(2) J. M. Feldman and H. E. Lebovitz, *Arch. Intern. Med.*, **123**, 314(1969).
(3) H. E. Lebovitz and J. M. Feldman, in "Diabetes Mellitus: Diagnosis and Treatment," vol. 3, S. S. Fajans and K. E. Sussman, Eds., American Diabetes Association, New York, N. Y., 1971, p. 147.
(4) M. Tishler, in "Molecular Modification in Drug Design; Advances in Chemistry Series 45," R. F. Gould, Ed., American Chemical Society, Washington, D. C., 1964, chap. 1.
(5) A. Korolkovas, "Essentials of Molecular Pharmacology," Wiley-Interscience, New York, N. Y., 1970, p. 59.
(6) G. Domagk, *Deut. Med. Wochenschr.*, **61**, 250(1935).
(7) W. B. Schwartz, *N. Engl. J. Med.*, **240**, 173(1949).
(8) A. A. Rubin, F. E. Roth, M. M. Winbury, J. G. Topliss, M. H. Sherlock, N. Sperber, and J. Black, *Science*, **133**, 2067(1961).
(9) J. G. Lombardino and E. H. Wiseman, *J. Med. Chem.*, **14**, 588(1971).
(10) B. R. Moffett and A. D. Rudzik, *ibid.*, **14**, 588(1971).
(11) H. Ulrich, *Accounts Chem. Res.*, **2**, 186(1969).
(12) N. P. Streptov, *Tr. Voronezh. Gos. Univ.*, **49**, 31(1958); through *Chem. Abstr.*, **56**, 378(1962).
(13) W. S. Hoffman, *J. Biol. Chem.*, **120**, 51(1937).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 5, 1973, from the *Department of Medicinal Chemistry, Ferris State College, Big Rapids, MI 49307, and the †Laboratory of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication March 1, 1973.

The authors thank the personnel of The Upjohn Co. for the pharmacological data. They also gratefully acknowledge many helpful discussions with Dr. Ronald L. Jacoby and Dr. Ronald H. Rynbrandt. T. J. Silhavy was the recipient of a National Science Foundation Undergraduate Research Participation Grant.

‡ Present address: Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

▲ To whom inquiries should be directed.

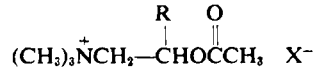
# Ketoxime Acetates: Substrates for Cholinesterases

**GEORGE M. STEINBERG▲, NORMAN C. THOMAS, MORTON L. MEDNICK, and VERNON BALTHIS**

**Abstract** □ Ketoxime acetates function as typical substrates for acetylcholinesterase. Several 3-pyridinium ketoxime esters have potential utility for spectrophotometric studies with the enzyme. Each is highly water soluble and gives a large change in absorbance upon hydrolysis. With each, the acylation step is rate limiting. They bind to the enzyme strongly; the lowest $K_{m(app)}$ observed is $1.5 \times 10^{-5}$ *M*. The substrates are hydrolyzed by butyrylcholinesterase. Several show great selectivity for butyrylcholinesterase over acetylcholinesterase.

**Keyphrases** □ Ketoxime acetates—as cholinesterase (acetyl and butyryl) substrates, binding constants, hydrolysis, absorbance changes, selectivity □ Cholinesterase substrates, acetyl and butyryl —ketoxime acetates, binding constants, hydrolysis, absorbance changes, selectivity □ Acetylcholinesterase—1-alkyl 3-pyridinium ketoxime halide esters as substrates □ Butyrylcholinesterase—1-alkyl 3-pyridinium ketoxime halide esters as substrates

A recent publication (1) reported the activity of aldoxime acetates as substrates for acetylcholinesterase (acetylcholine hydrolase, E.C. 3.1.1.7). The two principal purposes of that study were: (*a*) to gain information on the kinetic specificity and selectivity of the enzyme, and (*b*) to widen the range of spectrophotometrically useful substrates for studying enzyme properties. This note extends the work to ketoxime esters.

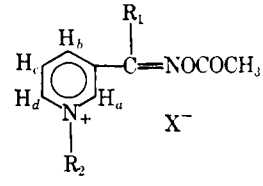


I: R = H
II: R = $CH_3$

Acetylcholinesterase is generally insensitive to bulking on the alcohol portion of the ester molecule. For example, methacholine (II) is hydrolyzed nearly as rapidly as acetylcholine (I) (2). The reverse pattern is observed with butyrylcholinesterase (acylcholine arylhydrolase, E.C. 3.1.1.8), where bulking near the ester linkage in a test substrate may markedly reduce or even completely abolish response to the enzyme (2). This study was initiated to learn if these characteristics extend to the oxime esters and to determine whether appreciable changes in $K_m$ could be provided by the simple expedient of changing the size (bulk) of the alkyl group attached to the pyridine nitrogen atom.

