S-ESTERS OF THIOHYDROXIMIC ACID ESTERS - A NOVEL CLASS OF CHOLINESTERASE REACTIVATORS

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Annual Report

by

Richard A. Kenley, Robert A. Howd, Carol W. Mosher, and John S. Winterle

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SRI International
333 Ravenswood Avenue
Menlo Park, California 94025

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Richard A. Kenley, Robert A. Howd, Carol W. Mosher, and John S. Winterle

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We have prepared a series of S-esters of thiohydroximic acid esters, given by the general formula $R'(0)C(NOH)S(CH_2)NR^2$HCl, where $R = CH_3; CH_2OC=CH_2, C_2H_5$; $n = 2,3'$; $R' = CH_3, C_2H_5$, or $i-C_2H_7$. The compounds were characterized with respect to acidity, nucleophilicity, and kinetics of reactivation of diisopropyl phosphoryl-acetylcholinesterase. The compounds were moderately active in reactivation of the enzyme, and activity is highly dependent on reactivator structure.
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We have prepared a series of thiohydroximic acid S-esters and evaluated them in vitro with respect to their ability to reactivate diisopropyl phosphoryl-acetylcholinesterase. The compounds conform to the general formula: \( RC(\cdot)C(\cdot)NOH)S(CH_2)_nNR_2 \cdot HCl \), where \( R = CH_3, C_6H_5, 4-CH_3OC_6H_4, 4-NO_2C_6H_4; n = 2,3; \) and \( R' = CH_3, C_2H_5, \) or \( i-C_3H_7. \) We also prepared \( 4-BrC_6H_4(\cdot)NOH)S(CH_2)N(C_2H_5)_2 \cdot HCl \) and \( 4-CH_3OC_6H_4C(\cdot)O)C(\cdot)NOH)S(CH_2)N(C_2H_5)_2 \cdot CH_3Cl \) for comparison. The thiohydroximates exhibit oxime acid dissociation constants \( (pK_A) \) in the range 6.9 to 8.4, bracketing the value of \( pK_a = 7.9 \) believed to be optimum for acetylcholinesterase reactivation. The compounds are also good nucleophiles; bimolecular rate constants \( (k_n) \) for reaction with p-nitrophenyl acetate follow the expression \( \log(k_n) = 6.7 - 0.69(14 - pK_A) \). The reactivation of diisopropyl phosphoryl-acetylcholinesterase is highly dependent on thiohydroximate structure: 4-h incubation of inhibited enzyme at \( pH = 7.6, 25^\circ C \) with \( 1 \times 10^{-3} M CH_3OC_6H_4C(\cdot)O)C(\cdot)NOH)S(CH_2)_3N(CH_3)_2 \cdot HCl \) gives no detectable restoration of activity, whereas \( 4-CH_3OC_6H_4C(\cdot)O)C(\cdot)NOH)S(CH_2)_3N(C_2H_5)_2 \cdot HCl \) restores inhibited enzyme activity to 58% of control under identical conditions. With thiohydroximate in excess over inhibited enzyme, the kinetics of reactivation are governed by a Michaelis constant \( (K_r) \) for binding thiohydroximate to the inhibited enzyme and a nucleophilic displacement rate constant \( (k_r) \) for attack on phosphorus.
Introduction

A variety of toxic organophosphorus esters owe their biological activity to phosphorylation\(^1\) of a serine hydroxyl at the active site of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), AChE.\(^2\) Inhibition of AChE leads to the accumulation of synaptic acetylcholine (ACh) and a concomitant disruption of nervous transmission.\(^3\) Therapy for anti-AChE agent intoxication is based on coadministration of anticholinergics (e.g., atropine) to antagonize the effects of accumulated ACh and of AChE "re-activators".\(^4\) These reactivators include nucleophilic species that displace inhibitor from the active surface and restore enzymatic activity.\(^5\)

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)

\[
\text{EOP} + \text{R} \xrightarrow{K_r} [\text{EOP} \cdot \text{R}] \xrightarrow{k_r} \text{HOH} \rightarrow \text{EOH} + \text{ROP}
\]

where: EOH is active enzyme, EOP is phosphorylated enzyme, R is a reactivator, ROP is phosphorylated reactivator, \([\text{EOP} \cdot \text{R}]\) is a complex between reactivator and inhibited enzyme, \(K_r (= [\text{EOP}][\text{R}]/[\text{EOP} \cdot \text{R}])\) is a Michaelis constant describing the affinity of the reactivator for the inhibited enzyme, and \(k_r\) is a rate constant for nucleophilic displacement of the inhibitor 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 later regard, compounds featuring the oximino (\(=\text{NOH}\)) 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 optimum value for the oxime acid dissociation constant (pK_a), known empirically 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, the pyridinium oximes, as a class, have been the compounds most studied. Specifically, 2-pyridinealdoxime methyl halides (2-PAM), 1,3-bis(4-hydroximinomethylpyridinium)propane dibromide (TMB-4), and 1,3-bis(4-hydroximinomethylpyridinium)dimethyl ether dichloride (toxogonin) are known to be powerful reactivators of phosphorylated AChE as well as effective antidotes for OP ester intoxication.8,11

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

The possibility that therapy of OP ester intoxication could be significantly improved through the use of more lipophilic reactivators has been appreciated by several investigators, and there have been various attempts to design reactivators that better penetrate biological membranes.

One approach has been to capitalize on the inherent activity of 2-PAM as a reactivator by modifying the general structure of the molecule. Thus Wilson\textsuperscript{30} and later Benschop et al\textsuperscript{18} quaternized 2-pyridinealdoxime with hydrophobic alkyl and aryl groups. Although these derivatives proved to be lipophilic, they were too toxic to be of any practical value. The tetrahydro-derivative of 2-PAM was prepared, but was devoid of activity as a reactivator.\textsuperscript{31} Higuchi and coworkers prepared 1,6-dihydro-2-PAM and found that the compound penetrates the blood-brain barrier and functions as a reactivator after in-vivo oxidation to 2-PAM.\textsuperscript{32-35} However, several studies have demonstrated that the conversion of 1,6-dihydro-2-PAM affords no increase in antidotal efficacy relative to 2-PAM itself.\textsuperscript{36-38}

There have also been several attempts to prepare nonquaternary AChE reactivators based on novel molecular structures unrelated to the pyridinium oximes. Examples include butanedioneoxime (DAM),\textsuperscript{39} 4-dimethylaminobutanedione 2-oxime (isonitrosine),\textsuperscript{18,40,41} 3-(diethylamino)propyl ester of oximinoacetic acid (OA3),\textsuperscript{18,42,43} 2-(diethylamino)ethyl S-ester of 4-bromobenzothiohydroximic acid (LA54),\textsuperscript{44-47} and 1,2,3-thiadiazole-5-aldoxime (TDA-5).\textsuperscript{48} These nonquaternary reactivators have demonstrated some limited advantages over the pyridinium oximes, but to date none have been proven to be clearly superior to 2-PAM in terms of overall therapeutic effectiveness.
Although different factors limit the utility of the individual non-quaternary 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. In vitro screening of nonquaternary reactivators has been largely confined to determination of the percent activity restored to inhibited AChE after incubation with one concentration of a reactivator for a given time interval. While such determinations identify the relative potencies of structurally related materials, they provide no insight into the molecular requirements for activity.

Schoene has used the determination of reactivation kinetics (the constants $K_r$ and $k_r$) to good advantage in establishing structure-activity relationships for bis-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 thiohydroximic acid esters, given by the formula:

$$\text{RC-CH-S(CH}_2\text{)}_n\text{NR}_2\text{OH}$$

Our choice of this general framework was based on considerations of synthetic flexibility, structural similarity to AChE substrates (e.g., ACh and acetyl thiocholine), 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 thiohydroximates and their characterization with respect to $pK_a$, nucleophilicity, and relative ability to reactivate diisopropyl phosphoryl-AChE. 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 2-PAM and BrC₆H₄C(:NOH)SCH₂CH₃N(C₂H₅)₂•HCl, a thiohydroximate that has been touted as a particularly effective reactivator.
Experimental Section

Melting points (uncorrected) were obtained with a Fischer-Johns apparatus, NMR spectra (in DMSO-d₆ solvent unless otherwise specified, Me₄Si 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 2500 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 MOPS buffer plus MgCl₂ (0.01 M), NaN₃ (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 $1.4 \times 10^3$ ACh units per milligram. Titration of the enzyme activity with ethyl p-nitrophenyl methylphosphonate indicated $6 \times 10^{-9}$ mol of active sites per milligram of enzyme, in good agreement with the molecular weight of $2.3 \times 10^5$ reported for the purified enzyme.

