rise and fall of the dissolution K values in the vicinity of the apparent CMC may be due to a surface tension effect. Further information about the micellar molecular weight of the drug–surfactant complex would be required to identify fully the mechanism responsible for this finding.

Studies of the dissolution of hydrocortisone using Model II equations were performed in six separate experiments. In three experiments, W_o was equal to 0.1 W_s; in the other three, W_o was equal to 0.6 W_s. Values of K are shown as a function of time in Fig. 5. It is evident that in both series of experiments, K decreased significantly with time.

The values of K are average rate constants as t_1 was set to zero in Eq. 9. Although there is a good deal of scatter in the results, values of K at later times, 28 sec., appear to be approaching a limiting value of the same order of magnitude as that determined using Model I.

Thus, the results obtained using both Models I and II show that although simple Noyes–Whitney kinetics may be usefully applied in dissolution at low levels of saturation, a more elaborate theoretical model is needed for systems that are more than about 40% saturated. The results of the Model II experiments show that the dissolution process under nonsink conditions is not described by the equations derived in this report.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 30, 1972, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication June 21, 1972.

Abstracted in part from a thesis submitted by M. P. Short to London University in partial fulfillment of the Doctor of Philosophy degree requirements.

* Present address: Sterling-Winthrop Research and Development, Newcastle-upon-Tyne, England.

† Present address: Portsmouth Polytechnic, Portsmouth, PO1 2D7, England.

▲ To whom inquiries should be directed.

Acetylcholinesterase Substrates: Acetoxymethylpyridines and Benzyl Acetate

GEORGE M. STEINBERG▲, NORMAN C. THOMAS, MORTON L. MEDNICK, and JOSEPH W. AMSHEY, Jr.

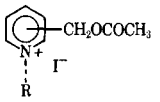
Abstract □ 2-Acetoxymethylpyridine (I) and 2-, 3-, and 4-acetoxymethylpyridinium methiodides (II, III, and IV, respectively) are spectrophotometrically useful substrates for acetylcholinesterase. Compounds III and IV are highly water soluble yet equal to phenyl acetate in resistance toward aqueous hydrolysis. Compound I and benzyl acetate are appreciably more stable. Kinetic constants for both enzymatic and nonenzymatic hydrolysis are reported. Comparison of the relative rates of acylation of acetylcholinesterase by Compounds I, III, and IV and phenyl acetate indicates considerable kinetic selectivity. Contrary to general expectations, the uncharged compounds, I and phenyl acetate, have the highest turnover rates.

Keyphrases □ Acetylcholinesterase substrates—acetoxymethylpyridines, benzyl acetate □ Hydrolysis rates, enzymatic, nonenzymatic—acetoxymethylpyridines □ Michaelis constants—acetoxymethylpyridines □ Acetoxymethylpyridine, methiodides—as acetylcholinesterase substrates, hydrolysis rates □ Benzyl acetate—as acetylcholinesterase substrate

As part of a program to develop spectrophotometrically useful substrates[1] for application in kinetic and mechanism studies with acetylcholinesterase (E.C. 3.1.1.7), results are reported here with four acetoxymethylpyridines (Table I) and benzyl acetate (V). The particular aim is to provide substrates having a range in kinetic constants, $K_{m(app)}$ and k_{cat}, and in useful wave-

[1] Earlier papers in this series include References 1 and 2.

Table I—Acetoxymethylpyridines



Compound	Ring Position of Substituent	R
I	2	—(free base)
II	2	CH_3
III	3	CH_3
IV	4	CH_3

lengths for kinetic measurement. Desirable adjunctive properties include high water solubility, stability toward nonenzymatic hydrolysis, and large values[2] of $\Delta\epsilon$.