## EXPERIMENTAL

The oxime acetates (III–VII) were prepared in conventional manner by oximation of the corresponding aldehydes or ketones, acetylation with acetic anhydride, and finally alkylation of the pyridine nitrogen atom with methyl iodide or benzyl bromide (3–7).



| | $R_1$ | $R_2$ |
|---|---|---|
| III: | $CH_3$ | $CH_3$ |
| IV: | $CH_3$ | $C_6H_5CH_2$ |
| V: | $C_2H_5$ | $CH_3$ |
| VI: | H | $C_6H_5CH_2$ |
| VII: | acetophenone oxime acetate | |

Table I—Elemental Analyses

| Compound | | Analysis, % C | H | Br | I | N | O |
|---|---|---|---|---|---|---|---|
| III | Calc. | 37.52 | 4.09 | — | 39.64 | 8.75 | 10.00 |
| | Found | 37.4 | 4.2 | — | 39.5 | 8.7 | 10.0 |
| IV | Calc. | 55.03 | 4.91 | 22.89 | — | 8.02 | 9.16 |
| | Found | 54.9 | 5.2 | 22.8 | — | 8.2 | 9.1 |
| V | Calc. | 39.53 | 4.52 | | 37.97 | 8.38 | 9.58 |
| | Found | 39.7 | 4.5 | — | 37.7 | 8.4 | 9.4 |
| VI | Calc. | 53.74 | 4.51 | 23.84 | — | 8.36 | 9.54 |
| | Found | 53.5 | 4.5 | 23.9 | — | 8.1 | 9.7 |
| 3-Acetylpyridine oxime acetate | | | | | | | |
| | Calc. | 60.66 | 5.65 | — | — | 15.72 | 17.96 |
| | Found | 60.4 | 5.5 | — | — | 15.7 | 17.8 |

NMR spectra[1] of the oxime acetates were determined in approximately 10% solutions containing internal standards. For the aromatic compounds, the solvent was $CDCl_3$ and the standard was tetramethylsilane. For the pyridine derivatives, $D_2O$ was used as the solvent and sodium 3-trimethylsilyl-1-propanesulfonate was the standard. The chemical shifts ($\delta$) reported are in parts per million.

The integration of peaks together with the sharpness of spectra indicates the absence of impurities. Hence, the purity of each compound is greater than 96–97% and *each exists as a single isomer* (1). For several compounds, elemental analyses were performed (C, H, N, O, and halogen, Table I).

Pertinent data[2] on the studied compounds are as follows: acetophenone oxime acetate, m.p. 56 57° [lit. (6) m.p. 55°], with NMR chemical shifts at 2.23 and 2.34 (acetate and C-methyl, respectively); *syn*-benzaldoxime acetate, m.p. < room temperature [lit. (7) m.p. 21°], with an NMR chemical shift at 2.12 (acetate methyl); IV, glass, with NMR chemical shifts at 2.43 (acetate methyl), 5.99 (C-methyl), 7.59 (benzene), 9.48 ($H_a$), 8.28 (t or 2d, $H_c$), 8.99 (d), and 9.13 (d, one is $H_b$ and the other $H_d$); 3-acetylpyridine oxime acetate, m.p. 37.8°, with NMR chemical shifts at 2.36 and 2.39 (acetate methyl and C-methyl, respectively), 7.55 (m, $H_c$), 8.18 (d, $H_b$), 8.69 (d, $H_d$), and 8.85 ($H_a$); III, m.p. 116–119° dec. [lit. (3) m.p. 115°], with NMR chemical shifts at 2.39 (acetate methyl), 2.60 (C-methyl), 4.55 ($^+$N-methyl), 8.25 (m, $H_c$), 9.00 (m, $H_b$ and $H_d$), and 9.35 ($H_a$); V, m.p. 112–115° dec., with NMR chemical shifts at 1.04 (C-methyl), 2.38 (acetate methyl), 3.08 (m, methylene), 4.54 ($^+$N-methyl), 6.95 (m, $H_c$), 7.64 and 7.77 ($H_b$ and $H_d$, respectively), and 8.06 ($H_a$); and VI, m.p. 126–128°, with NMR chemical shifts at 2.40 (acetate methyl), 6.02 (methylene), 7.65 (benzene), 8.30 (m, $H_c$), 8.90 (aldehydic proton), 9.10 (m, $H_b$ and $H_d$), and 9.46 ($H_a$).

