values comparable to those obtained by more complex physiological methods without the assumption of the model.

REFERENCES

(1) I. Sperber, *Pharm. Rev.*, **11,** 109(1959).

(2) A. Despopoulos, *J. Theoret. Biol.*, **8,** 163(1965).

(3) H. W. Smith, "The Kidney: Structure and Function in Health and Disease," Oxford University Press, New York, N. Y., 1951.

(4) K. Irmscher, D. Gabe, K. Jahnke, and W. Scholtan, *Arzneim.-Forsch.*, **16,** 1019(1966).

(5) K. A. McMahon and W. J. O'Reilly, *Food Cosmet. Toxicol.*, **7,** 493(1969).

(6) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128,** 537 (1939).

(7) S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," vol. 3, Academic, New York, N. Y., 1957, p. 76.

(8) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57,** 117(1968).

(9) O. W. Wendel, *Phys. Med. Biol.*, **5,** 411(1961).

(10) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams & Wilkins, Baltimore, Md., 1963, pp. 198, 205.

(11) K. A. McMahon and W. J. O'Reilly, *J. Pharm. Sci.*, **61,** 518 (1972).

(12) A. Falbriard, R. Busset, and R. Zender, *Helv. Physiol. Acta*, **25,** 123(1967).

(13) R. F. Pitts, "Physiology of the Kidney and Body Fluids," Year Book Medical Publishers, Chicago, Ill., 1963, p. 65.

(14) R. Zender, B. Blixenkrone, R. Borth, and A. Falbriard, *Helv. Med. Acta*, **31,** 696(1964).

(15) B. I. Kaplan and H. W. Smith, *Amer. J. Physiol.*, **113,** 354 (1935).

(16) H. M. Kaplan, "The Rabbit in Experimental Physiology," 2nd ed., Scholars Library, New York, N. Y., 1962, p. 94.

(17) T. A. Loomis, R. S. Hubbard, and G. F. Keopf, *Amer. J. Physiol.*, **139,** 197(1943).

(18) T. Arita, R. Hori, E. Owada, and K. Takahashi, *Chem. Pharm. Bull.*, **17,** 2526(1969).

(19) H. Laake, *Acta Med. Scand. Suppl.*, **168,** 1(1945).

(20) S. E. Dicker and H. Heller, *J. Physiol.*, **103,** 449(1945).

(21) J. Brod and J. H. Sirota, *Amer. J. Physiol.*, **157,** 31(1949).

(22) E. Nelson and I. O'Reilly, *J. Pharmacol. Exp. Ther.*, **129,** 368(1960).

(23) W. J. Jusko, G. Levy, S. J. Yaffe, and R. Gorodischer, *J. Pharm. Sci.*, **59,** 473(1970).

(24) "Handbook of Biological Data," W. S. Spector, Ed., W. B. Saunders, Philadelphia, Pa., 1956, p. 341.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 22, 1971, from the *Faculty of Pharmacy, University of Manitoba, Winnipeg, Canada.*

Accepted for publication December 6, 1971.

Supported by Medical Research Council of Canada Grant MA 3818.

The nonlinear curve-fitting program (NONLIN) was kindly provided by Dr. Carl Metzler, The Upjohn Co., Kalamazoo, Mich., and was run on the IBM 360-65 digital computer in the University of Manitoba Computer Centre.

▲ To whom inquiries should be directed. Present address: School of Pharmacy, S. A. I. T., Adelaide, Australia, 5049.

Oxime Acetates: Substrates for Acetylcholinesterase

G. M. STEINBERG*▲, L. M. BERKOWITZ*, N. C. THOMAS*, J. P. MADDOX*, and L. L. SZAFRANIEC†

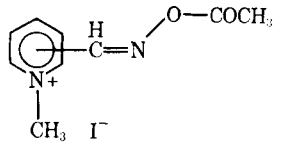
Abstract □ Oxime acetates function as typical substrates for acetylcholinesterase. Both acetate derivatives of *syn*-3- and *syn*-4-formyl-1-methylpyridinium iodide oximes are rapidly hydrolyzed. Both are highly water soluble and give large changes in absorbance upon hydrolysis. Hence, they have potential utility for spectrophotometric studies with the enzyme. The prior configurational assignment of the acetate derivative of *syn*-4-formyl-1-methylpyridinium iodide oxime has been confirmed by NMR spectroscopy. Vicinal aliphatic dioxime diacetates are hydrolyzed, but quite slowly, by acetylcholinesterase.

Keyphrases □ Acetylcholinesterase—oxime acetates as substrates □ *syn*-3- and *syn*-4-Formyl-1-methylpyridinium iodide oximes, acetate derivatives—configuration confirmation by NMR, acetylcholinesterase substrates □ Enzyme kinetics—oxime acetates as substrates for acetylcholinesterase □ 3-PAM acetate—reaction with isonitrosoacetone, substrate for acetylcholinesterase □ 4-PAM acetate—configuration, substrate for acetylcholinesterase □ NMR spectroscopy—configuration identification

It is becoming increasingly clear that the high selectivity and specificity of enzymes is a comparative rather than an absolute phenomenon. The substrate activity spectrum must ultimately be correlated with the enzyme's structure, conformation, and chemical mechanism. Hence, a knowledge of the activity spectrum provides information concerning these properties. It also provides flexibility in assay. We have observed that eel acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) catalyzes the hydrolysis of several *O*-acetyl oximes, including acetate derivatives of benzaldehyde oxime, *syn*-3- and *syn*-4-formyl-1-methylpyridinium iodide oximes[1] (*syn*-3- and *syn*-4-PAM acetates) (Structure I), and glyoxime and dimethylglyoxime (Structures II*a* and II*b*, respectively). In this article the kinetic constants for the enzymatic hydrolysis of the pyridinium compounds and semiquantitative results for the glyoximes are reported.