Augustinsson (3) earlier indicated that the compounds listed in Table I were effectively hydrolyzed by acetylcholinesterase and also butyrylcholinesterase (E.C. 3.1.1.8). His work was performed by titrimetric and manometric methods. In this work, their hydrolysis by acetylcholinesterase was examined spectrophotometrically and their kinetic constants and stability toward nonenzymatic hydrolysis were compared with reference substrates under identical experimental conditions. Compared with phenyl acetate, the most commonly used substrate for acetylcholinesterase spectrophotometric kinetic studies, 3-acetoxymethylpyridinium methiodide (III) and 4-acetoxymethylpyridinium methiodide (IV) offer the advantage of very high water solubility together with equal solution stability. 2-Acetoxymethylpyridine (I) and V are more resistant than phenyl acetate toward nonenzymatic hydrolysis.

EXPERIMENTAL

Enzyme kinetics were measured at pH 6.61, 25°, in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid[3] (VI) buffer (4), using Worthington purified eel cholinesterase (ECHP) on a spectrophotometer[4] fitted with thermostated cell compartment. For details, see Steinberg *et al.* (2). Experimental data were plotted by the method of Lineweaver and Burk to establish linearity. The kinetic constants were calculated by the Wilkinson (5) weighted regression method using a time-shared IBM 360-75 computer, programmed in BASIC language. The 95% confidence intervals (*CI*) were calculated from the standard error (*SE*) by multiplying by the appropriate Student's $t_{0.025}$ value (one tailed); degrees of freedom $= n - 2$, where n = number of data points used for the determination. Thus, 95% *CI* = average ± *SE* ($t_{0.025}$ value).

Hydrolysis rates (in absence of enzyme) were measured at pH 10.44, 25°, in 0.1 *M* cyclohexylaminopropanesulfonic acid[3] (VII) buffer. Substrate (*S*) concentrations were chosen on the basis of convenience; they varied between 0.9 and 6 × 10^{-4} *M*. Reactions were followed spectrophotometrically for 1–2 half times. A small volume of concentrated enzyme was then added to complete hydrolysis rapidly in order to obtain absorbance at the reaction end-point. The data, when plotted on semilog paper as $A_{inf} - A_t$ *versus* time, where A_{inf} and A_t are absorbance values at time infinity and t, respectively, gave straight lines. The value of $t_{1/2}$ was taken as the time interval over which the ordinate value fell by a factor of 2. The observed hydrolysis rate is computed from the relationship $k_{obs} = 0.693/t_{1/2}$. Alternatively, k_{obs} was calculated directly from initial slope data, using Eq. 3.

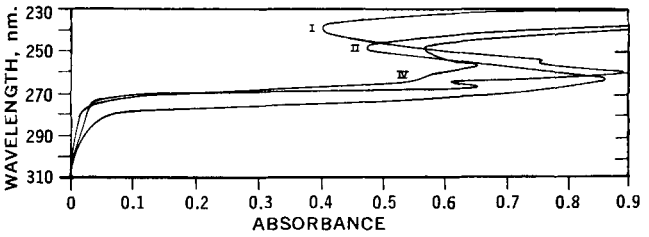


Figure 1—*Absorbance spectra of Compounds I, II, and IV in 0.1* M *buffer (VI). Concentrations: I, 2.44* × 10^{-4} M; *II, 1.20* × 10^{-4} M; *and IV, 1.43* × 10^{-4} M.

For the reaction: $S \rightarrow$ products, $-d[S]/dt = d[\text{products}]/dt = k_{obs}[S]$. So:

$$\frac{d\,[\text{products}]/dt}{[S]} = k_{obs} \qquad \text{(Eq. 1)}$$

However, the spectrophotometer records absorbance as a function of time. The differential expression, dA/dt, represents the slope of the tangent to the recorded hydrolysis rate curve. Initial rate data are taken as $\Delta A/\Delta t$, with the estimated slope of the rate curve tangent at zero time[5]. For the conversion of $S \rightarrow$ products, $A_{inf} - A_0 = \Delta A_{tot} = [S_0]\Delta\epsilon$; $\Delta\epsilon = (\Delta A_{tot})/[S_0]$, where A_0 is the value for initial absorbance and $[S_0]$ is the initial concentration of S. For partial conversion of S to products, it can be shown that $A_{inf} - A_t = [S]\Delta\epsilon$. $[S_0] = [S] + [\text{products}]$:

$$\frac{d[\text{products}]}{dt} \simeq \frac{\Delta[\text{products}]}{\Delta t} = \frac{[S_1] - [S_2]}{\Delta t} = \frac{(A_{inf} - A_1) - (A_{inf} - A_2)}{\Delta t\,\Delta\epsilon} = \frac{\Delta A}{\Delta t\,\Delta\epsilon} \qquad \text{(Eq. 2)}$$