*See appendix.
p-Nitrophenyl acetate (pNPA), 3-(N-morpholino)propanesulfonic acid (MOPS), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and acetyl thiocholine were used as supplied by the manufacturer (Sigma Chemical Co.). We prepared isopropyl nitrite in small amounts and stored it at 5°C until immediately before use. Where results of elemental analyses are given, only the symbols of the elements are indicated when experimental values agree within ±0.4% of theoretical.

Hydroximoyl Chlorides. The α-aroylhydroximoyl chlorides 4-RC₆H₄C(O)C(NOH)Cl, where R = H, CH₃, or NO₂, were prepared by treating the corresponding acetophenones with i-C₃H₇ONO and HCl as described by Brachwitz. CH₃C(O)C(NOH)Cl was similarly prepared from chloroacetone. p-Bromobenzohydroximoyl chloride was prepared by chlorination of p-BrC₆H₄CHNOH.

Table I gives selected data for the hydroximoyl chlorides.

S-(N,N-Dialkylamino)alkyl Acyl- and Aroylthiohydroximates (SR 2458, 2462, 3008, 3010, 3014, and 3015). All compounds were prepared from the corresponding hydroximoyl chlorides by the same general procedure. SR 3015 and 3014, were isolated, respectively, as the methyl chloride and oxalic acid salts. The remaining compounds were isolated as the hydrochloride salts. Table II presents selected data for the thiohydroximates. The preparation of S-(2-diethylamino)ethyl 4-methoxybenzoylthiohydroximate hydrochloride (SR 2461) 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 CHCl₃. 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
Table I. Selected Data for Hydroximoyl Chlorides

\[ RC(\cdot\text{NOH})\text{Cl} \]

<table>
<thead>
<tr>
<th>R</th>
<th>Yield, %</th>
<th>Recryst. Solvent</th>
<th>mp \degree C</th>
<th>NMR (\text{-NOH}), \delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{C}_6\text{H}_5\text{C}(:\text{O})^- )</td>
<td>52</td>
<td>( \text{C}_6\text{H}_5)-CCl₂</td>
<td>123-126</td>
<td>13.68</td>
</tr>
<tr>
<td>( 4-\text{NO}_2\text{C}_6\text{H}_4\text{C}(:\text{O})^- )</td>
<td>18</td>
<td>( \text{C}_6\text{H}_5)</td>
<td>136-140</td>
<td>13.86</td>
</tr>
<tr>
<td>( 4-\text{CH}_3\text{OC}_6\text{H}_4\text{C}(:\text{O})^- )</td>
<td>51</td>
<td>( \text{C}_6\text{H}_5)</td>
<td>131-133</td>
<td>13.40</td>
</tr>
<tr>
<td>( \text{CH}_3\text{C}(:\text{O})^- )</td>
<td>50</td>
<td>CCl₂</td>
<td>88-93</td>
<td>13.44</td>
</tr>
<tr>
<td>( 4-\text{BrC}_6\text{H}_4^- )</td>
<td>65</td>
<td>CCl₂</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Table II. Selected Data for Thiohydroximates
\( RCh(NH)(CH_3)_n \cdot R''X \)

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R )</th>
<th>( n )</th>
<th>( R' )</th>
<th>( R''X )</th>
<th>Yield, %</th>
<th>TLC*</th>
<th>mp, (^\circ)C</th>
<th>( \delta ) ((-NOH))</th>
<th>( \lambda_{max} )</th>
<th>( \epsilon ) cm(^{-1}) ( M^{-1} ) ( \times 10^{-4} )</th>
<th>( \text{pK}_a )</th>
<th>Anal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR 2458</td>
<td>C(_2)H(_5)C(O)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>HCl</td>
<td>32</td>
<td>0.56</td>
<td>136-141</td>
<td>12.94</td>
<td>262</td>
<td>1.06</td>
<td>7.60</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 2460</td>
<td>4-NO(_2)C(_2)H(_4)C(O)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>HCl</td>
<td>18</td>
<td>0.57</td>
<td>170-172.5</td>
<td>13.39</td>
<td>269</td>
<td>1.74</td>
<td>7.1</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 2461</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>HCl</td>
<td>65</td>
<td>0.65</td>
<td>139-140</td>
<td>12.69</td>
<td>230</td>
<td>1.12</td>
<td>7.8</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 2462</td>
<td>CH(_3)C(O)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>HCl</td>
<td>24</td>
<td>0.45</td>
<td>156-161</td>
<td>13.12</td>
<td>273</td>
<td>0.87</td>
<td>7.4</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 3008</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>2</td>
<td>CH(_3)</td>
<td>HCl</td>
<td>55</td>
<td>0.55</td>
<td>154-155.5</td>
<td>12.74</td>
<td>230</td>
<td>1.12</td>
<td>8.0</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 3009</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>2</td>
<td>(CH(_2))(_2)CH</td>
<td>HCl</td>
<td>70</td>
<td>0.64</td>
<td>179-184</td>
<td>12.70</td>
<td>230</td>
<td>1.13</td>
<td>8.0</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 3010</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>3</td>
<td>CH(_3)</td>
<td>HCl</td>
<td>d</td>
<td>0.30</td>
<td>c</td>
<td>12.50</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>SR 3014</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>3</td>
<td>CH(_3)</td>
<td>[HOC:(O)]</td>
<td>29</td>
<td>0.42</td>
<td>151-153.5</td>
<td>e</td>
<td>d</td>
<td>d</td>
<td>8.4</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>SR 3105</td>
<td>4-CH(_3)OC(_2)H(_4)C(O)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>CH(_3)</td>
<td>48</td>
<td>0.39</td>
<td>c</td>
<td>f</td>
<td>d</td>
<td>d</td>
<td>7.8</td>
<td>C(<em>n)H(</em>{n+5})Cl</td>
</tr>
<tr>
<td>LA54</td>
<td>4-BrC(_2)H(_4)</td>
<td>2</td>
<td>C(_2)H(_5)</td>
<td>HCl</td>
<td>41</td>
<td>0.63</td>
<td>146-149</td>
<td>12.16</td>
<td>233</td>
<td>1.41</td>
<td>9.2</td>
<td>C(<em>n)H(</em>{n+5})Cl, Br</td>
</tr>
</tbody>
</table>

aSilica gel, CHCl\(_3\)-MeOH (6:1).
bIn pH 7.6-0.13 M phosphate buffer.
cHygroscopic syrup.
dNot determined.
eBroad singlet, apparently including oxalate protons.
fIn CD\(_3\)OD solution, OH exchanged with OD.
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 using CHCl₃-MeOH (8:1) as eluting solvent. From the fractions that showed no impurities, we obtained 479 mg of a light yellow syrup; TLC, Rₚ 0.6 (CHCl₃-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 415 mg (60%) of SR 2461. TLC (CHCl₃-MeOH, 6:1) Rₚ 0.65; NMR δ 12.69 (s, 1H, =NOH), 10.55 (broad, 1H, NH), 7.93 (d, 2H, Ar), 7.4 (d, 2H, Ar), 3.87 (s, 3H, OCH₃), 2.7-3.4 (m, 8H, CH₂), 1.13 (t, 6H, CH₃); IR (nujol) ν 3215s, 2632s, 1587m, and 922s 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 (5%), mp 134°-135°C.