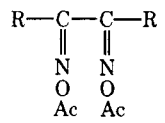
Acetylcholinesterase hydrolyses are quite sensitive to environmental conditions. Substrate turnover rates vary considerably with ionic strength, the presence or

[1] These compounds, which are closely related to the preferred oxime treatment compounds for organophosphonate poisoning (for example, pralidoxime chloride), have been generally referred to in the biochemical literature as 3-PAM and 4-PAM, respectively.



syn-3- and *syn*-4-PAM acetates

I



IIa: R = H

IIb: R = CH_3

absence of inorganic ions, pH, temperature, *etc.* (1). In general, positively charged organic ions are inhibitors while negatively charged ions are not. Studies were performed at pH 6.61 in 0.1 *M* 2-(*N*-morpholino)-ethanesulfonic acid (2), a zwitterionic buffer. This pH rather than the pH of maximum turnover rate (approximately 8) (3) was chosen to reduce the nonenzymatic hydrolysis of the substrates. This report also relates these results with those obtained with other acetylcholinesterase substrates under study in this laboratory.

EXPERIMENTAL

The *syn*-3- and *syn*-4-PAM acetates were prepared as described by Ginsburg and Wilson (4)[2]: 3-PAM acetate, m.p. 163–164° dec. [lit. (1) 151–161°, (2) 165°]; and 4-PAM acetate, m.p. 152–153° dec. [lit. (1, 2) 156°]. NMR spectra[3] were run in D_2O (concentrated approximately 10% by weight) using sodium 3-trimethylsilyl-1-propanesulfonate as an internal standard. For 4-PAM acetate the NMR chemical shifts (δ, p.p.m.) are: 2.37 (acetate methyl), 4.49 ($^+N—CH_3$), 8.41 (d)[4] ($H_{3,5}$), 8.86 (aldehydic proton), and 8.94 (d) ($H_{2,6}$). For 3-PAM acetate, the NMR chemical shifts (δ, p.p.m.) are: 2.32 (acetate methyl), 4.49 ($^+N—CH_3$), 8.16 (t or 2 d) (H_5), 8.78 (aldehydic proton), 8.92 (d) and 8.94 (d) (one is H_4 and the other H_6), and 9.24 (H_2). 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%[5].

Glyoxime diacetate and dimethylglyoxime diacetate were prepared by reaction of the corresponding oximes with acetic anhydride: glyoxime diacetate, m.p. 120–121° [lit. (8) 120°]; and dimethylglyoxime diacetate, m.p. 112–113° [lit. (8) 111°].

The acetylcholinesterase was a purified, stable, dry powder[6], 1000 units/mg. Ten milligrams was dissolved in 1.6 ml. of a previously boiled (5 min.) aqueous solution, pH approximately 7.4, containing KCl (0.225 *M*), 0.1% gelatin, and 0.02% sodium azide (KCl/gel) to give the enzyme concentrate, E_w. The concentration of active sites in E_w is estimated to be 2.0×10^{-5} *M* based upon the rate of hydrolysis of acetylcholine (9) and 1.9×10^{-5} *M* based upon titration with isopropyl methylphosphonofluoridate (sarin) and another organophosphorus anticholinesterase[7]. The concentrated solution, E_w, is stored in the frozen state. Dilutions of the concentrate that are used experimentally are stored at 5°. The enzyme is quite stable. Because of its extremely high catalytic activity, extensive dilution is required for its use. There has been some variability in the computed $k_{cat.}$ (Eq. 1) values. Whether the variation is due to changes in enzyme or simply to accumulated dilution errors is not known at this time. Therefore, phenyl acetate was used as a reference substrate. It has been studied in this laboratory with the same sample of diluted E_w used for the oxime acetates. Instead of giving computed values of $k_{cat.}$ in Tables I and II, the authors relate the $V_{max.}$ (Eq. 1) values to those of phenyl acetate.

At the time of the enzyme titration, the $K_{m(app.)}$ (Eq. 2) and $k_{cat.}$ values for phenyl acetate were 1.1×10^{-3} *M* and 6.4×10^5 min.$^{-1}$, respectively. One year later, for this study, the corresponding values were 1.08×10^{-3} *M* and 4.88×10^5 min.$^{-1}$.

Table I—Ester Hydrolysis by Eel Acetylcholinesterase[a]

Substrate	$K_{m(app.)}$, *M*	$\frac{V_{max.(s)}}{V_{max.(p)}} \times 100$[b]
3-PAM acetate	1.4×10^{-4}	10
4-PAM acetate	3.1×10^{-4}	2.0
Phenyl acetate	1.1×10^{-3}	100
Acetylcholine	(2.8×10^{-4})[c]	(88)[d]

[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 substrate and of phenyl acetate. [c] pH 7.4, 0.33 *M* KCl. See *Reference 9*. [d] Bovine erythrocyte; pH 7.5, 0.05 *M* phosphate. See *Reference 12*.