Hence, under initial conditions:

$$\frac{\Delta A}{\Delta t} \times \frac{1}{\Delta\epsilon} \times \frac{1}{S_0} = k_{obs} \qquad \text{(Eq. 3)}$$

Second-order reaction rate constants for the reaction of each ester with hydroxide ion were calculated using Eq. 4; the contribution, if any, of buffer VII to the hydrolysis rate was ignored:

$$k_{OH} = k_{obs}/[OH^-] \qquad \text{(Eq. 4)}$$

The pKa of Compound I is 3.7. Determination was made spectrophotometrically at 263 nm. using $\mu = 0.05$ acetate buffers, four pH values over pH range 3.51–4.74. Actual buffer pH values were determined at 25° using a pH meter[6]. The plot of pH *versus* log ([unprotonated]/[protonated]) gave a good straight line; pKa = pH, where log ([unprotonated]/[protonated]) = 0.

Synthesis—The tertiary pyridine 2-, 3-, and 4-carbinol acetates were prepared by reaction of the corresponding alcohols with acetic anhydride, followed by quaternization with methyl iodide. The procedure was substantially the same as previously reported by Augustinsson (6). Physical data given in parentheses were taken from that reference.

1. Carbinol acetates—b.p.: 2-, 146–147.5°/55 mm. (103–104°/15 mm.); 3-, 108°/7 mm. (118°/12 mm.); 4-, 118–119°/11 mm. (126°/20 mm.).

2. Quaternary carbinol acetates: m.p.: 2-, dec. >140°; 3-, slow dec. >118°, melted 137–140° dec.; and 4-, 130–132°. Elemental analyses (C, H, I, N, O) were performed on Compounds I, II, III, and IV. They conformed satisfactorily to the theoretical values.

3. Benzyl acetate—obtained commercially. Its NMR spectrum[7] indicated the absence of detectable impurities.

Spectra—Absorbance spectra were determined on the spectrophotometer under standard conditions, *i.e.*, 0.1 *M* (VI), pH 6.61, 25°, using the buffer solution as reference.

[2] $\Delta\epsilon = \epsilon_{pdts} - \epsilon_{substrate}$.

[3] VI and VII are commonly referred to as MES and CAPS. The latter compound, which was obtained from Calbiochem, has a pKa value of 10.4.

[4] Cary model 14.

[5] In the general case, one can estimate the slope of the tangent at any point on the curve from the slope of a chord taken over a very small fraction of the reaction process.

[6] Beckman Research.

[7] Varian A-60, neat.

Table II—Enzymatic Hydrolysis

Compound	$K_{m(app)}$[a], M	$\frac{V_{m(compound)}}{V_{m(phenyl\ acetate)}}$[b]	Concentration Range, M	λ, nm.	Δε[c], M^{-1} cm.$^{-1}$	k_m Reported[d], M
I	3.0×10^{-3} (1.3–4.6)	0.49	3–21 × 10^{-3}	276.0 269.5	38.7 378 (max)	5×10^{-3}
II	—	>0.5	3–11 × 10^{-4}	280.0	145	6.3×10^{-3}
III	2.3×10^{-4} (1.9–2.6)	0.35	6–45 × 10^{-5}	274.5	924 (max)	8.3×10^{-4}
IV	9.7×10^{-5} (3.5–16)	0.34	8.7–54 × 10^{-5}	267.5	780 (max)	5.4×10^{-4}
V	3.9×10^{-3} (2.1–5.8)	0.57	5.9–64 × 10^{-4}	262.5	35 (max)	—

[a] 95% confidence interval of coefficient in parentheses. [b] Ratio of V_m of compound to that of phenyl acetate measured under identical conditions. For this sample of enzyme, phenyl acetate, $K_{m(app)}$ = 1.08 (0.77 – 1.4) × 10^{-3} M; k_{cat} = 4.88 (4.55 – 5.41) × 10^5 min.$^{-1}$; for discussion, see *Reference 2*. [c] Max indicates a maximum in the difference spectra between ester and hydrolyzed product. [d] Torpedo acetylcholinesterase, pH 7.4; see *Reference 3*.

