KINETICS OF ACETYLCHOLINESTERASE FOR A SERIES OF AMINES AND AMIDES

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INTRODUCTION

Acetylcholinesterase is generally considered to have an anionic group which coincides with the binding site for the quaternary ammoniom head of acetylcholine. Recently, evidence has been presented by our group supporting the contention that this binding site is uncharged. This contention has led to the study of the active site with reversible inh inhibitors which contain an uncharged t-butly group in place of the quaternary ammonium group of acetylcholine. The present work was undertaken to study the active site by determining the K_i for a series of amines and amides.

This report includes a graph section, which gives the actual rates measured and shows the lines used to determine K_m, V_m, and K_i.

MATERIALS AND METHODS

The solutions were prepared as follows:

Substrate:

Acetylcholine iodide (Aldirch) was dissolved in triply distilled water to give 2.00×10^{-3}M solutions. This was done freshly for each determination.

Inhibitor:

The inhibitors used were t-butyl amine, neo-pentyl amine, neo- hexyl amine, t-butyl acetamide, neo-pentyl acetamide, and neo- hexyl acetamide. These inhibitors were prepared i n our lab. The amines were hydrochlorides.

Enzyme:

Acetylcholinesterase from electric organ of Electrophorus electricus (Sigma) was prepared by dissolving 1000 units of dry enzyme in 2.00 ml of 0.05M phosphate buffer, pH 7.00. 1.00 ml of this solution was then diluted to 10.00 ml with the same buffer solution.

Sigma characterized the enzyme as follows: Lot 46C-0895, 1168 units/ mg protein. Taking the molecular wt. of the enzyme to be 2.3×10^{5}, the enzyme solution was 1.86×10^{-7}M.

Reaction Mixture:

These were prepared in Rainin Radiometer pH stat vessels by mixing 5.00 ml of 0.80 M NaCl (prepared in our lab from Fischer NaCl) and appropriate amounts of substrate, inhibitor, and triply distilled water to give a final volume of 20.00 ml. All solutions were delivered from burets.

Procedure

The titrator was calibrated to pH 7.00 with phosphate buffer. The reaction mixture was placed in a thermostated jacket and maintained at 25°C. A stream of CO_2 free N_2 was passed into the reaction vessel. The reaction mixture was brought to pH 7.8 with 0.100N NaOH solution (Fischer). The reaction was started by injecting 0.05 ml of the enzyme solution into the reaction mixture with a Hamilton syringe equipped with a guide and a needle long enough to deliver the enzyme under the surface of the reaction mixture. The hydrolysis rate was followed for one minute. Runs were done in triplicate or duplicate. The substrate concentration was 2, 4, 6, 8, 10 x 10^{-4}M. The final enzyme concentration was between .8 x 10^{-10}M and 1.2 x 10^{-10}M.

Calculation of Km, V_m, and K_i.

Each K_i determination is the result of a preliminary K_i determination (performed to get a rough idea of the K_i) and a more precise determination. The precise determination included at least five different substrate and inhibitor concentrations, and each run was done in duplicate.

The averaged rates from the duplicate runs were linearized on Hanes plots (S /rate vs. rate). Points that did not fit the linearization well enough to give a correlation of .99 were dropped. In most cases, only one point was dropped. Each line was drawn with at least four points. The number of points used to draw the lines, as well as the correlation value, are tabulated with the results. In the Hanes plot, K_m is taken as the intercept of the Y axis divided by the slope, and V_m is the reciprocal of the slope. An example of this plot is given in Fig. 1.

K_i is determined by plotting the Y intercepts against the inhibitor concentration, and is taken as the negative X intercept. This plot is shown in Fig. 2.

RESULTS

Tables 1 through 6 show the V_m and K_m obtained at each inhibitor concentration for the six inhibitors. These tables also show the number of different substrate concentrations (points) used to draw each line, as well as the correlation value for each line. The errors given are approximate. Table 7 gives the K_i values for each inhibitor, the number of points used and the correlation value for each line, the percent inhibition (measured at the lowest substrate concentration), and the type of inhibition.