Enzyme Kinetics: Spectrophotometric[8]—Thermostatically controlled cell holders were maintained at 25 ± <0.1°. Full-scale deflection was adjusted to 0.1 absorbance unit. All runs were made in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. For 3- and 4-PAM acetates, concentrates were prepared in water and 10–150-μl. aliquots were added to cells containing 3.0 ml. of thermally preequilibrated buffer. In each case, absorbance of the ester was recorded prior to the addition of 20 μl. of suitably diluted E_w. Rapid mixing was achieved with a flat-faced stirring device. For phenyl acetate, a concentrate was prepared containing 50 μl. (precisely weighed)/50 ml. of buffer. Appropriate dilutions were made from the concentrate, which were then thermally equilibrated prior to addition to enzyme. The concentration ranges were: 4-PAM acetate, 1.1–16.5 $\times 10^{-4}$ *M*; 3-PAM acetate, 0.9–9 $\times 10^{-4}$ *M*; and phenyl acetate, 6.9–75 $\times 10^{-4}$ *M*.

At least two full runs (including at least five concentrations and 10 measurements) were made with each compound. Provisional examination of the resulting rate *versus* substrate concentration data was made using reciprocal plots (Lineweaver–Burk). Straight lines were obtained in each case; there was no indication of substrate inhibition. Values for $\Delta\epsilon$ (Table II, Footnote [b]) were calculated from the record of complete hydrolysis of two of the samples for each compound in each run. Contrary to the observations of nonlinearity by Schoene (10), both 3-PAM acetate and 3-PAM gave excellent Beer–Lambert law plots over the concentration range 2–10 $\times 10^{-4}$ *M*, 305 nm., pH 6.61. Computation of $K_{m(app.)}$ (Eq. 2) and $V_{max.}$ (or $k_{cat.}$) was made by the Wilkinson weighted regression method (11) using a time-shared computer[9], programmed in BASIC language.

Corrections were incorporated for dilution of the reaction solution due to added volumes of reagent solutions and also for hydrolysis of 3- and 4-PAM acetates in the aqueous concentrate during the course of study. Hydrolysis correction was unnecessary in the case of phenyl acetate and was of marginal value for 3-PAM acetate. It was established that the products of 4-PAM acetate hydrolysis gave no significant inhibition under the conditions of the experiment. The Wilkinson method permitted calculation of 95% confidence intervals of the kinetic constants for each individual run. Typical results are given in Table II. The ranges suggested by the 95% confidence intervals should be used with caution, since interrun errors may be significantly larger than intrarun errors. For example, compare relative values of $V_{max.}$ in Runs C and D.

Enzyme Kinetics: Titrimetric—For examination of the aliphatic oxime esters, substrate hydrolysis rates were measured[10] as described earlier (9)[11].

Spectra—Absorption spectra in the UV range were determined at 25°[12]. The experiment for comparison of the products of hydrolysis of 4-PAM acetate by alkali and by enzyme (Fig. 1) was performed as follows.

Curve B—To 3.00 ml. of 1×10^{-3} *M* 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 7.03, there was added 10 μl. of 4-PAM acetate concentrate (9.56×10^{-3} *M*). The final concentration of 4-PAM acetate was 3.18×10^{-5} *M*.

[2] For discussion and references on stereochemistry, see Blanch and Onsager (5).

[3] Varian A-60D.

[4] d = doublet, and t = triplet.

[5] For an example of the distinction of *syn*- and *anti*-aldoximes by NMR spectroscopy, see Poziomek *et al.* (6) and Tyson (7).

[6] Worthington Biochemicals, Code ECHP.

[7] G. M. Steinberg, to be published.

[8] Rate measurements were made on a Guilford spectrophotometer, model 2000.

[9] IBM 360-65.

[10] Using the Radiometer Autotitrator.

[11] Note the following errors in *Reference 9*. In Footnote 1, it should read "0.0073 *M* acetylcholine"; in Eq. 4, it should read "$K_{m(app.)} = (K_3k_3)/(k_2 + k_3)$."

[12] A Beckman model DK2 fitted with a thermostated cell holder was used.

Table II—Reproducibility of Computed Values[a]

Substrate	Run	$\Delta\epsilon$[b], 305 nm.	K_m, M^{-1}	$\frac{V_{max.(s)}}{V_{max.(p)}} \times 100$[c]
4-PAM acetate	A	3.33×10^3	3.0×10^{-4} (1.96–4.03)	1.94 (1.68–2.36)
	B	3.61×10^3	3.25×10^{-4} (2.2–4.3)	2.01 (1.72–2.29)
3-PAM acetate	C	1.96×10^3	1.38×10^{-4} (0.88–1.9)	11.9 (10.7–13.1)
	D	2.18×10^3	1.43×10^{-4} (1.13–1.74)	8.75 (8.20–9.29)

[a] Wilkinson's method (11); 95% confidence interval (of coefficient) in parentheses. [b] $\Delta\epsilon$ = change in molar absorbance resulting from complete hydrolysis of the substrate; $\Delta\epsilon = (\Delta A)/(S_0)$. [c] See Table I, Footnote [b].

Curve C—To the cell containing the solution of Curve B, there was added 15 μl. 5 *N* NaOH. The pH rose to above 12. The reaction was over "instantly." There was no change in spectrum over a 5-min. period.

Curve D—After the 5-min. waiting period, there was added to the cell 50 μl. of 4 *N* HCl. The pH fell to less than 2.

Curve E—To another 3.00-ml. aliquot of the original 4-PAM acetate solution in a cell mounted in the spectrophotometer, there was added 50 μl. $E_w/50$ (E_w diluted 1:50 with KCl/gel). The reaction was "instantaneous." There was no change in spectrum over a period of 10 min.