RESULTS AND DISCUSSION

Spectra—The spectra of Compounds I, II, and IV are given in Fig. 1. Measurements were made at pH 6.61. For Compounds II and IV, which undergo neither acidic nor basic dissociation, the spectra are representative for the entire pH range. For compound I, pKa 3.7, the species at pH 6.61 is unprotonated. Its spectrum is representative for pH values above approximately 5.5. Spectra of the hydrolyzed products have not been given. They are closely similar to those of the parent esters. Maximum values of Δε (Table II) generally coincide with high absorbance regions of the ester spectra. The corresponding wavelengths are useful only at low substrate concentrations. At high substrate concentrations, use is made of longer wavelengths, where A_0 is low. Although Δε may be less than maximum, linear initial slopes could be obtained with each compound (Table III).

Figure 2 contains the spectrum of Compound III, measured under standard conditions, together with its difference spectrum from hydrolyzed product. Like those of Compounds II and IV, these spectra are representative for the major part of the pH range. By analogy with other quaternary alcohols, the pKa for the product alcohol is estimated to be 12–13. Therefore, the product alcohol may begin to show signs of dissociation above pH 10–11, with resultant changes in spectrum.

Nonenzymatic Hydrolysis—Table IV contains the observed rates of reaction of Compounds I–IV and phenyl acetate with aqueous solvent, pH 10.44, 0.1 M buffer (VII). The values of k_{OH} were calculated from the hydroxide concentration (Eq. 4) assuming no significant contribution from Compound VI. The close identity between the calculated and reported values of k_{OH} for phenyl acetate supports this assumption. In several cases the reaction rate constants were calculated from both kinetic progress curves and from initial slopes. The results are in reasonable agreement. It is noteworthy that the uncharged compounds, I and V, are approximately seven times more stable hydrolytically than is phenyl acetate. The charged compounds, III and IV, are equal in stability to phenyl acetate and 100 times more stable than the charged quinolinium compound. The order of reactivity of the pyridinium isomers, II > IV > III, is normal and corresponds to the order of decreasing acidity of pyridinium aldoximes and hydroxamic acids (8).

Enzymatic Hydrolysis—The $K_{m(app)}$ and V_m values (Eqs. 5 and 6) are given in Table II. The $K_{m(app)}$ values differ significantly from the K_m values reported in *Reference 3*. The differences may reflect the different source of enzyme (Torpedo) and pH (7.4). The V_m values for Compounds I–V are presented as fractions of that obtained with phenyl acetate under the standard conditions. Since phenyl acetate is the most rapidly hydrolyzed substrate for acetylcholinesterase (7), Compounds I–V are "good" substrates.

The K_m and V_m values for Compound II could not be satisfactorily determined. The reaction progress records were appreciably curved and noisy and, hence, initial slope values (V_0) were difficult to estimate precisely. Nonetheless, the results clearly point to a constancy in v_0 over the range in S_0 values studied (Table II). Such constancy suggests either that over this range in S_0, $S_0 \gg K_m$ or, alternatively, that it represents a velocity maximum in a bell-shaped v_0 *versus* S_0 plot caused by substrate inhibition. Neither alternative correlates well with Augustinsson's results (3). He observed product inhibition with a sharp peak at $S_0 = 3 \times 10^{-3}$ M. Over our range in S_0, he observed only a marked increase in v_0 with S_0.