TABLE ONE

RESULTS t-Butyl Amine

Inhibitor ($\times 10^{-3}$ M)	K_m ($\times 10^{-4}$ M)	V_m ($\times 10^{-6}$ M/s)	Correlation	Points Used (Max = 5)
0	3.5 $\pm$ .6	2.78 $\pm$ .1	.99	4
1	5.7	2.43	.99	5
2	10.2	2.56	.98	5
3	17.47	2.98	.99	3
4	17.07	2.56	.98	5
5	20.18	2.56	.96	5

Enzyme Concentration: 1.60×10^{-10} M

TABLE TWO

RESULTS neo-Pentyl Amine

Inhibitor ($\times 10^{-4}$ M)	K_m ($\times 10^{-4}$ M)	V_m ($\times 10^{-6}$ M/s)	Correlation	Points Used (Max = 6)
00.0	2.20 ± .7	1.85 ± .07	.99	4
16.0	4.29	1.72	.99	5
32.0	6.53	1.67	.99	4
48.0	7.49	1.49	.99	4
64.0	14.0	1.78	.98	4
80.0	16.6	1.72	.99	4

Enzyme Concentration: 1.06×10^{-10} M

TABLE THREE

RESULTS , Neo- Hexyl amine

Inhibitor ($\times 10^{-4}$ M)	K_m ($\times 10^{-4}$ M)	V_m ($\times 10^{-6}$ M/s)	Correlation	Points Used Max = 8
0.00	2.04 $\pm$ .20	1.59 $\pm$ .04	.99	7
2.00	2.35	1.59	.98	.98
4.00	2.37	1.56	.99	8
8.00	2.52	1.56	.99	7
16.00	4.09	1.56	.98	5

Enzyme Concentration: 1.59×10^{-10} M

tetyl

TABLE FOUR

RESULTS t-butyl Acetamine

Inhibitor ($\times 10^{-3}$ M)	K_m ($\times 10^{-4}$M)	V_m ($\times 10^{-6}$M/s)	Correlation	Points Used (Max = 5)
0.00	2.59 ± .5	1.78 ± .1	1.00	4
5.00	4.68	1.39	.99	5
10.00	4.55	1.56	.99	5
15.00	5.91	1.33	.98	4
20.00	11.64	1.69	.99	4
25.00	8.27	1.19	.98	4

Enzyme Concentration: 1.00×10^{-10}M

TABLE FIVE

RESULTS neo-Pentyl Acetamide

Inhibitor ($\times 10^{-3}$ M)	K_m ($\times 10^{-4}$ M)	V_m ($\times 10^{-6}$ M/s)	Correlation	Points Used (Max = 5)
0.00	2.69 ± .4	2.38 ± .1	.99	5
5.00	3.77	2.27	1.00	5
10.00	2.42	2.33	1.00	5
15.00	4.48	2.08	1.00	4
20.00	4.14	1.79	.99	5
25.00	4.91	1.75	.99	4

Enzyme Concentration: 1.48×10^{-10} M

TABLE SIX

Results neo-Hexyl Acetamide

Inhibitor (x 10^{-4} M)	K_m (x 10^{-4} M)	V_m (x10^{-6} M/s)	Correl.	Points Used Max = 7
0.00	2.31 ± .20	1.35 ± .05	.99	6
4.00	2.32	1.32	.99	7
8.00	3.00	1.38	.99	7
12.00	3.10	1.39	.99	6
16.00	3.15	1.33	.99	7

Enzyme Concentration: 0.84 x 10^{-10} M

Inhibitor	K_i ($x 10^{-3}$ M)	Correl.	Pts.	% Inhibition	Inhibition Type
t-Butyl Amine	0.89 ± 0.17	.99	6	80	Competitive
neo-Pentyl Amine	0.64 ± 0.26	.99	5	75	Competitive
neo-Hexyl Amine	2.16 ± 1.49	.99	4	40	Competitive
t-Butyl Acetamide	5.51 ± 1.62	.98	5	70	Competitive-Noncompetive
neo-Pentyl Acetamide	18.20 ± 2.76	.97	5	70	Competitive-Noncompetivie
neo-Hexyl Acetamide	3.78 ± 0.54	.95	4	20	Competitive