S-(2-diethylamino)ethyl 4-Bromobenzothiohydroximate (LA54). 4-Bromo-benzothiohydroximoyl chloride (705 mg; 3.0 mmol) was treated with 2-diethyl-aminoethanethiol hydrochloride (510 mg; 3.0 mmol) and sodium methoxide (162 mg; 3.0 mmol) in 15 mL of isopropanol. The crude product was purified by "flash" chromatography on silica gel (CHCl₃-MeOH, 6:1) and final recrystallization from methanol-ether to give 450 mg (41% yield) of white crystalline product, mp 146°-149°C. TLC (CHCl₃-MeOH, 6:1) Rₚ 0.63 (UV and I₂ detected); NMR δ 12.6 (s, 1H, NOH), 10.42 (broad, 1H, NH), 7.69 (φ, 4H, Ar), 3.3-2.8 (m, 8H, CH₂), 1.13 (t, 6H, CH₃); IR (nujol) ν 3215s, 2632s, 1587m, and 922s cm⁻¹.

11
**Acetylcholinesterase Assay.** Two different spectrophotometric methods were used to determine AChE activity. The hydrolysis of PNPA to p-nitrophenolate\(^58,59\) \((\lambda_{\text{max}} = 402 \text{ nm}, \varepsilon = 1.50 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})^60\) was 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,\(^61\) monitoring production of 5-thio-2-nitrobenzoic acid \((\lambda_{\text{max}} = 412 \text{ nm}, \varepsilon = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})\). 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 follows.

**p-Nitrophenyl Acetate Method.** The lyophilized enzyme is dissolved in buffer at a nominal concentration of \(5 \times 10^2\) ACh units per mL and maintained at \(-10^\circ\)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 \(1.0 \times 10^9\) and 10 \(\mu\)L of substrate stock solution is added to 990 \(\mu\)L of diluted enzyme to give final concentrations of \(1.0 \times 10^{-3}\) M substrate and \(2 \times 10^{-9}\) 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 \(2.3 \times 10^{-6}\) M min\(^{-1}\).

**Ellman Method.** The 500 ACh units per mL stock solution is diluted by a factor of \(2.0 \times 10^3\) in MOPS buffer, DTNB is made to \(1.0 \times 10^{-2}\) M in pH 7, 0.1 M phosphate buffer, and acetyl thiocholine is made to \(7.5 \times 10^{-2}\) M in 1:9 \(\text{H}_2\text{O}:\text{EtOH}\). Combination of 20 \(\mu\)L of enzyme solution plus 30 \(\mu\)L 0.1 M phosphate buffer, pH 8, gives an analytical solution with final concentrations
of $2 \times 10^{-11}$ M AChE, $3.0 \times 10^{-4}$ M DTNB, and $7.5 \times 10^{-6}$ M acetyl thiocholine. 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 $7.6 \times 10^{-8}$ M min$^{-1}$.

**Acetylcholinesterase Inhibition and Reactivation**

**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 $1.0 \times 10^{-2}$ 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 $1.0 \times 10^{-2}$ M in water and diluted to $3.0 \times 10^{-6}$ to $2.0 \times 10^{-5}$ 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 $25^\circ \pm 0.2^\circ$ 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 $1.0 \times 10^{-2}$ M pNPA, and the enzyme activity is assayed. Thus the final concentrations in the assay solutions are as follows: $1 \times 10^{-6}$ M inhibited AChE, $3.00 \times 10^{-8}$ to $2.00 \times 10^{-8}$ M reactivator, and $1.0 \times 10^{-8}$ M substrate. Observed enzyme
activities are corrected (see Results Section) for spontaneous and re-activator-catalyzed hydrolysis of substrate to give net activities. 100% enzyme activity is defined as the activity restored by incubation of inhibited enzyme with $1.0 \times 10^{-3}$ M 2-PAM.

Elman 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 $1.0 \times 10^{-2}$ 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 $\times 10^{-6}$ to 2.00 $\times 10^{-3}$ 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: $1.0 \times 10^{-10}$ M AChE, 3.00 $\times 10^{-8}$ to 2.00 $\times 10^{-5}$ M reactivator, 3.0 $\times 10^{-4}$ M DTNB, and $7.5 \times 10^{-4}$ M acetyl thiocholine.
Results and Discussion

Synthesis, Structure, and Acidity. We prepared the thiohydroximates described in Table II by reaction (2) and (3)

\[
\begin{align*}
\text{RCCH}_3 + \text{iPrONO} \quad \overset{\text{HCl}}{\rightarrow} \quad \text{R-C} - \text{C} - \text{Cl} \\
\text{R-C} - \text{C} - \text{Cl} + \text{HS(CH}_2)_n \text{NR}_2 \quad \overset{\text{Et}_3\text{N}}{\rightarrow} \quad \text{R-C} - \text{C} - \text{S(CH}_2)_n \text{NR}_2
\end{align*}
\]

Unoptimized yields of reaction (2) ranged from 18% to 70%, based on the amount of hydroxamoyl chloride used.

Oxime acid dissociation constants ($pK_a$) were measured by spectrophotometric determination of oximate concentration in buffers of various pH.\(^{62}\)

From the data in Table II for the SR-series compounds, a plot (not shown) of $pK_a$ versus oxime proton NMR chemical shift is linear and conforms to equation (4)

\[
pK_a = (25.3 \pm 2.1) - (1.36 \pm 0.16) \delta
\]

For the aroylthiohydroximates (SR 2458, 2460, and 2461) a plot (not shown) of $pK_a$ versus Hammet substituent constant ($\sigma_p$)\(^{63}\) is also linear and conforms to equation (5)

\[
pK_a = (7.63 \pm 0.02) - 0.63 \pm 0.05) \sigma_p
\]

These correlations provide an indication of the validity of the $pK_a$ values, an important consideration because we have related thiohydroximate reactivity to the concentration of the dissociated form at a given pH and hence to
the acidity of the oxime functionality. We determined $pK_a = 7.99$ for 2-PAM, which is in good agreement with the value of 7.92 reported by Schoene and Strake.\textsuperscript{49} Our value of $pK_a = 9.28$ for LA54 is lower than that ($pK_a = 10.42$) determined potentiometrically by Krivenchuk et al.\textsuperscript{45} 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 syn and anti isomerization in the thiohydroximates insofar as the configuration of the oxime functionality can have a profound influence on the reactivation of inhibited AChE.\textsuperscript{10} Exner and coworkers find that benzohydroxamoyl chlorides\textsuperscript{6b} and methyl benzothiohydroximate\textsuperscript{65} adopt the syn configuration solution.

\begin{center}
\textit{syn}-benzohydroxamoyl chlorides
\end{center}
\begin{center}
\textit{syn}-methyl benzothiohydroximate
\end{center}

\textbf{Line Drawing 1}
These workers also report IR O-H stretching frequencies near 3560 cm⁻¹ for these compounds in CHCl₃. We find that LA54 and the hydroxamoyl chlorides listed in Table I exhibit O-H stretching frequencies at 3200 to 3300 cm⁻¹ for spectra taken in nujol mulls. By analogy, a syn configuration seems likely for these compounds. Contrasting this, the SR-series thiohydroximates nujol mull spectra show no sharp O-H bands in the 3000 to 4000 cm⁻¹ region. An IR spectrum of the free base of SR 2458 in CHCl₃, solution reveals a very broad band centered at 2564 cm⁻¹ that is typical of hydrogen-bonded O-H.⁶⁶ These observations suggest the anti configuration for the SR-series thiohydroximates, with six-center intramolecular hydrogen bonding of oximino proton to the α-carbonyl group.

\[ \text{anti-aryloxydithiohydroximate} \]

*Line Drawing 2*

**Nucleophilicity.** As a measure of the inherent reactivity of the thiohydroximates, we determined values for the bimolecular rate constant, \( k_n \), for reaction of the thiohydroximates with pNPA. We measured initial rates of production of p-nitrophenolate (pNP) from the reaction of \( 1.0 \times 10^{-3} \) M
pNPA with 10.0 to 100 x 10^-6 M thiohydroximate 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):

\[ + \frac{d[pNP]}{dt} = k_n [pNPA][OX] + k_o [pNPA] \]  

(6)

where [OX] is the concentration of the anionic form of the thiohydroximate, 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 (7) reduces to:

\[ k_{eQ} = +\frac{d[pNP]}{dt} [pNPA]^{-1} = k_n [OX] + k_o \]  

(8)

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 (9).