In Table III, Compounds I–IV are compared with phenyl acetate as spectrophotometric substrates for acetylcholinesterase at practical substrate concentrations, *i.e.*, phenyl acetate and I near maximum solubility. The results, although themselves precise, are only indicative of relative substrate sensitivities ($\Delta A/\Delta t$), since no effort was made to maximize observed rates. Phenyl acetate is considerably superior to the others. However, acetylcholinesterase is a very active enzyme. Under many conditions the others may be quite adequate for assay purposes. Compounds II, III, and IV have the marked advantage of very high water solubility without the attendant loss of stability toward hydrolysis as found, for example, in the quinolinium acetates (Table IV). Compound I, on the other hand, is considerably more resistant to hydrolysis. Hence, it may have utility in studies at elevated pH. Compounds III and IV have, in addition, large values of Δε. Hence, their kinetic constants can be determined with precision, so they may find use in kinetic and mechanistic studies. Finally, with all of the substrates reported here, the products of hydrolysis are nonacidic. Hence, the observed velocities ($\Delta A/\Delta t$) are relatively independent of pH, except insofar as the enzyme

Table III—Hydrolysis by Acetylcholinesterase[a] at High Substrate Concentration, pH 6.61 (Comparative Sensitivity)

Compound	Concentration, $M \times 10^2$	v, ΔA/min.	λ, nm.
Phenyl acetate	0.77	1.41	270
I	2.13	0.064	276
II	1.19	0.022	282.5
III	1.38	0.086	280
IV	1.52	0.036	275

[a] Approximate concentration of active sites = 6.8 × 10^{-9} M; see *Reference 2*.

Table IV—Nonenzymatic Hydrolysis[a]

Compound	k_{OH}[b], M^{-1} sec.$^{-1}$	$t_{1/2}$, sec.
I	0.43, 0.50, [0.38]	5040, 5820
II	7.3, 7.96, [6.36]	346, 315
III	2.42, [2.44]	1040
IV	3.48	720
Phenyl acetate	3.14 [3.24] (reported 3.7)[c]	—
Benzyl acetate	(Reported 0.13 × rate of phenyl acetate)[d]	—
Acetylcholine	(Reported 2.17)[e]	—
1-Methyl-6-acetoxyquinolinium iodide	[280][f]	—

[a] Measurement made at pH 10.44, 25°, buffer VII (0.1 M); rate contribution of buffer ignored. [b] k_{OH} values calculated from initial slopes have been placed in brackets. [c] T. C. Bruice, A. F. Hegarty, S. M. Felton, A. Donzel, and N. G. Kundu, *J. Amer. Chem. Soc.*, **92**, 1370 (1970). [d] E. Tommila and C. N. Hinshelwood, *J. Chem. Soc.*, **1938**, 1801. [e] M. R. Wright, *J. Chem. Soc., B*, **1968**, 545. [f] Calculated from data in A. K. Prince, *Arch. Biochem. Biophys.*, **113**, 195(1966).

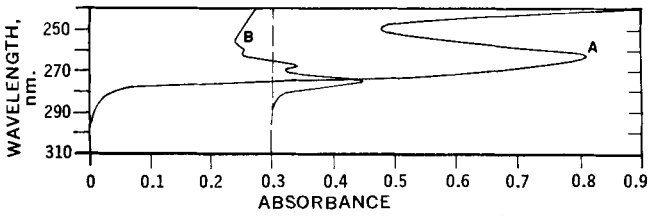


Figure 2—*Curve A, absorbance spectrum of Compound III; curve B, difference spectrum between hydrolyzed products and substrate. Buffer (VI) (0.1 M), pH 6.61, 25°; concentration 1.59 × 10⁻⁴ M was used. For the difference spectrum, absorbance at 310 nm. was arbitrarily set at 0.3.*

$$\text{enzyme} + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{enzyme}\cdot\text{ester} \xrightarrow{k_2} \underset{+\ \text{alcohol}}{\text{enzyme–acetyl}} \rightarrow \underset{+\ \text{acid}}{\text{enzyme}}$$

Scheme I

activity is pH dependent. With phenyl acetate, $\Delta\epsilon$ is highly sensitive to small changes in pH.