TABLE SEVEN

DISCUSSION

The K_i values reported in Table Seven are reliable for the following reasons:

1. Each determination is the result of a preliminary and and a precise determination.
2. Each run was done in duplicate, at least.
3. Each line was drawn with at least four points, in most cases 5 or more points were used.
4. All points fit a straight line with a correlation value of at least .95, in most cases .98.
5. Four of the K_i values were determined at 70% inhibition. Neo Hexyl Amine and Neo Hexyl Acetamine were determined at 40% and 20%, respectively, but the K_i value is not expected to change excessively if the inhibition is carried out to 70%.

There are two schemes for interpreting the K_i values for the amides. One scheme is to consider that the butyl and pentyl amides are equally effective inhibitors, and that they are approximately 2.5 times more efficacious inhibitors than the hexyl amide. The major difference in these three coumpounds is that the positive charge is uniformly displaced by one methyl group. If the t-butyl group binds to the trimethyl site and if an important contribution to binding comes from the electrostatic interaction between the positive charge and an anionic group somewhere on the active site, this interpretation implies that the anionic group can be placed such that it falls midway between the positive charge on the butyl and pentyl amides. The poorer binding for the hexyl amide is explained by the positive charge being too far from the anionic group to take full advantage of the electrostatic contribution t o the binding energy. See Figure 3.

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Hydrolysis by Acetylcholinesterase

APPARENT MOLAL VOLUMES AND *TRIMETHYL* AND *METHYL* SUBSITES*

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A study has been made of hydrolysis by acetylcholinesterase (EC 3.1.1.7) and by hydroxide of acetate ester $RCH_2CH_2OCOCH_3$, where, in the following compounds, R is: I, $(CH_3)_3\overset{+}{N}$—; II, $(CH_3)_3C$—; III, $(CH_3)_2\overset{+}{N}H$—; IV, $(CH_3)_2CH$—; V, $CH_3\overset{+}{N}H_2$—; VI, CH_3CH_2—; VII, $\overset{+}{N}H_3$—; VIII, CH_3—; IX, H; X, HO—; XI, CH_3O—; XII, Cl; XIII, Br; XIV, N≡C-. Acylation rate constants, k_2, are normalized for reactivity in hydrolysis by hydroxide, $k_{(OH)}$. Comparison of K_s within the pairs, Compounds III and IV, Compounds V and VI, Compounds VII and VIII, and probably Compounds I and II, and of K_s for Compounds X to XIV with Compounds V and VII indicate that positive charge makes little if any contribution to binding. Compounds I and II have similar normalized values of k_2 and bimolecular constants, $k_2(n)/K_s$. Compounds IV, VI, and VIII have greater normalized values than the charged analogues III, V, and VII. The effect of positive charge on k_{cat} and k_2 is attributed to the effect on intrinsic reactivity, $k_{(OH)}$. Positive charge is not specifically activating in acylation and is absent in the very efficient deacylation. The generally accepted "anionic" site is better considered an uncharged *Trimethyl* site, complementary to substrate $(CH_3)_3X$— groups. A linear correlation is found between log $(k_2(n)/K_s)$ for all 14 compounds and apparent molal volume, $\bar{V}^0$, of the β substituents, R. Enzymic reactivity is determined predominantly by precision of fit of the β substituent in the *Trimethyl* site and the acetyl methyl in its *Methyl* site, which limits mobility of substrate and desolvates the substrate-enzyme interface.

The major features considered to be involved in the interactions of acetylcholine, $(CH_3)_3\overset{+}{N}CH_2CH_2OCOCH_3$ (Compound I), with the enzyme which hydrolyzes it and the receptors on which it acts are the three *N*-methyl groups, the positive charge, and the ester group. The active site of acetylcholinesterase is generally characterized as comprising (1, 2) an anionic site at which the quaternary ammonium group binds and an esteratic site which is acetylated (3) at serine (4) in the course of the hydrolysis. In the receptor there would be a similar anionic site, and an esterophilic site (5).