Curve F—To the cell containing the solution of Curve E, there was added 15 μl. 5 *N* NaOH.

Curve G—To the solution of Curve F, 50 μl. 4 *N* HCl was added.

Reaction of 3-PAM Acetate with Isonitrosoacetone (1,2-Propandione 1-Oxime)—The reaction was followed spectrophotometrically using a spectrophotometer[13] fitted with a thermostatically controlled cell compartment. The conditions were: 0.1 *M* 2-*N*-(morpholino)-ethanesulfonic acid, pH 6.61, 25°, 305 nm. At this wavelength, isonitrosoacetone has no appreciable absorbance. Reactions were run over a range in concentrations of the two reactants. However, only one, at the highest concentration of reactants (Fig. 2), was subjected to detailed analysis. The others gave results that are semiquantitatively consistent.

RESULTS AND DISCUSSION

PAM Acetates: Configuration—In earlier work, Ginsburg and Wilson (4) suggested that the 3- and 4-formylpyridine oxime acetates and the corresponding methiodides had the *syn*-configuration. In each case, synthesis yielded only one isomer. Assignment of *syn*-configuration was based upon hydrolysis to the original oxime. However, assignment was not unequivocal (5).

In this work, NMR spectra were used for configurational assignment. As before, only a single isomer each of the 3- and 4-PAM acetates was obtained. Although both isomers of each would be necessary for unequivocal assignment, the evidence suggests strongly that 4-PAM acetate is *syn*. By analogy, it is assumed that the original *syn* assignment for 3-PAM acetate is valid. The NMR spectrum of each compound gave a single sharp peak corresponding to the "aldehydic" proton. As indicated in Table III, in related pairs of reference compounds the chemical shift of H_a for the *syn*-compound is in each case within the region of shift of the "aromatic" protons, whereas for the *anti*-compounds, H_a is always upfield. It is also considered highly unlikely that esterification of the oximino group would cause a shift downfield as great as 0.6 p.p.m. as required if 4-PAM acetate were *anti*, because of the considerable distance between aldehydic proton and the anisotropic carbonyl group[14].

PAM Acetates: Kinetics—Michaelis constants for 3-PAM acetate, 4-PAM acetate, and phenyl acetate are given in Table II. Both of the pyridinium oxime acetates are good substrates for acetylcholinesterase, although each is appreciably inferior in $V_{max.}$ to phenyl acetate [which has the highest reported kinetic constant (12)]. Both "bind" reasonably well to acetylcholinesterase, as reflected in the values of $K_{m(app.)}$. As discussed in an earlier paper (9 and references cited therein), however, acetylcholinesterase functions kinetically in a three-step reaction, so that the $K_{m(app.)}$ may represent a poor estimate of enzyme–substrate affinity. Enzymatic hydrolysis by acetylcholinesterase follows Michaelis–

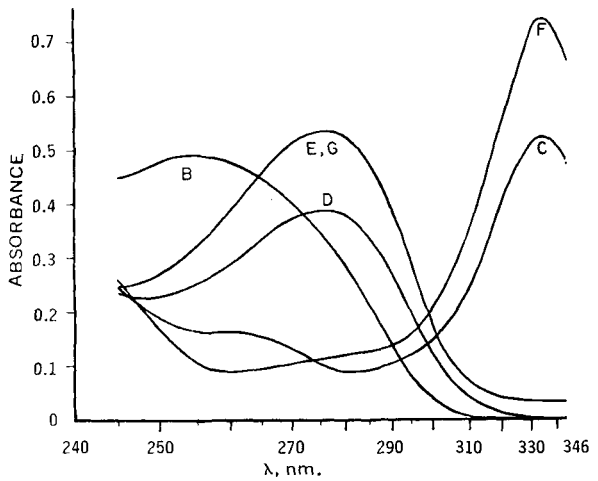


Figure 1—*Hydrolysis of 4-PAM acetate by alkali and by enzyme. Key: Curve B, original; Curves C and D, hydrolysis by alkali; and Curves E, F, and G, hydrolysis by enzyme.*

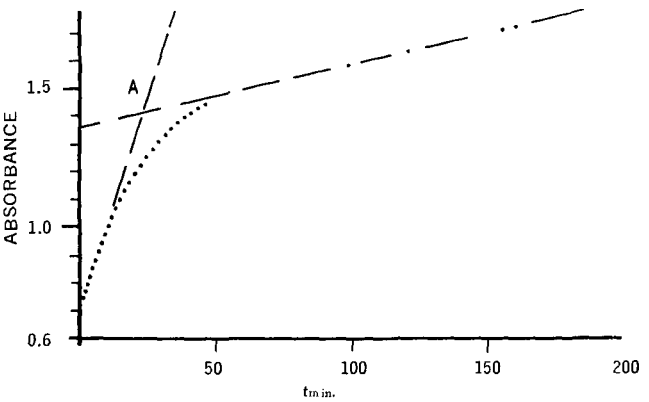


Figure 2—*Reaction between 3-PAM acetate (1.147×10^{-3} M) and isonitrosoacetone (1.555×10^{-3} M), pH 6.61, 0.1 M 2-(N-morpholino)ethanesulfonic acid, 25°. Reaction end-point (complete hydrolysis of 3-PAM acetate), absorbance = 2.958.*

[13] Cary model 14.