**Absorption Spectra**[3]—All measurements were made at 25.0 ± 0.1°, pH 6.61, in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, using buffer solution in the reference compartment (Table II). The oximes were obtained by enzymatic hydrolysis of the corresponding esters. Oxime configuration probably remains unchanged.

For the difference spectra, the ketoxime esters were placed in the reference beam. Hence, the positive values of $\Delta\epsilon$ represent an increase in absorbance upon hydrolysis. With *syn*-benzaldoxime acetate, the reverse is true; hydrolysis results in a reduction in absorbance. At pH 6.61, the oximes listed in Table II exist in the protonated form, since all have pKa values greater than 9 (3, 4).

**Enzyme Kinetics**—*Acetylcholinesterase*—Measurements were made spectrophotometrically as previously described (1). The enzyme source[4] and its storage were identical. Runs were made at 25 ± 0.1° in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. Computation of $K_{m(app)}$ and $V_{max}$ (or $k_{cat}$) was made by computer using the Wilkinson (8) weighted regression method. Estimates of the 95% confidence limits of the computed constants were calculated from their standard errors using the Student *t* table ($n - 2$ degrees of freedom). The number of experimental points taken varied from seven to 15, using a minimum of seven different concentrations of substrate. In each case, double-reciprocal plots (Lineweaver–Burk) gave good straight lines. With Compounds V and VI, the Beer–Lambert relationship was examined and the plots of both substrate and products, in each case, were linear (Table III).

[1] Varian A-60D.
[2] d = doublet, t = triplet, m = multiplet.
[3] Cary model 14.
[4] Worthington Biochemicals, Code ECHP.

Table II—Spectral Characteristics of Oxime Acetates and the Corresponding Oximes

| Compound | Acetate $\lambda_{max}$, nm. | Acetate $\epsilon_{max}$, $M^{-1}$ cm.$^{-1}$ × $10^{-3}$ | Oxime $\lambda_{max}$, nm. | Oxime $\epsilon_{max}$, $M^{-1}$ cm.$^{-1}$ × $10^{-3}$ | Difference Spectrum[a] $\lambda_{max}$, nm. | Difference Spectrum[a] $\Delta\epsilon_{max}$, $M^{-1}$ cm.$^{-1}$ × $10^{-2}$ |
|---|---|---|---|---|---|---|
| III | 254 | 5.17 | 258 | 6.04 | 260 | 8.5 |
| | | | | | 295 | 16.8 |
| IV | 241 | 10.2 | <240 | — | 263 | 11.0 |
| | | | 254 | 8.87 | 300 | 16.9 |
| V | <240 | — | <245 | — | 259 | 8.0 |
| | 268 | 4.9 | | | 297 | 18.1 |
| VI | 248 | 11.4 | 252 | 11.0 | 263 | 15.9 |
| | | | | | 305 | 15.9 |
| VII | 239 | 13.3 | 240 | 14.6 | — | — |
| *syn*-Benzaldoxime acetate | 257 | 5.8 | 253 | 4.8 | 262 | −12.4 |

[a] Ester is reference beam, $\Delta\epsilon = \Delta A/[\text{substrate}]$.

*Butyrylcholinesterase*[5]—A standard solution of the enzyme was prepared in potassium chloride-gel (1), which gave one-seventieth the activity of the acetylcholinesterase concentrate ($E_w$) when assayed with phenyl acetate, $7.71 \times 10^{-3}$ *M*, at pH 6.61 in the standard buffer. Comparative hydrolysis rates were determined for the two enzymes using appropriately diluted solutions to give the same hydrolysis rate with phenyl acetate.

Phenyl acetate was chosen as the basis of comparison instead of acetylcholine because its hydrolysis could be measured spectrophotometrically. However, the hydrolysis rates of the two substrates by these enzymes are closely similar, so extrapolation to the more conventional acetylcholine can be made[6].

Measurements were made at pH 6.61 in the standard buffer using the concentrations of the substrates indicated in Table IV.