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

(9)

In accordance with equation (8), plots (not shown) of \( k_{eQ} \) versus [OX] for the SR-series thiohydroximates, 2-PAM, and LA54 are linear with slope = \( k_n \) and intercept = \( k_o \). Table III 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 (9), the \( k_o \) values are essentially independent of the reactivator used. The average for all values of \( k_o \) from Table III is \( 2.3 \pm 0.4 \times 10^{-3} \text{ min}^{-1} \), which is in good agreement with the value of \( k_o = 2.1 \times 10^{-3} \text{ min}^{-1} \) reported by Jencks and Gilchrist.\(^6\) For 2-PAM, the value of \( k_n = (9.6 \pm 0.8) \times 10^2 \text{ M}^{-1} \) compares to the value \( k_n = 4.0 \times 10^2 \text{ M}^{-1} \text{ min}^{-1} \).
Table III. Rate Constants for Hydrolysis of p-Nitrophenyl Acetate (pNPA) in the Presence of Thiohydroximates$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_c$</th>
<th>$k_d$</th>
<th>p$K_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M^{-1} \text{min}^{-1} \times 10^{-2}$</td>
<td>$\text{Min}^{-1} \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>SR 2458</td>
<td>$3.9 \pm 0.2$</td>
<td>$2.0 \pm 0.4$</td>
<td>6.40</td>
</tr>
<tr>
<td>SR 2460</td>
<td>$1.4 \pm 0.1$</td>
<td>$2.8 \pm 0.4$</td>
<td>6.86</td>
</tr>
<tr>
<td>SR 2461</td>
<td>$6.1 \pm 0.02$</td>
<td>$2.0 \pm 0.4$</td>
<td>6.18</td>
</tr>
<tr>
<td>SR 2462</td>
<td>$2.7 \pm 0.1$</td>
<td>$2.8 \pm 0.6$</td>
<td>6.60</td>
</tr>
<tr>
<td>SR 3008</td>
<td>$5.1 \pm 0.6$</td>
<td>$1.7 \pm 0.4$</td>
<td>6.00</td>
</tr>
<tr>
<td>SR 3009</td>
<td>$5.6 \pm 0.4$</td>
<td>$2.4 \pm 0.3$</td>
<td>5.99</td>
</tr>
<tr>
<td>SR 3014</td>
<td>$13.4 \pm 0.05$</td>
<td>$2.2 \pm 0.2$</td>
<td>5.58</td>
</tr>
<tr>
<td>SR 3015</td>
<td>$3.9 \pm 0.03$</td>
<td>$1.8 \pm 0.3$</td>
<td>6.18</td>
</tr>
<tr>
<td>LA54</td>
<td>$2.4 \pm 0.2$</td>
<td>$2.6 \pm 0.3$</td>
<td>4.72</td>
</tr>
<tr>
<td>2PAM</td>
<td>$9.6 \pm 0.8$</td>
<td>$2.9 \pm 1.2$</td>
<td>6.01</td>
</tr>
</tbody>
</table>

$^a$ Determined from equivalent first-order rates, $k_{eq}$, for p-nitrophenolate production from $1.0 \times 10^{-3} \text{ M pNPA}$ in the presence of $10$ to $100 \times 10^{-6} \text{ M test compound}$ at pH 7.6 and 25°C in 0.1 M MOPS buffer; see equation (7).

$^b$ See Table II.

$^c$ Nucleophilic displacement rate constant from slope of plot of $[\text{OX}]$ vs $k_{eq}$; see equations (8) and (9).

$^d$ Spontaneous hydrolysis rate constant, from intercept of plot of $[\text{OX}]$ vs $k_{eq}$; see equations (8) and (9).

$^e pK_B = 14 - pK_A$. 
determined by Bergmann and Govrin\textsuperscript{68} at pH = 8.0 and 25°C.

Figure 1 is a Bronsted plot (log $k_n$ versus $pK_B$) of the data of Table III. The data for the SR-series compounds conform to equation (10):

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

where the $\beta$-value (slope) of 0.69 ±0.08 compares to the value of $\beta = 0.8$ commonly observed for reaction of pNPA with oxygen nucleophiles.\textsuperscript{67,69}

Thus the thiohydroximates 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 Methods 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^{-5}) of pNPA used in the assay, the text 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. 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 substrate hydrolysis were smaller, and could be made by running blanks with reactivator in an
FIGURE 1  CONJUGATE BASE IONIZATION CONSTANTS (pKₐ) VERSUS LOGARITHM OF BIMOLECULAR NUCLEOPHILIC DISPLACEMENT RATE CONSTANT (kₐ) FOR HYDROLYSIS OF p-NITROPHENYL ACETATE IN THE PRESENCE OF VARIOUS REACTIVATORS AT pH = 7.6, 25°C
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 (11):

\[ \text{EOP} \xrightarrow{k_a} \text{EOP}' \]  

(11)

where EOP is diisopropyl phosphoryl-AChE and EOP' is a dealkylated-, inhibited-enzyme in accordance with the accepted mechanism for this phenomenon. We determined \( k_a \) under our experimental conditions by incubating the inhibited enzyme for time intervals before reactivating with \( 1.0 \times 10^{-3} \) M 2-PAM. Semilog plots of \( A_t/A_0 \) versus time (where \( A_t \) is AChE activity after incubation for \( t \) min before reactivation with 2-PAM, and \( A_0 \) 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 \((1.61 \pm 0.08) \times 10^{-3} \text{ min}^{-1}\), 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 x 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 SR 3009, none of the compounds gave any evidence of inhibition of the enzyme using this procedure. For SR 3009 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 thiohydroximates 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 thiohydroximates as reactivators of diisopropyl phosphoryl-AChE, we added the DFP-inhibited enzyme to $1.00 \times 10^{-3}$ M solution of the test compounds and determined enzyme activity after 2-, 4-, and 24-h incubation periods. Percent reactivation is calculated according to equation (12):

$$\% r_t = 100 \left(1 - \frac{E_t}{E_o}\right)$$

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 IV summarizes the data for the SR-series thiohydroximates, plus 2-PAM and LA54.

From the table, it is seen that the SR-series thiohydroximates 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 (SR 3008, 3009, and 2461), activity decreases with increasing size of the alkyl substituent, indicating a steric effect on the reactivation. It is also significant that the quaternary thiohydroximate SR 3015 is actually less reactive than the tertiary analog SR 2461. We anticipated that the tertiary alkylamine functionality would be completely protonated at pH = 7.6 and that the
Table IV. Percent Reactivation of (\%\text{t}) of Diisopropyl Phosphoryl-AChE after Incubation with $1.00 \times 10^{-3}$ M Reactivator at $25^\circ$C, pH = 7.6