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Initially, the positive charge appeared to be the most significant feature of Compound I, and it was proposed that the negative charge in the anionic site increases enzymic activity by attracting, binding, and orienting cationic substrates (6). Support for this was found in that protonated dimethylaminoethyl acetate, $(CH_3)_2\overset{+}{N}HCH_2CH_2OCOCH_3$, was bound better and hydrolyzed faster than both the uncharged conjugate base and the uncharged carbon analogue, isoamyl acetate $(CH_3)_2CHCH_2CH_2OCOCH_3$ (2). Similarly, change in charge of inhibitor with pH led to a change in binding (2, 6). The differences were less than 1 order of magnitude, and the origins of the effects are not unequivocal. A larger effect was seen in the 30-fold more favorable inhibition by hydroxyethyldimethylammonium ion than by the isosteric isoamyl alcohol (6), interpreted as possibly arising from interaction of positive and negative charges at a distance of 5 Å, corresponding to close contact of tetraalkylammonium and carboxylate ions (7). However, effects of change in structure and charge on binding of inhibitors may not be identical with those in reactions of substrates (8).

The *N*-methyl groups were important, as successive methylation of alkylammonium ions starting with methylamine and ethanolamine led to improved inhibition (6), and *N*-methylation of substrates starting with β-aminoethyl acetate increased both binding and reactivity (9). The methyl groups contributed more to binding than did coulombic attraction (6, 7). Increased size of alkyl groups led to increased binding of quaternary ammonium ion inhibitors (8), which may be essentially hydrophobic (10), but led to decreased binding and reactivity of substrates related to acetylcholine (8). Less than 10% of the binding of tetraalkylammonium ions appeared due to their charge, and it was suggested that there is no negative charge in the "anionic" binding site (11). It was noted (7) that the calculation did not take into account decrease in solvation by water of ammonium ions with increasing methylation (12). Further evidence as to the importance of such methyl groups was found when substitution of methyl groups, in the series from ethyl acetate to neohexyl acetate, $(CH_3)_3CCH_2CH_2OCOCH_3$ II, the uncharged carbon analogue of acetylcholine, led to progressively increasing reactivity toward acetylcholinesterase (13–15), analogous to that in the β-aminoethyl acetate series (9). A recent study (16) indicated a value of k_{cat}/K_m for II of $3.2 \times 10^3\ s^{-1}/1.4 \times 10^{-3}\ M = 2 \times 10^6\ M^{-1}\ s^{-1}$, 10^3-fold greater than that for ethyl acetate but significantly lower than that for acetylcholine. While doubt has been expressed (11), the presence of a negative charge in the "anionic site" of the enzyme is widely and firmly accepted (5, 7, 17, 18), as it is for receptors (5, 19).

Detailed descriptions of the active site of the enzyme have been offered (7, 11, 18). Hydrophobic areas and anionic sites

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TABLE I

Hydrolysis of $RCH_2CH_2OCOCH_3$ by acetylcholinesterase (pH 7.8, 25°C, 0.18 M NaCl) and by hydroxide

