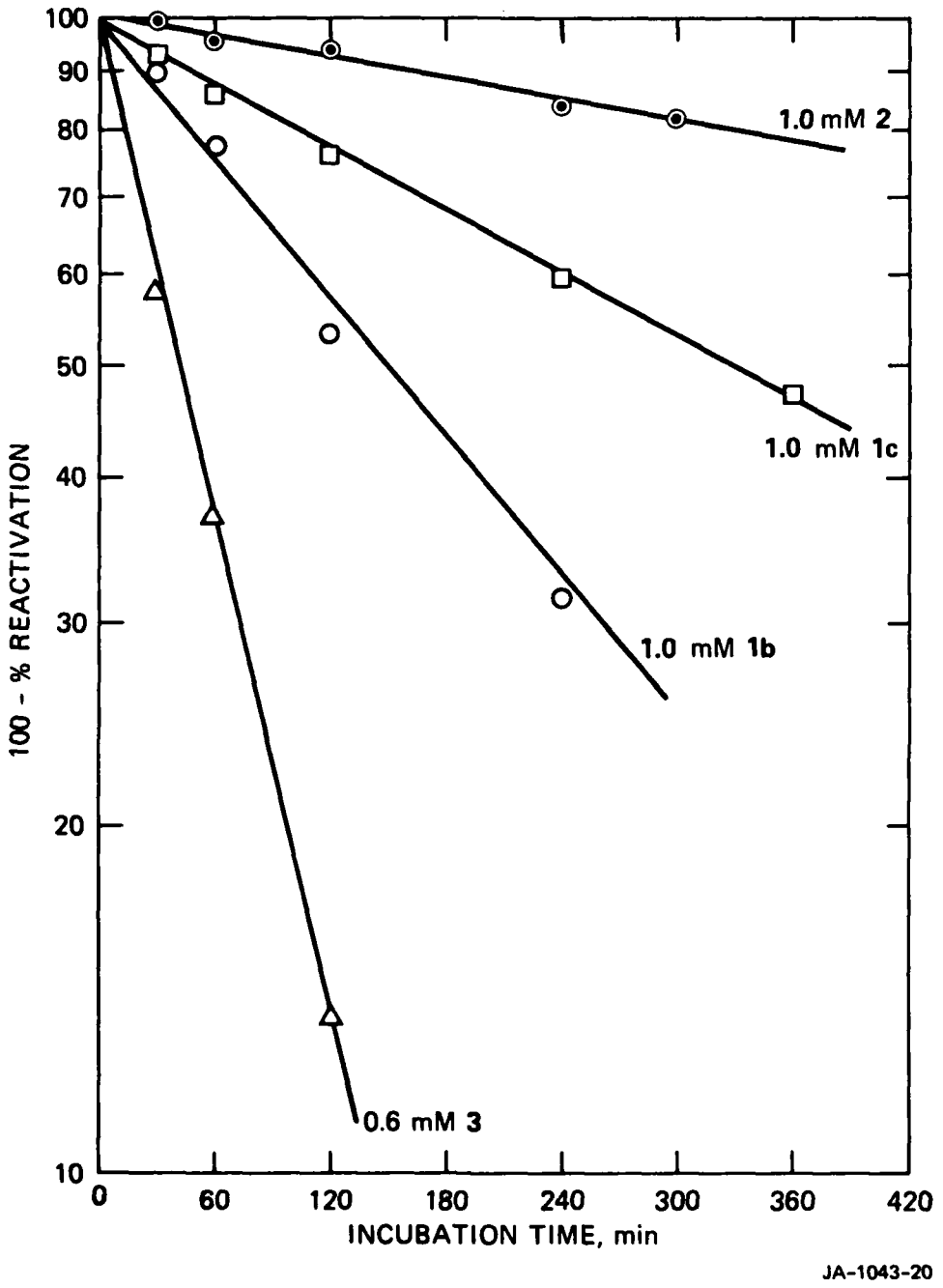


FIGURE 3 SEMILOGARITHMIC PLOT OF (100 - % REACTIVATION) VERSUS INCUBATION TIME FOR REACTION OF ETHYL METHYLPHOSPHONYL-AChE WITH COMPOUNDS: **1b, 1c, 2, AND 3**

To relate observed reactivation kinetics to the mechanism for reactivation shown in reaction (13), we treated the data of Table 3 according to equation (19):

$$(k_{obs})^{-1} = \frac{K_r}{k_r}[OX]^{-1} + \frac{1}{k_r} \qquad (19)$$

where [OX] is the concentration of oximate ion at pH 7.6, calculated from the concentration of added test compound, [HOX] as in equation (20)

$$[OX] = [HOX]\ \{1 + \text{antilog}[pK_a - 7.6]\}^{-1} \qquad (20)$$

The derivation of equation (20) is straightforward and has been described elsewhere.[25-28] We used the concentration of oximate (instead of [HOX]) in equation (19) because the protonated form of the oxime is essentially unreactive as a nucleophile or as a reactivator.

According to equation (19), a plot of $(k_{obs})^{-1}$ versus $[OX]^{-1}$ is linear with slope = K_r/k_r and intercept = $1/k_r$. Such plots therefore permit the calculation of the individual rate constants for reactivation, k_r and K_r. It can also be shown[27-28] that in the limit of low reactivator concentration ($[OX] \ll K_r$) the ratio of k_r to K_r is equivalent to an apparent bimolecular rate constant for reactivation, i.e.:

$$k_r/K_r = k_b \qquad (21)$$

The bimolecular reactivation rate constant, k_b, is a measure of the <u>inherent</u> activity of an oximate as a reactivator of the inhibited enzyme. Because various oximes and thiohydroximates ionize to different extents at pH 7.6, we define an effective rate constant for reactivation of inhibited AChE as the product of k_b and the fraction of added test compound present as oximate at pH 7.6, i.e.,

$$k_{eff} = k_b[1 + \text{antilog}(pK_a - 7.6)]^{-1} \qquad (22)$$

Table 4 summarizes values for reactivation rate constants

Table 4

RATE CONSTANTS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS AT 25°C, AT pH 7.6

Compound[a]	[HOX] mM	$[OX]^{-1}$[b] $M^{-1} \times 10^{-3}$	$(k_{obs})^{-1}$ min	Slope[c] M-min $\times 10^2$	Intercept[c] min	Correlation[c] Coefficient	k_r[d] $min^{-1} \times 10^3$	K_r[d] $M \times 10^4$	k_b[e] M^{-1} min^{-1}	k_{eff}[f] M^{-1} min^{-1}
1a	0.0300	56.4	613.0	1.05 ± 0.14	41.5 ± 35	0.958	24.1	2.53	95.3	56.3
	0.0600	28.2	403.0							
	0.100	16.9	142.0							
	0.200	8.46	226.0							
	0.300	5.64	56.2							
	5.00	3.38	84.7							
	10.0	1.69	30.3							
1b	0.0300	88.5	2060							
	0.100	26.6	763	2.68 ± 0.052	133 ± 24	0.999	7.52	2.02	37.2	14.0
	0.300	8.85	362							
	1.00	2.66	212							
1c	0.100	76.1	2630	3.23 ± 0.36	133 ± 170	0.994	7.52	2.43	30.9	4.06
	0.300	25.4	813							
	1.00	7.61	483							
1d	0.00300	715	14700	2.05 ± 0.0082	34.4 ± 35.4	0.999	29.1	5.96	48.8	22.8
	0.0100	215	4480							
	0.100	21.5	452							
2	0.500	14.6	2760							
	1.00	7.31	1720	11.4 ± 1.3	1050 ± 113	0.986	0.952	1.09	8.77	1.26
	2.00	3.65	1510							
	3.00	2.44	1370							
3	0.0300	230	442	0.188 ± 0.0162	16.4 ± 19.7	0.998	61.2	1.15	534	77.4
	0.100	68.9	179							
	0.200	34.4	62.9							
	0.600	11.5	29.9							
4	0.000500	6910.0	735	0.0104 ± 0.00064	27.8 ± 24	0.996	36.0	0.0374	9630	2780
	0.00150	2300.0	307[g]							
	0.00400	864.0	109							
	0.0100	345.0	43.5[h]							

[a] See Table 1 for structures and pK_a data.
[b] Calculated from equation (20).
[c] From linear least-squares linear regression of data according to equation (19).
[d] From equation (19).
[e] From equation (21).
[f] From equation (22).
[g] Average of four determinations, see Table 3.
[h] Average of five determinations, see Table 3.

calculated from the data of Table 3 using equations (19) through (22). Figure 4 is a double-reciprocal plot of the data according to equation (19) for 1a through 1c, 2, and 3. Figure 4 does not show data for 1d and 4 because of the differences in scale. In examining Table 4, it may be helpful to recall that K_r, by definition, equals $[OX][EOP(O)(Me)OC_2H_5]/[EOP(O)(Me)OC_2H_5 \cdot OX]$ and that a low value for K_r reflects a high proportion of oximate present as the oximate/inhibited enzyme complex.

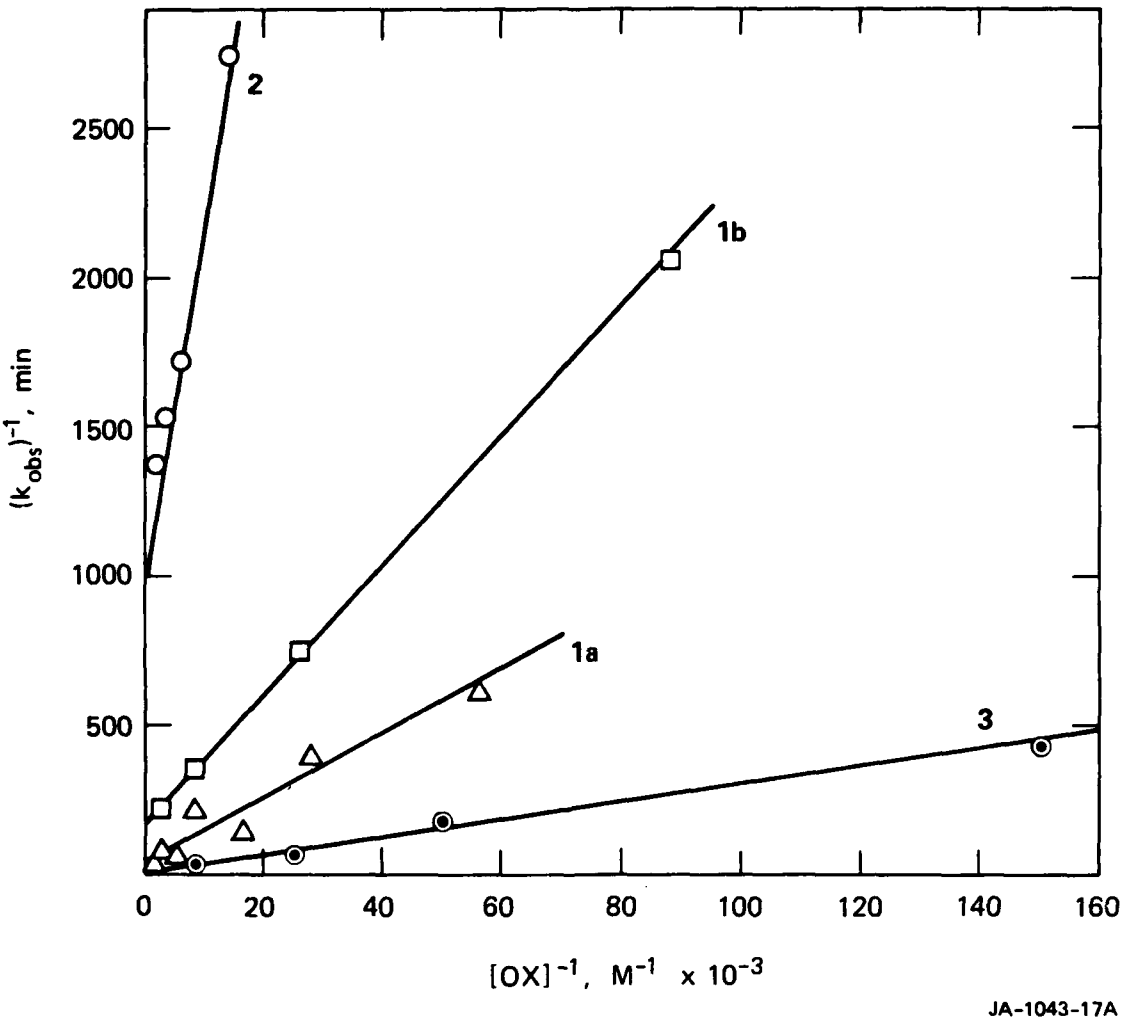


FIGURE 4 RECIPROCAL OBSERVED RATE CONSTANT FOR REACTIVATION, $(k_{obs})^{-1}$, VERSUS RECIPROCAL OXIMATE CONCENTRATION FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY **1a**, **1b**, **2**, AND **3**

Table 4 and Figure 4 demonstrate that the kinetic data conformed well to equation (19). Least-squares linear regression of the data for experiments 1 through 8 gave slopes with reasonably small relative uncertainty. Relative uncertainties were larger in calculations of intercept values, but least-squares correlation coefficients indicated a good fit of the data to equation (19).

The precision of the kinetic data demonstrates the general utility of ethyl methylphosphonyl-AChE as an in vitro model for evaluating AChE reactivators. Other inhibitors have been investigated as in vitro models for reactivation, but most of the literature models suffer important disadvantages. Examples of phosphylated AChE's and complicating factors are 3,3-dimethyl-2-butyl methylphosphonyl-AChE (rapid dealkylation[28,29], incomplete reactivation[30]), cyclopentyl methylphosphonyl-AChE (rapid dealkylation,[31] reinhibition by phosphonyl-oxime[32]), diisopropyl phosphoryl-AChE (rapid dealkylation[20]), ethyl dimethylphosphoramido-AChE (dealkylation[33], low rates of reactivation[34]), and diethyl phosphoryl-AChE (reinhibition[35,36]).

Similarly, for any in vitro model system, different approaches to determining of reactivation kinetics may or may not provide meaningful data. For example, determining percent activity restored to inhibited AChE after incubation with one concentration of reactivator for a single time period is convenient for preliminary screening of novel reactivators,[37,38] but cannot be used to calculate reactivation rates. Determination of observed pseudo-first-order rate constants for reactivation at a single concentration of reactivator is sometimes used to calculate bimolecular rate constants, assuming a simple second-order kinetic expression for the reactivation reaction.[39-41] However this assumption is clearly invalid in view of the well-established[25-28] mechanism for reactivation shown in reaction (13). It is also invalid to calculate rate constants for reactivation according to equation (19) if reinhibition of the enzyme by phosphonyl oxime is demonstrably important under the experimental conditions used.[42]

In summary, there is a definite need for a well-characterized in vitro model system for determining kinetics of reactivation of phosphylated-AChE. Of the many systems described in the literature, few, if any, are wholly satisfactory. By contrast, ethyl methylphosphonyl-AChE proves to be exceptionally useful for kinetic determinations. Under the conditions that we

have chosen, reactivation of ethyl methylphosphonyl-AChE by oximates proceeds without substantial interference from complicating side reactions. The system conforms well to a simple rate expression, and accurate reactivation kinetics can be conveniently determined. The inhibitor (EPMP), enzyme (eel AChE), and standard reactivator (4) reported here are all obtainable conveniently and in high purity, and this should facilitate comparison of data obtained in different laboratories using the single model system.

Structure-Activity Relationships

Having developed a useful *in vitro* model, we wished to examine structure-activity relationships for the thiohydroximates. The goal was to relate specific chemical and structural properties of the nonquaternary reactivators to the kinetic constants k_r, K_r, and k_b. We anticipated, for example, that k_r values might correlate with the inherent nucleophilicity (and hence to the basicity)[18] of the oximates and that K_r values would vary with structural changes (such as replacing the diethylamino moiety in 1a with a methyl group, as found in 2).

Surprisingly, however, the individual values of k_r and K_r did not vary in a systematic fashion with oximate structure or inherent reactivity. Compounds 2 and 3 for example exhibited identical pK_a values, and the two oximates should feature similar Bronsted nucleophilicities. However, compounds 2 and 3 showed, respectively, the lowest and highest k_r values of all the reactivators tested.

Similarly, we expected 2 to exhibit a distinctly low affinity for inhibited enzyme (and hence an unusually high value for K_r) because of all the reactivators reported here only 2 does not feature a protonated or quaternary amine functionality to provide coulombic interactions with the enzyme anionic region. We found, however, the K_r value for 2 to be identical (within a factor of two) with K_r values for 1 and 3.

Thus we conclude that k_r and K_r values cannot be considered independently in elucidating structure-activity relationships for AChE reactivators, that k_r and K_r are coupled, and that only k_b values genuinely reflect the reactivity of oximate reactivators.

To date, the literature has not adequately addressed the interdependence of k_r and K_r values and the mechanistic implications of our findings deserve further comment. In this regard, it is important to recall that K_r is not truly a direct measure of the fraction of total oximate bound to the anionic region of the inhibited enzyme. Rather, K_r derives from observed rate constants for restoration of enzymatic activity, and the value of K_r therefore only reflects the fraction of bound oximate that contributes to reactivation.

Any molecule (including any oximate/inhibited enzyme complex) exists in a finite number of low energy conformations that are accessible at ambient temperature. For a particular oximate/inhibited enzyme complex only those conformations that feature the $=NO^-$ moiety suitably positioned to approach phosphylated serine will result in reactivation. Also, the inherent nucleophilicity of the oximate will determine the rate at which nucleophilic attack on phosphylated serine proceeds for any conformer of reactivator/inhibited enzyme complex. Thus k_r and K_r values depend not only on oximate reactivity but also on geometry of the oximate/inhibited enzyme complex. This interdependence between oximate reactivity and structure necessarily couples k_r and K_r values and precludes interpretation of individual values of these constants.

Differences in oximate/inhibited enzyme complex conformations may also explain the generally lower activity of the nonquaternary reactivators compared with 4. Table 4 shows that K_r for 4 is lower by a factor of approximately 10^2 than K_r values for 1, 2, and 3. In view of the foregoing discussion, we do not believe that the low K_r value for 4 indicates a particularly high affinity of the pyridinium oxime for the inhibited enzyme. Rather, we consider that, as a first approximation, 1, 3 and 4 form oximate/inhibited enzyme complexes equally well. Two observations support this view. First, we observed previously[18] that 1b and its methyl quaternary analog, $MeOC_6H_4C(O)C(NOH)CH_2CH_2N(C_2H_5)\cdot CH_3I$, do not differ greatly in reactivity toward phosphylated AChE. Second, Table 4 demonstrates that 2, which is devoid of an amine functionality, is markedly inferior to 1 and 3 as a reactivator. Thus the protonated dialkylaminoalkyl moiety common to 1 and 3 does provide electrostatic binding of oximate to the inhibited enzyme. Because simple coulombic interactions between the AChE aspartate and the

(quaternary or protonated) ammonium functionalities of 1, 3, and 4 should be roughly equivalent, it seems unreasonable to attribute the comparatively high reactivity of 4 to enhanced affinity toward inhibited AChE. Rather, it is likely that the nonquaternary reactivators differ from 4 primarily with respect to conformational degrees of freedom. Because 1 and 3 are rather flexible, we suggest that the nonquaternary reactivators combine with phosphonylated AChE to yield an oximate/inhibited enzyme complex featuring many low energy conformations, only a small fraction of which have the oximate moiety positioned close to phosphylated serine. In the case of the more rigid molecule, 4, a higher proportion of oximate/inhibited enzyme complex conformations have geometries that favor nucleophilic attack on phosphylated serine.

Thus in terms of free energy of activation for reactivation of phosphylated-AChE, we propose that the nonquaternary reactivators with dialkylaminoalkyl groups and 4 do not exhibit appreciably different enthalpies of activation; the binding affinities are approximately equal in both cases. Rather, the differences in reactivity apparently relate to entropic factors, i.e., to degrees of freedom for the oximate/inhibited enzyme complexes.

CONCLUSIONS

The search for AChE reactivators for treating organophosphorus ester poisoning has continued unabated for the last 25 years. Despite considerable research in this field, surprisingly few investigators have examined details of the in vitro activity of candidate drugs using a well-characterized model system. We believe that accurate determination of reactivation kinetics for structurally related compounds is critical to the rational development of improved therapeutics. Of the many enzyme/inhibitor combinations reported in the literat re, we believe the ethyl p-nitrophenyl methylphosphonate/eel AChE model system described here is the only combination that has been demonstrated to be substantially free of complicating side reactions (such as dealkylation, reinhibition, and spontaneous reactivation) that otherwise interfere with measurement of reactivation kinetics. EPMP is easily synthesized and, though quite toxic, can be safely and

conveniently used to prepare inhibited AChE samples for kinetic experiments. We strongly recommend that the ethyl methylphosphonylated eel AChE be considered as a model system for characterization of any experimental AChE reactivators.

The thiohydroximates reported here proved to be modest AChE reactivators compared with 2-hydroxyiminomethyl-1-methylpyridinium iodide: the thiohydroximates are less reactive toward ethyl methylphosphonyl-AChE by factors of 10^1 to 10^3. An examination of structure-activity relationships for the nonquaternary reactivators suggests that entropic factors (as opposed to low affinity for inhibited AChE) limit the activity of the thiohydroximates. We have argued that the thiohydroximates bind strongly to the anionic region of inhibited AChE, but that only a relatively small fraction of the low energy conformations of the reactivator/inhibited enzyme complex feature an oximate/phosphonyl serine geometry suitable for reactivation of the enzyme. Thus we include rigidity with other important factors (such as nucleophilicity, geometry, and cationic functionality) as a requirement for oximate activity in reactivating of inhibited AChE.

EXPERIMENTAL DETAILS

Materials

Diethyl methylphosphonate (Specialty Organics, Inc.), p-bromobenzophenone (Aldrich Chemical Co), and glyoxime (Aldrich) were used as supplied by the manufacturer. Ethyl p-nitrophenyl methylphosphonate (EPMP) was prepared by reaction of p-nitrophenol with diethyl methylphosphonate and purified by vacuum distillation.[43] CAUTION! EPMP is an extremely toxic anticholinesterase agent. It must be handled with gloves and in a fume hood or at high dilutions at all times.

2-(Diethylamino)ethyl p-bromobenzoylthiohydroximate hydrochloride (1a) was prepared per our previously described method[18] by conversion of p-bromoacetophenone to α-chloro-α-hydroxyimino-p-bromoacetophenone followed by esterification with 2-(diethylamino)ethane thiol and

treatment with HCl. Yield, 29%; m.p. 161-165°C; TLC, R_F = 0.58 ($CHCl_3$:MeOH, 6:1). NMR (DMSO-d_6), δ 13.00 (br, s, 1H, =NOH), 10.64 (br, 1H, NH^+), 7.80 (m, 4H, Ar), 3.4-2.9 (m, 8H, CH_2), 1.18 (t, 6H, CH_3). IR(nujol), ν , 2640(s), 1660(s), 1600(s), 985(s) cm^{-1}. Anal. C,H,N,Cl.

3-(Dimethylamino)propyl p-bromobenzoylthiohydroximate (1d) was prepared as described for 1a, substituting 3-(dimethylamino)propane thiol in the esterification step. Yield, 32%; m.p. 104-106°C; TLC, R_f = 0.45 ($CHCl_3$-MeOH, 6:1). The NMR of 1d in DMSO-d_6 showed no separate resonances for hydroxyimino or oxalic acid protons. However, D_2O exchange removed three proton signals occurring near the aromatic protons at 7.7 ppm. To show the presence of the =NOH proton in 1d, the NMR of the free base of 1d was also determined in $CDCl_3$. NMR data in both solvents follow. DMSO-d_6, δ 7.80 (m, 7H, Ar, =NOH, oxalate), 2.98 (m, 4H, $2CH_2$), 2.65 (s, 6H, $2CH_3$), 1.7 to 2.0 (m, 2H, CH_2). $CDCl_3$, δ 11.40 (s, br, 1H, =NOH), 7.73 (q, 4H, Ar), 2.93 (m, 2H, CH_2), 2.52 (m, 2H, CH_2), 2.18 (s, 6H, CH_3), 1.6 to 1.9 (m, 2H, CH_2).

n-Propyl p-bromobenzoylthiohydroximate (2) was prepared by esterification of α-chloro-α-hydroxyimino-p-bromoacetophenone with propane thiol after purification by flash and thick layer chromatography in silica gel using $CHCl_3$-MeOH (20:1). Yield 15%; m.p. 67-69°C; TLC, R_f = 0.66 ($CHCl_3$:MeOH, 20:1). NMR(DMSO-d_6) δ 12.60 (s, 1H, =NOH), 7.81 (m, 4H, Ar), 2.83 (t, 2H, CH_2), 1.47 (m, 2H, CH_2), 0.86 (t, 3H, CH_3); IR ($CHCl_3$) ν, 3400 (br, m), 1690, (m), 1590, (m), 1220, (s), cm^{-1}. Anal. C, H, N, Cl.