<table>
<thead>
<tr>
<th>Compound</th>
<th>(%\text{t}) After Incubation for t hr</th>
<th>(t = 2)</th>
<th>(t = 4)</th>
<th>(t = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR 2458(^c)</td>
<td>63</td>
<td>83</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>SR 2460</td>
<td>18</td>
<td>20</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>SR 2461</td>
<td>31</td>
<td>58</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>SR 2462</td>
<td>13</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>SR 3008</td>
<td>56</td>
<td>65</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>SR 3009</td>
<td>4.3</td>
<td>2.8</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>SR 3014</td>
<td>0.0</td>
<td>0.0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>SR 3015</td>
<td>5.6</td>
<td>9.5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>LA54(^c)</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2PAM</td>
<td>109</td>
<td>105</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)See Table II.  
\(^b\)\(\%\text{t}\) calculated from equation (12).  
\(^c\)Reactivator concentration $= 2.00 \times 10^{-3}$ M.
protonated species would provide the coulombic interaction with the anionic subsite necessary for enhanced affinity of the reactivator for the inhibited enzyme. If this were not the case, the quaternary ammonium analog would predictably exhibit considerably higher activity than the nonquaternary reactivator. The fact that SR 2461 is actually more reactive than the methylated SR 3009 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-(dimethylamino)ethyl and 3-(dimethylamino)propyl S-esters of p-methoxybenzoylthiohydroximic acid (SR 3008, and 3014, 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 SR 3014 (Table II) is 0.6 log units higher than that of SR 2461. Therefore at pH 7.6, SR 2461 is 40% ionized [equation (9)], whereas SR 3014 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 method are also apparent when 2-PAM is compared with the thiohydroximates. Because reactivation by 2-PAM is complete within 2 h, it is impossible to use the data of Table IV to quantitatively compare it with the thiohydroximates. 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 LA54 deserve comment. We find LA54 to be considerably inferior to 2-PAM as a reactivator of diisopropyl phosphoryl-
AChE and attribute the lack of activity of LA54 primarily to its high 
$\text{pK}_a$ (= 9.42) 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.\textsuperscript{45} who obtained 35% 
and 41% reactivation of ethyl methylphosphonylbutyrylcholinesterase after 
incubation with 2-PAM and LA54, respectively.

Even considering the differences in inhibited enzymes used, it is 
difficult to rationalize the large differences in the relative activities 
of LA54 and 2-PAM reported by us and by Krivenchuk and coworkers. In the 
absence of additional details about the experimental method employed 
by Krivenchuk et al., we cannot rule out other reasons for this 
apparent discrepancy. In any case, 2-PAM proves to be a very poor reactivator 
in our experiments, and we believe that published reports of its antidotal 
effectiveness\textsuperscript{44-47} should be interpreted with some caution.

**AChE Reactivation Kinetics.** The work of several authors\textsuperscript{70,71} makes 
it clear that the kinetics of reactivation of phosphorylated AChE cannot be 
adequately described by a simple bimolecular substitution reaction mechanism. 
Rather, the mechanism involves reversible formation of a Michaelis complex 
between reactivator and inhibited enzyme followed by a nucleophilic dis- 
placement reaction on phosphorus, as shown in equation (1). For the 
particular case of reactivation of diisopropyl phosphoryl-AChE, dealkylation 
of the inhibited enzyme [reaction (11)] is sufficiently rapid that this 
reaction must also be taken into account in determining reactivation kinetics.

De Jong and Wolring\textsuperscript{70,71} have devised a kinetic scheme that allows 
for parallel processes of reactivation and dealkylation. The scheme 
consists of reactions (1), (11), and (13):
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 (13) for dealkylation of the Michaelis 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:

\[
\ln (\%_{\text{react}} - \%_t) = \ln (\%_{\text{react}}) - k_{\text{obs}} t
\]  

\[
k_{\text{obs}} - k_a = \frac{k_{\max}[\text{OX}]}{K_r + [\text{OX}]}  
\]  

\[
k_{\max} = k_a' - k_a + k_r  
\]  

\[
k_a' = \left(\frac{k_{\max} + k_a}{k_a}\right)[\text{EOP'}]_{\infty}/[\text{EOH}]_{\infty} - k_{\text{obs}}[\text{OX}]^{-1}(1 + [\text{EOP'}]_{\infty}/[\text{EOP}]_{\infty})^{-1}  
\]  

\[
[\text{EOP'}]_{\infty}/[\text{EOH}]_{\infty} = (100 - \%_{\text{react}})/\%_{\text{react}}  
\]  

where $k_{\text{obs}}$ is the pseudo-first-order (oximate in excess) rate constant for reactivation; $\%_{\text{react}}$ is the maximum attainable percent reactivation; $k_{\max}$ is defined as in equation (16); $[\text{EOP'}]_{\infty}$ is the amount of nonreactivatable enzyme at infinite time; $[\text{EOH}]_{\infty}$ is the concentration of active enzyme at infinite time; and $k_a$ is determined in an independent experiment according to equation (11).

Rearranging equation (17) gives
Similarly, rearranging equation (15) gives

\[
\frac{[\text{EOP}']_{\text{CC}}}{[\text{EOH}]_{\text{CC}}} = \frac{k_a k_r [\text{OX}]^{-1}}{k_r} + \frac{k'_a}{k_r}
\]  

(19)

Thus a plot of ln (ZrCC) versus time gives slope = -kobs and intercept = ln (ZrCC); a plot of [EOP']/[EOH] vs [OX]^{-1} gives slope = k K_r/k_r', and intercept = k'_a/k_r; and a plot of (kobs - k_a)^{-1} versus [OX]^{-1} gives slope = K_r/kmax and intercept = 1/kmax. Because k_a is independently known (vide supra), the determination of ZrCC and Zr as a function of time and reactivator concentration permits calculation of the important kinetic constants K_r and k_r. Table V summarizes the necessary derivations. Wang and Braid\textsuperscript{7c} have shown that at low reactivator concentration, equation (21) holds:

\[
k_b = \frac{k_r}{K_r}
\]  

(21)

where k_b is a bimolecular rate constant for reactivation in the limit of low reactivator concentration. Finally, we define k\textsubscript{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 (9) and (21), k\textsubscript{eff} is given by equation (22):

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

(22)

To probe these relationships, we determined ZrCC and Zr as a function
Table V. Formulas for Calculating Kinetic Constants for Reactivation of Inhibited AChE

<table>
<thead>
<tr>
<th>Plot</th>
<th>Abscissa</th>
<th>Ordinate</th>
<th>Slope (n)</th>
<th>Intercept (n)</th>
<th>Calculate</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>t</td>
<td>ln((%r_\to - %r_t))</td>
<td>(S_1 = -k_{\text{obs}})</td>
<td>(I_1 = \ln(%r_{oo}))</td>
<td>([EOP']/[EOH] = \frac{100 - \exp[I_1]}{\exp[I_1]})</td>
<td>(14), (18)</td>
</tr>
<tr>
<td>2</td>
<td>([\text{OX}]^{-1})</td>
<td>([EOP']</td>
<td><em>{oo}/[EOH]</em>{oo})</td>
<td>(S_2 = k_a K_r / k_r)</td>
<td>(I_2 = k'_a / k_r)</td>
<td>(k'_a = I_2 k_r)</td>
</tr>
<tr>
<td>3</td>
<td>([\text{OX}]^{-1})</td>
<td>((k_{\text{obs}} - k_a)^{-1})</td>
<td>(S_3 = K_r / k_{\text{max}})</td>
<td>(I_3 = 1/k_{\text{max}})</td>
<td>(k_r = S_3 / I_3)</td>
<td>(16), (20)</td>
</tr>
</tbody>
</table>

\(k_{\text{obs}}\) is the observed rate constant.

\(k_a\) is the reversible inhibition rate constant.

\(K_r\) is the equilibrium dissociation constant for the inhibitor.

\(I\) is the inhibitor concentration.

\([\text{OX}]\) is the concentration of the oxidant.

\(r\) is the reaction rate.

\(\%r_{oo}\) is the percentage of free enzyme.

\(\%r_t\) is the percentage of total enzyme.

\(\%r_{oo}\) is the percentage of inhibited enzyme.
of time for various concentrations of 2-PAM, SR 2458, 2461, and 3008. Figure 2 is a sample plot of equation (14) for 1.00 x 10^{-3} M 2-PAM, and SR 2461, and 0.200 x 10^{-3} M SR 3008. The linearity of the plots indicates that complicating factors, such as reinhibition of the enzyme by phosphorylated reactivator, are unimportant under the experimental conditions. Table VI summarizes the values of k_{obs} and ln(\%_{CC}) 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 SR 2458, 2461, and 3008. The plot for 2-PAM is not shown because of the difference in scale for [OX]^{-1}, but the data for 2-PAM conform reasonably well to equation (20). A similar plot (not shown) of [OX]^{-1} versus [EOP']^{-1} versus [EOP']_{CC}/[EOH]_{CC} conforms to equation (19) for the reactivators investigated. Data from Table VI and from least squares treatment of the data according to equations (19) and (20) are summarized in Table VII along with kinetic constants calculated as in Table V and equations (21) and (22).