Substrate: Compound number	Substrate: R	Concentration	10^9 [E]	$10^{-2} \times k_{cat}$	K_m	$10^{-4} \times k_{cat}/K_m$	$10^{-2} \times k_2$	K_s	k_{OH^-}	$10^{-2} \times k_2(n)$	$10^{-4} \times k_2(n)/K_s$	R, $\bar{V}^0_{25}$
		mM	*M*	s^{-1}	*mM*	$M^{-1}\,s^{-1}$	s^{-1}	*mM*	$M^{-1}\,s^{-1}$	s^{-1}	$M^{-1}\,s^{-1}$	*ml/mol*
I	$(CH_3)_3\overset{+}{N}$—	0.036–0.24	0.22–0.40	160	0.33 ± 0.07	4800	1100	1.2[a]	2.8			62.8
II	$(CH_3)_3C$–	0.16–3.0	0.53–1.73	31 ± 3	2.6 ± 0.3	120	37	3.1	0.11	950	3100	73.6
III	$(CH_3)_2\overset{+}{N}H$—	0.25–5.0	0.14–0.24	48 ± 2	1.1 ± 0.1	440	64	1.4	2.7[b]			45.9
IV	$(CH_3)_2CH$–	0.23–3.3	1.1–2.9	12 ± 2	2.6 ± 0.7	46	13	2.8	0.11	330	1200	58.4
V	$CH_3\overset{+}{N}H_2$—	2.6–13	2.7–5.5	22 ± 1	20 ± 1	11	24	22				27.5
VI	CH_3CH_2–	0.31–15	0.7–1.9	13 ± 2	12 ± 2	11	14	13	0.11	350	270	42.4
VII	$\overset{+}{N}H_3$—	3.2–16	1.2	19 ± 2	24 ± 3	8.0	21	27				9.3
VIII	CH_3–	2.6–91	1.8–18	2.6 ± 0.3	33 ± 5	0.80	2.6	33	0.11	67	20	26.4
IX	H–	36–270	13	3.7 ± 0.4	220 ± 29	0.17	3.8	230	0.11	97	4.3	10.4
X	HO–	0.9–44	6.5–33	1.6 ± 0.3	22 ± 4	0.71	1.6	22	0.46	9.5	4.3	12.2
XI	CH_3O–	0.15–100	0.9–9.0	8.3 ± 1.2	4.7 ± 0.7	18	8.6	4.9	0.38	64	130	31.9
XII	Cl–	4.6–23	13	15 ± 0.9	32 ± 2	4.8	16	35	0.31	150	43	19.0
XIII	Br–	0.7–6.7	13	9.6 ± 0.5	9.9 ± 0.6	9.7	10	10	0.42	68	65	26.6
XIV	N≡C–	2.0–16	5.3	11 ± 1	17 ± 2	6.2	11	18	1.3	25	14	21.6

[a] Estimated value (see "Results"). [b] Determined at pH 7.8.

rate constants for the reactivity of the substrates toward the enzyme are listed as $k_2(n)/K_s$. Values of $k_{(OH)}$ are considered equal for the positively charged substrates, Compounds I, III, V, and VII (see "Materials and Methods"), and values of k_{cat}/K_m are used for them.

The value of k_{cat} for Compound I is 5 times that of Compound II; K_m is more favorable by a factor of 8. Normalization for the lower intrinsic hydrolytic reactivity of Compound II leads to a $k_2(n)$ 9.5×10^4 s^{-1} for Compound II, similar to that for Compound I, and to a high value of $k_2(n)/K_s$, 65% as great as k_{cat}/K_m for Compound I.

2-Dimethylaminoethyl acetate (Compound III) with one less methyl than Compound I shows a decrease in k_{cat} by a factor of 3.3 and a similar increase in K_m; k_2 becomes much smaller and k_3 is no longer rate-limiting; K_s = 1.4 mM, and k_{cat}/K_m is 1 order of magnitude less than for Compound I. Isopentyl acetate (Compound IV) has k_{cat} and k_2 smaller than the values of Compound II by factors of ~2.5, while K_m and K_s are essentially unchanged. While the observed reactivity is less than that of 2-dimethylaminoethyl acetate, the normalized value of k_2 is 5 times greater and the normalized reactivity ($k_2(n)/K_s$) is greater by a factor of 2.6.

2-Methylaminoethyl acetate (Compound V), with the second *N*-methyl group replaced by H, shows a further 2.4-fold decrease in k_{cat} and a large, 18-fold, increase in K_m as compared with Compound III. With removal of the last *N*-methyl group, 2-aminoethyl acetate (Compound VII) shows no further decrease in k_{cat}, a small increase in K_m, and a small decrease in reactivity as compared with Compound V.