2-(Dimethylamino)ethyl thioglyoximate hydrochloride (3) was prepared from monochloroglyoxime.[44] To a solution of crude monochloroglyoxime (2.5 g, ~ 2 x 10^{-2} mol) and triethylamine (4.0 g, 4.0 x 10^{-2} mol) in 75 mL of $CHCl_3$ was added to 2-(dimethylamino)ethanethiol hydrochloride (2.8 g, 2.0 x 10^{-3} mol). A slight exotherm was noted and the mixture was stirred 2 hours at ambient temperature. The reaction mixture was then concentrated and triturated with two 50-mL portions of diethyl ether. The ether triturations were combined, dried over anhydrous $MgSO_4$, and concentrated to yield 2.0 g of oil, containing oxime as indicated by IR. The residue from the trituration was dissolved in water and extracted repeatedly with CH_2Cl_2. No oxime was

evident (IR) in the CH_2Cl_2 extract. The ether triturations were column chromatographed (20% CH_3OH:80% acetone), and oxime-containing fractions were combined and concentrated, affording an oil insoluble in $CHCl_3$ and acetone but soluble in CH_3OH and dimethyl sulfoxide (DMSO). The oil was dissolved in 10 mL of DMSO, and 100 mL of $CHCl_3$ was added. The solution was cooled to -20°C and scratched to yield 0.150 g (10%) of a white crystalline precipitate. The solid material exhibited a broad melting point range (55-134°C). This suggests that the compound readily loses water on heating in the melting point apparatus. Consistent with this, the field ionization mass spectrum of 1c shows no molecular ion, but a parent peak at mass 141 corresponding to 4-(2-dimethylamino)ethyl-1,2,5-oxadiazole, the product resulting from elimination of HCl and H_2O from 1c. NMR (DMSO-d_6), δ12.47 (br, s, 1H, =NOH); 11.84 (s, 1H, CHNOH) 7.80 (s, 1H, CHNOH); 3.33 (m, 4H, CH_2) and 2.73 ppm (s, 6H, CH_3). IR (KBr), 3300 (s), 2940 (s), 2700 (s), 1460 (s), 1390 (s) and 955 (s) cm^{-1}. Analysis for $C_6H_{14}N_3O_2SCl$. Calc'd: C, 31.65; H, 6.20; N, 18.45; S, 14.08; Cl, 15.56. Found: C, 31.64; H, 6.23; N, 18.44; S, 14.08; Cl, 15.56.

Methods

Acetylcholinesterase determinations in vitro. 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 (0.1%). Enzyme activities were assayed by the Ellman method[45] on a Gilford-modified DU Spectrophotometer coupled to a Digital Electronics Corp. MINC-11 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 (Worthington) 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 determine respectively, rates of enzyme denaturation and spontaneous reactivation. Control experiments demonstrated that

inhibition of AChE by reactivators in the assay solutions and decomposition of reactivators were negligible under the reaction conditions. Observed activities were corrected to net activities by subtracting rates of spontaneous and reactivator-induced substrate hydrolysis.

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 per mL of enzyme solution with 110 μL MOPS buffer to give enzyme "stock" solution. For determining uninhibited AChE activity, dilute 25 μL of stock solution to 20 mL with MOPS buffer 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, and dilute to 4.0 mL in MOPS and assay 50 μL.

For reactivation studies, dilute 100 μL of inhibited AChE solution to 1.0 mL with MOPS buffer, remove 25 μL (for each incubation), and dilute to 1.0 mL with MOPS plus reactivator at known (including zero) concentrations. Remove 50 μL for assay.

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 bis-dithionitrobenzoic acid, plus 10 μL of 0.075 M acetylthiocholine, and monitor increased absorbance at 412 nm versus time.

ACKNOWLEDGMENT

This work was supported by the US Army Medical Research and Development Command Contract DAMD17-79-C-9178. The authors gratefully acknowledge Dr. Brennie Hackley of the US Army Medical Research Institute for Chemical Defense and Dr. Edward Acton of SRI International

for many helpful suggestions. We also thank Dorris Taylor of SRI for pK_a determinations and Dr. Ronald Fleming for developing the MINC program for enzyme kinetics.

LITERATURE CITED

1. Heath, D. F. Organophosphorus Poisons - Anticholinesterases and Related Compounds, Pergamon Press, New York, 1961.

2. Spear, R. C.; Jenkins, D. L.; Milby, T. H.; Environ. Sci. Technol., 1975, 9: 308-313.

3. Milby, T. H.; J. Am. Med. Assoc., 1971, 216: 2131-2133.

4. Koller, W. C.; Klawans, H. L.; Handb. Clin. Neurol. 1979, 37: 541-52.

5. Baker, E. L.; Warren, Mc.; Zack, M.; Dobbin, R. D.; Miles, J. W.; Miller, S.; Alderman, L.; Teeters, W. R.; Lancet, 1978, 1: 31-34.

6. Koelle, G. B.; in The Pharmacological Basis of Therapeutics, L. Goodman and A. Gilman (eds.), MacMillan, New York, 1965; pp. 404-444.

7. Sim, V. M.; in Drill's Pharmacology in Medicine, 3rd Ed., McGraw-Hill, New York, 1965; pp. 971-982.

8. Harris, B. L.; Shanty, F.; Kirk-Othmer Encyclopedia of Chemical Technology, 3rd Ed., Vol. 5, J. Wiley and Sons, New York, 1980; pp. 393-416.

9. Meselson, M.; Robinson, J. P.; Sci. Amer., 1980, 242: 39-47.

10. "CB Weapons Today," in The Problems of Chemical and Biological Warfare, Stockholm International Peace Research Institute, Humanities Press, New York, 1973; Vol. 2, pp. 17-115.

11. Karczmar, A. G.; Int. Encycl. Pharmacol. Ther., 1970, 1(13): 1-40.

12. Usdin, E.; Int. Encycl. Pharmacol. Ther., 1970, 1(13): 47-356.

13. Englehard, N.; Prchal, K.; Nenner, M.; Angew Chem. Internat. Ed., 1967, 6: 615-626.

14. Wills, J. H.; Int. Encycl. Pharmacol. Ther., 1970, 1(13): 357-469.

15. Namba, T.; Nolte, C. T.; Jackrel, J.; Grob, D.; Am. J. Med., 1971, 50: 475-492.

16. Ellin, R. I.; Wills, J. H.; J. Pharmaceut. Sci., 1964, 53: 995-1007.

17. McNamara, B. P.; "Oximes As Antidotes in Poisoning By Anticholinesterase Compounds," Edgewood Arsenal Special Publication. 5B-SP-76004, avail. NTIS AD-A0/23243, 1976.

18. Kenley, R. A.; Howd, R. A.; Mosher, C. W.; Winterle, J. S.; J. Med. Chem., 1981, 24: 1124-1133.

19. Kosower, E. M.; An Introduction To Physical Organic Chemistry, J. Wiley and Sons, New York, 1968, p. 49.

20. Aaron, H. S.; Michel, H.O.; Witten, B.; and Miller, J. I.; J. Am. Chem. Soc., 1958, 80: 456-458.

21. Ooms, A.J.J.; Boter, H. L.; Biochem. Pharmacol., 1965, 14: 1839-1846.

22. Keijer, J. H.; Wolring, G. Z.; Biochim. Biophys. Acta, 1969, 185: 465-68.

23. Schoene, K.; in Medical Protection Against Chemical Warfare Agents, Almquist and Wiksell, Uppsala, 1976, pp. 88-100.

24. Berry, W. K.; Davies, D. R.; Biochem. J., 1966, 100: 572-576.

25. Green, A. L.; Smith, H. J.; Biochem. J., 1958, 68: 28-31.

26. Green, A. L.; Smith, H. J.; Biochem. J., 1958, 68: 32-35.

27. Wang, E.I.C.; Braid, P. E.; J. Biol. Chem., 1967, 242: 2683-2687.

28. Schoene, K.; Steinhanses, J.; Wertmann, A.; Biochim. Biophys. Acta., 1980, 616: 384-388.

29. Michel, H. O.; Hackley, B. E.; Berkowitz, G. L.; Hackley, E. B.; Gillilan, W.; Pandau, M.; Archiv. Biochem. Biophys., 1967, 121: 29-34.

30. De Jong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol.; 1980, 29: 2379-2387.

31. Keijer, J. H.; Wolring, G. Z.; De Jong, L.P.A.; Biochem. Biophys. Acta, 1974, 334: 146-155.

32. DeJong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol. 1978, 27, 2229.

33. De Jong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol., 1978, 27: 2911.

34. Schoene, K.; Oldiges, H.; Archiv. Int. Pharmacodyn. Ther., 1973, 204: 110-23.

35. Schoene, K.; Strake, E. M.; Biochem. Pharmacol., 1971, 20: 1041-1051.

36. Schoene, K.; Biochem. Pharmacol., 1972, 21: 163-170.

37. Hagedorn, I.; Stark, I.; Lorenz, H. P.; Angew. Chem. Int. Ed., 1972, 11: 307-309.

38. Dirks, V. E.; Scherer, A.; Schmidt, M.; Zimmer, G. Arzneim.-Forsch., 1970, 20, 197-200.

39. Krivenchuk, V. E.; Brizgailo, L. I.; Petrunkin, V. E. Biokhim. 1974, 39, 1163-7.

40. Maksimovic, M. Acta. Pharm. Jugoslav., 1976, 26, 233-237.

41. Simon, V.; Wilhelm, K.; Granov, A.; Besarovic-Lazarev, S.; Buntic, A.; Fajdetic, A.; Binenfeld, Z. Archiv. Toxicol., 1979, 1, 301-306.

42. Skrinjaric-Spoljar, M.; Kralj, M. Archiv. Toxicol., 1980, 45, 21-27.

43. Fukuto, T. R.; Metcalf, R. L. J. Am. Chem. Soc., 1959, 80, 372-377.

44. Gaudiano, G.; Ricca, A. Gazz. Chim. Ital., 1959, 89, 587.

45. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M.; Biochem. Pharmacol., 1961, 7, 88-95.

Chapter III

α-HETEROAROMATIC ALDOXIMES AND THIOHYDROXIMATES AS REACTIVATORS OF ETHYL METHYLPHOSPHONYL-ACETYLCHOLINESTERASE

INTRODUCTION

Many organophosphorus (OP) compounds irreversibly inhibit acetylcholinesterase (AChE).[1-7] Therapy of intoxication by these nerve poisons relies on coadministration of cholinolytics (e.g., atropine) and so-called cholinesterase reactivators.[8-11] The latter drugs combine strong reversible binding to inhibited AChE with high inherent nucleophilicity to effect rapid displacement of inhibitor from the enzyme. For all practical purposes, pyridinium aldoximes, such as 2-hydroxyiminomethyl-1-methylpyridinium halide (2-PAM) are the only cholinesterase reactivators currently available for clinical or emergency first-aid application. As quaternary ammonium salts, the pyridinium aldoximes penetrate poorly from the serum into hydrophobic cell membranes. Limited tissue distribution (especially within the central nervous system)[12-14] for pyridinium oximes is a potentially serious disadvantage that could be improved upon, in principle, by development of nonquaternary cholinesterase reactivators.

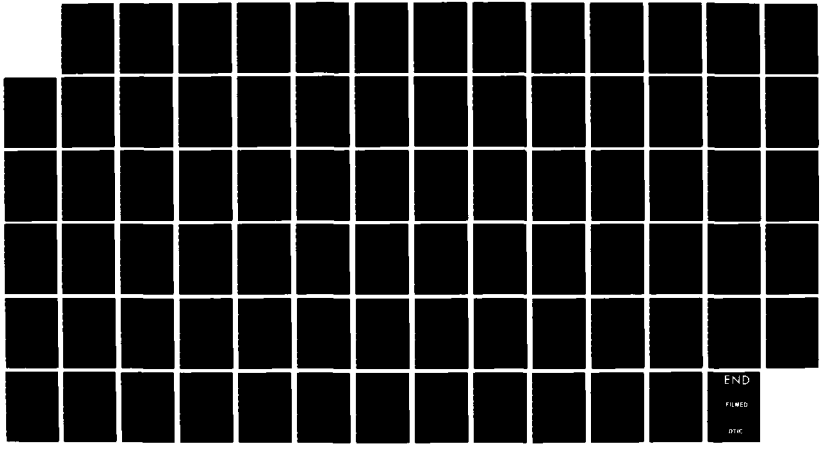
In our search for improved anticholinesterase agent antidotes, we prepared and evaluated[15,16] a series of α-ketothiohydroximic acid dialkylaminoalkyl S-esters, 1.

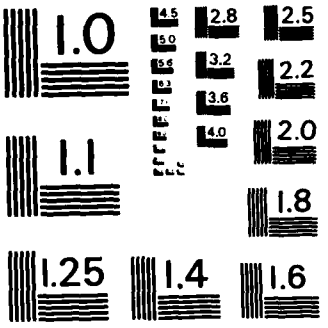
$$RC(:O)C(:NOH)S(CH_2)_nN(R^{1'})_2 \cdot HCl$$

Our objective was to incorporate into a single molecule the following elements: a good nucleophile (the hydroxyiminomethyl moiety); a protonated dialkylaminoalkyl chain to mimic substrate (acetylcholine) binding characteristics; and an electron-withdrawing group (the

AD-A160 562 NONQUATERNARY CHOLINESTERASE REACTIVATORS(U) SRI INTERNATIONAL MENLO PARK CA R A KENLEY ET AL. 30 AUG 82 DAMD17-79-C-9178 2/2

UNCLASSIFIED F/G 6/1 NL



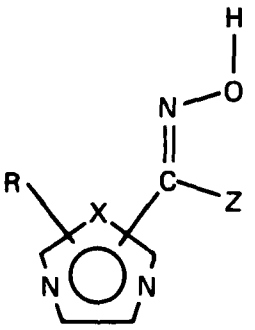


MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963 - A

α-carbonyl) to bring the hydroxyiminomethyl acid dissociation constant near the optimal[6] value of pK_a ~ 8.

We evaluated the type 1 compounds with respect to kinetics of reactivation of diisopropyl phosphoryl-AChE[15] and of ethyl methylphosphonyl-AChE.[16] All of the type 1 compounds investigated reactivate phosphylated[17] AChE, and we established useful structure-activity relationships for the series. However, none of the type 1 compounds compares favorably with 2-PAM in vitro; the best of the nonquaternary compounds, $BrC_6H_4C(:O)C(:NOH)CH_2CH_2N(C_2H_5)_2 \cdot HCl$ (1a) is one fiftieth as potent as 2PAM toward ethyl methylphosphonyl AChE.

In the following sections, we describe the synthesis and characterization of two different classes of cholinesterase reactivators: α-heteroaromatic aldoximes, 2, and the corresponding α-heteroaromatic thiohydroximates, 3. The reactivators are given by the general formula:



2(Z = H) and 3(Z = $SCH_2CH_2NEt_2$)

where X = O, S, or C_6H_5N, and R = H, CH_3, or C_6H_5. Our selection of the 5-membered heterocyclic functionality is based on the report by Benschop et al.[18] that certain thiadiazolyl aldoximes do function as cholinesterase reactivators, although only to a moderate degree. We wished to extend the original work of Benschop et al. to a greater variety of ring systems in order to better define the molecular parameters that govern reactivity of the type 2 compounds toward phosphylated AChE.

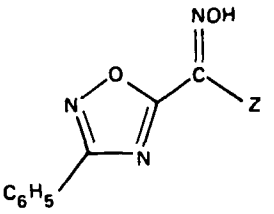
For the type 3 compounds, we anticipated that the protonated dialkylaminoalkyl functionality would contribute binding interactions with anionic regions[7] of the AChE active site and therefore enhance the

inherent reactivity of the hydroxyiminomethyl-heteroaromatic systems. As described in the following sections, we find strict geometric and functional group requirements for reactivity of the heteroaromatic aldoximes toward phosphonylated AChE. We also observe that type 3 compounds that feature a dialkylaminoalkyl moiety exhibit particularly high affinity for the AChE active site. However, this affinity does not necessarily mean that the compounds are potent AChE reactivators.

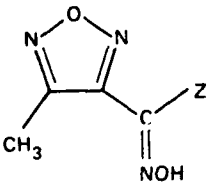
RESULTS AND DISCUSSION

Synthesis and Structure

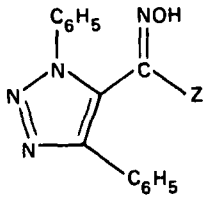
We prepared eight pairs of type 2 and type 3 compounds given by the following structures



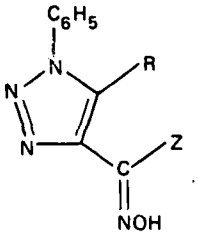
2a (Z = H)
3a (Z = $SCH_2CH_2NEt_2 \cdot HCl$)



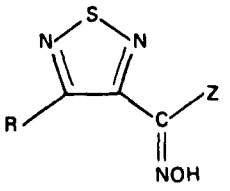
2b (Z = H)
3b (Z = $SCH_2CH_2NEt_2 \cdot HCl$)



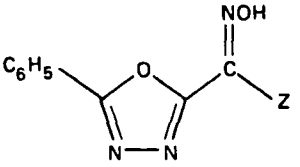
2c (Z = H)
3c (Z = $SCH_2CH_2NEt_2$)



2d (R = C_6H_5, Z = H)
3d (R = C_6H_5, Z = $SCH_2CH_2NEt_2$)
2e (R = H, Z = H)
3e (R = H, Z = $SCH_2CH_2NEt_2$)



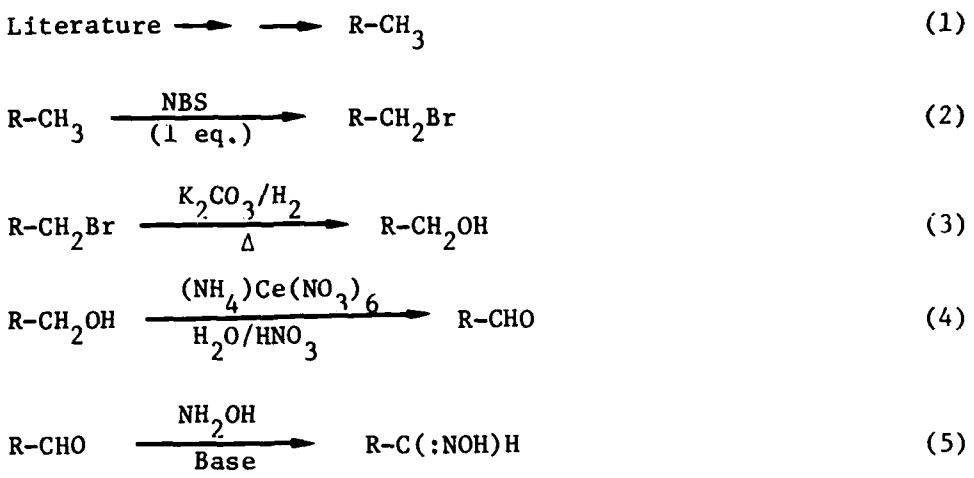
2f (R = H, Z = H)
3f (R = H, Z = $SCH_2CH_2NEt_2$)
2g (R = CH_3, Z = H)
3g (R = CH_3, Z = $SCH_2CH_2NEt_2 \cdot HCl$)



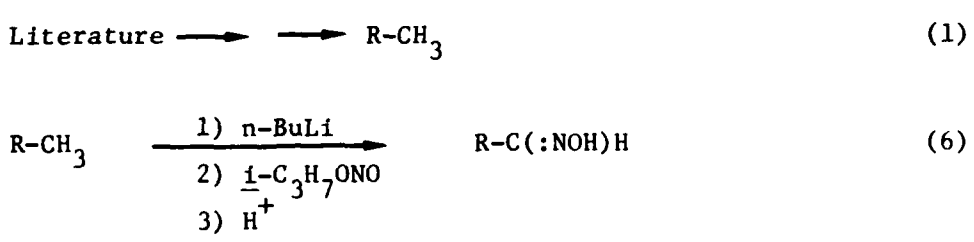
2h (Z = H)
3h (Z = $SCH_2CH_2NEt_2 \cdot HCl$)

We used three different routes to the desired type 2 compounds. These are shown below, where R represents the various heteroaromatic ring systems.

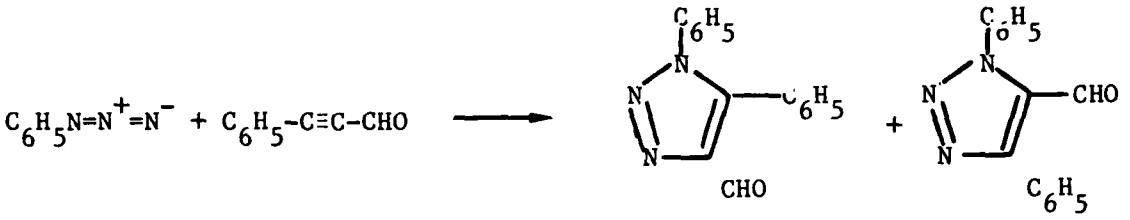
Method A (2b, 2f, 2g)



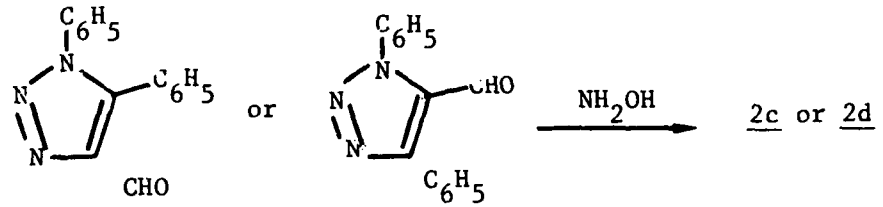
Method B (2a, 2h)



Method C (2c, 2d)



(7)



(5)

We used reactions (4) and (5) of Method A to prepare 2e from the known 1-phenyl-4-hydroxymethyl-1,2,3-triazole.

The experimental section references literature routes to appropriate starting materials and details the preparation of target compounds.

The type 3 compounds were obtained from the corresponding aldoximes via chlorination [reaction (8)] to the hydroximoyl chlorides (type 4 compounds) and subsequent thioesterification [reaction (9)]:

$$R\text{-}C(:NOH)H \xrightarrow{NCS} R\text{-}C(:NOH)Cl \qquad (8)$$

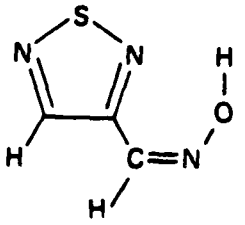
4

$$4 + HSCH_2CH_2NEt_2 \longrightarrow R\text{-}C(:NOH)SCH_2CH_2NEt_2 \qquad (9)$$

Table 1 provides selected data on the type 2 and type 3 compounds prepared to date.

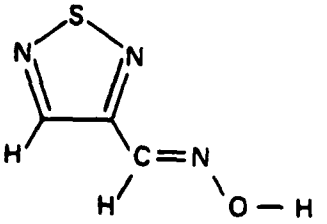
Generally, all three synthetic routes gave low yields of the target compounds. Reactions (2) and (6) proved to be particularly difficult, giving low yields of desired compounds and numerous impurities and unidentified side products. Nevertheless, we successfully obtained enough of the pure test compounds to support our *in vitro* investigations.

Of the eight heteroaromatic aldoximes shown in Table 1, only one (2f) exhibited two sets of NMR proton signals, indicating a mixture of E- and Z-hydroxyiminomethyl isomers. The major component (80%) of the mixture exhibited proton resonances at δ 12.10 (=NO-H), 9.12 (=C-H), and 8.43 (Ar-H), whereas the minor isomer showed peaks at δ 12.53 (=N-OH), 9.50 (=C-H), and 7.95 (Ar-H). For a wide variety of aromatic aldoximes, the isomers with the hydroxyimino hydroxyl group cis to the methine hydrogen exhibit methine proton resonances that are shifted upfield, and ortho-aromatic ring protons that are shifted downfield, from the comparable resonances exhibited by the isomer with hydroxyl group trans to the aldehydic =C-H.[19-22] By analogy, we assign the Z- and E-configurations, respectively, to the major and minor components of the 2f isomer mixture.