At this point, some discussion of the precision of the measurements seems appropriate. Accurate determination of \%_{CC} is essential for reliable calculation of values for k_{obs} and especially for the ratio [EOP']_{CC}/[EOH]_{CC}. We did not make replicate determinations of \%_{CC} 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 (19). These intercepts are used to calculate k_{a}', and we view the values for k_{a}' in Table VII as tentative. The intercepts calculated from equation (19) are also subject to a degree of uncertainty, and this must be taken into consideration when interpreting values for k_{r}. 
FIGURE 2  NATURAL LOGARITHM OF PERCENT MAXIMUM REACTIVATION MINUS REACTIVATION AT TIME t, $\ln(\%_{max} - \%_t)$ VERSUS TIME AT 25°C, pH = 7.6 FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE WITH $1.0 \times 10^{-3}$M 2PAM (□—□), $1.0 \times 10^{-3}$M SR2461 (○ — ○), AND $0.20 \times 10^{-3}$M SR3008 (▲ — ▲).
FIGURE 3 DOUBLE RECIPROCAL PLOT OF OBSERVED FIRST-ORDER REACTIVATION RATE CONSTANT MINUS DEALKYLATION RATE CONSTANT, \((k_{\text{obs}} - k_a)^{-1}\) VERSUS OXIMATE CONCENTRATION \(([OX]^{-1})\) FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE BY SR2458 (■ ■ ■), SR2461 (○ — C), AND SR3008 (△ — △)
Table VI. 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>[HOX], M x 10^{-4}</th>
<th>[OX], M x 10^{-4}</th>
<th>$k_{obs}$, min^{-1} x 10^3</th>
<th>ln($%r_{\infty}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR 2458</td>
<td>2.00</td>
<td>1.00</td>
<td>5.90 ± 0.8</td>
<td>3.19 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>2.00</td>
<td>6.82 ± 1.7</td>
<td>3.39 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>10.0</td>
<td>11.0e</td>
<td>4.42</td>
</tr>
<tr>
<td>SR 2461</td>
<td>2.00</td>
<td>0.752</td>
<td>3.02 ± 0.16</td>
<td>3.30 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>1.52</td>
<td>4.40 ± 0.62</td>
<td>3.49 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>2.26</td>
<td>5.13 ± 1.0</td>
<td>3.79 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td>2.63</td>
<td>7.12 ± 0.27</td>
<td>4.10 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>3.76</td>
<td>7.24 ± 0.26</td>
<td>4.05 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>4.51</td>
<td>6.90 ± 0.17</td>
<td>4.22 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>7.52</td>
<td>7.65 ± 0.47</td>
<td>4.30 ± 0.06</td>
</tr>
<tr>
<td>SR 3008</td>
<td>2.00</td>
<td>0.569</td>
<td>7.46 ± 0.41</td>
<td>3.15 ± 0.063</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>1.71</td>
<td>8.17 ± 1.1</td>
<td>3.83 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>2.85</td>
<td>10.3 ± 1.5</td>
<td>4.15 ± 0.23</td>
</tr>
<tr>
<td>2PAM</td>
<td>0.0300</td>
<td>0.000870</td>
<td>6.57 ± 0.38</td>
<td>3.93 ± 0.049</td>
</tr>
<tr>
<td></td>
<td>0.0800</td>
<td>0.0232</td>
<td>9.31 ± 0.11</td>
<td>4.20 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>0.0289</td>
<td>19.6 ± 0.80</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>0.160</td>
<td>0.0463</td>
<td>20.2 ± 1.1</td>
<td>4.42 ± 0.083</td>
</tr>
<tr>
<td></td>
<td>0.200</td>
<td>0.0571</td>
<td>29.8 ± 1.4</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>2.89</td>
<td>125 ± 10</td>
<td>4.49 ± 0.12</td>
</tr>
</tbody>
</table>

*aSee Table II.

bCalculated from [HOX] and equation (9).

cCalculated from slope of least squares plot of data according to equation (14).

dCalculated from intercept of least squares plot of data according to equation (14).

eCalculated from three points, including $\%r_{\infty}$, no valid statistical data.

fNo infinity point measured. Slopes calculated from initial points by the method of Guggenheim (ref. 72).
Table VII. Kinetic Constants for Reactivation of Diamonopropyl
Phosphoryl-d3G at 25°c, pH = 7.6

<table>
<thead>
<tr>
<th>Component</th>
<th>( [\text{M}]] )</th>
<th>( 10^{14} )</th>
<th>( [\text{M}] \times 10^{-6} )</th>
<th>( \text{K}_d \times 10^{12} )</th>
<th>( \text{K}_{d1} \times 10^{12} )</th>
<th>( \text{K}_{d2} \times 10^{12} )</th>
<th>( \text{K}_{d3} \times 10^{12} )</th>
<th>( \text{K}_{d4} \times 10^{12} )</th>
<th>( \text{K}_{d5} \times 10^{12} )</th>
<th>( \text{K}_{d6} \times 10^{12} )</th>
<th>( \text{K}_{d7} \times 10^{12} )</th>
<th>( \text{K}_{d8} \times 10^{12} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>d3G (a)</td>
<td>2.00</td>
<td>10.0</td>
<td>231</td>
<td>3.12</td>
<td>0.21 ± 0.07</td>
<td>3.2 ± 1.3</td>
<td>1.4 ± 0.16</td>
<td>103 ± 24</td>
<td>2.0</td>
<td>9.3</td>
<td>1.3</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>5.00</td>
<td>190</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>1.00</td>
<td>168</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d3G (b)</td>
<td>2.00</td>
<td>13.3</td>
<td>689</td>
<td>2.69</td>
<td>0.17 ± 0.18</td>
<td>2.1 ± 0.30</td>
<td>4.6 ± 0.35</td>
<td>66 ± 22</td>
<td>2.4</td>
<td>14</td>
<td>0.9</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>6.67</td>
<td>353</td>
<td>2.05</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>4.61</td>
<td>181</td>
<td>1.28</td>
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<td></td>
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<td></td>
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</tr>
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<td></td>
<td>7.00</td>
<td>3.80</td>
<td>180</td>
<td>0.651</td>
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<tr>
<td></td>
<td>10.0</td>
<td>2.66</td>
<td>126</td>
<td>0.742</td>
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<tr>
<td></td>
<td>13.0</td>
<td>2.20</td>
<td>187</td>
<td>0.470</td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>1.33</td>
<td>184</td>
<td>0.330</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>d3G (c)</td>
<td>2.00</td>
<td>12.6</td>
<td>170</td>
<td>3.29</td>
<td>-0.018 ± 0.11</td>
<td>1.9 ± 0.10</td>
<td>0.32 ± 0.19</td>
<td>117 ± 21</td>
<td>0.19</td>
<td>10</td>
<td>0.27</td>
<td>380</td>
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<td></td>
<td>4.00</td>
<td>5.96</td>
<td>155</td>
<td>1.47</td>
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<td></td>
<td>6.00</td>
<td>4.60</td>
<td>133</td>
<td>0.996</td>
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<tr>
<td></td>
<td>10.0</td>
<td>3.19</td>
<td>115</td>
<td>0.576</td>
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<td></td>
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<tr>
<td>d3G (d)</td>
<td>0.0000</td>
<td>1450</td>
<td>200</td>
<td>0.994</td>
<td>0.11 ± 0.050</td>
<td>0.007 ± 0.007</td>
<td>0.17 ± 0.003</td>
<td>13 ± 14</td>
<td>6.7</td>
<td>61</td>
<td>0.11 ± 0.007</td>
<td>5500</td>
</tr>
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<td></td>
<td>0.00004</td>
<td>426</td>
<td>129</td>
<td>0.508</td>
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<td>0.508</td>
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<td></td>
<td>0.0010</td>
<td>246</td>
<td>55.5</td>
<td>n</td>
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<td>0.0016</td>
<td>216</td>
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<td>0.0020</td>
<td>175</td>
<td>35.4</td>
<td>n</td>
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<td></td>
<td>0.0000</td>
<td>1.50</td>
<td>8.13</td>
<td>0.112</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aSee Table II.
*bCalculated from [NAD] and equation (17).
*cFrom [NAD] values of Table IV, \( b_{NAD} = 1.81 \times 10^{-1} \text{ min}^{-1} \). See text.
*dFrom \( 1/\text{[NAD]} = (1 - \text{[E]}_{\text{NAD}}) / (\text{[E]}_{\text{NAD}}) \), \( \text{[E]}_{\text{NAD}} \) is taken from Table VI.
**dIntercept of least squares plot of data according to equation (19).
*Calculated as shown in Table V.
*Calculated as shown in Table V.
*Calculated as shown in Table V.
*Calculated from equation (21).
*Calculated from equation (22).
*See Table III.
These caveats notwithstanding, the kinetic constants given in Table VII 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 x \( 10^{-3} \) min\(^{-1} \) for SR 3008, 2461, and 2462. These compare to \( k_a = 1.61 \times 10^{-3} \) min\(^{-1} \) for the inhibited enzyme in the absence of reactivator and indicate no significant effect of the thiohydroximates on the rate of dealkylation. For 2-PAM, however, \( k'_a = 6.7 \times 10^{-3} \) min\(^{-1} \), suggesting that 2-PAM accelerates the aging of DFP-inhibited enzyme. Similar effects of pyridinium oximes on dealkylation of ethyl N,N-dimethylamino phosphoramidocyanidate-inhibited AChE were reported by De Jong and Wolring. \(^7\) The possibility that accelerated dealkylation is a general phenomenon for reactivation of phosphorylated AChE by pyridinium oximes is interesting and should be investigated further.