[14] In a parallel case, $(CH_3)_2C{=}NOCONH_2$, where the distance between carbonyl group and methyl protons in molecular models is somewhat shorter than the carbonyl–aldehydic proton distance in 4-PAM acetate, the difference in chemical shifts for the *syn*-methyl protons and *anti*-methyl protons = 0.045 p.p.m. (L. L. Szafraniec, unpublished data).

Menten kinetics; however, the kinetic constants are complex functions (Scheme I and Eqs. 1 and 2):

$$\text{enzyme} + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{enzyme} \cdot \text{ester} \xrightarrow{k_2} \text{enzyme} - \text{acyl} \xrightarrow{k_3} \underset{\text{"alcohol"}}{+} \text{enzyme} + \text{acid}$$

Scheme I

$$V_{max.} = k_{cat.}\, E_0; \qquad k_{cat.} = \frac{k_2 k_3}{k_2 + k_3} \qquad \text{(Eq. 1)}$$

$$K_{m(app.)} = \frac{K_s k_3}{k_2 + k_3}; \qquad K_s = \frac{k_2 + k_{-1}}{k_1} \qquad \text{(Eq. 2)}$$

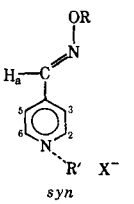
Thus, dissociation of the substrate complex is measured by K_s; but, again, K_s represents the equilibrium dissociation constant for enzyme + ester $\rightleftharpoons$ enzyme·ester, only if $k_{-1} \gg k_2$. While k_{-1} may not be $\gg k_2$ in all cases, the use of this assumption is worthwhile since it permits the development of relationships between substrate structure and binding which, if not quantitatively precise, are probably good approximations and useful.

It will be assumed that the deacylation step in the enzymatically mediated hydrolysis reactions with each of the substrates is kinetically identical; that is, there are no conformational or other changes due to the presence of unhydrolyzed substrate (13). Then the k_3 value for phenyl acetate, which is approximately equal to $k_{cat.}$ since $k_2 > k_3$ (14), can be used to calculate relative values of k_2 and also of K_s for the other substrates.

Table IV presents for each of the substrates studied here and also for acetylcholine the computed values of K_s and k_2/k_3 and the reported rates of their reactions with hydroxide. It can be seen that both of the PAM acetates bind to the enzyme considerably more effectively than does the natural substrate acetylcholine or the neutral aromatic ester phenyl acetate. The greater binding probably results from the combination of electrostatic and hydrophobic (and/or charge transfer) interactions with the protein. Because of the large distance between the positively charged nitrogen atom and the ester carbonyl group (*vide infra*), it is unlikely that either of the PAM acetates occupies precisely the same site as acetylcholine. This hypothesis is strongly supported by the very poor correlation between k_2 (the acylation rate constant) and k_{OH} (Table IV, Footnote [b]). The failure to observe substrate inhibition with the PAM acetates is consistent with their low k_2/k_3 ratios; that is, in each case, acylation is rate limiting (9).

Both are potentially useful substrates for spectrophotometric acetylcholinesterase assay, although 3-PAM acetate is superior because of its greater resistance to aqueous hydrolysis and its

Table III—NMR Chemical Shifts (δ, p.p.m.) for 4-PAM Acetate and Related Compounds



syn

Compound	Configuration	H_a	Chemical Shift $H_{3,5}$	$H_{2,6}$
4-PAM acetate (R = $COCH_3$, R′ = CH_3)	*syn*	8.86	8.41	8.94
4-Pyridine aldoxime[a] (R = H)	*syn*	8.62	7.98	8.97
	anti	8.02	8.32	9.10
4-PAM[a] (R = H, R′ = CH_3)	*syn*	8.80	8.66	9.30
	anti	8.26	8.93	9.37
4-Pyridine aldoxime *O*-methyl ether[b] (R = CH_3)	*syn*	8.39	8.22	8.82
	anti	8.23	8.66	8.82
4-PAM *O*-methyl ether[b] (R = R′ = CH_3)	*syn*	8.35	8.19	8.79
	anti	8.25	8.65	8.85

[a] *Reference 6.* [b] *Reference 7.*

Table IV—Hydrolysis by Eel Acetylcholinesterase[a]

Substrate	K_s, *M*	k_2/k_3[b]	k_{OH}[c], M^{-1} sec.$^{-1}$
3-PAM acetate	1.55×10^{-4}	0.117	80[d]
4-PAM acetate	3.1×10^{-4}	0.026	170[d]
Phenyl acetate	$>10^{-2}$	$\gtrsim 10$[e]	3.7[f]
Acetylcholine	(2.3×10^{-3})[g]	5–10[e]	2.17[h]

[a] 25.0°, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. [b] Since k_3 is the rate constant for a common step in the reaction sequence, these numbers represent relative values for the individual reaction rate constants, k_2. [c] k_{OH}, second-order rate constants for hydrolysis by OH^-. [d] See *Reference 5.* [e] See *Reference 14.* [f] T. C. Bruice, A. F. Hegarty, S. M. Felton, A. Donzel, and N. G. Kundu, *J. Amer. Chem. Soc.*, **92**, 1370(1970). [g] See Footnote [c], Table I. [h] M. R. Wright, *J. Chem. Soc., Ser. B*, **1968**, 545.

greater $V_{max.}$. Each is highly water soluble and offers a modest range in wavelength in the UV where, as hydrolysis proceeds, the weakly absorbing ester is converted to the strongly absorbing oxime. In 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.61, each hydrolyzes approximately three times as fast as the rate calculated from the k_{OH} values given in Table IV. At this pH, 3-PAM acetate is adequately stable. For 4-PAM acetate, a somewhat lower pH (perhaps 6) might be preferable.