Z-2f

(Major Component)

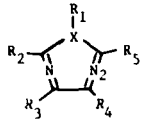


E-2f

(Minor Component)

Table 1

SELECTED DATA FOR α-HETEROAROMATIC ALDOXIMES AND THIOHYDROXIMATES



Code	Compound No.	Method[a]	X	N_1[b]	N_2[b]	R_1	R_2	R_3	R_4	R_5	Yield[c] %	M.P. °C	NMR (=NOH)[d] δ	pK_a[e]	Analysis[f]
4185-17	2a	B	O	2	4	g	g	C_6H_5	g	C(NOH)H	8	161-163	12.87(br)	7.88	C,H,N
4185-54	3a	B	O	2	4	g	g	C_6H_5	g	C(NOH)X[h]	27	199-201(d)	13.88	6.32	C,H,N
4185-94	2b	A	O	2	5	g	g	C(NOH)H	CH_3	g	24	54-56	12.47	9.15	C,H,N
4185-96	3b	A	O	2	5	g	g	C(NOH)X[h]	CH_3	g	41	121-122	13.14	7.59	C,H,N,S,Cl
4185-18	2c	C	N	2	3	C_6H_5	g	g	C_6H_5	C(NOH)H	31	163-165	12.10(br)	9.25	C,H,N[i]
4185-43	3c	C	N	2	3	C_6H_5	g	g	C_6H_5	C(NOH)Y[j]	28	152-155	12.58(br)	k	C,H,N,S
4185-19	2d	C	N	2	3	C_6H_5	g	g	C(NOH)H	C_6H_5	63	194-196	11.47	10.2	C,H,N
4185-41	3d	C	N	2	3	C_6H_5	g	g	C(NOH)Y[j]	C_6H_5	35	185-187	12.13	k	C,H,N,S
4185-85	2e	A	N	2	3	C_6H_5	g	g	C(NOH)H	H	29	171-173	12.17	10.5	C,H,N
4185-88	3e	A	N	2	3	C_6H_5	g	g	C(NOH)Y[j]	H	46	241-243	k	k	C,H,N,S[i]
11879-10	2f	A	S	2	5	g	g	H	C(NOH)H	g	16	148-149	12.10[l] 12.53	9.45	C,H,N,S
11879-14	3f	A	S	2	5	g	g	H	C(NOH)Y[j]	g	18	122-124	k	7.69	C,H,N,S[i]
11879-7	2g	A	S	2	5	g	g	CH_3	C(NOH)H	g	73	131-134	12.05	9.61	C,H,N,S
11879-12	3g	A	S	2	5	g	g	CH_3	C(NOH)X[h]	g	39	179-182	12.72	8.65[m]	C,H,N,S,Cl
4811-5	2h	B	O	3	4	g	C_6H_5	g	g	C(NOH)H	3	189-190	11.37(br)[n]	7.90	C,H,N
4811-28	3h	B	O	3	4	g	C_6H_5	g	g	C(NOH)Y[j]	52	214-215	10.95(br)[o]	6.35	C,H,N,S

a. See text for description of general routes.

b. N_1, N_2 indicates ring positions of heteroatoms.

c. Yield for production of target compound from immediate precursor.

d. In DMSO-d_6 unless otherwise noted.

e. Determined spectrophotometrically in 0.1 M buffer.

f. Analysis agrees within ±0.4% of theoretical unless otherwise noted.

g. No substituent.

h. X = $SCH_2CH_2N(C_2H_5)_2$.

i. See experimental details section.

j. Y $CH_2CH_2N(C_2H_5)_2 \cdot HCl$.

k. Not determined.

l. Peak area ratio 80:20 for 12.53: 12.10 singlets.

m. Determined potentiometrically in 0.1 M $NaClO_4$.

n. In CD_3COCD_3.

o. In $CDCL_3$.

Aldoximes with cis-hydroxyl and aldehydic =C-H groups typically exhibit hydroxyimino proton chemical shifts at higher field than the trans isomers.[19,20] The hydroxyimino resonances for Z- and E-isomers of 2f occurred at high and low field, respectively, opposite to the trend expected by analogy with reported compounds. We consider that the assignment of Z- and E-configurations as shown above for 2f are valid on the basis of NMR data for the methine and ring protons. The anomalous chemical shift observed for the hydroxyimino proton in Z-2f could be a result of intramolecular hydrogen bonding to the ring nitrogen.

We hoped to establish configurations for the other examples of type 2 compounds in Table 1, but such assignments are treacherous in the absence of pairs of E- and Z-isomers or crystallographic data.[21-24] Therefore, at present we do not assign isomeric configurations for hydroxyiminomethyl aldoximes other than 2f.

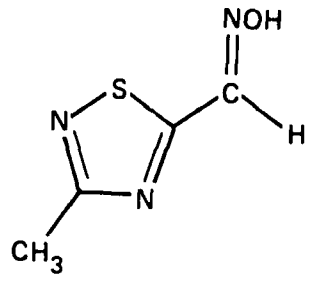
Acidity, Nucleophilicity, and Stability

High nucleophilicity is a primary requirement for cholinesterase reactivators. In accordance with the usual[25,26] Bronsted relationships, nucleophilicities of oximate anions increase with increasing pK_a of the conjugate acid hydroxyiminomethyl groups. The relationship between oximate nucleophilicity and basicity coupled with the requirement for a high degree of dissociation to the oximate form at physiological pH combine to dictate an optimal $pK_a \sim 8$ for cholinesterase reactivators.[6]

We determined pK_a values (using a spectrophotometric technique)[27] for all eight of the type 2 compounds and for five of the type 3 compounds listed in Table 1. We could not determine pK_a valuesfor triazolyl thiohydroximates 3c-3e: these compounds were poorly soluble in water and showed small spectral changes as a function of pH.

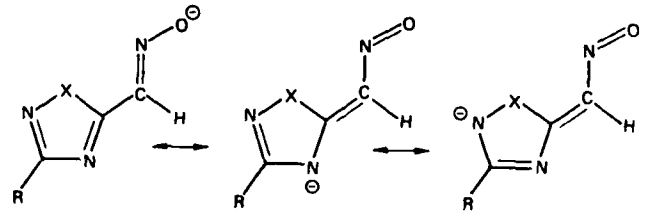
Table 1 shows that the type 2 and 3 compounds spanned a wide range of acidities--from a low of $pK_a = 6.3$ (3a, 3b) to a high of pK_a greater than 10 (2d, 2e). Of the 13 compounds tested, only four (2a, 3b, 3f, 2h) exhibited pK_a values in the useful range 7.5 to 8.5. Typically,

converting type 2 compounds to the corresponding thiohydroximic acid esters lowered the pK_a by approximately 1.5 units. We previously[16] observed this trend for glyoxime (pK_a = 9.9) and the corresponding thioglyoximate, $H(HON:)CC(:NOH)SCH_2CH_2N(Me_2)_2$ (pK_a = 8.4). The strong electron-withdrawing characteristics of the sulfur atom also extend to the heteroaromatic ring systems. Benschop et al.[18] report pK_a = 6.97 for the 1,2,4-thiadiazole 2i, whereas the similar 1,2,4-oxadiazole 2a exhibited pK_a = 7.88.

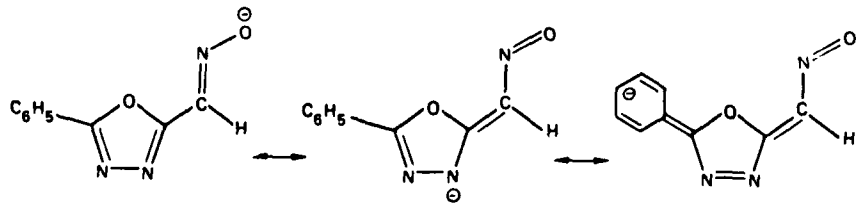


2i

Of the nine substituted heteroaromatic ring systems reported here and in reference 18, aldoximes 2a, 2h, and 2i exhibited the lowest pK_a values. We attribute the highly-electron withdrawing properties of these ring systems to delocalization of negative charge onto two heterocyclic nitrogens (2a, 2i) or onto a ring nitrogen and a phenyl ring (2h).



2a, 2i



2h

To provide a direct measure of the inherent nucleophilicities of the type 2 and type 3 compounds (and also as a control in our AChE assays), we determined bimolecular rate constants for reaction of the compounds with acetylthiocholine (AcSCh).

We incubated three or more concentrations of each test compound with excess AcSCh and monitored thiocholine production versus time. We followed the reactions to very low conversions and observed pseudo-zero-order kinetics. Under these conditions, the rate of thiocholine production obeyed equation (10):

$$+d[\text{thiocholine}]/dt \cdot [\text{AcSCh}]^{-1} = k_n[\text{OX}] + k_o \qquad (10)$$

where $+d[\text{thiocholine}]/dt$ is the observed zero-order rate of product formation; k_o is the pseudo-first-order rate constant for spontaneous hydrolysis of AcSCh; k_n is the bimolecular rate constant for attack by oximate on AcSCh; [OX] is the concentration of added test compound, [HOX], present in the dissociated (oximate) form, and equation (11) governs the [HOX]/[OX] equilibrium:

$$[\text{OX}] = [\text{HOX}] \cdot [1 + \text{antilog}\,(pK_a - pH)]^{-1} \qquad (11)$$

Table 2 summarizes k_n and k_o values for reaction of AcSCh with the type 2 and type 3 compounds.

Linear regression of the data in Table 2 gave the Bronsted relationship, equation (12):

$$\log(k_n) = -3.46\ (\pm\ 0.97) + 0.59\ (\pm\ 0.12)\ pK_a \qquad (12)$$

Within the limits of experimental uncertainty the slope (β value) of equation (12) equaled the value ($\beta = 0.69 \pm 0.08$) previously[15] observed for reaction of type 1 compounds with p-nitrophenyl acetate. These β values are typical[27-29] of reactions of hydroxyiminomethy derivatives

Table 2

RATE CONSTANTS FOR REACTION OF ACETYLTHIOCHOLINE WITH WATER (k_o) AND WITH TYPE 2 AND TYPE 3 COMPOUNDS (k_n) AT 25°C, pH = 8

Compound[a]	pK_a	k_n[b,c] M^{-1}-min^{-1}	k_o[b,d] min^{-1} x 10^3
2a	7.88	51.8 ± 1.3	0.31 ± 0.01
2b	9.15	128 ± 9.0	0.27 ± 0.014
2c	9.25	e	e
2d	10.2	493 ± 8.1	0.26 ± 0.003
2e	10.5	e	e
2f	9.45	207 ± 17	0.51 ± 0.25
2g	9.61	237 ± 33	0.62 ± 0.38
2h	7.90	42.8 ± 1.0	0.15 ± 0.011
3a	6.32	4.35 ± 0.35	0. 6 ± 0.006
3b	7.59	37.7 ± 0.62	0.28 ± 0.0079
3c	e	e	e
3d	e	e	e
3e	e	e	e
3f	7.69	25.6 ± 0.44	0.28 ± 0.005
3g	8.65	71.2 ± 0.36	0.28 ± 0.002
3h	6.35	2.28 ± 0.56	0.12 ± 0.02
			mean = 0.30 ± 0.15

a. See Table 1 for structures.

b. Error limits are ± S.D. from linear least-squares regression analysis.

c. k_n is the bimolecular rate constant for reaction of oximate with 7.5×10^{-4} M AcSCh in 0.1 M phosphate buffer. Calculated according to equations (10) and (11).

d. k_o is the rate constant for spontaneous hydrolysis of 7.5×10^{-4} M AcSCh in 0.1 M phosphate buffer.

e. Not determined.

with acetate esters and demonstrate that the types 1, 2, and 3 compounds behave as nucleophiles in the anticipated fashion.

To check the hydrolytic stability of our test compounds, we determined k_n values for AcSCh hydrolysis as a function of time. For each of the compounds reported in Table 2, observed thiocholine production rates (d[thiocholine]/dt) were invariant to within S.D. = ± 10% over the four- to six-hour incubation period normally used in our in vitro assay. On the basis of these results, we exclude the possibility of significant hydrolytic degradation of the test compounds under the conditions employed for our investigation.

Reversible Acetylcholinesterase Inhibition

We directly determined the degree to which type 2 and type 3 compounds reversibly inhibited AChE activity for two reasons: first to correct for reversible inhibition in our AChE assay and, second, to probe possible correlations between test compound affinity for the enzyme active site and reactivity toward phosphonylated AChE.

We incubated AChE with three or more concentrations of each test compound and assayed for activity at least three times between 30 min and 4 h incubation periods. In all cases, observed activities were invariant with time and we calculated activities as the mean values (± S.D.) for all three time points. For some compounds, inhibition was negligible at concentrations up to 10^{-3} and in these cases, we averaged activities over the range of concentrations tested.

From the observed activities, we calculated the percentage enzyme inhibition, I, according to equation (13):

$$I = 100(A_o - A_I)/A_o \qquad (13)$$

where A_o and A_I, respectively, are AChE activities in the absence and presence of added test compounds. For compounds that showed a strong dependence of AChE activity on inhibitor concentration, we calculated the concentration of inhibitor giving 50% enzyme inhibition (I50 values)

by linear least-squares regression of I versus log[HOX] data. Table 3 summarizes the inhibition data.

From the table, it is clear that the two classes of compounds differed substantially with respect to their ability to reversibly inhibit the enzyme: each of the type 3 compounds inhibited more strongly than the corresponding type 2 compound. As previously[15,16] observed for the type 1 compounds, type 2 compounds did not appreciably inhibit AChE at the concentrations used in our assays. Except for 2d, I50 values were >1 mM for each of the type 2 compounds tested. By comparison, several of the type 3 compounds were rather potent AChE inhibitors. Compound 3a, in particular, was a powerful inhibitor with I50 = 7.5 μM.

To further characterize AChE inhibition by 3a, we determined AChE activities as a function of the concentration of added 3a at various substrate (AcSCh) concentrations.

Figure 1 is a Lineweaver-Burke[30] plot for inhibition by 3a. Linear least-squares regression analysis of the data shown in the figure gave a y-intercept value of 0.139 (± 0.028) X $10^{-6}M^{-1}$-min, indicating that 3a acts as a competitive, reversible inhibitor.

To calculate the equilibrium constant, K_i, for reversible dissociation of the enzyme-inhibitor complex, we used the relationship shown in equation (14)[30]

$$\frac{(\text{slope})_{HOX}}{(\text{slope})_{HOX=0}} = 1 + [HOX] \cdot K_i^{-1} \qquad (14)$$

where $(\text{slope})_{HOX}$ and $(\text{slope})_{HOX=0}$ are, respectively, the slopes of the 1/v versus $[AcSCh]^{-1}$ lines in the presence and absence of added 3a. From the data of Figure 1 and equation (14), we calculated K_i = 0.98 (± 0.03) x 10^{-6} M.

Reactivation of Phosphonylated AChE

We previously[16] investigated the interactions of type 1

Table 3

PERCENTAGE REVERSIBLE INHIBITION (I) OF AChE ACTIVITY
BY TYPE 2 AND TYPE 3 COMPOUNDS

Type 2				Type 3			
Compound[a] No.	[HOX][b] μM	I	I_{50}[c] mM	Compound[a] No.	[HOX][b] μM	I	I_{50}[c] mM
2a	10.0 50.0 100	1.7 ± 3[d]	>1	3a	1.50 5.00 15.0 40.0 50.0	20.3 ± 4[e] 64.3 ± 12 62.1 ± 5 80.6 ± 2 84.0 ± 2	0.0075
2b	10.0 50.0 100	8.7 ± 2[d]	>1	3b	10.0 50.0 100	8.4 ± 1[e] 26.9 ± 1 39.3 ± 1	0.25
2c	f	f	f	3c	2.00 5.00 10.0	5.6 ± 4[d]	>1
2d	2.00 5.00 10.0	0.0 ± 2[e] 3.6 ± 2 17.9 ± 1	0.25	3d	2.00 5.00 10.00	0.0 ± 2[e] 14.9 ± 0.7 24.1 ± 2	0.055
2e	2.00 5.00 10.0	4.8 ± 3[d]	>1	3e	f	f	f
2f	10.0 50.0 100	5.7 ± 0.5[d]	>1	3f	2.00 5.00 10.0	4.0 ± 0.4[e] 13.4 ± 0.72 11.4 ± 0.1	>1
2g	10.0 50.0 100	7.5 ± 2[d]	>1	3g	10.0 50.0 100	9.97 ± 0.5 15.4 ± 1 25.9 ± 0.4	1.1
2h	f	f	f	3h	2.00 5.00 10.0	2.53 ± 0.9 16.9 ± 3 27.0 ± 2	0.039

a. See Table 1 for structures.

b. [HOX] is the concentration of added test compound in the assay solution.

c. I_{50} is the concentration of HOX that inhibits 50% of AChE activity.

d. Mean value for all three concentrations.

e. Mean value for one concentration at three incubation intervals.

f. Not determined.

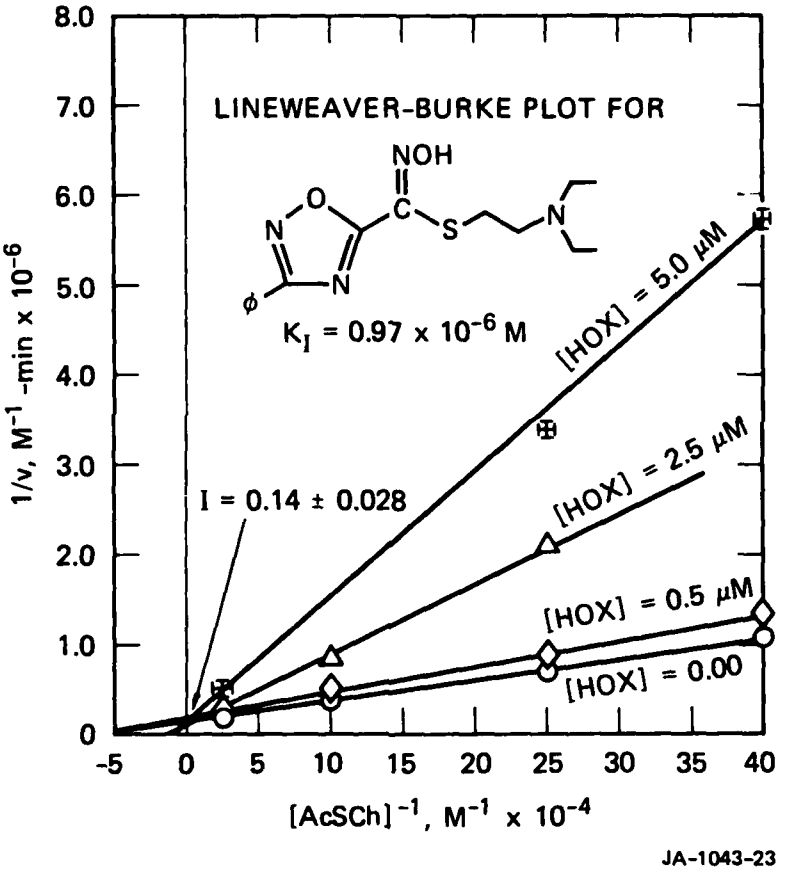


FIGURE 1 LINEWEAVER-BURKE PLOT FOR COMPOUND **3a**

nonquaternary reactivators with ethyl methylphosphonyl-AChE. Using reaction conditions identical to those reported here, we demonstrated that potentially complicating side reactions, such as AChE denaturation, dealkylation ("aging") of ethyl methylphosphonylated AChE, and enzyme inhibition by phosphonyl oxime, proceed at negligibly slow rates compared with rates of oximate-induced reactivation.

Thus, the chemistry of the AChE/ethyl p-nitrophenyl methylphosphonate/oximate system that we used in the current investigations can be satisfactorily described by the reaction set in equations (15)-(20):

$$(C_2H_5O)CH_3P(O)OC_6H_4NO_2 + EOH \xrightarrow{k_i} EOP(O)CH_3(OC_2H_5) \qquad (15)$$

$$EOP(O)CH_3(OC_2H_5) + OX \underset{}{\overset{K_r}{\rightleftharpoons}} [EOP(O)CH_3(OC_2H_5)\cdot OX] \qquad (16)$$

$$[EOP(O)CH_3(OC_2H_5)\cdot OX] \xrightarrow{k_r} EOH \qquad (17)$$

$$EOH + HOX \overset{K_i}{\rightleftharpoons} [EOH\cdot HOX] \qquad (18)$$

$$EOP(O)CH_3(OC_2H_5) + H_2O \xrightarrow{k_{sp}} EOH \qquad (19)$$

$$HOX \overset{K_a}{\rightleftharpoons} OX + H^+ \qquad (20)$$

For this scheme, the following definitions apply:

- EOH = active enzyme
- $EOP(O)CH_3(OC_2H_5)$ = phosphonylated enzyme
- $[EOP(O)CH_3(OC_2H_5)\cdot OX]$ = [phosphonyl enzyme/oximate] complex
- $[EOH\cdot HOX]$ = [enzyme•oxime] complex
- K_r = [phosphonyl enzyme/oximate] dissociation constant
- k_r = [phosphonyl enzyme/oximate] displacement rate constant
- K_i = [enzyme/oxime] complex dissociation constant
- k_{sp} = rate constant for spontaneous reactivation of inhibited enzyme
- K_a = oxime/oximate acid dissociation constant

To determine reactivation kinetics (see the Experimental Details Section and Reference 16 for details), we inhibited AChE to approximately 90% of control activity, then incubated the phosphonylated enzyme with various concentrations of test compounds. At timed intervals, we withdrew aliquots, assayed for activity, and then made appropriate corrections for spontaneous and oximate-induced hydrolysis of the substrate. With reactivator present in large excess over ethyl methylphosphonyl-AChE, restoration of enzyme activity followed pseudo-first-order kinetics according to equation (21):

$$\ln(100 - R) = k_{obs} \cdot t \qquad (21)$$

where k_{obs} is the observed rate constant for oximate-induced reactivation. In equation (21), R is the observed percent reactivation at time t given by equation (22)

$$R = 100 \frac{\{A_t[100/(100 - I)] - A_i\}}{A_c - A_i} \qquad (22)$$

where: A_c, A_i, and A_t, respectively, are observed activities for uninhibited (control) enzyme, enzyme after reaction with ethyl p-nitrophenyl methylphosphonate, and ethyl methylphosphonyl-AChE after incubation for t min with reactivator. In equation (22), observed A_t values are multipled by the factor 100/(100 - I) to correct for inhibition of AChE by added test compound in the assay solution [see equation (13)].

Spontaneous reactivation [reaction (19)] proceeded slowly but at a nonnegligible rate in our system, and we corrected observed reactivation rate constants as in equation (23):

$$(k_{obs})_c = k_{obs} - k_{sp} \tag{23}$$

where k_{sp} was calculated according to equation (24)

$$\ln\left(\frac{A_c - A_i}{A_c}\right) = k_{sp} \cdot t \tag{24}$$

We screened all 16 compounds in Table 1 for activity as reactivators of ethyl methylphosphonyl-AChE, but only seven of the compounds restored enzyme activity at a significant rate. For these 7 compounds, we determined reactivation kinetics. Table 4 summarizes k_{obs} values and related data for these experiments.

Typically, data treatment by linear least-squares regression showed good adherance to equation (21): semilogarithmic plots of 100 - R versus time were linear to high conversions and AChE reactivation was complete on long incubation with high concentrations of the reactivators. Thus, the system was kinetically well-behaved,[16] and we can exclude enzyme denaturation, dealkylation, and reinhibition (by phosphonyl oxime) as significant sources of error in our determinations.