Insofar as reactivation is concerned, 2-PAM is characterized both by a strong affinity for the inhibited enzyme and high nucleophilicity toward phosphorus. Our values of \( K_r = 0.11 \times 10^{-6} \) M and \( k_r = 61 \times 10^{-3} \) min\(^{-1} \) for 2-PAM 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, 2-PAM is 200 times more effective than SR 2461 and approximately 15 times more active than SR 3008, the most effective of the SR-series thiohydroximates. It is interesting to note that for the thiohydroximates, 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, an inhibitor that yields a phosphorylated enzyme that undergoes neither dealkylation nor spontaneous reactivation to a significant degree at 25°C. Investigations along these lines are in progress and will be reported in a separate communication.
Conclusions

The SR-series thiohydroximates are moderately active reactivators of diisopropyl phosphoryl-AChE. In this respect they should be compared to reactivators such as TDA-$5^8$ and HS6$^9,75$ that are inferior to 2-PAM, TMB-4, or toxogonin as reactivators, but that nevertheless are effective in therapy of organophosphorus agent intoxication. If the thiohydroximates combine significant activity as reactivators with low toxicity, prolonged biological half-life, and improved tissue penetration, they should prove to be useful antidotes. However, the toxicological and pharmacological behavior of the thiohydroximates remains to be established.

We regard the thiohydroximates 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 thiohydroximates 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 readily 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 thiohydroximates and that these can be quantitated through determination of reactivation kinetics.

The appreciation 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 thiohydroximates can be applied similarly to the design of improved therapeutics based on nonquaternary reactivators.
Literature Cited

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


68. F. Bergmann and H. Gorvin, Biochimie, 55, 515 (1973).


Appendix

MINC-11 COMPUTER PROGRAM FOR ENZYME KINETICS
MINC ENZYME KINETICS PROGRAM (ENKIN)

The program ENKIN is designed to determine initial rates of enzyme reactions. Data are treated by the method of least squares. The program is written for the MINC-11 and controls the acquisition and manipulation of absorbance data from a Gilford 2000 spectrophotometer. Permanent storage of the data in a disc file is provided for. The program is divided into the following sections:

1. Input of identification data and operating parameters.

The main purposes for this section are to set up the disc file with identification and a label and to acquire the input parameters "SCALE FACTOR" and "DATA POINTS PER CUVEtte." The disc file setup requires the following responses from the operator.

a. NAME. The first line of the disc file includes the operator's name. There is no restriction on the type of characters except that they must fit on one line (80 character maximum).

b. IDENTIFIER. As part of the identification of the data for the disc storage file, the time and data are automatically stored after the operator's name. In addition, a further experiment IDENTIFIER is provided for. It consists of up to 80 characters of any type that the operator chooses to aid in the identification of the particular experiment.

c. FILE NAME. The file name is the name that the MINC uses to identify the data file. It appears in the directory. The FILE NAME may have up to six alphanumeric characters, but the first character must be
a letter. The program assigns the file to the left-hand disc (SYO:) and assigns the extension .DAT to the file unless otherwise specified. The complete file name is thus SYO:xxxxxx.DAT. The right-hand disc (SYI:) can be specified, and the extension may take any other three-letter value that the operator includes as part of FILE NAME.

The next operation is acquisition of two operating parameters.

d. SCALE FACTOR. The program ENKIN takes electrical signals from the recorder input of the Gilford 2000 spectrophotometer. Whatever value of absorbance that gives a full-scale reading on the recorder gives a value of 1.000 to the program. Thus if full scale on the recorder is adjusted to be 0 to 1.0 absorbance units, the SCALE FACTOR should be 1.0. If full scale is 0.0 to 0.1, the SCALE FACTOR is 0.1. The SCALE FACTOR can be used to convert absorbance into concentration units. To accomplish this, the operator must enter the concentration that corresponds to full scale on the recorder.

e. DATA POINTS PER CUVETTE. The program provides for up to 100 data points for each cuvette. The actual number of points must be specified by the operator. The program examines each cuvette, records data if available, and increments a counter if the data were recorded. This process is repeated until one of the counters has the same value specified in DATA POINTS PER CUVETTE, at which point the program ceases to take data. The actual number of data points is recorded for each cuvette.

2. Accumulation of data and updating of display.

For the accumulation of data, two interfaces are used. The first is the MINC preamplifier channel A. Its input must be connected in parallel with the 50-mV input to the chart recorder. The preamplifier is placed
on the left side of the MINC analog-to-digital converter (ADC), and the preamplifier output is thus routed to channel 8 of the ADC.

The second interface consists of four photodarlington pilot light detectors. The pilot light detectors require +5V and ground. The detectors for pilot lights 1, 2, 3, and 4 are connected to input channels 4, 5, 6, and 7, respectively, of the ADC. A signal of greater than 1 volt is detected if the pilot light is on. (The signal is actually a ripple voltage, the shape of which depends on the particular light source.)

Data from the first interface are acquired using a subroutine which returns the sum of 20 samples of the ADC. Since full-scale input to the recorder is 50 mV, the sum of 20 samples of a full-scale reading is 1.0 V. There is thus no adjustment required in order to represent fraction of full scale. The time (in minutes) is evaluated after the first ten samples and before the second ten samples are summed.

Data from the second interface are also acquired by a subroutine. The subroutine samples each pilot light consecutively. If a pilot light is found on, the lamp indicator variable is set appropriately and control is returned to the main program. If no pilot light is found on after the last one is checked, the checking procedure is started over at pilot light No. 1.