3-PAM Acetate: Reaction with Isonitrosoacetone—To explore the general utility of 3-PAM acetate as an acetylcholinesterase substrate, its reaction was examined with isonitrosoacetone, a powerful nucleophile chemically related to such effective reactivators of phosphonylated aectylcholinesterase as 2-PAM and 1,1′-trimethylene-[bis(4-formyl)pyridinium] dibromide dioxime (TMB-4) (15). As indicated in Fig. 2, at pH 6.61 there is an appreciable reaction. Under the same conditions of pH and concentration, phenyl acetate gives little or no reaction. Hence, the oxime acetates would be less desirable substrates than phenyl acetate for enzyme studies in the presence of nucleophiles (for example, in reactivation of inhibited enzyme).

In Fig. 2, the increase in absorbance reflects the conversion of 3-PAM acetate to 3-PAM. At complete conversion, the absorbance value was calculated to be 2.958. Upon mixing the two ingredients, there is a rapid initial reaction which then subsides to a slower steady state. This pattern is consistent with a "leaky" equilibrium situation, which is most probably described by Scheme II.

Several other reactions may occur, producing nitriles and further hydrolysis products (16–19). However, these would be minor and will be ignored. From the data of this study, one can obtain approximate values of the bimolecular rate constants for the forward and reverse reactions, k_1 and k_{-1}. For this estimate, k_1 was calculated from initial conditions, Eq. 3:

$$\left(\frac{\Delta[\text{3-PAM acetate}]}{\Delta t}\right)_0 = k_1[\text{3-PAM acetate}]_0 \times [CH_3COCH{=}NOH]_0 \qquad \text{(Eq. 3)}$$

and k_{-1} was calculated from the equilibrium condition, which was approximated as the intersection A in Fig. 2, Eq. 4:

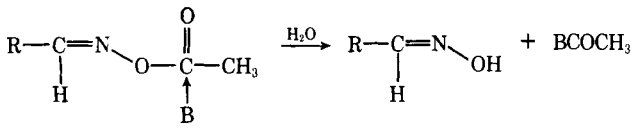
$$k_1[\text{3-PAM acetate}]_{eq.}\,[CH_3COCH{=}NOH]_{eq.} = k_{-1}[\text{3-PAM}]_{eq.}[CH_3COCH{=}NOCOCH_3]_{eq.} \qquad \text{(Eq. 4)}$$

The subscripts 0 and eq. in Eqs. 3 and 4 refer to conditions at zero time and at equilibrium, respectively, and protonated and unpro-

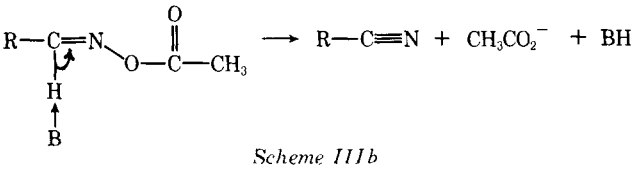
$$\begin{array}{ccc} CH_3COO^- & & CH_3COCH{=}NOH \\ + & & \downarrow\uparrow \\ \text{3-PAM}^- & & H^+ \\ \uparrow & & + \\ \text{3-PAM acetate} & + & CH_3COCH{=}NO^- \end{array} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}$$

$$\begin{array}{ccc} \text{3-PAM} & + & CH_3COCH{=}NOCOCH_3 \\ \downarrow\uparrow & & \downarrow OH^- \\ H^+ & & CH_3COCH{=}NO^- \\ + & & + \\ \text{3-PAM}^- & & CH_3COO^- \end{array}$$

Scheme II



Scheme IIIa



Scheme IIIb

tonated forms of the oximes are not distinguished. Using these approximations, $k_1 = 7.3\ M^{-1}$ min.$^{-1}$ and $k_{-1} = 52\ M^{-1}$ min.$^{-1}$ at pH 6.61.

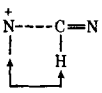
4-PAM Acetate: Products of Enzymatic Hydrolysis—4-PAM acetate reacts with hydroxide ion to give a mixture of 74% 4-PAM and 26% nitrile (or its hydrolysis products) (5). The corresponding isopropyl methylphosphonylated 4-PAM reacts with hydroxide ion and also with other bases to yield similar products (16). The reactions in each case have been interpreted as nucleophilic attack by the base (B) on carbonyl (or phosphonyl) to give oxime (Scheme III*a*) and on the "aldehydic" proton to give nitrile (Scheme III*b*).

Isopropyl methylphosphonylated 4-PAM is a powerful phosphonylating inhibitor of acetylcholinesterase; hence, the phosphonate group is nucleophilically attacked by the enzymes active site. It is also known that the ratio of the rate of attack on phosphorus to the rate of attack on the "aldehydic" proton in isopropyl methylphosphonylated 4-PAM in the nonenzymatic reaction is increased as one increases the nucleophilicity of the base. However, even such a superior nucleophile as 4-PAM does cause some elimination to the nitrile. With isopropyl methylphosphonylated 4-PAM, it was not possible in the earlier work (16) to determine the oxime–nitrile product ratio for the enzymatically mediated reaction because of the small quantity of enzyme available. Since 4-PAM acetate is a substrate, and therefore "turns over," it was possible to explore this characteristic of the enzyme to determine whether it would react exclusively as a nucleophile (and attack only the carbonyl) or whether it could participate as a general base (to give nitrile).