To demonstrate structure-activity relationships among the various reactivators, we examined the dependence of reactivation rate on oximate concentration. For the reaction set given by equations (15) through (20), it can be shown[15,16,31-36] that equation (25) follows:

$$(k_{obs})_c^{-1} = \frac{1}{k_r} + [OX]^{-1} \frac{K_r}{k_r} \tag{25}$$

The constants K_r and k_r define the reactivation process with respect to formation of [inhibited enzyme/oximate] complex and transformation of the complex to active enzyme. In the limit of low reactivator concentration equation (26) follows:

$$k_b = k_r/K_r \tag{26}$$

Table 4

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) AND RELATED DATA FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS[a]

Compound[b] No.	[HOX] in Incubation Solution mM	[HOX] in Assay Solution μM	A_c μM-min^{-1}	A_I μM-min^{-1}	k_{sp}[c] min^{-1} x 10^3	k_{obs}[d] min^{-1} x 10^3
2a	0.500	25.0	13.1	0.746	0.141	43.6 ± 5.3
	0.200	10.0				35.1 ± 0.46
	0.0800	4.00				12.3 ± 0.49
	0.0300	1.50				5.49 ± 0.28
2h	1.00	50.0	8.53	0.68	0.191	6.89 ± 0.86
	0.500	25.0				2.48 ± 0.056
	0.100	5.00				1.05 ± 0.38
	0.050	2.50				0.756 ± 0.056
3a	1.00	50.0	11.8	0.36	0.108	7.69 ± 0.42
	0.300	15.0				2.94 ± 0.069
	0.100	5.00				2.15 ± 0.10
	0.0300	1.50				0.691 ± 0.018
3b	1.00	50.0	11.8	2.28	0.221	2.97 ± 0.30
	0.300	15.0				1.71 ± 0.15
	0.100	5.00				0.848 ± 0.031
	0.0300	1.500				0.482 ± 0.023
3f	1.00	50.0	12.0	2.21	0.191	13.6 ± 0.56
	0.300	15.0				7.41 ± 0.19
	0.100	5.00				1.63 ± 0.34
	0.030	1.50				0.862 ± 0.069
3g	1.00	100	11.6	1.23	0.145	4.25 ± 0.20
	0.300	30				1.94 ± 0.11
	0.100	10				0.890 ± 0.045
	0.0300	3.0				0.300 ± 0.023
3h	1.00	50.0	9.73	1.98	0.196	2.24 ± 0.15
	0.500	25.0				2.03 ± 0.094
	0.100	5.00				0.774 ± 0.027
	0.0500	2.50				0.490 ± 0.028

[a] At pH 7.6, 25°C.

[b] See Table 1 for structures.

[c] k_{sp} is the pseudo-first-order rate constant for spontaneous reactivation of inhibited AChE; see equation (24).

[d] k_{obs} is the observed pseudo-first-order rate constant for induced reactivation of inhibited AChE; see equation (21).

where k_b is a bimolecular rate constant for reactivation and a measure of the inherent reactivity of the oximate form of the reactivator. To account for the degree of dissociation of added test compound to oximate, we define the "effective" bimolecular rate constant, k_{eff} as

$$k_{eff} = k_b \, [1 + \text{antilog}(pK_a - pH)]^{-1} \qquad (27)$$

Table 5 summarizes kinetic constants for reactivation of ethyl methysphosphonyl-AChE by type 2 and type 3 compounds. For comparison, the table also gives data obtained previously for 1a and 2-PAM.

The table demonstrates the range of activities of the compounds investigated: k_{eff} values varied from a low of 6.42 M^{-1}-min^{-1} (3h) to a high of 2780 M^{-1} min^{-1} (2-PAM). Of the nonquaternary reactivators, 2a was the most effective compound. Relative k_{eff} values for 1a:2a:2-PAM were 1:3.3:51, thus 2a exceeded the activity of the best type 1 compound by a factor of three but was 17-times less reactive than 2-PAM.

Interestingly, the thiadiazolyl oxime, 2i, (which shares the 1,2,4-ring substitution pattern with 2a) also proved to be the most effective of the three heteroaromatic aldoximes studied by Benschop et al.[18] Apparently, the 1,2,4-substitution pattern offers significant advantages for reactivation of phosphonylated AChE. The following section examines nonquaternary reactivator structure-activity relationships in greater detail.

Structure-Activity Relationships

The extremely low activities of compounds 2b through 2f as cholinesterase reactivators relates to the very high pK_a values exhibited by these oximes. At pH = 7.6, these reactivators were less than 1% dissociated into the nucleophilic oximate species. Compounds 3c through 3e were poorly soluble in water and, even at saturation concentrations, these compounds did not significantly reactivate ethyl methylphosphonyl-AChE.

Table 5

KINETIC CONSTANTS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE BY VARIOUS TEST COMPOUNDS[a]

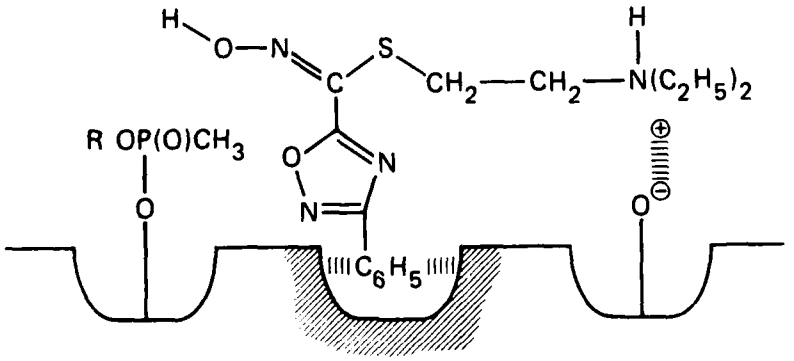
Compound[a] No.	[HOX][b] mM	$[OX]^{-1}$[c] M^{-1}	$(k_{obs})_c^{-1}$[d] min	Slope[e] M-min x 10^2	Intercept[f] min	k_r min^{-1} x 10^3	K_r M x 10^4	k_b[g] M^{-1} min^{-1}	k_{eff}[h] M^{-1} min^{-1}
2a	0.500	5,990	23.0	0.179	9.32 ±	107	1.92	557	186
	0.200	15,000	28.6	0.098	5.27				
	0/0800	37,400	82.2						
	0.0300	99,800	187.						
2h	1.00	3,340	149.	2.44 ±	206 ±	4.85	1.18	40.9	12.2
	0.500	6,690	436.	0.31	115				
	0.100	33,400	1,164						
	0.050	66,900	1.770						
3a	1.00	1,050	132.	4.57 ±	102 ±	9.80	4.48	21.9	20.8
	0.300	3,500	1,353	0.35	64				
	0.100	10,500	490.						
	0.0300	35,000	1,720.						
3b	1.00	1,,930	364.	5.40 ±	360 ±	2.78	1.54	18.5	10.6
	0.300	6,440	671.	0.32	107				
	0.100	19,300	1,590.						
	0.0300	64,400	3,796.						
3f	1.00	2,230	74.6	1.51 ±	75.0 ±	13.3	2.01	66.1	5.41
	0.300	7,430	139.	0.080					
	0.100	22,300	695.						
	0.0300	74,300	1,490.						
3g	1.00	12,100	243.	0.991 ±	135 ± 21	7.41	7.34	101	8.26
	0.300	40,700	556.	0.028					
	0.100	122,000	1,340.						
3h	1.00	1,020	490.	15.2 ±	265 ±	3.77	5.74	6.57	6.42
	0.500	2,040	546.	0.54	62.				
	0.100	10,200	1,730.						
	0.0500	20,400	3,400						
1a	i	i	i	1.05 ± 0.12	36.8 ± 29	27.2	2.85	95.3	56.2
2-PAM	i	i	i	0.0104 ± 0.00064	27.8 ± 24	36.0	0.0374	9630	2780

[a] See Table 1 for structures. 1a is p-$BrC_6H_5C(:NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$.
[b] [HOX] is concentration of added test compound.
[c] Calculated according to equation (11).
[d] Calculated according to equation (23).
[e] Linear least-squares regression slope (± S.D.) according to equation (25).
[f] Linear least-squares regression intercept (± S.D.) according to equation (25).
[g] From equation (26).
[h] From equation (27).
[i] Data from Reference 16.

More informative structure-activity relationships apply to compounds 2a, 3b, 3f, and 2h. Each of these compounds exhibited acid dissociation constants near the optimal value of $pK_a \approx 8$. Nevertheless, reactivation potencies varied substantially: in terms of k_b values, 2a was 10 to 25 times more reactive than 3b, 3f, or 2h toward phosphonylated AChE. Because inherent nucleophilicities for 2a, 3b, 3f, and 2h were essentially identical [see Table 2 and equation (12)], AChE binding forces and geometries must dictate the relative reactivities of these four compounds. For compounds 2a and 2b, we conclude that the 15-fold difference in relative k_b values derives from strict geometric requirements for the phosphonyl enzyme/reactivator complex. Both 2a and 2h feature phenyl-substituted oxadiazolyl aldoxime functionalities, but differ with respect to the relative orientations of the phenyl and hydroxyiminomethyl groups. These two compounds probably bind equally well to the phosphonylated enzyme, in which case the enhanced activity observed for 2a requires that in the [phosphonyl enzyme/reactivator] complex the hydroxyiminomethyl group of 2a is better positioned to attack at phosphorus. Strict geometric requirements are well-known for 2-PAM and the isomeric 3- and 4-hydroxyiminomethyl-1-methylpyridinium reactivators.[6] We believe, however, that similar requirements have not been previously reported for nonquaternary reactivators.

We originally predicted that incorporating a protonated dialkylaminoalkyl functionality into the hydroxyiminomethyl-heteroaromatic structure would enhance reactivator binding to phosphonylated AChE and, hence, reactivation potency. Comparing the relative reactivities of 2a, 3b, and 3f, reveals, however, that this is not necessarily the case. Whether conversion of an α-heteroaromatic aldoxime to the corresponding dialkylaminoalkyl thiohydroximate confers additional affinity of reactivator for the enzyme active site remains unclear (see below), but our data establish that the presence of a dialkylaminoalkyl group is neither a necessary nor a sufficient condition for providing high reactivity toward phosphonylated enzyme.

A final point concerns specific binding interactions for 2a and 3a. Because it features no cationic moieties, 2a cannot bind to AChE via coulombic interactions with anionic centers at the enzyme active site. This leaves hydrophobic forces as the only possible binding interaction between phosphonylated AChE and 2a. It is well-established that hydrophobic regions near the AChE active site control binding of organophosphorus[37,38] and other[39-41] inhibitors as well as binding of N-alkylpyridinium reactivators.[35,36] Therefore it is reasonable for hydrophobic interactions to govern binding of nonquaternary reactivators. In the case of 3a, the unusually high potency of the thiohydroximate as a reversible, competitive inhibitor suggests multiple binding interactions. Specifically, we propose (as shown below) that 3a binds to AChE both via hydrophobic interactions and via electrostatic interactions between the protonated diethylamino group and the AChE anionic site. If so, 3a resembles several adiphenine derivates [e.g., $(C_6H_5)_2CHC(:O)OCH_2CH_2N(C_2H_5)_2 \cdot HCl$] that also exhibit "two-site" binding to AChE.[42]



Possible AChE Binding Interactions for SR4185-54

JA-1043-22

CONCLUSIONS

We prepared a series of α-heteroaromatic aldoximes and thiohydroximates and evaluated the compounds as reactivators of ethyl methylphosphonylated AChE. On the basis of this work and earlier investigations by ourselves[15,16] and others,[18] we can now identify some of the molecular parameters that govern the activity of nonquaternary cholinesterase reactivators.

Hydroxyiminomethyl acid dissociation constants primarily dictate the inherent reactivity of nonquaternary reactivators. Nonquaternary reactivators do not differ from pyridinium aldoximes in this regard: compounds for which $7 < pK_a > 9$ simply do not reactivate phosphonylated AChE at near physiological pH. Our work does elucidate substituent types and patterns that can provide reactivators with pK_a values in the useful range. We find, for example, that the 1,2,4-oxadiazolyl, 1,2,4-thiadiazolyl, and 2-aryl-1,3,4-oxadiazolyl ring systems are highly electron-withdrawing. We also find that converting an α-heteroaromatic aldoxime to the corresponding dialkylaminoalkyl thiohydroximate lowers the hydroxyiminomethyl pK_a value by approximately 1.5 units.

We also observe strict geometrical requirements for α-heteroaromatic aldoximes. Of the ring systems investigated so far, the 3-substituted-5-hydroxyiminomethyl-1,2,4-oxadiazole or 1,2,4-thiadiazole derivatives provide the highest reactivity toward phosphonylated AChE. For these heteroaromatic aldoximes, it appears that hydrophobic forces control binding of the compounds to inhibited enzyme.

Conversion of α-heteroaromatic aldoximes to thiohydroximic acid dialkylaminoalkyl S-esters can enhance affinity of the compounds for the enzyme active site, as evidenced by the relative abilities of the compounds to reversibly inhibit AChE. This enhanced affinity, however, does not necessarily result in increased reactivation potency.

Of the 16 heteroaromatic compounds that we have reported, only one (3-phenyl-5-hydroxyiminomethyl-1,2,4-oxadiazole, 2a) exhibits pronounced

activity as a reactivator of ethyl methylphosphonyl-AChE. Compound 2a is approximately 10 times more reactive than any of the other heteroaromatics we have studied and at least 3 times more reactive than any of the α-ketothiohydroximates reported earlier.[15,16]

In absolute terms, 2a is only a modest reactivator; 2-PAM is 17 times more reactive toward ethyl methylphosphonylated AChE. Whether improved tissue distribution or other pharmacological properties will compensate for the relatively low inherent reactivity of 2a remains to be determined. More significant than the absolute activity of 2a is the fact that we have developed clearer guidelines for the design of improved nonquaternary reactivators. Specifically, we consider that the 1,2,4-oxadiazolyl system provides a likely target for future work. Modifications of the basic 1,2,4-oxadiazolyl system (for example, to increase hydrophobicity) should lead to new compounds with enhanced reactivity.

EXPERIMENTAL DETAILS

Materials

Melting points were obtained in glass capillaries with a Laboratory Devices Mel-Temp apparatus or on a Fisher-Johns melting point apparatus and are presented uncorrected. Infrared spectra were determined with Perkin-Elmer Models 281 and 735B spectrophotometers. Nuclear magnetic resonance spectra were obtained with a Varian Associates EM-360 spectrometer, and chemical shifts are reported in parts per million (δ) relative to an internal tetramethylsilane reference. Microanalyses were performed with a Perkin-Elmer 240 elemental analyzer (C,H,N). Other elements were analyzed at Stanford University analytical laboratory. Elemental analytical results are reported below only for compounds whose observed values were not within ± 0.4% of theoretical.

Materials not described below were prepared or obtained as previously described.[15,16]

Literature methods were used to prepare the following starting materials (desired type 2 compound indicated parenthetically): 3-phenyl-5-methyl-1,2,4-oxadiazole (2a);[43] 3,4-dimethyl-1,2,5-oxadiazole (2b);[44] 1,4-diphenyl-5-formyl-1,2,3-triazole (2c);[45] 1,5,-diphenyl-4-formyl-1,2,3-triazole (2d);[45] 1-phenyl-4-formyl-1,2,3-triazole (2c);[46] 3-methyl-1,2,5-thiadiazole (2f);[47] and 3,4-dimethyl-1,2,5-thiadiazole (2g);[47] 2-methyl-5-phenyl-1,3,4-oxadiazole (2h).[48] As described in the Results and Discussion Section, we prepared the heteroaromatic aldoximes, 2, and converted these to the thiohydroximates, 3, via the hydroximoyl chlorides, 4. The synthesis of each type 2, 3, and 4 compound is detailed below and is given in the order of the individual ring substitution patterns a through h (see Table 1).

3-Phenyl-5-hydroxyiminomethyl-1,2,4-oxadiazole (2a). 5-Methyl-3-phenyl-1,2,4-oxadiazole (1.6 g, 0.01 mol) was dissolved in 40 mL of freshly distilled tetrahydrofuran and cooled to -78°C. To this solution, t-butyllithium (2.0 M, in 6 mL pentane, 0.012 mol) was added slowly.

Then, addition of i-C_3H_7ONO (1.1 g, 0.012 mol) followed by acidification of the solution with 30 mL of 4 N HCl, extraction with three 20-mL portions of ether, drying the combined ether extracts over $MgSO_4$, and removal of solvent yielded 1.2 g of 2a contaminated with starting material. Sublimation of the crude product gave 0.150 g (7.9%) of 2a m.p. 161-163°C. NMR (DMSO-d_6), δ12.87 (br, s, 1H, N-OH); 8.37 (s, 1H, CHNOH); 8.00 (m, 2H, phenylaromatic) and 7.56 ppm (m, 3H, phenylaromatic). IR (KBr) 3225 (s), 2960 (m), 1540 (m), 1430 (s), 1345 (s), 1035 (s) and 990 (s) cm^{-1}. Analysis: C, H, N.

3-Phenyl-1,2,4-oxadiazol-5-yl hydroximoyl chloride (4a). Compound 2a (0.94 g, 5.0 mmol) dissolved in 30 mL of dimethylformamide was treated with 0.67 g (5.0 mmol) of N-chlorosuccinimide. The resulting mixture was stirred 15 min followed by bubbling approximately 15 mL of HCl (g) through the solution. The solution was stirred an additional 15 min,and then was heated to 55°C and allowed to cool slowly (approximately 1 h). The resulting mixture was poured into 200 mL of water and extracted with two 100-mL portions of ether. The ether extracts were combined, washed with three 100-mL portions of water, dried ($MgSO_4$), filtered, and the solvent was evaporated yielding 0.94 g (85%) of a white amorphous solid; m.p. 155-159°C. The crude reaction product was assigned the hydroximoyl chloride structure of 4a, based on the following: NMR (acetone-d6) δ12.70 (s, 1H, NOH), 8.15 (m, 2H, phenyl), and 7.57 ppm (m, 3H, phenyl). Analysis for $C_9H_6N_3O_2Cl$. Cal'd: C, 48.32; H, 2.68; N, 18.79. Found: C, 51.03; H, 2.93; N, 18.24.

3-Phenyl-1,2,4-oxadiazol-5-ylthiohydroximic acid 2-(Diethylamino) ethyl S-Ester Hydrochloride (3a). To the crude hydroximoyl chloride 4a (0.90 g, 4.0 mmol) was added 0.68 g (4.0 mmol) of diethylaminoethanethiol hydrochloride dissolved in 75 mL of chloroform that contained 2 mL of freshly distilled triethylamine. The total reaction volume was increased to 100 mL by adding an additional 25 mL of chloroform, and the resulting solution was stirred overnight. The reaction mixture was then washed with two 100 mL portions of water,

dried ($MgSO_4$) and filtered, and the solvent removed on the rotary evaporator, yielding 1.21 g of 3a. The crude material was purified by column chromatography (silica gel, ether). Fractions of 40 mL were collected and examined by TLC, like fractions were combined and concentrated, yielding 0.35 g (27%) of an analytically pure yellow oil. Analysis: C,H,N.

Purified 3a was converted to its HCl salt by dissolving the chromatographed product in ether followed by the dropwise addition of an ether solution saturated with HCl gas. White crystals of the hydrochloride precipitated from solution and were filtered and dried in vacuum: m.p. 199-201°C (dec); NMR (DMSO-d_6) δ13.88 (br s, 1H, NOH), 8.10 (m 2H, phenyl), 7.69 (m, 3H, phenyl), 3.63 (m, 2H, SCH_2), 3.17 (m, 6H, CH_2), and 1.17 ppm (t, 6H, CH_3).

3-Bromomethyl-4-methyl-1,2,5-oxadiazole*. In a 500-mL round bottom flask were placed 24.5 g (0.25 mol) of freshly distilled 3,4-dimethyl-1,2,5-oxadiazole, 44.5 g (0.25 mol) of N-bromosuccinimide, 1.5 g of benzoyl peroxide, and 600 mL of carbon tetrachloride. The resulting slurry was heated at reflux overnight, filtered hot into 1 L of water and the organic layer subsequently washed with three 250-mL portions of water. The organic layer was dried ($MgSO_4$) filtered and concentrated, yielding 33.5 g of crude product. The NMR of the crude material showed approximately 60% conversion. The monobromide can be distilled at 96-100°C under a water aspirator vacuum; however, the crude material was used in subsequent reactions without further purification: NMR ($CDCl_3$) δ4.59 (s, 2H, CH_2Br) and 2.50 ppm (s, 3H, CH_3).

3-Hydroxymethyl-4-methyl-1,2,5-oxadiazole. In a 500-mL round bottom flask were placed 33.5 g of crude 3-bromomethyl-4-methyl-1,2,5-oxadiazole, 25 g of potassium carbonate, and 350 mL of water. The

*Caution: 3-Bromomethyl-4-methyl-1,2,5-oxadiazole is a severe lachrymator and should be handled accordingly.

resulting mixture was heated to 90°C and stirred for 90 minutes. The resulting aqueous solution was decanted from a small amount of residual oil, saturated with sodium chloride, and extracted with three 75-mL portions of ether. The combined ether extracts were dried ($MgSO_4$), filtered and concentrated, yielding 11.95 g of the crude alcohol. The NMR of the crude alcohol showed a 30% contamination with 3,4-dimethyl-1,2,5-oxadiazole. The 3-hydroxymethyl compound was used without further purification: NMR ($CDCl_3$) δ4.88 (s, 2H, CH_2), 4.55 (br s, 1H, OH) and 2.47 ppm (s, 3H, CH_3).

3-Formyl-4-methyl-1,2,5-oxadiazole. To 100-mL of 3.5N nitric acid was added 11 g of the crude 3-hydroxy-methyl-4- methyl-1,2,5-oxadiazole. To this solution, preheated to 50°C, was added 132 g of ceric ammonium nitrate in 300 mL of 3.5N nitric acid, and the resulting mixture was stirred at 50° for 6 hr. The resulting mixture was cooled and extracted with three 75-mL portions of methylene chloride; the combined extracts were washed with water (2 x 100 mL), 1 N $NaHCO_3$ (100 mL), and brine (100 mL), and dried ($MgSO_4$. Evaporation of solvent yielded 5.0 g of of crude aldehyde contaminated with unreacted 3,4-dimethyl-1,2,5-oxadiazole: NMR ($CDCl_3$) δ10.40 (s, 1H, C(O)H) and 2.62 ppm (s, 3H, CH_3).

3-Hydroxyiminomethyl-4-methyl-1,2,5-oxadiazole (2b). 3-Formyl-4-methyl-1,2,5-oxadiazole (1.12 g, 10 mmol) dissolved in 100 mL of absolute ethanol was treated with 0.7g (10 mmol) of hydroxylamine hydrochloride and 0.79g (10 mmol) of pyridine. The resulting mixture was refluxed for 2 h. The ethanol was removed under aspirator vacuum and the residue taken up in 100-mL of ether. The ether solution was washed with two 50-mL portions of 1 N HCl, dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 750 mg of a white amorphous solid, which proved to be a mixture of the desired oxime contaminated with 3,4-dimethyl-1,2,5-oxadiazole. Recrystallization from 30-60°C petroleum ether yielded 300 mg (23.6%) of analytically pure 2b: m.p. 54-56°C; NMR (DMSO-d_6) δ12.42 (s, 1H, NOH), 8.40 (s, 1H, CHNO), and 2.50 ppm (s, 3H, CH_3). Analysis: C,H,N.

4-Methyl-1,2,5-oxadiazol-3-yl Hydroximoyl Chloride (4b). To 3-

hydroxyiminomethyl-4-methyl-1,2,5-oxadiazole (2b) (230 mg,1.8 mmol) dissolved in 15 mL of dimethylformamide was added with stirring 240 mg (1.8 mmol) of N-chlorosuccinimide. The resulting mixture was stirred at room temperature for 20 min, and 15 mL of HCl gas was bubbled into the reaction mixture. After 30 min, the reaction mixture was heated to 50°C and allowed to slowly cool for 40 min. The mixture was poured into 60 mL of water, and the aqueous solution was extracted with two 50-mL portions of ether. The combined ether extracts were washed with three 50-mL portions of water, dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 280 mg (96%) of the crude hydroximoyl chloride. NMR showed approximately 80% conversion: NMR ($CDCl_3$) δ12.10 (S, 1H, NOH), and 2.50 ppm (S, 3H, CH_3). This material was used without further purification.