Enzyme Kinetics—Acetylcholinesterase functions kinetically in a three-step reaction (Scheme I). Michaelis–Menten kinetics are followed (1 and references cited therein); however, the kinetic constants are complex functions (Eqs. 5 and 6):

$$v = \frac{V_{max}S}{S + K_{m(app)}} \quad \text{where } V_{max} = k_{cat}E_0 \qquad \text{(Eq. 5)}$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3} \qquad K_{m(app)} = \frac{K_sk_2}{k_2 + k_3} \qquad K_s = \frac{k_2 + k_{-1}}{k_1} \qquad \text{(Eq. 6)}$$

where k_1, k_{-1}, k_2, and k_3 are rate constants; v is the observed rate of product formation; and E_0 and S are the concentrations of enzyme and substrate, respectively.

Per the discussion in *Reference 1*, it is noteworthy that the ratios of $V_{m(compound)}/V_{m(phenyl\ acetate)}$ are high and yet not greater than 1. For phenyl acetate, current evidence suggests that deacylation is the rate-limiting step and that $k_2 \gg k_3$ (7). Hence, if the three-step mechanism is valid, the V_m for acetate esters cannot exceed the V_m for phenyl acetate. These results conform to the three-step mechanism. The high V_m ratios indicate that both acylation and deacylation steps are sufficiently close in rate that they both contribute to the observed rate constant. If one assumes that $V_{m(phenyl\ acetate)}$ is directly proportional to k_3 and that k_3 is identical for all of the substrates, one can calculate k_2/k_3 for each compound from the V_m ratios using Eqs. 5 and 6. Further, one can calculate the value of K_s from the observed $K_{m(app)}$ and the values of k_2 and k_3. Finally, if one assumes that the "intrinsic" reactivity of each ester is measured by its reactivity toward hydroxide, one can examine the kinetic specificity of the enzyme. In Table V, the values of k_2/k_3, K_s, and $k_2/k_3/k_{OH}$ are listed for Compounds I, III, IV, and V and phenyl acetate.

The values of K_s for the three uncharged compounds, I, V, and phenyl acetate, are approximately 50 times greater than those of the two positively charged compounds, III and IV, which is in conformity with the binding selectivity of acetylcholinesterase. However, the rate of the transfer step, k_2, is higher for I and V than for the charged compounds. In terms of reactivity toward hydroxide ($k_2/k_3/k_{OH}$), the transfer rate for both I and V is considerably superior, being 10 and 14 times greater than that for III and IV, respectively. Such differences in relative k_2 values have been variously attributed to preferred orientation of the acetyl group at the esteratic subsite—

Table V—Kinetic Constants for Enzymatic Hydrolysis

Substrate	k_2/k_3	K_S, M	$(k_2/k_3)/k_{OH}$, M min.
I	1	10^{-2}	2.15
III	0.54	3.5×10^{-4}	0.223
IV	0.54	1.5×10^{-4}	0.155
V	1.3	9×10^{-3}	~3.2[a]
Phenyl acetate	10[b]	10^{-2}	—

[a] Calculated from data in E. Tommila and C. N. Hinshelwood, *J. Chem. Soc.*, **1938**, 1801. [b] *Reference 7.*

permitting easier passage into the transition state—as a result of different positions of binding to the protein of the charged and uncharged "alcohol ends" of the respective molecules (9), to the competitive inhibition of substrate hydrolysis by catalytically unproductive additional modes of binding by the substrate to the enzyme (10), and to conformational accommodation of the enzyme protein upon binding with the substrate in the initial (or subsequent) Michaelis complex (11). Whether any or all of these explanations apply in this case, we cannot say. However, the results do demonstrate considerable kinetic selectivity for the *uncharged* substrate by the enzyme.

Finally, these results represent a further critical study of neutral substrates of acetylcholinesterase. Lenz and Hein (12) pointed out that phenyl acetate may be an anomaly in that it is an uncharged substrate with $k_2 > k_3$, and they suggested its reexamination for this reason. The high values of k_2 for neutral Compounds I and V provide additional examples of this phenomenon and, hence, suggest that it is a general property of the enzyme and not one uniquely limited to a single anomalous substrate.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 25, 1972, from the *Biochemistry Section, Basic Sciences Branch, Biomedical Laboratory, Edgewood Arsenal, MD 21010*

Accepted for publication June 13, 1972.

▲ To whom inquiries should be directed.