A brief flow chart of the section 2 program operation is shown in Figure A-1. Cuvette number has the symbol C. Q and Q9 are temporary values of C. V2 is a temporary value of absorbance, and Y is either absorbance or concentration, depending on the value and units of the SCALE FACTOR. The symbol X is used to represent time in those calculations that involve summations. Similarly, N is used to indicate point count. The
following formulas are used to calculate slope, intercept, correlation coefficient (R), and standard deviations of slope and intercept [S (slope) and S (intercept)].

\[
\text{slope} = \frac{N\Sigma XY - \Sigma X \Sigma Y}{N\Sigma X^2 - (\Sigma X)^2}
\]

\[
\text{intercept} = \frac{\Sigma X^2 \Sigma Y - \Sigma X \Sigma XY}{N\Sigma X^2 - (\Sigma X)^2}
\]

\[
R = \frac{N\Sigma XY - \Sigma X \Sigma Y}{(N\Sigma X^2 - (\Sigma X)^2)^{1/2} (N\Sigma Y^2 - (\Sigma Y)^2)^{1/2}}
\]

\[
S (\text{slope}) = \left( \frac{T\Sigma X^2}{N\Sigma X^2 - (\Sigma X)^2} \right)^{1/4}
\]

\[
S (\text{intercept}) = \left( \frac{T\Sigma X^2}{N\Sigma X^2 - (\Sigma X)^2} \right)
\]

where

\[
T = \frac{\Sigma Y^2 - (\Sigma Y)^2 \Sigma X^2 - 2\Sigma XY \Sigma X Y + N(\Sigma XY)^2}{N\Sigma X^2 - (\Sigma X)^2}
\]

\[
N - 2
\]

3. Data Storage.

After NAME, time, date, IDENTIFIER, and SCALE FACTOR, the kinetic data are stored. For each cuvette, the least squares slope and intercept, the correlation coefficient, and the number of data points are stored. Next, the standard deviations of the slope and intercept are stored for each cuvette. This is followed by a list of times (in minutes) and absorbances (or concentrations depending on the SCALE FACTOR) for each cuvette.
FIGURE A1  FLOW CHART OF SECTION 2 OF ENKIN
The operator can examine these data using the command TYPE followed by the FILE NAME. Hard copy of the data files is not possible unless a line printer is available. For printed copies, the floppy discettes can be transported to any LSI-11 computer that has a line printer and RX02 disc drive.


Data may be displayed on the terminal using MINC graphics routines. To skip this feature, the operator must enter a zero. Otherwise the cuvette number must be entered. All four cuvettes can be examined by sequentially typing the appropriate cuvette numbers.
ENKIN is a program for determining initial rates of enzyme catalyzed reactions. It takes absorbance data from a Gilford 2000 spectrophotometer and treats it by the method of least squares.

Section 1: Input of ID data and operating procedures

```plaintext
10 REM - ENKIN is a program for determining initial rates of enzyme catalyzed reactions. It takes absorbance data from a Gilford 2000 spectrophotometer and treats it by the method of least squares.
50 REM

60 REM - Section 1 Input of ID data and operating procedures
70 DIM X(3), Y(3), X2(3), Y2(3), X9(3), N(3)
80 DIM A(3), B(3), C(3), S1(3), S2(3)
90 DIM P(3, 100), O(3, 100)
100 PRINT 'ENTER YOUR NAME PLEASE'
110 INPUT NS
120 PRINT 'ENTER IDENTIFIER FOR THIS EXPERIMENT'
130 INPUT ES
140 PRINT 'ENTER FILE NAME FOR THE DATA'
150 INPUT F$
160 OPEN F$ FOR OUTPUT AS FILE *1
170 PRINT *1, N$, CLK$, DAT$
180 PRINT 01, E$
190 PRINT 'ENTER SCALE FACTOR'
200 INPUT F5
210 REM

Section 2: Accumulation of data and updating of display

220 DISPLACE CLEAR
230 MOVE_CURSOR(1, 1)
240 PRINT 'Kinetic Parameters (in minutes) for Cuvettes 1 to 4'
250 PRINT 'Slope', 'Intercept', 'R', 'N'
260 MOVE_CURSOR(10, 1)
270 PRINT 'Cuvette', 'Time', 'Absorbance (or concentration)'
280 START_TIME('CHZ')
290 REM
300 GOSUB 1110
310 GOSUB 1110
320 GOSUB 1110
330 GOSUB 1110
340 REM
350 REM - C is cuvette number, X1 is time, V is absorbance (or conc.)
360 GOSUB 1110
370 G1=C
380 GOSUB 1110
390 09=C
400 IF C=G1 THEN GO TO 380
410 N(C)=N(C)+1
420 IF N(C)-N1 GO TO 700
430 GOSUB 1230
440 PAUSE(-1)
450 V2=V
460 GOSUB 1230
470 GOSUB 1110
480 IF C<0 THEN N(C)=N(C)-1 GO TO 370
490 IF ABS(V-V2)>01 THEN GO TO 440
500 V=V+F5
510 P(C)=N(C)=V
520 Q(C)=N(C)=X1
530 MOVE_CURSOR(11+1)
540 PRINT C1*X1+V$
550 X(C)=X(C)+X1
560 Y(C)=Y(C)+V
570 X2(C)=X2(C)+X1*X1
580 Y2(C)=Y2(C)+V*V
590 X9(C)=X9(C)+X1*V
600 IF N(C)=1 GO TO 370
```
610 D=N(C)*X2(C)-X(C)*X(C)
620 A(C)=(X2(C)*Y(C)-X(C)*X9(C))/D
630 R(C)=(N(C)*X9(C)-X(C)*Y(C))/SQR(D*(N(C)*Y2(C)-Y(C)*Y(C)))
640 PRINT B(C),A(C),R(C),N(C)
670 GO TO 370
680 REM
690 REM - Data gathering loop ends here
700 N(C)=N(C)-1
710 MOVE_CURSOR(13+1)
720 PRINT 'S(slope)' ','S(intercept)'  
730 FOR L=0 TO 3
740 T=(Y(L)*X2(L)-2*X9(L)*X(L)*N(L)*X9(L)+2)/(N(L)*X2(L)-X(L)t2)
750 S1(L)=SOR(T*X2(L)/((N(L)*X2(L)-X(L)t2))
760 S2(L)=SGR(T*N(L))/((N(L)*X2(L)-X(L)t2))
780 PRINT S2(L),S1(L)
810 NEXT L
800 REM
810 REM - Section 3 Data storage
820 PRINT #1,'Slope','Intercept,' 'R',' N'
830 FOR L=0 TO 3
840 PRINT #1,B(L),A(L),R(L),N(L)
850 NEXT L
860 PRINT #1,'S(slope)' ','S(intercept)'  
870 FOR L=0 TO 3
880 PRINT #1,S2(L),S1(L)
890 NEXT L
900 FOR L=0 TO 3
910 PRINT #1,'Time','Absorbance' 'Cuvette ' 'L+1
920 PRINT #1,'(or concentration)'
930 FOR C=1 TO N(L)
940 PRINT #1,Q(L,C),P(L,C)
950 NEXT C
960 NEXT L
970 CLOSE #1
980 REM
990 REM - Section 4 Graphics
1000 PRINT 'ENTER CUYETTE ' '
Enter zero to skip graphic routines'
1010 INPUT N4
1020 IF N4=0 THEN DISPLAY_CLEAR \ GO TO 1350
1030 IF N4>4 THEN PRINT 'TYPE 1, 2, 3, OR 4' \ GO TO 1000
1040 DISPLAY_CLEAR
1050 GRAPH "LINES",N(N4-1),Q(N4-1,1),P(N4-1,1))
1060 PRINT 'DATA FROM CUVETTE ' 'N4
1070 GO TO 1000
1080 GO TO 1350
1090 REM
1100 REM - Subroutine determines position of cuvette
1110 H=1
1120 AIN(#1,4+)
1130 IF W>H THEN C=0 \ RETURN
1140 AIN(#1,5+)
1150 IF W>H THEN C=1 \ RETURN
1160 AIN(#1,6+)
1170 IF W>H THEN C=2 \ RETURN
1180 AIN(#1,7+)
1190 IF W>H THEN C=3 \ RETURN
1200 GO TO 1120

52
1210 REM
1220 REM - Subroutine determines absorbance and time
1230 V=0
1240 FOR L=1 TO 10
1250 AIN(+V1,+,8r)
1260 W=W+V1
1270 NEXT L
1280 GET_TIME(X1)
1290 X1=X1/60
1300 FOR L=1 TO 10
1310 AIN(+V1,+,8r)
1320 W=W+V1
1330 NEXT L
1340 RETURN
1350 END