4-Methyl-1,2-5-oxadiazol-3-ylthiohydroximic acid 2-(Diethylamino-ethyl S-ester Hydrochloride (3b) To the 230 mg (1.4 mmol) of hydroximoyl chloride dissolved in 50-mL of chloroform was added 240 mg (1.4 mmol) of diethyl-aminoethanethiol hydrochloride and 290 mg (2.8 mmol) of triethylamine. The resulting mixture was stirred overnight, then washed with two 50-mL portions of water, dried over anhydrous $MgSO_4$, filtered, and solvent removed, to yield 350 mg of viscous yellow oil. This material was chromatographed on 125 g of silica gel using ether as eluent, yielding 150 mg (41%) of the free amine 3b as a clear oil. The hydrochloride salt of 3b was prepared by dissolving the oil in 25 mL of ether and adding dropwise an ether solution saturated with HCl gas. White crystals of the hydrochloride precipitated from solution. The crystals were filtered, and dried under vacuum, yielding 170 mg of analytically pure 3b hydrochloride: m.p. 121-122°C; NMR (DMSO-d_6); δ13.14 (s, 1H, NOH), 3.20 (m, 8H, CH_2) and 1.22 ppm (t, 6H, CH_3). Analysis: C,H,N,S,Cl.

1,4-Diphenyl-5-hydroxyiminomethyl-1,2,3-triazole (2c). Phenyl azide[49] (12.0 g, 0.096 mol) and phenylpropargyl aldehyde (12.5 g, 0.1 mol) were added to 65 mL of toluene and heated at reflux overnight. The solvent was removed, and the intermediate 1,4-diphenyl-5-formyl-1,2,3-triazole was separated from the isomeric 1,5-diphenyl-4-formyl-1,2,3-triazole by silica gel chromatography with 4:1 CH_2Cl_2:30-60°C petroleum ether eluent.

Subsequent treatment of the 1,4-diphenyl compound (4.5g, 0.018 mol) with one equivalent of hydroxylamine hydrochloride and recrystallization from CH_3OH per the method of Sheehan and Robinson[45] yielded 1.5 g (31%) of 2c. Recrystallization from 95% ethanol afforded the analytical sample: mp 163-165°C; NMR (DMSO-d_6) δ12.10 (br s, 1H, NOH); 8.22 (s, 1H, CHNOH); 7.88 (m, 2H, phenyl); and 7.60 ppm (m, 8H, phenyl). IR (KBr) 3000 (br, s), 1580 (m), 1480 (s), 1440 (s), 970 (s) and 825 (s) cm^{-1}. Analysis for $C_{15}H_{12}N_4O$. Calc'd: C, 68.17; H, 4.58; N, 21.20. Found: C, 68.05; H, 4.53; N, 20.53.

1,4-Diphenyl-1,2,3-triazol-5-yl hydroximoyl chloride (4c). 1,4-Diphenyl-5-hydroxyiminomethyl-1,2,3-triazole, 2c (1.07 g, 4 mmol) dissolved in 30 mL of dimethylformamide was treated with 0.54 g (4 mmol) of N-chlorosuccinimide. Reaction and workup, similar to those described for 4a, yielded 1.07 g (88%) of a white crystalline material; m.p. 164-168°C. The crude reaction product was assigned the hydroximoyl chloride structure 4c based on the following: NMR (DMK-d_6), δ12.53 (s, 1H, NOH), 7.95 (m, 2H, phenyl) and 7.60 ppm (m, 8H, phenyl). Analysis for $C_{15}H_{11}N_4Cl$. Cal'd: C, 60.30; H, 3.69; N, 18.76; Cl, 11.89. Found: C, 61.50; H, 3.93; N, 18.45; Cl, 10.13.

1,4-Diphenyl-1,2,3-Triazol-5-yl Thiohydroximic Acid 2-(Diethylamino)ethyl S-Ester (3c). Treatment of hydroximoyl chloride 4c (1.029) with diethylaminoethanethiol hydrochloride, as described previously for 4a and 4b, yielded 0.50 g of crude 3c. Recrystallization from 95% ethanol yielded 0.38 g (28%) of analytically pure 3c; m.p. 152-155°C; NMR (DMSO-d_6) δ12.58 (s, 1H, NOH) 7.92 (m, 2H, phenyl), 7.67 (m, 8H, phenyl), 2.20 (m, 8H, CH_2) and 0.70 ppm (t, 6H, CH_3). Analysis: C,H,N,S.

1,5-Diphenyl-4-hydroxyiminomethyl-1,2,3-triazole (2d). Compound 2d was prepared from the corresponding 1,5-diphenyl-4-formyl-1,2,3-triazole (5.0g, 0.020 mol) as described above for 2c. Recrystallization from 95% C_2H_5OH yielded 3.35g (63%) of the desired material: m p. 194-196°C; NMR (DMSO-d_6) δ11.4 (s, 1H, NOH), 8.16 (S, 1H, CHNOH), and 8.20 ppm (m, 10 H,

phenyl); IR (KBr), 3175 (s), 1480 (s), 980 (s), 960 (s), 830 (s), 755 (s) and 680 (s) cm^{-1}. Analysis: C,H,N.

1,5-Diphenyl-1,2,3-Triazol-4-yl Hydroximoyl Chloride (4d). 4-Hydroxyiminomethyl-1,5-diphenyl-1,2,3-triazole, 2d (2.64 g, 10 mmol) was dissolved in 50 mL of dimethylformamide with stirring and N-chlorosuccinimide (1.34 g, 10 mmol) was added. The resulting mixture was stirred 15 min followed by bubbling 15 mL of HCl gas through the solution. After 15 min the solution was heated to 55°C and then allowed to cool slowly to ambient temperature (about one hr). The resulting mixture was poured into 200-mL of water and extracted with two 100-mL portions of ether. The ether extracts were combined, washed with three 100-mL portions of water, dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 2.76 g (92%) of a white crystalline compound: m.p. 179-184°C. The solid material was assigned the hydroximoyl chloride structure 4d based on the following: NMR (DMK-d_6), δ11.25 (s, 1H, NOH) and 7.38 ppm (m, 10H, C_6H_5). Analysis for $C_{15}H_{11}N_4Cl$. Cal'd: C, 60.30; H, 3.69; N, 18.76; Cl, 11.89. Found: C, 61.18; H, 3.72; N, 18.81; Cl, 10.15.

1,5-Diphenyl-1,2,3-Triazol-4-yl Thiohydroximic Acid 2-(Diethylamino)ethyl S-Ester (3d). The hydroximoyl chloride 4d (2.62 g, 8.8 mmol) in 75 mL of chloroform with 1.48 g (9.0 mmol) diethylaminoethanethiol hydrochloride and 6 mL of triethylamine (excess) and the resulting solution stirred overnight. Concentration the crude reaction solution on the rotary evaporator yielded 3.18 g of a white crystalline product. The crude precipitate was triturated with a 60% ethanol/40% water and filtered. The precipitate was dried over P_2O_5, yielding 2.94 g (85%). Two recrystallizations from a 75% chloroform/25% methanol solution yielded analytically pure 3d: NMR (DMSO-d_6) δ12.13 (br s, 1H, NOH), 7.48 (m, 10H, phenyl), 2.55 (m, 8H, CH_2) and 0.89 ppm (t, 6H, CH_3). Analysis: C,H,N,S.

4-Formyl-1-phenyl-1,2,3-triazole. To 5.0 g (28.5 mmol) of 4-hydroxymethyl-1-phenyl-1,2,3-triazole (prepared by the cycloaddition of 2-propyn-1-ol with 1 equivalent of phenyl azide,[38]) was added a 0.5-M solution of ceric ammonium nitrate (32.9 g, 2.1 equivalents) in 120-mL of

water. The reaction mixture was heated to 75°C for 90 min, cooled, and extracted with three 100-mL portions of ether. The combined ether extracts were washed with two 100-mL portions of 1-N $NaHCO_3$ solution, dried over anhydrous $MgSO_4$, filtered, and the solvent was removed yielding light yellow crystals of the crude aldehyde. Column chromatography on silica gel using a methylene chloride as eluent yielded 1.77g (35.4%) of analytically pure aldehyde, m.p. 97-99°C (Lit. m.p. 99-100°C).[46] Analysis: C,H,N.

4-Hydroxyiminomethyl-1-phenyl-1,2,3-triazole (2e). 4-Formyl-1-phenyl-1,2,3-triazole (1.77 g, 10.2 mmol) dissolved in 150 mL of absolute ethanol was treated with 0.77 g (10.2 mmol) of hydroxylamine hydrochloride and 0.81 g of pyridine. The resulting mixture was heated for 2 h, the ethanol evaporated, and the residue taken up in 150 mL of ether. The ether was washed with two 100-mL portions of water, followed by the addition of 100-mL of diethyl ether saturated with HCl gas to remove residual pyridine. Subsequent washing with two additional 100-mL portions of water, drying over anhydrous $MgSO_4$, filtering, and solvent removal yielded 750 mg of a white crystalline product. The material was recrystallized twice from 95% ethanol, yielding 550 mg (29%) of an analytically pure sample of 2e: m.p. 171-173°C; NMR (DMSO-d_6) δ12.17 (s, 1H, NOH), 9.24 (s, 1H, CHNO), 7.98 (m, 2H, phenyl), 7.88 (s, 1H, triazolyl) and 7.55 ppm (m, 3H, phenyl). Analysis: C,H,N.

1-Phenyl-1,2,3-triazol-4-yl Hydroximoyl Chloride (4e). To 2e (250 mg, 1.3 mmol) dissolved in 15 mL of dimethylformamide, 180 mg (1.3 mmol) of N-chlorosuccinimide was added. After 20 min of stirring, the solution was heated to 50°C and allowed to cool; then 10 mL of HCl gas was bubbled into the reaction mixture. After 20 min, the crude reaction mixture was poured into 60-mL of ice-water. Extraction with two 40-mL portions of ether followed by washing the combined extracts with three 50-mL portions of water, drying over anhydrous $MgSO_4$, filtering, and concentrating yielded 260 mg (89%) of a white crystalline product: NMR showed essentially quantitative conversion to the desired hydroximoyl chloride product: NMR ($CDCl_3$), δ11.53 (s, 1H, NOH); 8.8 (s, 1H, CH triazolyl), 7.85 (m, 2H, phenyl) and 7.50 ppm (m, 3H, phenyl). This material was used without further purification.

1-Phenyl-1,2,3-triazol-4-yl Thiohydroximic Acid. 2-(Diethylaminoethyl S-Ester (3e). To 260 mg (1.2 mmol) of 4e dissolved in 50 mL of chloroform, was added 0.20 g of diethylaminoethanethiol hydrochloride and 0.5 g (excess) of triethylamine. The resulting mixture was stirred for two hours, then washed with two 50-mL portions of water. A solid that remained suspended in the chloroform solution dissolved after the addition of 50 mL of ether. The mixture was dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 170 mg (46%) of a white crystalline compound. The crude product was recrystallized twice from petroleum ether (30-60°C), yielding off-white needles of 3e: m.p. 241-243°C; NMR (DMSO-d_6) δ9.15 (s, 1H, CH, triazolyl), 7.95 (m, 2H, phenyl), 7.60 (m, 3H, phenyl), 2.90 (m, 2H, S, CH_2), 2.33 (m, 6H, CH_2) and 0.80 ppm (t, 6H, CH_3). Analysis for $C_{15}H_{21}N_5OS$. Cal'd: C, 56.40; H, 6.63; N, 21.93; S, 10.04. Found: C, 55.41; H, 6.00; N, 23.02; S, 7.52.

3-Bromomethyl-1,2,5-thiadiazole. In a 250-mL round bottom flask was placed 15.30 g (15.3 mmol) of 3-methyl-1,2,5-thiadiazole 29.96 g (16.8 mmol) of N-bromosuccinimide, 0.4-g of benzoyl peroxide, and 150 mL of carbon tetrachloride, and the reaction mixture was heated at reflux overnight. The crude reaction mixture was washed with two 75-mL portions of 1 N sodium thiosulfate and two 50-mL portions of water. The resulting solution was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated, yielding 20.57 g of crude product. An NMR of the product showed a mixture of starting material, plus mono-, di-, and tribromo-substituted products formed during the reaction in a ratio of 15:50:25:10, respectively. This crude product was used in subsequent reactions without further purification. NMR ($CDCl_3$), δ8.63 (s, 1H, CH) and 4.72 ppm (s, 2H, CH_2Br).

3-Hydroxymethyl-1,2,5-thiadiazole. The crude 3-bromomethyl-1,2,5-thiadiazole (prepared above) was treated with 20 g of potassium carbonate in 150 mL of water. The resulting mixture was heated to 100°C and stirred

for 90 min. The crude reaction mixture was then saturated with sodium chloride and extracted with three 100-mL portions of diethyl ether. The combined ether extracts were dried over anhydrous $MgSO_4$, filtered and concentrated, yielding 5.25 g of the desired alcohol, contaminated with a small quantity of the dibromo compound. NMR ($CDCl_3$) δ8.60 (s, 1H, CH), 4.92 (br s, 2H, CH_2) and 4.95 ppm (br s, 1H, OH).

3-Formyl-1,2,5-thiadiazole. 3-Hydroxymethyl-1,2,5-thiadiazole (5.25 g, 45 mmol) was treated with 52 g of ceric ammonium nitrate dissolved in 190 mL of water. The resulting solution was heated to 75°C for 45 min, and the crude reaction mixture was extracted with two 50-mL portions of ether. The combined ether extracts were dried over anhydrous $MgSO_4$, filtered, and the solvent was removed, yielding the desired aldehyde. Isolation of the aldehyde was difficult because the material was volatile and codistilled with ether. As a result of this volatility, the aldehyde was converted to the desired oxime without isolation. NMR ($CDCl_3$) δ10.25 (2, 1H, C(O)H) and 9.10 ppm (s, 1H, CH).

3-Hydroxyiminomethyl-1,2,5-thiadiazole (2f). In a 1-L flask were placed approximately 5.7 g (50 mmol) of crude 3-formyl-1,2,5-thiadiazole in 200 mL of diethyl ether, 3.5 g (50 mmol) of hydroxylamine hydrochloride, 3.9 g of pyridine and 500 mL of absolute ethanol. The resulting mixture was refluxed overnight, concentrated, and then taken up in 200 mL of ether. The ether solution was washed with two 150-mL portions of 1 N HCl followed by two 150-mL portions of water, dried over anhydrous $MgSO_4$, filtered, and concentrated, yielding 1.05 g (16%) of 2f as a white crystalline material. The NMR of the crude product was consistent with an assigned structure of the E- and Z-aldoximes (see Results and Discussion).

Recrystallization from 25% diethyl ether/75% petroleum ether (30-60°C) yielded an analytically pure sample of the mixed E- and Z-aldoximes: m.p. 148-149°C; NMR ($DMSO-d_6$) for Z-aldoxime (major component), δ12.10 (s, 1H, NOH), 9.12 (s, 1H, CHNO), and 8.43 ppm (s, 1H, CH); E-aldoxime (minor component), δ12.53 (s, 1H, NOH), 9.50 (s, 1H, CHNO), and 7.95 ppm (s, 1H, CH). Analysis: C,H,N,S.

1,2,5-Thiadiazol-3-yl Hydroximoyl Chloride (4f). Aldoxime 2f (150 mg, 1.2 mol) dissolved in 15 of mL dimethylformamide was treated with N-chlorosuccinimide (160 mg, 1.1 mmol). Reaction and workup identical to those described previously for 4a yielded 190 mg (100%) of the desired hydroxyimoyl chloride: NMR (DMK-d_6), δ12.07 (s, 1H, NOH), and 9.00 ppm (s, 1H, CH). This material was converted to 3f without further purification or characterization.

1,2,5-Thiadiazol-3-yl thiohydroximic acid 2-(Diethylamino)ethyl S-Ester (3f). To 190 mg (1.2 mmol) of 4f (prepared above in 50 mL of chloroform) was added 210 mg (1.2 mmol) of diethylaminoethanethiol hydrochloride and 250 mg (2.2 mmol) of triethylamine. The reaction mixture was stirred for 2.5 h, then washed with two 100-mL portions of water, dried over $MgSO_4$, filtered and concentrated, yielding a yellow oil.

The oil slowly solidified, was triturated with diethyl ether, and filtered, yielding 55 mg (18%) of 3f as white crystalline material, m.p. 122-124°C. An analytical sample of 3f was obtained by column chromatography on silica gel using ether as eluent: NMR (DMK-d_6) δ9.07 (s, 1H, CH), 2.10 (m, 2H, SCH_2), 2.50 (m, 6H, CH_2) and 0.95 (t, 6H, CH_3). Analysis for $C_9H_{16}N_4S_2$: Cald: C, 41.54; H, 6.15; N, 20.77. Found: C, 41.13; H, 6.26; N, 21.62.

3-Bromomethyl-4-methyl-1,2,5-thiadiazole*. The subject material was prepared in a manner similar to that described above for 3-bromomethyl-1,2,5-thiadiazole. An NMR of the crude reaction product showed a mixture of starting material and monobromide, with an approximate 60% conversion. The material was converted to the alcohol with further characterization. NMR ($CDCl_3$) δ4.55 (2, 2H, CH_2Br) and 2.50 ppm (s, 3H, CH_3).

---

*Caution: 3-Bromomethyl-4-methyl-1,2,5-thiadiazole is a severe lachrymator and should be handled accordingly.

3-Hydroxymethyl-4-methyl-1,2,5-thiadiazole. The crude monobromide, described above, was treated as described previously for 3-bromomethyl-1,2,5-thiadiazole with potassium carbonate in water at 90°C, yielding the crude monoalcohol contaminated with only small quantities of the dibromide. NMR ($CDCl_3$), δ4.80 (br, s, 2H, CH_2OH), and 2.50 ppm (s, 3H, CH_3). The material was oxidized to the corresponding aldehyde without further purification.

3-Formyl-4-methyl-1,2,5-thiadiazole. The crude monoalcohol (12.75 g 0.1 mmol) was treated with 104 g, (2.1 equivalents) of ceric ammonium nitrate dissolved in 400 mL of water. Reaction and workup were as described for the previously prepared aldehydes. Because of the aldehyde's volatility it was converted to the desired oxime without isolation. NMR ($CDCl_3$), δ10.27 (s, 1H, C(O)H) and 2.80 ppm (s, 3H, CH_3).

3-Hydroxyiminomethyl-4-methyl-1,2,5-thiadiazole (2g). In a 1-1L flask were placed 6.4 g (50 mmol) of the freshly prepared aldehyde described above in 225 mL ether, 3.5 g (50 mmol) of hydroxylamine hydrochloride, 3.9g of pyridine and 500 mL of absolute ethanol. The resulting mixture was heated at reflux for two h, and the solvent was evaporated. The residue was dissolved in 75 mL of ether, and the ether solution was washed with two 150-mL portions of 1N HCl followed by 150 mL of water and dried ($MgSO_4$). Removal of solvent afforded 5.2 g (73%) of 2g. An analytical sample was obtained by repeated recrystallization from a 1:1 petroleum ether (30-60°C)/diethyl ether mixture: m.p. 131-134°C. NMR (DMSO-d_6) δ12.05 (s, 1H, NOH), 8.48 (s, 1H, CH), and 2.73 (s, 3H, CH_3). Analysis: C,H,N,S.

4-Methyl-1,2,5-thiadiazol-3-yl Hydroximoyl chloride (4g). A 1.73-g (12 mmol) sample of 2g, (prepared above) dissolved in 60 mL of dimethylformamide, was treated with 1.62 g (12 mmol) of N-chlorosuccinimide. The hydroximoyl chloride 4g was obtained in a manner similar to that described for the previous hydroximoyl chlorides. The NMR indicated an approximate 85% conversion to the hydroximoyl chloride. NMR (DMK-d_6), δ12.88 (s, 1H, NOH) and 2.80 ppm (s, 3H, CH_3). The material was converted to 3g without further purification.

4-Methyl-1,2,5-thiadiazol-3-yl Thiohydroximic acid 2-(Diethylamino)ethyl S-Ester Hydrochloride (3g). To a solution of 1.55 g (8.7 mmol) of 4g in 75 mL of chloroform were added 1.48 g (8.7 mmol) of diethylaminoethanoethiol hydrochloride and 1.76 g (17.4 mmol) of triethylamine and the resulting solution was stirred overnight. The crude reaction mixture was then washed with 100 mL of water, dried over anhydrous $MgSO_4$, filtered, and concentrated. TLC examination of the residue (silica gel, ether) indicated three components. The crude material was purified on 125 g of silica gel using ether as eluent. The first fraction proved to be 2g; NMR and melting point were identical to authentic sample. The second fraction, 0.93 g (39%) of a yellow oil, proved to be the free amine of the desired 3g. The hydrochloride salt was prepared by dissolving the oil in 25 mL of ether and adding dropwise an ether solution saturated with HCl gas. The white crystals which precipitated were filtered and dried under vacuum, yielding 0.98 g of analytically pure 3g: m.p. 179-182°C; NMR (DMSO-d_6) δ12.72 (s, 1H, NOH), 3.18 (m, 8H, CH_2), 2.63 (s, 3H, CH_3) and 1.10 ppm (t, 6H, CH_3). Analysis: C,H,N,S,Cl.

2-Hydroxyiminomethyl-5-Phenyl-1,3,4-Oxadiazole (2h). n-Butyllithium (7.5 ml, 18 mmol of 2.4M solution) was added to a solution of 2.41 g (15 mmol) of 2-methyl-5-phenyl-1,3,4-oxadiazole in 40 ml of tetrahydrofuran at -60°C (temperature rising to -30°C). The dark red solution was allowed to warm to -20° over a 5-min period, then cooled to -60°C. After 10 min, 3.02 mL (22.5 mmol) of isoamyl nitrite was added. After 5 min at -60°, the solution was allowed to warm to 20°C over a 45-min period. Then the solution was cooled to 0°C and 25 mL of 3 M HCl was added, turning the solution yellow. After 1 h of stirring, the layers were separated, and the aqueous layer was extracted with ether (2 x 40 mL). Combined organic layers were washed with water (50 mL) and brine (50 mL) and dried (Na_2SO_4). Removal of solvent yielded 2.35 g of material that was partitioned between 100 mL of saturated sodium carbonate solution and dichloromethane (3 x 50 mL). The dark red aqueous solution was cooled to 0°C, treated with 3 M HCl to pH 6, and extracted with dichloromethane (3 x 50 mL). The organic layer was washed with 50 mL of brine and then dried

(Na_2SO_4). Removal of solvent yielded 0.59 g of material, 0.49 g of which was placed on three silica gel preparative TLC plates and developed with 3% methanol/dichloromethane (3X). The higher R_f major band was extracted with acetone to furnish 0.16 g of solid. This material was placed on two silica gel preparative TLC plates, and the plates were developed with ether. Extraction of the uppermost band with acetone yielded 82.6 mg (3%) of oxime. Recrystallization from acetone/hexanes gave the analytical sample: mp 189-190°C; IR(KBr) 3210, 3150, 3070, 2985,2885, 1610, 1550, 1480, 1455, 1040, 995, 850, and 685 cm^{-1}; NMR (CD_3COCD_3) (δ) 7.4-8.3 (m,5,arom), 8.38 (s,1,H-C=NOH), and 11.37 ppm (br, s, 1, H-C=NOH). Anal: C,H,N.

5-Phenyl-1,3,4-Oxadiazol-2-yl Hydroximoyl Chloride (4h). A mixture of 65.0 mg (0.344 mmol) of 2h, 46.0 mg (0.344 mol) of N-chlorosuccinimide, and a trace of HCl gas in 5 mL of DMF was heated at 50°C for 0.5 h. After cooling, the mixture was poured into 50 mL of water and extracted with ether (2 x 50 mL). The combined ether layers were washed with water (2 x 50 mL and brine (50 mL and dried (Na_2SO_4).
Removal of solvent yielded 62.7 mg (82%) of crude hydroximoyl chloride as a white solid. The material was used without further purification.

5-Phenyl-1,3,4-Oxadiazole-2-yl Thiohydroximic Acid 2-(Diethylamino)ethyl S-Ester Hydrochloride (3h). To a stirred solution of 62.7 mg (0.28 mmol) of 4h and 47.6 mg (0.28 mmol) of 2-diethylaminoethanethiol hydrochloride in 10 ml of dichloromethane was added 0.3 mL of triethylamine. The solution immediately turned yellow.The solution was stirred 1.5 h, diluted to 50 mL with dichloromethane, washed with 50 mL portions of 1M sodium bicarbonate solution and brine, and dried (Na_2SO_4). Removal of solvent yielded 94 mg of material, which was placed on one preparative silica gel TLC plate. After the plate was developed with ether, the lower R_f band was extracted with acetone to provide 46.4 mg (52%) of thiohydroximate as an oil: NMR($CDCl_3$) δ1.11 (t,6,$2CH_3$), 2.50-3.63 (m, 8, $4CH_2$), 7.55 (m, 3, arom), 8.10 (m, 2, arom) and 10.95 ppm (br s, 1, C=NO-H). The thiohydroximate was dissolved in 5 mL

of ethe and ether saturated with HCl gas was added until no further precipitation resulted. Collection of the precipitate afforded 42.1 mg of hydrochloride salt, mp 214-215°C. Anal: C, H, N, S.