In Fig. 1, spectral comparison is made between the products of 4-PAM acetate hydrolysis by aqueous base and by acetylcholinesterase. Enzymatic hydrolysis gives only 4-PAM. Curve B is the spectrum of 4-PAM acetate at pH 7.03. Curve E represents the products of enzymatic hydrolysis at pH 7.03 (4-PAM). Curves F and G are, respectively, spectra of the alkaline form of the enzymatic hydrolysis product (4-PAM) and its acidic form *after* the prior (F) alkaline treatment. Alkaline treatment *after* enzymatic hydrolysis has no effect on products since E and G are identical. Spectra E, F, and G are clearly consistent with the reported properties of 4-PAM under the cited conditions (5). At pH 7.03, 4-PAM exists in the protonated form (pKa 8.4) (4, 16), hence the virtual identity of spectra E and G. Treatment of 4-PAM acetate with alkali (Curve C) confirms the earlier report of parallel pathways. The elevated section in the 250–270-nm. region represents products *via* Scheme III*b* [the nitrile is further hydrolyzed to pyridone and other products (16–18)]. The region at the 334-nm. maximum (and also the 275-nm. maximum in Curve D) corresponds to 4-PAM. The yield of 4-PAM (calculated from Curves C and F and D and G) is 71% [reported (5) 74%].

Binding and Mechanistic Implications—The active site of acetylcholinesterase consists of a binding subsite (anionic) and a reactive subsite (esteratic) (20). From pH profile and other studies, the reactive or esteratic subsite is believed to contain an unprotonated imidazole group (histidine) plus a serine hydroxyl group and a basic group of pH approximately 10. The anionic subsite is negatively charged and probably contains a carboxylate group. The distance between the two subsites should correspond to the $^+$N- - -C(=O) distance in acetylcholine and hence be 5Å (Table V). Both 3- and 4-PAM acetates have a much greater $^+$N- - -C(=O) distance (Table V). If the N^+ and ester groups are regarded as separate binding determinants, it is impossible for the PAM acetates to fit productively

Table V—Intraatomic Distances[a]

Substance		
Acetylcholine	4.9–5.0	—
3-PAM acetate	7.2–7.7	4.5
4-PAM acetate	8.5	4.9–5.0

[a] In Å; measured on Stuart–Briegleb models.

(hydrolysis to occur) the active site in the same fashion as acetylcholine. It can be envisioned that: (*a*) the active site is distended to accommodate the fit of the two separate substrate determinants, or (*b*) the ester determinant prevails and the N^+ and ring bind to the protein surface in an area other than at the anionic subsite. Either hypothesis would be consistent with the relatively poor acylation rates of the PAM acetates compared to that of acetylcholine (Table IV). In either case, there would be a difference in their reaction energy profile from that of acetylcholine, with a most likely increase in activation energy.

One can also hypothesize a nonproductive fit, determined by the binding of the N^+ group to the anionic subsite which would occur within the "acetylcholine" cleft. Such a fit would require that parts of the PAM acetates extend beyond that of the acetate methyl group; in fact, it would correspond to the extended length of butyryl- or valerylcholine. Butyrylcholine is a substrate, albeit poorer than acetylcholine (21), so that such a fit should be possible. Under these circumstances, the imidazole group might well be in a position to attack the aldehydic proton in 4-PAM acetate (Scheme III*b*) since its distance from the anionic subsite is similarly 5 Å. This is not the case since the enzymatic reaction gives only oxime.

Vicinal Dioxime Diacetates—Both Compounds II*a* and II*b* are hydrolyzed by acetylcholinesterase, albeit rather slowly. At a substrate concentration of $1.25 \times 10^{-3}\ M$ (pH 7.4, 0.225 *M* KCl, titrimetric assay), acetylcholine is hydrolyzed approximately 1000 times as fast as II*b* which, in turn, is hydrolyzed 2–3 times as fast as II*a*. As reported by Milone (8) and confirmed in this study, Compound II*b* hydrolyzes sequentially (he reported that $k_1 = 30 \times k_2$). In this laboratory, at pH 10.2 the values of $K_{obs.}$ (first-order rate constant) are 0.41 and 6.3×10^{-2} min.$^{-1}$, respectively. With the enzyme, there is no indication of two separate hydrolytic steps in the production of 2 moles of acid. Evidently, the diester and monoester are closely similar in their response as substrates.

REFERENCES

(1) "Handbuch Exp. Pharmakol," vol. 15, G. B. Koelle, Sub. Ed., Springer-Verlag, Berlin, Germany, 1963, p. 312.

(2) N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. Singh, *Biochemistry*, **5**, 467(1966).

(3) F. Bergmann, S. Rimon, and R. Segal, *Biochem. J.*, **68**, 493 (1958).

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

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

(6) E. J. Poziomek, D. N. Kramer, W. A. Mosher, and H. O. Michel, *J. Amer. Chem. Soc.*, **83**, 3916(1961).

(7) B. C. Tyson, Jr., Ph.D. thesis, University of Delaware, Newark, Del., 1969, p. 53.

(8) M. Milone, *Gazz. Chim. Ital.*, **62**, 868(1932); through *Chem. Abstr.*, **27**, 706(1933).

(9) G. M. Steinberg, M. L. Mednick, R. E. Rice, and J. P. Maddox, *J. Pharm. Sci.*, **59**, 1107(1970).

(10) K. Schoene, *Arzneim.-Forsch.*, **18**, 1350(1968).