## RESULTS AND DISCUSSION

Comparison of the kinetic constants of the 1-methylpyridinium Compounds III and V (Table III) with those of *syn*-3-formyl-1-methylpyridinium iodide oxime acetate, VIII (1), indicates that the bulking methyl and ethyl groups have little effect on either $K_{m(app)}$ or $V_{max}$ for the enzyme acetylcholinesterase. Elimination of the positive charge, as in VII, resulted in an increase in $K_{m(app)}$ without change in $V_{max}$. Replacement of the 1-methyl group by the larger 1-benzyl group, as in Compounds IV and VI, decreased $K_{m(app)}$ by one order of magnitude and produced a corresponding reduction in $V_{max}$. The decrease in $K_{m(app)}$ may be attributed to increased hydrophobic binding in the region just beyond the anionic binding site (9). The decrease in $V_{max}$ cannot clearly be attributed to any specific parameters. Speculation on its cause at this point seems unwarranted.

The hydrolytic stability of the pyridinium ketoxime acetate, III, is approximately twice that of the corresponding aldoxime ester, VIII (Table III). The hydrolysis rates of the pairs III and IV and VI and VIII are probably very similar, since the effect of increased bulk at the remote *meta*-position should be very small. Similarly, Compound V, with the bulkier ethyl group in place of the methyl group in Compound III, may be expected to be equal to or even a bit more resistant to hydrolysis than III. Elimination of the charge, as in VII, considerably reduces the hydrolysis rate of this oxime ester.

[5] Worthington horse serum cholinesterase, code CHE.
[6] H. Michel, unpublished. Using acetylcholine, pH 7.4, 0.224 *M* KCl, 0.1% gelatin, 25°, titrimetrically, acetylcholinesterase had a $K_m = 3 \times 10^{-4}$ *M* and $V_{max} = 1.63 \times 10^{-4}$ meq./min. and horse serum cholinesterase had a $K_m = 1.85 \times 10^{-3}$ *M* and $V_{max} = 1.4 \times 10^{-4}$ meq./min. Using phenyl acetate, pH 7.2, 0.01 *M* morpholinopropanesulfonic acid, 0.01 *M* magnesium sulfate, 0.01% gelatin, $10^{-3}$ *M* Versene, 25°, spectrophotometrically, acetylcholinesterase had a $K_m = 1.8 \times 10^{-3}$ *M* and $V_{max} = 1.4 \times 10^{-4}$ meq./min. and horse serum cholinesterase had a $K_m = 1.6 \times 10^{-3}$ *M* and $V_{max} = 3.2 \times 10^{-4}$ meq./min.

**Table III**—Ester Hydrolysis by Eel Acetylcholinesterase[a]

| Substrate | $K_m$, $M \times 10^4$ | $\frac{V_{max(s)}}{V_{max(p)}} \times 100$[b] | Concentration Range, $M \times 10^4$ | λ, nm. | Δε, $M^{-1}$ cm.$^{-1}$ $\times 10^{-3}$ | $k_{OH^-}$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|---|---|
| III | 1.7 (0.54–2.9) | 5.0 | 2.5–22 | 302.5 | 1.6 | 42[c] |
| IV | 0.16 (0.11–0.20) | 0.60 | 0.2–2.6 | 300 | 1.69 | — |
| V | 2.5 | 5.1 | 5–35 | 300 | 1.75 | — |
| VI | 0.15 (0.10–0.20) | 0.76 | 0.2–4.7 | 305 | 1.59 | — |
| VII | 25 (11–39) | 4.4 | 7–25 | 295 | 0.355 | 2.46[d] |
| VIII[e] | 1.55 | 10 | — | 305 | 2 | 80[c] |

[a] 25.0°, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. [b] $V_{max(s)}$ and $V_{max(p)}$ refer, respectively, to $V_{max}$ values of the individual substrates and of phenyl acetate. [c] *Reference 3.* [d] *Reference 4.* [e] *Reference 1.*

**Table IV**—Comparative Hydrolysis Rates: Butyrylcholinesterase *versus* Acetylcholinesterase[a]

| Substrate | $v_{ChE}/v_{AChE}$ | Concentration of Substrate, $M \times 10^3$ |
|---|---|---|
| Phenyl acetate | 1.0 | 7.71 |
| III | 20.8 | 8.35 |
| IV | 15.4 | 6.0 |
| V | 6.7 | 9.03 |
| VI | 57 | 5.89 |

[a] $v_{ChE}$ and $v_{AChE}$ refer, respectively, to the substrate hydrolysis rates catalyzed by horse serum cholinesterase and eel acetylcholinesterase.