Methods

AChE inhibition and reactivation studies were conducted in accordance with the specific method described in reference 16. Briefly, the method involves reacting a slight (10 to 15%) excess of enzyme with ethyl p-nitrophenyl methylphosphonate and incubating the inhibited enzyme with known concentrations of test compound at 25°C in pH 7.6, 0.1 M morpholinopropane sulfonic acid buffer plus NaN_3(0.002%), $MgCl_2$ (0.01 M) and bovine serum albumin (0.01%). At timed intervals, aliquots of the reaction mixture were withdrawn and diluted into pH 8, 0.1 M phosphate buffer for assay of AChE activity using acetyl thiocholine (7.5×10^{-4} M) as substrate and the colorimetric method of Ellman et al.[50]

Reversible inhibition of AChE by thest compounds was measured as above, except that phosphonylation of the enzyme was omitted. Enzyme was incubated with test compounds at three concentrations for 60, 120, and 180 min before aliquots were withdrawn for assay. In all cases, the degree of inhibition was found to be independent of incubation periods, and reported activities for each concentration of test compound are mean values (± standard deviation--S.D.) for all three time points.

Kinetic reactions of test compounds with AcSCh were determined under the conditions used for assaying AChE and therefore served also as correction factors in our reactivation experiments. The experiments were performed under pseudo-zero-order conditions, and rate constants were calculated as described in the Results and Discussion Section.

LITERATURE CITED

1. Heath, D. F. Organophosphorus Poisons--Anticholinesterases and Related Compounds, Pergamon Press, New York, 1961.

2. Koelle, G. B. Chapter 22 in "Anticholinesterase Agents," The Pharmacological Basis of Therapeutics, L. Goodman and A. Gilman (eds.), MacMillan, New York, 1965; pp. 404-444.

3. Sim, V. M. Chapter 60 in "Chemicals Used as Weapons in War," Drill's Pharmacology in Medicine, 3d ed., McGraw-Hill, New York, 1965; pp. 971-982.

4. Harris, B. L.; Shanty, F.; Wiseman, W. J.; Kirk-Othmer Encyclopedia of Chemical Technology, 3d Ed., Vol. 5, J. Wiley and Sons, New York, 1980; pp. 393-416.

5. Karczmar, A. G.; Int. Encycl. Pharmacol. Ther., 1970, 1(13),1-40.

6. Usdin, E.; Int. Encycl. Pharmacol. Ther., 1970, 1(13), 47-356.

7. Englehard, N.; Prchal, K.; and Nenner, M.; Angew. Chem. Internat. Ed., 1967, 6 615-626.

8. Wills, J. H.; Int. Encycl. Pharmacol. Ther. 1970 1(13), 357-469.

9. Namba, T.; Nolte, C. T., Jackrel, J.; Grob, D.; Am. J. Med., 1971, 50, 475-492.

10. Ellin, R. I.; Wills, J. H.; J. Pharmaceut. Sci., 1964, 995-1007.

11. McNamara, B. P.; "Oximes As Antidotes in Poisoning by Anticholinesterase Compounds," Edgewood Arsenal Special Publication 5B-SP-76004.

12. Hobbiger, F.; Vojvodic, V. Biochem. Pharmacol., 1967, 16, 455-462.

13. Milosevic, M. P.; Andjelkovic, D., Nature (Lond.), 1966, 210, 206.

14. Bajgar, J.; Jakl, A.; Hrdina, V.; Biochem. Pharmacol., 1971, 20, 3230-3233.

15. Kenley, R. A.; Howd, R. A.; Mosher, C. W.; Winterle, J. S.; J. Med. Chem.; 1981, 24, 1124-1133. See Chapter I of this report.

16. Kenley, R. A.; Bedford, C. D.; Howd, R. A.; Jackson, S.; Mosher, C. W.; Part II of this Report.

17. We use the term "phosphylation" when we do not distinguish between "phosphonylation" and "phosphorylation."

18. Benschop, H. P.; van den Berg, G. R.; Van Hooidonk, C.; De Jong, L.P.A.; Kientz, G. E.; Berends, F.; Kepner, L. A.; Meeter, E.; Visser, R.P.L.S.; J. Med. Chem., 1979, 22, 1306-1313.

19. Kleinspehn, G. G.; Jong, J. A.; Studniare, S. A.; J. Org. Chem., 1967, 32,460-462.

20. Benschop, H. P.; Van Oosten, A. M.; Platenburg, D.H.J.M.; Van Hooidonk, C.; J. Med. Chem., 1970, 13, 208-1212.

21. Poziomek, E. J.; Kramer, D. N.; Fromm, B. W.; Mosher, W. A.; J. Org. Chem.,1961, 26, 423-427.

22. Poziomek, E. J.; Kramer, D. N.; Mosher, W. A.; Michel, H. O.; J. Am. Chem. Soc., 1961, 83, 3917.

23. Kitz, R. J.; Ginsburg, S.; Wilson, I. B.; Biochem. Pharmacol., 1965, 14, 471-1477.

24. Smolikova, J.; Exner, O.; Barbaro, G.; Macciantelli, D.; Dondoni, A.; J. Chem. Soc. Perkin II, 1980, 1051-1056.

25. Kosower, E. M., "An Introduction to Physical Organic Chemistry," Wiley, New York, 1968.

26. Fina, N. J.; Edwards, J. O.; Int. J. Chem. Kin., 1973, 5, 1.

27. Albert, A.; Sergeant, E. P.; Ionization Constants of Acids and Bases," J. Wiley and Sons, Inc., New York, 1962.

28. Jencks, W. P.; Gilchrist, M.; J. Am. Chem. Soc., 1968, 90,2622.

29. Guillot-Edelheit, G.; Laloi-Diard, M.; Eisenstein, O.; Tetrahedron, 1978, 34, 523.

30. Lehninger, A. L., "Biochemistry," Worth Publ. Inc., New York, 1970, p. 161.

31. Green, A. L.; Smith, H. J.; Biochem. J., 1958, 68, 32.

33. Wang, E.I.C.; Braid, P. E.; J. Biol. Chem., 1967, 242, 2683.

34. Schoene, K.; Strake, E. M. Biochem. Pharmacol.; 1971, 20, 2527.

35. DeJong, L.P.A.; Wolring, G. Z.; Biochem. Pharmacol., 1978, 27, 2911.

36. DeJong, L.P.A.; Wolring, G. Z. Biochem. Pharmacol., 1978, 27, 2229.

37. Bracha, P.; O'Brien, R. D.; Biochem., 1968, 7, 1545.

38. Kabachnik, M. I.; Brestkin, A. D.; Godovikov, N. N.; Michelson, M. J.; Rozengart, E. V.; Rozengart, V. I.; Pharmacol. Rev., 1970, 22, 355.

39. Belleau, B.; J. Med. Chem., 1964, 7, 776.

40. Belleau, B.; DiTullio, V.; J. Am. Chem. Soc., 1970, 92, 6320.

41. Ingraham, L. L.; Alspach, J. D.; J. Med. Chem., 1977, 20, 161.

42. Weigand A. W.; Kuhnen, H.; Haller, R.; Biochem. Pharmacol., 1976, 25, 1719.

43. Stolle, R.; Chem. Ber., 1899, 32, 797.

44. Behr, L. C.; Brent, J. T.; Organic Synthesis; Coll. Vol. IV, John Wiley and Sons, New York, 1963, p. 342.

45. Sheehan, J. C.; Robinson, C. A.; J. Am. Chem. Soc., 1951, 73, 1207.

46. Hottel, R.; Chem. Ber., 1941, 24B, 1680.

47. Weinstock, L. M.; Davis, P.; Handelsman, B.; Tull, R.; J. Org. Chem., 1967, 32, 2823.

48. Weidinger, H.; Kranz, J.; Chem. Ber., 1963, 96, 1049.

49. Lindsay, R. O.; Allen, C.F.H.; Organic Synthesis; Coll. Vol. III, John Wiley and Sons, New York, 1955, p. 710.

50. Ellman, G. L.; Courtney, K. D.; Andres, V.; and Featherstone, R. M.; Biochem. Pharmacol., 1961, 7, 88.

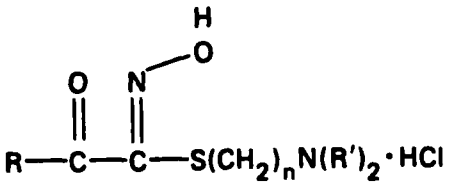
Chapter IV

3-SUBSTITUTED-1,2,4-OXADIAZOL-5-YL ADOXIMES AND THIOHYDROXIMIC ACID 2-(DIETHYLAMINO)ETHYL S-ESTERS AS REACTIVATORS OF ETHYL METHYLPHOSPHONYL-ACETYLCHOLINESTERASE

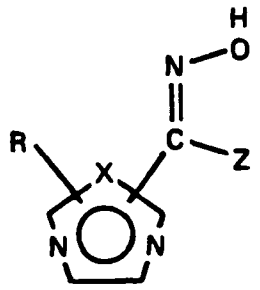
Introduction

Various organophosphorus esters irreversibly inhibit acetylcholinesterase (AChE) by phosphylating a serine hydroxyl at the enzyme active site.[1-7] Appropriately designed nucleophiles can reversibly bind to phosphylated AChE and displace the phosphorus atom thereby restoring enzymatic activity. The use of such cholinesterase "reactivators" in therapy of organophosphorus anticholinesterase agent poisoning is well known.[8-11] Although the literature provides many examples of fundamentally different types of cholinesterase reactivators, only pyridinium aldoximes, such as 2-hydroxyiminomethyl-1-methylpyridinium halide (2-PAM), have enjoyed widespread clinical application. As a class, the hydrophilic quaternary pyridinium aldoximes suffer a serious limitation with respect to penetration through hydrophobic cell membranes into various tissue regions (especially the central nervous system). In an attempt to develop improved anticholinesterase agent antidotes, we have investigated various types of nonquaternary cholinesterase reactivators.[12-14]

Our previous work focused on α-ketothiohydroximates, 1, α-heterocyclic aldoximes, 2, and α-heterocyclic thiohydroximic acid 2-(diethylamino)ethyl S-esters, 3.

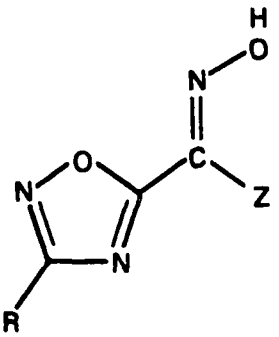


1



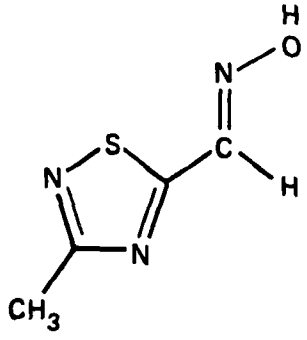
2 (Z = H)
3 (Z = $SCH_2CH_2NEt_2$)

We prepared more than 25 examples of type 1, 2, and 3 compounds and evaluated them with respect to reactivation of phosphylated AChE in vitro. Of these 25 compounds, 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole, 2a, is the most potent reactivator of ethyl methylphosphonyl-AChE. The corresponding 3-phenyl-1,2,4-oxadiazol-5-yl thiohydroximic acid 2-(diethylamino)ethyl S-ester, 3a, is a weaker reactivator but a powerful reversible inhibitor of AChE.

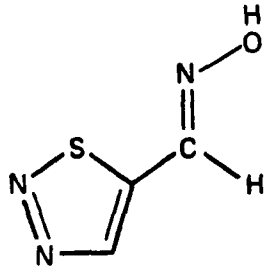


2a (R = C_6H_5, Z = H)
3a (R = C_6H_5, Z = $SCH_2CH_2NEt_2$)

The unusual properties of 2a and 3a recommended further investigations of the 3-substituted-1,2,4-oxadiazolyl system. In this chapter, we describe the synthesis and evaluation of six new type 2 and type 3 compounds wherein we replaced the phenyl group of 2a and 3a with R = CH_3, t-C_4H_9, or 1-naphthyl. For comparison, we have also included the following compounds: a type 1 compound, p-$BrC_6H_4C(O)C(:NOH)SCH_2N(C_2H_5)_2$ HCl (1a); and two; thiadiazolyl aldoximes, 4 and 5, that Benschop et al.[15] previously investigated as cholinesterase reactivators.



4



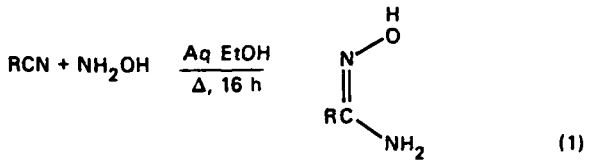
5

We find that all four of the type 2 1,2,4-oxadiazolyl oximes are significantly superior to 4 and 5 with respect to reactivation of ethyl methylphosphonyl AChE. Indeed, 3-(1-naphthyl)-5-hydroxyiminomethyl-1,2,4-oxadiazole (compound 2d below) is 40 times more reactive than 4 and only 5 times less reactive than 2-PAM toward this inhibited enzyme. Additionally, we find that incubating micromolar concentrations of 3a with AChE antagonizes irreversible inhibition of the enzyme by subsequent addition of ethyl p-nitrophenyl methylphosphonate (EPMP) and simultaneously restores activity to ethyl methylphosphonyl-AChE that does form.

RESULTS AND DISCUSSION

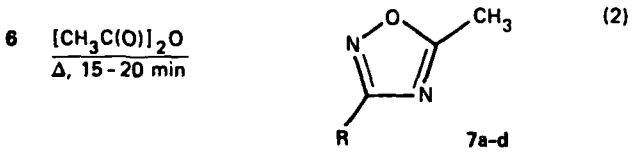
Synthesis and Structure

We prepared the aldoximes, 2, and the corresponding thiohydroximates 3 via reactions (1) through (5):

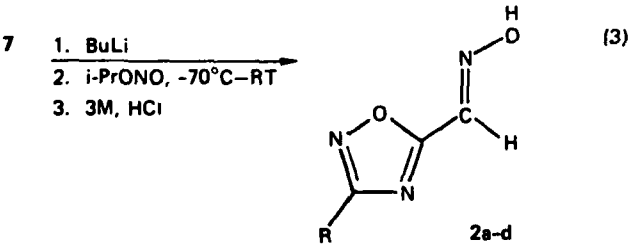


(1)

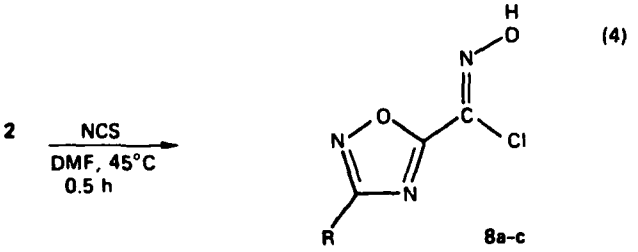
6a, R = C_6H_5
b, R = t-C_4H_9
c, R = CH_3
d, R = 1-naphthyl



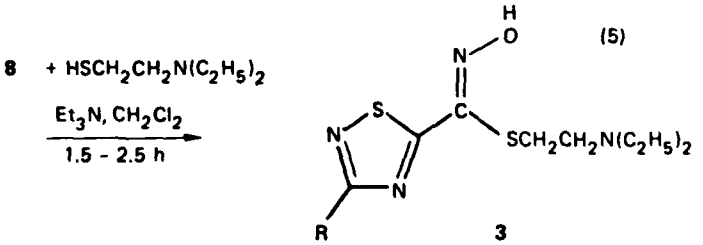
7a-d (2)



2a-d (3)



8a-c (4)



3 (5)

Generally reactions (1), (2), (4), and (5) proceeded in good yield. As previously noted,[14] however, reaction (3) typically gave poor yields of the desired product and many side products. The experimental section details the syntheses of the target compounds. Table 1 provides selected data for compounds 2 through 5.

Acidity, Nucleophilicity, and Stability

We determined hydroxyiminomethyl acid dissociation constants (pK_a values) for each of the type 2 and type 3 compounds in Table 1. All the type 2 compounds exhibited acid dissociation constants near $pK_a = 8$, the empirically known optimal value for cholinesterase reactivators. All the type 3 compounds exhibited $pK_a \sim 6.5$. This reduction of the pK_a was previously[13,14] noted for other aldoxime/thiohydroximate conversions. As a measure of the inherent nucleophilicities of the target compounds (and also as a control in our AChE assays), we determined rate constants for reaction of the compounds shown in Table 1 with acetylthiocholine (AcSCh). We also determined rate constants for reaction of ethyl p-nitrophenyl methylphosphonate (EPMP) with 2PAM, 1a, 3a, and 3-methyl-4-hydroxyiminomethyl-1,2,5-oxadiazole (compound 2e, $pK_a = 9.15$[14]).

These experiments were conducted as previously described[14] with EPMP or AcSCh in large excess over test compound. We followed these reactions to low conversions and observed pseudo-zero-order kinetics according to equations (6) and (7):

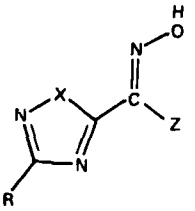
$$+d[\text{thiocholine}]/dt \cdot [\text{AcSCh}]^{-1} = k_n[OX] + k_o \quad (6)$$

$$+d[\text{p-nitrophenolate}]/dt \cdot [\text{EPMP}]^{-1} = k_n'[OX] + k_o' \quad (7)$$

where $+d[X]/dt$ are observed zero-order rates for product formation; k_n and k_n', respectively, are bimolecular rate constants for reaction of the oximate form (OX) of the test compounds with AcSCh and EPMP; and k_o and k_o' are pseudo-first-order rate constants for spontaneous hydrolysis

Table 1

SELECTED DATA FOR HETEROAROMATIC COMPOUNDS



Compound No.	X	R	Z	Yield, %	m.p., °C	NMR (=NOH), δ	pK_a[a]	Anal.[b]
2a	O	C_6H_5	H	7.9	161-163	12.87[c]	7.88	C,H,N
2b	O	t-C_4H_9	H	10	130.5-132	13.01[c] 12.07[d]	7.91	C,H,N
2c	O	CH_3	H	6.9	163.5-164	13.02[c] 12.22[d]	8.11	C,H,N
2d	O	1-naphthyl	H	14	153-155	13.25[c] 12.26[d]	8.05	C,H,N
3a	O	C_6H_5	$SCH_2CH_2NET_2$	27	199-201(d)[e]	13.88[c]	6.32	C,H,N
3b	O	t-C_4H_9	$SCH_2CH_2NET_2$	81	87-88.5	9.78[d]	6.61	C,H,N,S
3c	O	CH_3	$SCH_2CH_2NET_2$	63	92-93.5	6.73[d]	6.32	C,H,N,S
3d	O	1-naphthyl	$SCH_2CH_2NET_2$	84	193.5-196[e]	9.55[d]	-	C,H,N,S[e]
4	S	CH_3	H	f	f	13.77[c,f]	6.97[f]	f
5	S	g	H	f	f	13.63[c,f]	7.64[f]	f

[a]Determined spectrophotometrically in 0.1 M buffer.

[b]Analytical data for indicated elements within ±0.4% of theoretical.

[c]In $(CD_3)_2SO$.

[d]In $(CD_3)_2CO$.

[e]Hydrochloride salt.

[f]Data of reference 15.

[g]Compound 5 is 5-hydroxyiminomethyl-1,2,3-triazole.

AcSCh and EPMP, respectively. We calculated the fraction of added test compound, HOX, dissociated to the oximate form at the pH of the experiment according to equation (8):

$$[OX] = [HOX]\ [1 + \text{antilog}\ (pK_a - pH)]^{-1} \qquad (8)$$

Table 2 summarizes rate constants for reactions of test compounds with AcSCh and EPMP.

According to the usual[16] Bronsted relationship for oximate anions, a plot of hydroxyiminomethyl pK_a versus $\log(k_n)$ should be linear with the slope (β value) of the plot proportional to the oximate nucleophilicity. Table 3 summarizes Bronsted relationships for attack by nonquaternary oximates on various reactants. The table shows good adherence to the Bronsted relationship for all the reactants and nucleophiles investigated. Interestingly, the β values were in all cases essentially identical within the limits of experimental uncertainty (mean $\beta = 0.70 \pm 0.07$). The equivalent β values indicate essentially identical relative nucleophilicities for the type 1, 2, and 3 compounds toward the reactants studied.

To check the possible hydrolytic degradation of the test compounds under the conditions of our experiments, we measured k_n values for reaction of the compounds with AcSCh over the 4- to 6-hour incubation period used in the reactivation experiments. None of the compounds exhibited significant trends toward higher or lower k_n values with time: the observed k_n values were constant to within ±20%. We conclude that hydrolysis of test compounds was negligible under the conditions used in our experiments.

Reversible AChE Inhibition

The compounds listed in Table 1 reversibly inhibited AChE to varying degrees. To elucidate possible binding interactions between the test compounds and AChE (and also as a control for our AChE assays), we determined the dependence of AChE activity on the concentration of added

Table 2

RATE CONSTANTS FOR REACTIONS OF ALDOXIME AND THIOHYDROXIMATE ANIONS WITH ACETYLTHIOCHOLINE (AcSCh) AND WITH ETHYL p-NITROPHENYL METHYLPHOSPHONATE (EPMP)

EPMP[b] Compound No.	Reactions with AcSCh[a] k_n[c] $M^{-1}min^{-1}$	pK_a	Compound No.	Reactions with k'_n,[d] $M^{-1}-min^{1}$	pK_a
2a	51.8[e]	7.88[e]	2-PAM	5.70	7.99[f]
2b	54.8	7.91	3a	0.293	6.32[e]
2c	91.4	8.11	1a[g]	1.12	7.44[f]
2d	70.8	8.05	2e[h]	13.5	9.05[e]
3a	4.35[e]	6.32[e]			
3b	5.13	6.61			
3c	3.73	6.32			
3d	i	i			
4	5.68	6.97			
5	42.7	7.64			

[a]At 25°C in pH 8, 0.1 M phosphate buffer.

[b]At 25°C in pH 7.6, 0.1 M morpholinopropanesulfonate buffer.

[c]Calculated according to equation (6).

[d]Calculated according to equation (7).

[e]Data of reference 14.

[f]Data of reference 13.

[g]1a is $BrC_6H_5C(:O)C(:NOH)SCH_2CH_2$ Et_2.HCl.

[h]2e is 3-methyl-4-hydroxyiminomethyl-1,2,5-oxadiazole.

[i]Not determined.

Table 3

BRONSTED RELATIONSHIPS[a] FOR REACTIONS OF ALDOXIME AND THIOHYDROXIMATE ANIONS WITH VARIOUS REACTANTS AT 25°C

Nucleophiles	Reactant	A[a,b]	β[a,c]	Reference
Type 1 compounds	p-nitrophenyl acetate	-3.04(±0.34)	0.69(±0.08)	12
Types 2 and 3 compounds[d]	acetyl thiocholine	-4.19(±1.0)	0.67(±0.12)	14
2a-2d, 3a-3d, 4, 5[e]	acetyl thiocholine	-4.49(±0.51)	0.79(±0.069)	this work
1a, 3a, 2e, 2PAM[f]	ethyl p-nitrophenyl methylphosphonate	-4.55(±0.76)	0.64(±0.09)	this work
		Average	0.70 ± 0.07	

a. $\log(k_n) = A + \beta \cdot pK_a$.

b. Linear lease-squares intercept (±S.D.) of Bronsted plot.

c. Linear least-squares slope (±S.D.) of Bronsted plot.

d. Data of Table 3 in reference 14, excluding the compounds 2a and 3a shown in Table 1 of this report.

e. See Table 1.

f. See Tables 1 and 2.

test compound in the assay solution. Table 4 summarizes the results of these experiments. To check the possibility of progressive AChE inhibition by the test compounds, we assayed for activity at 60-, 120-, and 180-min incubation intervals. In all cases, AChE activity was constant over the three-hour incubation period, and we report observed enzyme activities as the mean value (±S.D.) of the three time points for each concentration of test compound used. For some of the test compounds, observed AChE activities were also independent of concentration of added compound over the range of concentrations used. For these compounds, we also averaged observed activities for all concentrations employed. Table 4 expresses the data as percentage inhibition, I, of enzyme activity where:

$$I = 100(A_o - A_I)/A_o \qquad (9)$$

and A_o and A_I are AChE activities in the absence and presence, respectively, of added test compound. Additionally, we calculated I50 values (i.e., the [HOX] at which I = 50) from linear least-squares regression analysis of *I versus log[HOX]* data.