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

(12) R. M. Krupka, *Biochem. Biophys. Res. Commun.*, **19**, 531 (1965).

(13) R. M. Krupka, *Biochemistry*, **3**, 1749(1964).

(14) J. K. Stoops, Ph.D. thesis, Northwestern University, Evanston, Ill., 1966, p. 265.

(15) R. I. Ellin and J. H. Wills, *J. Pharm. Sci.*, **53**, 995(1964).

(16) G. M. Steinberg and S. Solomon, *Biochemistry*, **5**, 3142 (1966).

(17) J. W. Patton, Ph.D. thesis, University of Wisconsin, Madison, Wis., 1961.
(18) R. I. Ellin, J. S. Carlese, and A. A. Kondritzer, *J. Pharm. Sci.*, **51**, 141(1962).
(19) J. J. O'Neill, H. Kohl, and J. Epstein, *Biochem. Pharmacol.*, **8**, 399(1961).
(20) I. B. Wilson, in "The Enzymes," vol. 4, P. D. Boyer, H. Lardy, and K. Myrbach, Eds., Academic, New York, N. Y., 1961, p. 301.
(21) L. A. Mounter and V. P. Whittaker, *Biochem. J.*, **47**, 525 (1950).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 16, 1971, from the **Biochemistry Section, Basic Medical Sciences Branch, Biomedical Laboratory, Edgewood Arsenal, MD 21010*, and the *†Materials Engineering Lab Section, Physical Chemistry Branch, Chemical Laboratory, Edgewood Arsenal, MD 21010*

Accepted for publication December 10, 1971.

The authors thank Jay Amshey for helpful discussion and editorial comment.

▲ To whom inquiries should be directed.

Survey of Microbial Contamination of Ophthalmic Ointments▲

FRANCES W. BOWMAN, EVERETT W. KNOLL, MACK WHITE, and PHILIP MISLIVEC

Abstract □ A survey was conducted by the Food and Drug Administration to obtain a profile of the microbial load in all antimicrobial ophthalmic ointments manufactured in the United States. Eighty-two batches of ointments from 27 manufacturers were tested. Contamination was detected in 16 batches (19.5%).

Keyphrases □ Ophthalmic ointments—survey of microbial contamination in antibiotic and nonantibiotic commercial preparations □ Microbial contamination of ophthalmic ointments—survey of commercial preparations □ Antibiotic ophthalmic ointments—survey of microbial contamination in commercial preparations □ Sterility of ophthalmic ointments—survey of commercial preparations

The *Antibiotic Regulations* of the Food and Drug Administration (FDA), the USP, and the NF require all ophthalmic solutions and suspensions to be sterile, and they specify sterility tests for these preparations. None of these, however, requires sterility tests for ophthalmic ointments, except for certain antibiotic ophthalmic ointments when these are labeled sterile (1).

From 1947 to 1950, the *Antibiotic Regulations* (2) required a microorganism count to be performed on each batch of antibiotic ophthalmic ointments. (The viable counting method involved smearing the ointment directly on the surface of an agar plate.) During that time, even though the *Regulations* specified that the count should not exceed five viable organisms per gram of ointment, the incidence of contamination detected was very low. This was undoubtedly due to the poor sensitivity of the method, and the test was deleted from the *Regulations* in 1950 (3).

In 1958, Vander Wyk and Granston (4) reported that of 28 samples of antibiotic ophthalmic ointments tested, 17 were nonsterile. The method used included a dispersion step in which the ointment was shaken for 1 hr. at room temperature with sterile glass beads in 25 ml. of distilled water. Three 1.0-ml. aliquots were then placed in petri dishes, mixed with melted blood agar, and incubated at 37° for 24 hr.

The finding of contamination in over 50% of the batches tested prompted a survey by Bowman and Holdowsky (5), who used the same method described by Vander Wyk and Granston (4).

Forty-six samples representing 19 batches of ophthalmic ointments were tested in the survey, and only two batches (10%) were found to be contaminated. Although this method is an improvement over the earlier one (1), its weaknesses are twofold when used to test antibiotic ophthalmic ointments. First, some organisms may become encased in oil or fat and thus be denied access to nutrients required for survival and multiplication. Second, any organisms that may be released from the antibiotic ointment onto the culture medium encounter the antimicrobial activity of the antibiotic from the product, and this activity might prevent their growth. Thus, a better method for use with petrolatum-based ointments is needed.

The use of isopropyl myristate as a diluent for petrolatum-based ointments was first reported by Sokolski and Chidester (6), who improved the recovery of viable cells from ointments by a filtration technique. Isopropyl myristate dissolves certain sterile petrolatum-based antibiotic ointments so that these preparations can be tested for sterility by membrane filtration. With the advent of this improved procedure for recovering microorganisms from petrolatum-based ointments, another survey was conducted in 1968 to reinvestigate the incidence of microbial contamination in antibiotic ointments. Although contamination was detected in eight batches (7%) of the 114 batches tested, no *Pseudomonas aeruginosa* or any other Gram-negative bacilli were recovered.

In 1964, eight cases of severe eye disorder were reported in Sweden in workmen treated with a contaminated antibiotic ointment (7). As a result of the infections, one patient lost an eye and the other patients suffered reduced visual acuity. *P. aeruginosa* of identical characteristics was isolated both from the infected eyes and from the ointment used in the treatment. Since this unfortunate incident, Sweden has imposed a sterility requirement on ophthalmic ointments. Similar un-