Following the discussion in *Reference 1* for the three-step mechanism involved in substrate hydrolysis by acetylcholinesterase, the data in Table III lead to the conclusions that acylation is the rate-limiting step and that the $K_{m(app)}$ values are closely similar to $K_s$ for the compounds examined here. A comparison of the reaction rates of Compounds III and VII shows that VII, the uncharged ester, reacts equally rapidly with acetylcholinesterase while its reaction rate with hydroxide ion is only one-twentieth that of III. With Compound VII, kinetic specificity is reflected in loss of affinity (increased $K_m$) and with a relative increase in reaction rate.

The pyridinium ketoxime esters have similar kinetic constants to the corresponding pyridinium aldoxime esters. They are more resistant to aqueous hydrolysis. Furthermore, experience has shown that the ketoximes are substantially more stable during storage than the aldoximes, and it is assumed that this property extends to the oxime esters. Hence, in the absence of other considerations, the ketoxime esters would be preferred.

Table IV indicates the comparative ease of hydrolysis of Compounds III–VI by butyrylcholinesterase and acetylcholinesterase, using phenyl acetate as the reference standard. The oxime acetates are considerably more selective than phenyl acetate as substrates for butyrylcholinesterase. Compound VI is particularly outstanding in this regard, and the corresponding butyrate might be even more useful as a highly selective substrate for serum (butyryl) cholinesterase.

*syn*-Benzaldoxime acetate is hydrolyzed by acetylcholinesterase. However, the spectra of ester and oxime are quite similar. Thus, at 262 nm., the wavelength of maximum absorbance change, the ester absorbance is very high, $\epsilon = 5.7 \times 10^3$. At longer wavelengths where the ester absorbance falls, $\Delta\epsilon$ falls correspondingly. Thus, this compound is not particularly useful for spectrophotometric purposes and its properties were not studied further.

## SUMMARY


1. 3-Substituted 1-alkyl pyridinium ketoxime acetates are useful substrates for acetylcholinesterase.

2. Among the 1-alkyl pyridinium compounds, replacement of the methyl group by the benzyl group decreases $K_{m(app)}$ approximately 10-fold and reduces $V_{max}$ ($k_{cat}$) to approximately the same extent.

3. The oxime acetates are good substrates for butyrylcholinesterase. 1-Benzyl-3-formylpyridinium halide oxime acetate is hydrolyzed considerably more rapidly by butyrylcholinesterase than by acetylcholinesterase. This compound or the corresponding butyrate might be useful as a highly selective substrate for butyrylcholinesterase.


## REFERENCES


(1) G. M. Steinberg, L. M. Berkowitz, N. C. Thomas, J. P. Maddox, and L. L. Szafraniec, *J. Pharm. Sci.*, **61**, 527(1972).

(2) "Handbuck Exp. Pharmakol," vol. 15, G. B. Koelle, Sub. Ed., Springer-Verlag, Berlin, Germany, 1963, p. 103.

(3) J. H. Blanch and O. T. Onsager, *J. Chem. Soc., Ser. B*, **1965**, 3729. 3734.

(4) J. H. Blanch and J. Andersen, *ibid.*, **1968**, 1969.

(5) S. Ginsburg and I. B. Wilson, *J. Amer. Chem. Soc.*, **79**, 481 (1957).

(6) E. Beckman, *Chem. Ber.*, **20**, 2580(1887).

(7) A. Hantzsch, *ibid.*, **24**, 36(1891).

(8) G. N. Wilkinson, *Biochem. J.*, **80**, 324(1961).

(9) M. I. Kabachnik, A. P. Brestkin, N. N. Godovikov, M. J. Michelson, E. V. Rosengart, and V. I. Rosengart, *Pharmacol. Rev.*, **22**, 355(1970).


## ACKNOWLEDGMENTS AND ADDRESSES


Received December 12, 1972, from the *Medical Research Division, Biomedical Laboratory, Department of the Army, Edgewood Arsenal, MD 21010*

Accepted for publication March 15, 1973.

The authors thank Mrs. Linda L. Szafraniec for the NMR measurements and the spectral analyses, the Analytical Department of these laboratories for the elemental analyses, and Dr. Jay Amshey for editorial comment.



▲ To whom inquiries should be directed.