Table 4 shows that neither the type 2 compounds nor the thiadiazolyl aldoximes 4 and 5 significantly inhibited AChE at the concentrations tested. Our previous[14] investigation also showed type 2 compounds generally to be poor inhibitors. The type 3 compounds exhibited varying degrees of anti AChE activity: compounds 3a and 3d were very powerful inhibitors whereas 3b and 3c were weak inhibitors. Compound 3d is the most inhibitory compound tested to date.

Reactivation of Ethyl Methylphosphonyl-AChE

We inhibited AChE with EPMP and investigated the kinetics of AChE reactivation as a function of the concentration of added test compound. The experimental methods and kinetic derivations were as previously described.[13,14] Definitions of relevant kinetic and experimental constants follow:

Table 4

PERCENTAGE REVERSIBLE INHIBITION (I) OF AChE ACTIVITY
BY TEST COMPOUNDS AT 25°C, pH 7.6

Compound[a] No.	[HOX] in Assay,[b] μM	I,[c] %	I_{50},[d] mM
2a	10.00 50.00 100	1.7 ± 3[e,f]	>1
2b	2.00 5.00 10.0	-2.0 ± 9[e]	>1
2c	2.00 5.00 10.0	1.0 ± 2	>1
2d	2.00 5.00 10.0	3.6 ± 5[e]	>1
3a	1.50 5.00 15.0 40.0 50.0	20.3 ± 4[f,g] 64.3 ± 12 62.1 ± 5.1 80.6 ± 2.2 84.0 ± 1.8	0.0075
3b	2.00 5.00 10.0	1.1 ± 0.4[e]	>1
3c	10.0 25.0 50.0	19.0 ± 2[g] 22.2 ± 3 34.9 ± 2	0.31
3d	1.00 2.50 5.00 10.0 25.0 50.0	24 ± 0.2 47 ± 0.3 58 ± 1.5 76 ± 1.1 89 ± 1.3 95 ± 0.4	0.0033
4	2.00 5.00 10.0	-1.1 ± 3[e,h]	>1
5	2.00 5.00 10.0	-4 ± 2.5[e,h]	>1

[a]See Table 1 for structures.

[b][HOX] is the concentration of added test compound in the assay solution.

[c]See Equation (9).

[d]I_{50} is the concentration of added test compound that inhibits AChE to 50% of control activity.

[e]I = mean value (± S.D.) for all indicated [HOX].

[f]Data of Reference 14.

[g]I = mean value (± S.D.) over three time points at each indicated [HOX].

[h]Reference 15 reports I < 5 for [HOX] = 80 μM.

$[HOX]$ = concentration of added test compound

$[OX]$ = concentration of added test compound present as anionic (oximate) form = $[HOX]\,[1 + \text{antilog}\,(pK_a - 7.6)^{-1}]$

A_c = control (uninhibited) enzyme activity

A_i = enzyme activity after incubation with EPMP for 20 min

A_t = (observed enzyme activity at time t) $\cdot$ [100/(100-I)]; see equation (9)

R_t = percent reactivation = $100(A_t - A_i)/(A_c - A_i)$

k_{obs} = observed pseudo-first-order rate constant for oximate-induced reactivation = $[\ln(100 - R_t)]\,t^{-1}$

k_{sp} = pseudo-first-order rate constant for spontaneous (hydrolytic) reactivation = $[\ln(A_c - A_i)]\,t^{-1}$

$(k_{obs})_c$ = corrected first-order reactivation rate constant = $k_{obs} - k_{sp}$

K_r = dissociation constant for [reactivator/inhibited enzyme] complex

k_r = nucleophilic displacement rate constant for [reactivator/inhibited enzyme] complex

k_b = bimolecular rate constant for reactivation in the limit of low oximate concentration

k_{eff} = effective bimolecular rate constant corrected for fraction of added test compound present as oximate = $k_b[1 + \text{antilog}\,(pK_a - 7.6)]^{-1}$

Table 5 summarizes k_{obs} and k_{sp} values for the various compounds. In all cases, semilogarithmic plots of $\ell n(100 - R_t)$ versus time were linear, and parallel control experiments with 2-PAM demonstrated that R_t approached 100 at long incubation times. Thus, the <u>in vitro</u> assay was kinetically well-behaved, and complicating side reactions such as enzyme denaturation, dealkylation, and reinhibition by phosphonyl oxime are not significant sources of error in our determinations.

Table 5

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) AND RELATED CONSTANTS[a] FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT pH 7.6, 25°C

Compound[b] No.	[HOX] (incubation), mM	[HOX] (assay), μM	A_C, μM min^{-1}	A_I, μM min^{-1}	k_{sp},[c] $min^{-1} \times 10^3$	k_{obs},[c] $min^{-1} \times 10^3$
2a	0.0300	1.50	13.1	0.746	0.141 ± 0.010	5.49 ± 0.28
	0.0800	4.00				12.3 ± 0.49
	0.200	10.0				35.1 ± 0.46
	0.500	25.0				43.6 ± 5.3
2b	0.0500	2.50	10.9	1.64	0.0764 ± 0.07	1.94 ± 0.087
	0.100	5.00				3.68 ± 0.12
	0.500	25.0				17.2 ± 0.85
	1.00	50.0				28.5 ± 7.3
2c	0.0500	2.50	13.3	1.38	0.114 ± 0.001	7.06 ± 1.7
	0.100	5.0				15.9 ± 0.74
	0.500	25.0				46.2 ± 5.3
	1.00	50.0				67.6 ± 12.4
2d	0.0500	2.50	8.56	0.845	0.206 ± 0.01	19.8 ± 2.2
	0.100	5.00				34.1 ± 1.2
	0.500	25.0				48.6 ± 5.0
3a	0.0300	1.50	11.8	0.360	0.108 ± 0.001	0.691 ± 0.018
	0.100	5.00				2.15 ± 0.10
	0.300	15.0				2.94 ± 0.069
	1.00	50.0				7.69 ± 0.42
3b	0.0500	2.50	12.3	1.56	0.0711 ± 0.0003	0.278 ± 0.020
	0.100	5.00				0.297 ± 0.025
	0.500	25.0				0.135 ± 0.012
	1.00	50.0				0.106 ± 0.021
3c	0.0500	2.50	11.9	0.570	0.0718 ± 0.007	0.324 ± 0.035
	0.100	5.00				0.419 ± 0.0028
	0.500	25.0				0.978 ± 0.16
	1.00	50.0				2.09 ± 0.096
3d	e	e	e	e	e	e
4	0.0395	1.98	12.3	1.05	0.130 ± 0.003	0.680 ± 0.029
	0.0790	3.95				1.07 ± 0.038
	0.395	19.8				4.00 ± 0.12
	0.790	39.5				7.52 ± 0.32
5	0.0384	1.92	11.6	1.47	0.0671 ± 0.001	0.333 ± 0.034
	0.0767	3.84				0.558 ± 0.024
	0.384	19.2				2.61 ± 0.13
	0.767	38.4				4.43 ± 0.46

[a]See text for definitions of terms.

[b]See Table 1 for structures.

[c]Values reported with ± S.D. as determined by least-squares linear regression.

[d]Data of Reference 14.

[e]Not determined at this time.

For a variety of reactivators, reactivation kinetics depend on reactivator concentration as in equation (10)[17-21]

$$(k_{obs})_c^{-1} = \frac{K_r}{k_r}[OX]^{-1} + \frac{1}{k_r} \qquad (10)$$

where K_r reflects affinity of the reactivator for reversible binding to inhibited enzyme and k_r is the rate at which enzyme-bound reactivator displaces organophosphorus inhibitor from the AChE active surface. In the limit of low reactivator concentration, the ratio k_r/K_r equals a bimolecular rate constant, k_b, and this value represents the inherent reactivity of any oximate toward phosphylated enzyme. Finally, as a measure of the effective activity of a test compound, we define k_{eff} as the product of k_b and the fraction of added test compound present in the anionic (oximate) form:

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

Table 6 summarizes kinetic constants for reactivation of ethyl methylphosphonyl-AChE by type 2 and type 3 compounds, plus compounds 4, 5, and 2-PAM. The table reveals that all the type 2 compounds tested significantly surpassed the previously known thiadiazolyl oximes 4 and 5 with respect to inherent (k_b) and effective (k_{eff}) reactivities toward phosphylated AChE. Compound 2d, in particular, exhibited pronounced activity as a reactivator. In terms of k_{eff} values, 2d was 40-times more reactive than 4 and only 5-fold less reactive than 2-PAM. Compound 2d was the most effective nonquaternary reactivator that we have studied to date.

By comparison, the type 3 compounds generally showed low reactivity toward phosphonylated AChE: k_b values for the type 3 compounds were 25 to 140 time lower than values for the corresponding type 2 compounds. Our previous study[14] also revealed a substantial increase in the inherent reactivities of heteroaromatic aldoximes compared with analogous thiohydroximate derivatives.

Table 6

KINETIC CONSTANTS FOR REACTIVATION OF ETHYL METHYLPHOSPHONYL-AChE AT pH 7.6, 25°C[a]

Compound No.	[HOX] mM	$[OX]^{-1}$ M^{-1}	$(k_{obs})^{-1}$ min	Slope M-min x 10^2	Intercept min	k_r min^{-1} x 10^3	K_r M x 10^4	k_b M^{-1}-min^{-1}	k_{eff} M^{-1}-min^{-1}
2a	0.0300	99,800	187	0.179 ± 0.098	9.32 ± 5.27	107	1.92	557	186
	0.0800	37,400	82.2						
	0.200	15,000	28.6						
	0.500	5,990	23.0						
2b	0.0500	60,600	515	0.848 ± 0.018	57.6 ± 6.2	17.4	1.47	116	38.5
	0.100	30,300	272						
	0.500	6,060	58.1						
	1.00	3,030	25.6						
2c	0.0500	83,300	144	0.161 ± 0.012	5.67 ± 5.7	176	2.84	630	151
	0.100	41,700	63.4						
	0.500	8,330	21.7						
	1.00	4,170	14.8						
2d	0.0500	76,300	51.0	0.0446 ± 0.0079	15.6 ± 3.9	64.1	0.286	2240	587
	0.100	38,200	29.5						
	0.500	7,630	20.7						
3a	0.0300	35,000	1720	4.57 ± 0.35	102 ± 64	9.80	4.48	21.9	20.8
	0.100	10,500	490						
	0.300	3,500	353						
	1.00	1,050	132						
3b	0.500	21,900	28,700	117 ± 5.1	2170 ± 625	0.461	5.39	0.855	0.778
	0.100	11,000	15,600						
	0.500	2,190	4,430						
	1.00	1,100	4,830						
3c	0.0500	21,100	3,970	17.8 ± 27	457 ± 2.7	2.19	3.89	5.62	5.34
	0.100	10,500	2,880						
	0.500	2,110	1,100						
	1.00	1,050	495						
3d	b	b	b	b	b	b	b	b	b
4	0.0395	31,300	1,820	5.66 ± 0.33	88.5 ± 57	11.3	6.40	17.7	14.3
	0.0790	15,600	1,060						
	0.395	3,130	258						
	0.790	1,560	135						
5	0.0384	54,300	3,760	6.90 ± 0.20	57.5 ± 62	17.4	12.0	14.5	7.02
	0.0767	27,300	2,050						
	0.384	5,430	393						
	0.767	2,730	229						
2-PAM[c]	0.000500	6,910,000	735	0.0104 ± 0.00064	27.8 ± 24	36.0	0.0374	9630	2780
	0.00150	2,300,000	307						
	0.00400	804,000	109						
	0.0100	345,000	43.5						

[a] See Table 1 for structures, text for term definitions.
[b] Not determined yet.
[c] Data of reference 13.

Protection Against Phosphonylation of AChE

Compounds 3a and 3d are powerful reversible AChE inhibitors. We previously[14] demonstrated the competitive nature of the inhibition for 3a and concluded that 3a and substrate (AcSCh) both bind at the enzyme active site. To extend this work we investigated the degree to which 3a also competes with an organophosphonate (EPMP) for binding to the enzyme. To do this, we incubated AChE with excess 3a prior adding EPMP (which was also in excess over AChE) and assayed AChE activities as a function of 3a concentration and incubation interval after adding EPMP.

Figure 1 shows the dependence of AChE activity on incubation interval for the reaction of the enzyme with 20 nM EPMP in the absence of 3a and in the presence of 300 μM 3a. In the figure, the percentage observed activity ($100 \cdot A_t/A_c$) includes a correction for enzyme inhibition by 3a in the assay using the expression A_t = (observed activity)100/(100-I). The figure reveals pseudo-first-order loss of AChE activity in the absence of 3a. We repeated the inhibition experiment in the absence of 3a three times and calculated a bimolecular rate constant, k_i, for AChE inhibition by EPMP according to equations (11) and (12):

$$\ell_n(A_t/A_c) = k_{11} \cdot t \qquad (11)$$

$$k_{11} = k_i[\text{EPMP}] \qquad (12)$$

For the three runs, we found $k_i = 1.1(\pm 0.25) \times 10^5\ M^{-1}\text{-}min^{-1}$.

Figure 1 also reveals that incubating AChE with 300 μM 3a before adding EPMP markedly reduced the degree of inhibition at any incubation interval after addition of EPMP. The data for AChE activity in the presence of 3a showed a rapid initial loss of enzyme activity, followed by a plateau in activity corresponding to establishment of a steady-state.

We also determined the AChE activity after adding EPMP as a function of 3a concentration. In these experiments, we preincubated

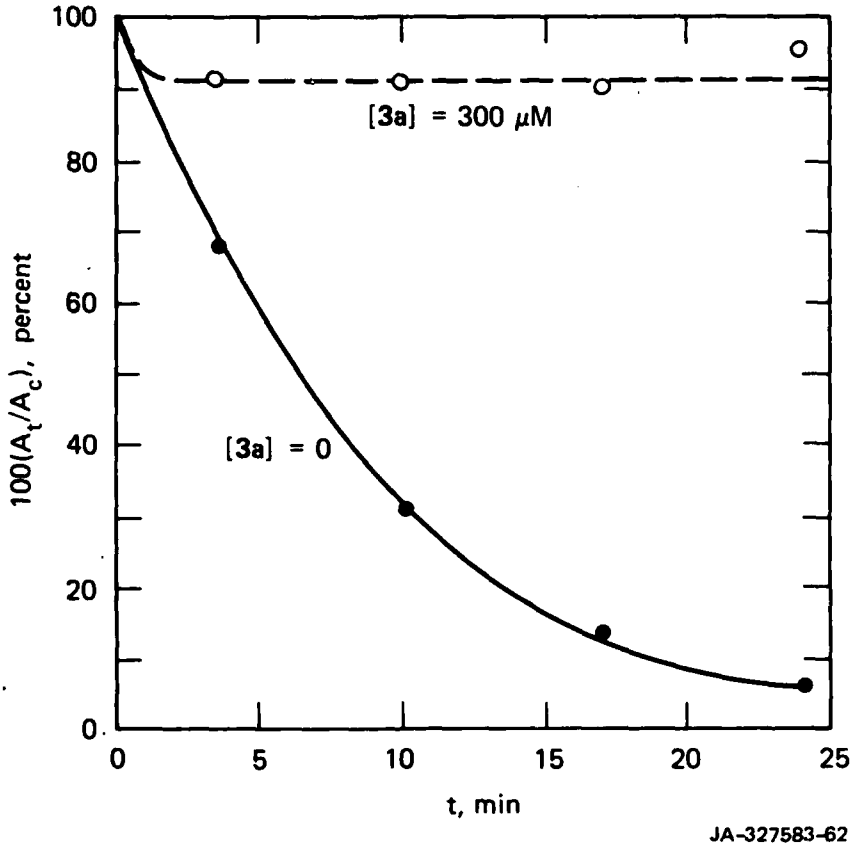


FIGURE 1 PERCENTAGE OF CONTROL ACETYLCHOLINESTERASE ACTIVITY, $100(A_t/A_c)$, VERSUS INCUBATION INTERVAL, t, FOR REACTION OF ENXYME (A_c = 5.11 μM min^{-1}) WITH 20 nM ETHYL p-NITROPHENYL METHYLPHOSPHONATE IN THE ABSENCE, ● , OR PRESENCE, ○ , OF INITIALLY ADDED **3a**

In 0.1 M pH 7.6 buffer at 25°C.

AChE with 300 to 3.00 μM 3a, added 20 nM EPMP, and determined the percentage AChE at an incubation interval (t = 17 to 33 min) corresponding to the plateau region in Figure 1. Table 7 summarizes these data. Here, the relationship between AChE activity and [3a] was clear: residual AChE activity increased monotonically with [3a]. Even at the lowest concentration of 3a (= 3 μM), AChE activity did not decrease below 40% of control during the experiment.

To more quantitatively (and mechanistically) describe the protection of AChE by 3a, we considered the reaction set given by equations (13) through (18)

$$EOH + EPMP \xrightarrow{k_i} EOP \quad (13)$$

$$EOH + HOX \underset{}{\overset{K_i}{\rightleftharpoons}} [EOH \cdot HOX] \quad (14)$$

$$EOP + OX \underset{}{\overset{K_r}{\rightleftharpoons}} [EOP \cdot OX] \quad (15)$$

$$[EOP \cdot OX] \xrightarrow{k_r} EOH + Products \quad (16)$$

$$EPMP + OX \xrightarrow{k_n'} Products \quad (17)$$

$$HOX \underset{}{\overset{K_a}{\rightleftharpoons}} H^+ + OX \quad (18)$$

where EOH is active enzyme, EOP is ethyl methylphosphonyl-AChE, [EOH•HOX] is [reactivator/enzyme complex], [EOP•OX] is [reactivator/phosphonylated enzyme] complex, and HOX is the protonated form and OX is the anionic form of 3a.

When we substitute into equations (13) through (18), the initial concentrations of EPMP and 3a and our experimentally determined values for k_i (above); K_r, k_r (Table 6); k_n' (Table 2); K_a (Table 1), and K_i (= 0.98 μM, Reference 14) and solve for the concentration of EOH as a function of time by numerical integration, we calculate much smaller

Table 7

PLATEAU AChE ACTIVITIES AFTER REACTION OF ENZYME WITH ETHYL p-NITROPHENYL METHYLPHOSPHONATE AND INITIALLY ADDED 3a[a]

[3a] μM	Incubation Interval Min	$100 \cdot (A_t/A_c)$[b]
100	25	102
30.0	25	91
10.0	33	79
3.00	33	46

a. Reaction conditions were: pH = 7.6 in 0.1 M morpholinopropanesulfonate buffer, T = 25°C, [EPMP] = 20 mM, A_c = 4.96 μM^{-1} min^{-1}.

b. A_t corrected for AChE inhibition by 3a in the assay solution according to the expression A_t = (observed activity)·(100/(100 - I).

enzyme activities than those observed in Table 7. Furthermore, the rate and equilibrium constants for reactions (13) through (18) predict that activities will slowly, but, monotonically decrease with time, whereas we observed a steady state. These discrepancies suggest that equations (13) through (18) do not properly describe the true reaction and that additional chemistry is occurring.

In effect, the data require that when 3a is preincubated with AChE, reactivation of phosphonylated AChE by 3a occurred considerably more rapidly than the k_r and K_r values of Table 6 predict. Although mechanistic conclusions are speculative at this time, the above analysis suggests that prior binding of 3a to AChE followed by phosphonylation yields a [reactivator/phosphonyl-AChE] complex with a different conformation than the complex that occurs when phosphonylation precedes equilibrium binding of the reactivator.

Structure-Activity Relationships

Within the series of compounds 2a through 2d, reactivities toward ethyl methylphosphonylated AChE (k_b values) varied by a factor of 20. Because 2d (where R = 1-naphthyl) was the most reactive of the series and because the type 2 compounds do not feature cationic moieties it seems likely that hydrophobic interactions strongly influence the affinities of the type 2 reactivators for the phosphonylated enzyme.

For the type 2 versus type 3 compounds, the relative reactivities toward phosphonylated AChE primarily depend on relative nucleophilicities toward phosphorus. Tables 2 and 3 demonstrate the dependence of oximate nucleophilicity on hydroxyiminomethyl acidity. Because the type 3 compounds feature relatively low pK_a values, they must also react relatively slowly with ethyl methylphosphonyl-AChE. We prepared the type 3 thiohydroximates partly to demonstrate the degree to which the protonated diethylaminoethyl functionality influences reactivation potency. On the basis of the data in Table 6 and data previously cited[14] for α-heteroaromatic thiohydroximates, we find no evidence for enhanced reactivity toward ethyl methylphosphonyl-AChE in

type 3 versus type 1 compounds. Of course, the generally lower nucleophilicities of the type 3 compounds may mask any otherwise inherently higher reactivies for this class of reactivator. Whether structurally analogous type 2 and type 3 compounds featuring equivalent pK_a values would reveal higher reactivity for the type 3 reactivators remains an interesting, but as yet unanswered, question.

It is also interesting to compare compound 4 with the type 2 reactivators. Relative k_b values for 4 versus the analogous 2c differ by a factor of 36, with 2c being more reactive. Here again, we attribute the low reactivation potency of 4 to its low pK_a value and correspondingly low nucleophilicity. In a practical context, the comparisons with compound 4 are valuable because De Jong[22] has determined relative reactivation potencies of 4 and several other well-known nonquaternary reactivators. For these reactivators, De Jong determined the percentage reactivation of isopropyl methylphosphonyl-AChE as a function of reactivator concentration and incubation interval. De Jong's experimental conditions (25°C, pH = 7.5) bovine erhthrocyte AChE, were similar to ours, and we can therefore meaningfully compare the two sets of data.

Table 8 summarizes reactivation data obtained by De Jong for isopropyl methylphosphonyl-AChE and by us for ethyl methylphosphonyl-AChE. In the table, we calculated percentage reactivation, R, of ethyl methylphosphonyl-AChE by 2-PAM, 2d, and 4 using the data of Table 6 plus equation (10) and the expression $\ell n(100 - R) = k_{obs} \cdot t$. For 2-PAM and 4, we can directly compare reactivation data for the two inhibited enzymes. Doing so reveals for both 2-PAM and 4 that the ethyl methylphosphonylated enzyme is 1.5 to 2 times less difficult to reactivate than the isopropyl methylphosphonyl-AChE. Steric bulk of the ethyl versus the isopropyl group readily accounts for the relative susceptibilities of the two phosphonylated enzymes toward reactivation.

To estimate the reactivity of 2d toward isopropyl methylphosphonyl-AChE, we assumed a 1.5-fold reduction in the k_{obs} value calculated for 0.03 mM reactivator from the data of Table 6. Thus for k_{obs} = 9.2 x

Table 8

PERCENTAGE REACTIVATION OF $RO(CH_3)P(O)AChE$ AT 25°C, pH 7.6

Compound	Code	[HOX] mM	% Reactivation at t min for					
			$R = C_2H_5$ [a]			$R = i\text{-}C_3H_7$ [b]		
			t = 120	360	1440	120	360	1440
4	3-Me-TDA-5	1.0	53	90	100	40	70	85
2d	4811-26	1.0	99	100	100	67[c]	96[c]	100[c]
		0.03	76	100	100			
		0.005	24	56	96			
2PAM	2-PAM	0.005	67	96	100	35	55	70
$CH_3C(O)C(NOH)H$	MINA	1	-	-	-	25	55	80
$CH_3C(O)C(NOH)CH_3$	DAM	1	-	-	-	<5	<5	<5
$CH_3C(O)C(NOH)CH_2N(CH_3)_2$	Isonitrosine	1	1	1	1	15	35	65
$(C_2H_5)_2NCH_2CH_2CH_2C(O)C(NOH)H$	OA3	1	-	-	-	95	100	100
		0.03	-	-	-	20	40	50

a. Calculated according to equation (10) and the expression $\ln(100-R) = k_{obs} \cdot t$ using data of Table 6.

b. Reported in Reference 22.

c. Calculated from data for $R = C_2H_5O$, assuming that when $R = i - C_3H_7$, the inhibited enzyme is 1.5 times more difficult to reactivate.

10^{-3} min^{-1}, we calculate R = 67, 96, and 100%, respectively, at 120-, 360-, and 1440-minute incubation intervals. Comparison of these estimates with De Jong's data in Table 8 reveals that 2d is considerably more reactive than any of the other reactivators except 2-PAM. Indeed, on the basis of this evaluation, we believe that 2d is the most reactive nonquaternary reactivator ever reported. This α-heteroaromatic aldoxime may represent a major advance in the search for improved anticholinesterase agent antidotes.

A final point concerns the ability of 3a and possibly 3d to protect AChE against inhibition by the organophosphonate EPMP. There are several known[23-30] examples of reversible AChE inhibitors that antagonize irreversible inhibition by organophosphonates either by competitively binding to the enzyme active site or by inducing conformational changes in the enzyme. That 3a also protects AChE against phosphonylation is not surprising. It is significant, however, that 3a competes with EPMP for the AChE active site and simultaneously reactivates any phosphonylated enzyme that does form. With 3a and EPMP both in excess over AChE, the parallel processes of phosphonylation and reactivation constitute a steady state, and the observed residual enzyme activity depends on the relative concentrations of reactivator and organophosphonate in solution. Indeed, our preliminary analysis of the steady-state data suggest that incubating AChE with 3a before adding EPMP effectively increased the reactivating potency of the compound relative to the case when the order of addition was reversed. Mechanistically, this implies distinctly different conformations for the [reactivator/phosphonyl-AChE] complexes formed from combining reactivator with phosphonylated AChE or from reacting EPMP with [reactivator/AChE] complex. Regardless of the mechanistic inferences, our data have practical significance insofar as the dual protection/reactivation properties of 3a suggest the potential use of this drug as pretreatment against organophosphonate intoxication.

CONCLUSIONS

We prepared a series of 3-substituted-1,2,4-oxadiazolyl-5-aldoximes and thiohydroximic acid 2-(diethylamino)ethyl S-esters and evaluated them as reactivators of ethyl methylphosphonyl AChE.

The 3-(1-naphthyl)-1,2,4-oxadiazolyl aldoxime (compound 2d) is a powerful reactivator. Compound 2d is only 5 times less reactive than 2-PAM toward ethyl methylphosphonyl-AChE, and 2d is 40 times more reactive than 3-methyl-5-hydroxyiminomethyl-1,2,4-thiadiazole (4). The comparison between 2d and 4 indicates that 2d is probably the most powerful nonquaternary reactivator ever reported. Because 4 and other weak nonquaternary reactivators have demonstrated antidotal efficacy versus isopropyl methylphosphonoflouridate intoxication *in vivo*,[15,22] it seems likely that 2d will exhibit particularly useful therapeutic properties.

The 1,2,4-oxadiazol-5-yl thiohydroximates, 3a and 3d, also exhibit interesting properties *in vitro*. They are a weak reactivators of ethyl methylphosphonyl-AChE primarily beause the high acidity of the hydroxyiminomethyl group dictates inherently low nucleophilicity for the conjugate base oximate anion compound. Compound 3a and presumably 3d is a powerful competitive reversible cholinesterase inhibitor and preincubation of AChE with micromolar concentrations of 3a protects against irreversible phosphonylation of the enzyme. Thus 3a simultaneously antagonizes AChE phosphonylation and reactivates phosphonylated AChE that does form. These properties recommend the evaluation of 3a and 3d for pretreatment of organophosphonate poisoning. Whether other modifications to these structures can successfully enhance the reactivation potency of the basic structure while retaining the strong reversible anti-AChE activity of the molecule remains an interesting question.

EXPERIMENTAL DETAILS

Materials

Nuclear magnetic resonance spectra were recorded on a Varian Associates EM-360 spectrometer; chemical shifts are reported in parts per million (δ) from internal tetramethylsilane. Splitting patterns are designated as: s, singlet; t, triplet; q, quartet; m, multiplet; br, broad. Infrared (IR) spectra were obtained on a Perkin-Elmer Model 735B spectrophotometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Microanalyses were performed with a Perkin Elmer 240 elemental analyzer (C,H,N) and by Galbraith Laboratories, Inc., Knoxville, Tennessee.

Tetrahydrofuran (THF) was distilled from calcium hydride and stored under nitrogen over 4A molecular sieves. Metalation reactions were performed under a positive pressure of dry nitrogen. Analytical thin layer chromatography (TLC) was performed on Analtech Uniplate silica gel GF (scored 10 by 20 cm plates, 250 micrometer). Preparative TLC was performed on Uniplate silica gel GF(20 x 20 cm plates, 2000 micrometer). Column chromatography was done on silica gel reagent (90-200 mesh) obtained from Accurate Chemical and Scientific Corp. Compounds 2a and 3a were prepared as previously described.[14] 5-Hydroxyiminomethyl-1,2,3-thiadiazole (4) and 3-methyl-5-hydroyiminomethyl-1,2,4-thiadiazole (5) were kindly provided by Dr. L.P.A. De Jong of TNO, Netherlands. Both compounds exhibited sharp melting points near the literature[15] values and neither showed any impurities in TLC analysis.

As described in the Results and Discussion Section, we prepared the heteroaromatic aldoximes (2) via the corresponding amidoximes (6) and heteroaromatic methyl derivatives (7). Conversion of the aldoximes to the hydroximyl chlorides (8) was followed by thioesterification to yield the desired type 3 compound. The syntheses of the new target compounds and intermediates are detailed below in order of the basic 3-substituted-heteroaromatic structure b through d (see Table 1).

Trimethyl acetomidoxime (6b). Trimethyl acetamidoxime was prepared on a 0.50-molar scale by the method of M. Freund and F. Lenze.[31] The crude product was recrystallized from toluene giving 26.1 g (45%) of 1b, mp 112-114°C (lit[31] mp 115-116°C).

3-(t-Butyl)-5-methyl-1,2,4-oxadiazole (7b). Compound 7b was prepared by the procedure used for 7d (see below). Trimethylacetamidoxime (21.33 g, 0.184 mol) was converted to 23.2 g (90%) of 7b, isolated as a colorless liquid. A 5.00 g portion was distilled to furnish the analytical sample: bp 147°=148°C (760 torr); IR (neat) 2975, 2930, 2905, 2875, 2590, 2510, 1475, 1460, 1400, 1355, 1275, 1195, and 890 cm^{-1}; NMR ($CDCl_3$)δ 1.39 (s, 9, t-Bu) and 2.58 ppm (s, 3, CH_3).

3(t-Butyl)-5-hydroxyiminomethyl-1,2,4-oxadiazole (2b). The procedure for preparing 2d (see below) was followed using 6.10 g (43.5 mmol) of 2b. The crude acidic product (2.18 g) was placed on a column containing 100 g of silica gel. Elution with 10% ether/hexane gave 0.73 g (9.9%) of 2b, m.p. 128.5-130°C. Two recrystallizations from acetone/hexane afforded the analytical sample: mp 130.5-132°C; IR (KBr) 3220, 3150, 3005, 2970, 2875, 1595, 1570, 1355, 1270, 1195, 1060, and 1000 cm^{-1}, NMR (CD_3COCD_3) δ1.41 (s, 9, tBu), 8.30 (s, 1, H-C = NOH), and 12.07 ppm (s, 1, NOH; NMR (DMSO-d_6) δ1.36 (s, 9, tBu), 8.38 (s, 1, H-C = NOH), and 13.01 ppm (s, 1, NOH). Anal. Calcd. for $C_7H_{11}N_3O_2$: C, 49.69; H, 6.55; N, 24.84. Found: C, 49.73; H, 6.63; N, 24.77.

General Procedure for Preparing Hydroximoyl Chlorides (8).[32] One equivalent for N-chlorosuccinimide was added to a solution of the appropriate oxime 2 (0.9-1.5 mmol) in 6 to 12 mL of DMF at 23-24°C. After 5 min, 10 mL of gas from the headspace of a concentrated hydrochloric acid bottle was added via syringe. After 10 min, the solution was heated to 45°C and stirred at 45°C for 30 min. The cooled mixture was poured into 50 mL of water and extracted with ether (3 x 50 mL). The combined ether layers were washed with water (2 x 50 ml) and brine (50 mL) and dried (Na_2SO_4). Removal of solvent furnished the crude hydroximoyl chloride 8 (94-96% yield), which was used in the subsequent reaction without further purification.

3(t-Butyl)-1,2,4-oxadiazol-5-yl thiohydroximic acid 2-(diethylamino) ethyl S-ester (3b). Compound 3b was prepared in the same fashion as 3d (see below) using 283 mg (1.39 mmol) of 8b. The crude product was purified by preparative TLC (silica gel, ether) to give 340 mg (81%) of 5b, m.p. 86-87.5°C. Recrystallization from acetone/hexane and acetone afforded the analytical sample: m.p. 87-88.5°C; NMR (CD_3COCD_3) δ 1.00 (t, 6, J = 7.5 Hz, CH_2 CH_3), 1.43 (s, 9, tBu), 2.56 (q, 4, J = 7.5 Hz, CH_2 CH_3), 2.56-3.53 (m, 4, CH_2 CH_2), and 9.78 ppm (br s, 1, NOH). Anal. Calcd for C_{13} H_{24} N_4 O_2 S: C, 51.97; H, 8.05; N, 18.65; S, 10.67. Found: C, 52.15; H, 8.01; N, 18.82; S, 10.49.

Acetamidoxime (6c). Compound 6c was prepared on a 1.0-molar scale by the method of Nordmann[33] and was isolated as the hydrochloride salt in 46% yield, mp 140-142°C (lit.[33] m.p. 140°C).

3,5-Dimethyl-1,2,4-oxadiazole (7c)[34,35] Compound 7c was prepared by the same procedure used for 7d (see below). Acetamidoxime hydrochloride 20.0 g (0.181 mol) was converted to 10.6 g (60%) of 7c, which was isolated as a colorless liquid.

3-Methyl-5-hydroxyiminomethyl-1,2,4-oxadiazole (2c). The procedure for the preparing of 2d (see below) was followed, using 7.50 g (76.4 mmol) of 7c. The crude acidic product (2.05 g) was placed on a column containing 100 g of silica gel. Elution with 20% ether/hexane provided 0.67 g (6.9%) of 2c. Two recrystallizations from acetone/hexane afforded the analytical sample: mp 163.5-164°C; NMR (CD_3COCD_3) δ2.42 (s, 3, CH_3), 8.29 (s, 1, H-C=NOH), and 12.22 ppm (s, 1, NOH); NMR (DMSO-d_6)δ 2.43 (s, 3, CH_3), 8.34 (s, 1, H-C = NOH), and 13.02 ppm (br s, 1, NOH). Anal. Calcd. for $C_4H_5N_3O_2$: C, 37.79; H, 3.97; N, 33.07. Found: C, 37.61; H, 4.05; N, 33.40.

3-Methyl-1,2,4-oxadiazol-5-yl thiohydroximic acid 2-(diethylamino) ethyl S-ester (3c). Compound 3c as in the same manner as 3d below using 128 mg (0.79 mmol) of 8c. Preparative TLC (silica gel, ether) of the crude product furnished 128.5 mg (63%) of 3c, m.p. 87-89°C. Recrystallization from acetone/hexane afforded the analytical sample: m.p. 92-93.5°C; NMR (CD_3COCD_3) δ 0.97 (t, 6, J = 7Hz, CH_2CH_3), 2.45 s,

3, CH_3), 2.54 (q, 4, J = 7Hz, CH_2CH_3) 2.54-3.47 (m, 4, CH_2CH_2), and 6.73 ppm (brS, 1, NOH). Anal. Calcd for $C_{10}H_{18}N_4O_2S$: C, 46.49; H, 7.02; N, 21.69; S, 12.41. Found: C, 46.81; H, 7.14; N, 21.82; S, 12.26.

1-Naphthamidoxime (6d).[35,36] A solution of 10 g (0.25 mol) of sodium hydroxide in 50 mL of water was added to a mixture of 17.4 g (0.25 mol) of hydroxylamine hydrochloride and 1.00 mL of 95% ethanol. 1-Cyanonaphthalene (38.3 g, 0.25 mol) was added, followed by 50 mL of 95% ethanol. The mixture was heated under reflux for 18 h. After cooling the solvent was removed in vacuo at 60°C. The residue was treated with 150 mL of 3 M HCl and the mixture heated to boiling. Insoluble material was filtered and the cooled filtrate was extracted wth dichloromethane (2 x 100 mL). The aqueous layer was treated with concentrated ammonium hydroxide to pH 8. The precipitate was collected and recrystallized from 60% aqueous ethanol to give 25.6 g (55% of 1-naphthamidoxime), m.p. 149.5-151.5°C (lit.[36] mp 148-149°C). In two subsequent preparations, 67% yields were obtained.

3-(1-Naphthyl)-5-methyl-1,2,4-oxadiazole (7d).[36,37] A mixture of 20.0 g (0.107 mol) of 6d and 50 mL of acetic anhydride was heated at reflux for 15 min. After cooling, 200 mL of water was added and concentrated ammonium hydroxide was added at 0°C to pH = 8. The mixture was extracted with ether (2 x 200 mL). Combined ether layers were washed with brine (200 mL) and dried ($MgSO_4$). Removal of solvent afforded 25.0 g of material which was placed on a column containing 450 g of silica gel. Elution with dichloromethane provided 17.0 g (75%) of 2a, m.p. 28-29.5°C. Recrystallization from petroleum ether gauge needles melting at 29-30°C (lit.[36] m.p. 36°C).

3(1-Naphthyl)-5-hydroxyiminomethyl-1,2,4-oxadiazole (2d). n-Butyllithium (3.8 mL, 9.1 mmol of 7.4 M solution) was added to a solution of 1.60 g (7.61 mmol) of 7d in 20 mL of THF at -70°C over a 5-min period (temperature rising to -45°C). The dark-red solution was warmed to -20°C over a 10-min period, then recooled to -70°C. After 5 min, 1.20 mL (11.4 mmol) of isopropyl nitrate[38] was added. After 5 minutes, at -70° → -60°C, the solution was warmed to 18°C. After the addition of 25

mol of 3 M HCl, the mixture was extracted with ether (3 x 50 mL). The ether layer was washed with 50 mL of brine and dried (Na_2SO_4). Removal of solvent yielded 3.42 g of material which was partitioned between ether (50 ml) and 0.5 N NaOH (2 x 50 ml). The aqueous solution was adjusted to pH 6 with 3 M HCl and extracted with dichloromethane (2 x 50 ml). The organic solution was washed with 50 mL of brine and dried (Na_2SO_4); removal of solvent gave 0.68 g of material, which was placed on a column containing 60 g of silica gel. Elution with 20% ether/hexane afforded 0.25 g (13.7%) of crude oxime, m.p. 147-149°C. Two recrystallizations from hexane plus a trace of acetone furnished the analytical sample: m.p. 153-154°C; IR (kBr) 3220, 3165, 3060, 3010, 2885, 1570, 1005, and 775 cm^{-1}; NMR (CD_3COCD_3) δ7.4-8.4 (m, 5, arom), 8.48 (s, 1, H-C = NOH), 8.98 (m, 2, arom), and 12.20 ppm (s, 1, NOH). Anal. Calcd for C_{13} H_9 N_3 O_2: C, 65.26; H, 3.79; N, 17.57. Found: C, 65.34; H, 3.82; N, 17.68.

3-1-Naphthyl)-1,2,4-oxadiazol-5-yl thiohydroximic Acid 2-(Diethylamine)ethel S-Ester (3d).[39] Triethylamine (1.0 mL) was added to a stirred solution of 323 mg (1.18 mmol) of 8d and 200 mg (1.18 mmol) of 2-diethylaminoethanethiol hydrochloride in 20 mL of dichloromethane. After 2 h, the solution was diluted to 50 mL with dichloromethane and washed with 50-mL portions of 1 M sodium bicarbonate solution and brine and dried (Na_2 SO_4). Removal of solvent gave 546 mg of crude product, which was chromatographed on three silica gel preparative TLC plates using ether as developing solvent. Extraction of the lower R_f UV active band with acetone yielded 366 mg (84%) of 3d as a colorless oil: NMR (CD_3COCD_3) δ0.95 (t, 6, J = 8 Hz, CH_3), 2.55 (q, 4, J = 8 Hz, CH_2CH_3), 2.7-3.7 (m, 4, CH_2CH_2), 7.48-9.25 (m, 7, arom) and 9.55 ppm (s, 1, NOH).

A solution of 338 mg of 3d in 25 mL of ether was treated with ether saturated with HCl gas to provide 333 mg (90%) of hydrochloride salt: m.p. 193.5-196°C (dec). Anal. Calcd. for C_{19} H_{23} N_4 O_2 S Cl: C, 56.08; H, 5.70; N, 13.77; S, 7.88. Found: C 56.53; H, 5.66; N, 13.85; S, 8.10.

An attempted preparation of 2d using ether as solvent, as described in the literature,[39] failed.

Methods

AChE inhibition and reactivation studies were conducted in accordance with the specific method described in reference 14. Briefly, the method involves reaction of a slight (10 to 15%) excess of enzyme with ethyl p-nitrophenyl methylphosphonate and incubation of the inhibited enzyme with known concentrations of test compound at 20°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%). At timed intervals, aliquots of the reaction mixture were withdrawn and diluted to pH 8, 0.1 M phosphonate buffer for assay of AChE activity using acetyl thiocholine (7.5×10^{-4} M) as substrate and the colorimetric method of Ellman et al.[40]

Reversible inhibition of AChE by test compounds was measured using the same procedure described above, except that phosphonylation of the enzyme was omitted. Enzyme was incubated with test compounds and aliquots withdrawn at three time points between 0.5 and 4-h incubation periods. In all cases, the degree of inhibition was found to be independent of incubation periods, and reported activities for each concentration of test compound are mean values (± standard deviation--S.D.) for all three time points.

Kinetics of reactions of test compounds with AcSCh were determined under the conditions used for assaying AChE (i.e., 25°C in pH 8, 0.1 M phosphate buffer) and therefore served also as correction factors in our reactivation experiments. The experiments were performed under pseudo-zero-order conditions, and rate constants were calculated as described in the Results and Discussion Section.

Rate constants for reaction of test compounds with ethyl p-nitrophenyl methylphosphonate (EPMP) were conducted as described in the preceding paragraph, substituting EPMP for AcSCh and pH 7.6, 0.1 M MOPS buffer for phosphate buffer. Product (p-nitrophenolate) production was monitored spectrophotometrically at 402 nM.

The reaction of EPMP with AChE in the presence and absence of compound 3a was examined using the general procedures described for the reactivation experiments. To determine the effective concentration of AChE in solution, we added known concentrations of EPMP to a stock solution of enzyme and recorded AChE activities after the phosphonylation reaction had gone to completion. AChE activity equivalent to 17 $\mu M\ min^{-1}$ was inhibited to 50% by 10 nM of EPMP. To determine the rate constant for inhibition of AChE by EPMP, we added EPMP latter in >10-fold molar excess to a stock solution of AChE in pH 7.6 buffer, withdrew aliquots at timed intervals and diluted 140-fold into pH 8 buffer for assay. Experiments with 3a present were performed as described above except that 3a was incubated at known concentrations with AChE before adding EPMP.

LITERATURE CITED

1. Heath, D. F.; Organophosphorus Poisons - Anticholinesterases and Related Compounds, Pergamon Press, New York, 1961.

2. Koelle, G. B.; in The Pharmacological Basis of Therapeutics, L. Goodman and A. Gilman (eds.), MacMillan, New York, 1965; pp. 404-444.

3. Sim, V. M.; in Drill's Pharmacology in Medicine, 3rd Ed., McGraw-Hill, New York, 1965; pp. 971-982.

4. Harris, B. L.; Shanty, F. Kirk-Othmer Encyc. Chem. Technol. Encylopedia of Chemical Technology, 3rd Ed., Vol. 5, J. Wiley and Sons, New York, 1980; pp. 393-416.

5. Karczmar, A. G.; Int. Encycl. Pharmacol. Ther., 1970, 1 (13):1.

6. Usdin, E.; Int. Encycl. Pharmacol. Ther., 1970, 1(13):47.

7. Englehard, N.; Prchal, K.; Nenner, M.; Angew Chem. Internat. Ed., 1967, 6:615.

8. Wills, J. H.; Int. Encycl. Pharmacol. Ther., 1970 1(13):357.

9. Namba, T.; Nolte, C. T.; Jackrel, J.; Grob, D. Am. J. Med., 1971, 50:475.

10. Ellin, R. I.; Wills, J. H.; J. Pharmaceut. Sci., 1964, 53:995.

11. McNamara, B. P.; "Oximes As Antidotes in Poisoning By Anticholinesterase Compounds," Edgewood Arsenal Special Publication. 5B-SP-76004, avail. NTIS AD-AO/23243, 1976.

12. Kenley, R. A.; Howd, R. A.; Mosher, C. W.; Winterle, J. S.; J. Med. Chem., 1981, 24:1124. Chapter I of this report.

13. Kenley, R. A.; Bedford, C.D.; Howd, R. A.; Jackson, S.; Mosher, C. W.; submitted to J. Med. Chem. Chapter II of this report.

14. Kenley, R. A.; Bedford, C. D.; Howd, R. A.; Miller, A.; Dailey, O.; submitted to J. Med. Chem. Chapter III of this report.

15. Benschop, H. P.; Vandenberg, G. R.; Van Hooidonk, C.; De Jong, L.P.A.; Kientz, C. E.; Berends, F.; Kepner, L. A.; Meeter, E.; and Visser, R.P.L.S. J. Med. Chem., 1979, 22:1306.

16. Kosower, E. M.; An Introduction to Physical Organic Chemistry, J. Wiley and Sons, New York, 1968, p. 49.

17. Schoene, K.; in Medical Protection Against Chemical Warfare Agents, Almquist and Wiksell, Uppsala, 1976, pp. 88-100.

18. Berry, W. K.; Davies, D. R.; Biochem. J., 1966, 100:572.

19. Green, A. L.; Smith, H. J.; Biochem J., 1958, 68:28.

20. Green, A. L.; Smith, H. J.; Biochem J., 1958, 68:32.

21. Wang, E.I.C.; Braid, P. E.; J. Biol. Chem., 1967, 242:2683.

22. De Jong, L.P.A.; "Reactivating Potency of Some Oximes Toward Phosphylated Acetylcholinesterase," TNO Report 1974-12. Avail. NTIS 29744/OST.

23. Belleau, B.; DiTullio, V.; Tsai, Y.-H.; Mol. Pharmacol, 1970, 6:41.

24. Maayani, S.; Weinstein, H.; Ben-Zvi, N.; Cohen, S.; Sokolovsky, M.; Biochem. Pharmacol., 1974, 23:1263.

25. Dawson, R. M.; Bladen, M. P.; Biochem. Pharmacol, 1979, 28:2211.

26. Heilbronn, E.; Acta Chem. Scand., 1061, 15:1380.

27. Tonkopii, V. D.; Prozorovskii,Z.B.; Byull. Exsp. Biol. Med., 1978, 82:823.

28. Tonkopii, V. D.; Ardabev, T. V.; Byull. Exsp. Biol. Med., 1978, 86:441.

29. Harris, L. W.; Heyl, W. C.; Stitcher, D. L.; Broomfield, C. A.; Biochem. Pharmacol., 1978, 27:758.

30. Schoene, K.; Steinhanses, J.; Oldiges, H.; Biochem. Pharmacol., 1976, 25:1955.

31. Freund, M.; Lenze, F. Chem. Ber., 1891, 24:2150.

32. Liu, K.-C.; Shelton, B. R.; Howe, R. K. J. Org. Chem., 1980, 45:3916.

33. Nordmann, E.; Chem. Ber., 1884, 17:2746.

34. Barrans, J.; C. R.,Hebd. Seances Acad Sci., 1959, 249:1096.

35. Ekstrand, A. G. Chem Ber., 20, 1887, p. 219.

36. Richter, E.; Chem. Ber., 1889, 22:2249.

37. Tiemann, F.; Kruger, P. Chem. Ber., 1884, 17:1685.

38. Blatt, A. H. and Noyes, W. A.; Organic Syntheses, Coll. Vol. II, John Wiley and Sons, Inc., New York, N.Y., 1943, p. 108.

39. Benn, M. H.; Can. J. Chem, 1964, 42:2393.

40. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M.; Biochem. Pharmacol, 1961, 7:88.

DISTRIBUTION LIST

12 copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20307-5100
4 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, Maryland 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END

FILMED

12-85

DTIC