Replacement of the second C-methyl group by H, leading to *n*-butyl acetate (Compound VI), has a small effect, leading to no change in k_{cat} and a 4-fold increase in K_m as compared with isopentyl acetate (Compound IV). Observed reactivities (k_{cat}/K_m) are now equal in the charged and uncharged pair, Compounds V and VI. Normalization leads to a $k_2(n)$ for Compound VI 15 times greater and a $k_2(n)/K_s$ 20 times greater than for the charged analogue, Compound V. *N*-Propyl acetate (Compound VIII), with the third C-methyl group replaced, shows a 5-fold decrease in k_{cat} and a 3-fold increase in K_m, as compared with Compound VI. Its observed reactivity (k_{cat}/K_m) is lower by 1 order of magnitude than that of the charged analogue, Compound VII, largely due to lower k_{cat}; normalization leads to values of $k_2(n)$ and $k_2(n)/K_s$ which are greater for uncharged Compound VIII than for Compound VII.

Replacement of the positively charged $\overset{+}{N}H_3$ in Compound VII by an uncharged hydrophilic —OH in β-hydroxyethyl acetate (Compound X) leads to a 1 order of magnitude decrease in k_{cat} and to no change in K_m and K_s. Normalization decreases the difference and values of $k_2(n)$ and $k_2(n)/K_s$ are about half those for VII. The absence of any β substituent in ethyl acetate (Compound IX) leads to k_{cat} slightly greater than that of Compound X but to a 1 order of magnitude increase in K_m. Normalization leads to $k_2(n)/K_s$ equal to that of Compound X.

Electronegative β substituents of varying hydrophobicity, CH_3O—, Cl—, Br—, —C≡N (Compounds XI, XII, XIII, and XIV respectively), lead to values of k_{cat} and k_{cat}/K_m similar to those for *n*-butyl acetate (Compound VI) and greater than those arising from the β-CH_3 and β-OH substituents in Compounds VIII and X. These substrates have higher intrinsic hydrolytic reactivity and lower normalization factors than the alkyl acetates, and their normalized reactivities lie between those of Compounds VI and VIII.

The value of K_s for acetylcholine is not readily ascertained. Equation 2 is valid if there is rapid equilibrium binding preceding acylation. While satisfactory for the other substrates, it may not be valid for Compound I because of the high value of k_2, 1.1×10^5 s^{-1}. Instead, a steady state expression may be applicable; Equation 3, which reduces to Equation 2 when k_{-1} is much greater than k_2.

$$k_{cat}/K_m = k_2 \Big/ \left(\frac{k_{-1} + k_2}{k_1}\right) \qquad (3)$$

This leads, for acetylcholine, to 2.3 mM = $K_s + (k_2/k_1)$. If association of Compound I with the enzyme is diffusion controlled, $k_1 \sim 10^9$ M^{-1} s^{-1} and K_s = 2.2 mM, similar to K_m. If the association is slower by 1 order of magnitude, $k_1 \sim 10^8$ M^{-1} s^{-1} and K_s would be 1.2 mM. This would correspond to a very efficient process, one of every two binding interactions leading to acylation. The value of k_1 must be equal to, or greater than, that of k_{cat}/K_m, $\geq 5 \times 10^7$ M^{-1} s^{-1}. A value only twice as great is not unreasonable and would lead to an estimated value of K_s of 1.2 mM, which is more favorable than that of the uncharged analogue, Compound II, by a factor of ~2.5, and similar to that of Compound III. It is of course conceivable that hydrolysis of Compound I is even more efficient; if 90% of association led to reaction, K_s = 0.23 mM.

During the analysis of these results it became desirable to calculate the apparent molal volumes, $\bar{V}^0_{25}$, of the β substitu-

external to the primary binding site were inferred from increased inhibition from longer alkyl chains in mono- and bisquaternary ammonium compounds (20–22). Binding of cationic ligands at anionic groups external to the acetylcholine binding site may cause conformational changes and affect binding and reaction at the primary site (23–26). Consideration of the action of activators and inhibitors has led to the proposal that there are three quite separate binding sites (27), but the need for this has been questioned (28). Binding of aromatic cations has been attributed to charge-transfer interaction with indole of tryptophan (29), located ~5 Å from the carboxylate of the primary anionic binding (28), and further support for hydrophobic interactions at areas outside the anionic subsite has been offered (30). It has also been proposed that interactions at the anionic subsite and hydrophobic area lead to induced-fit conformational change which is essential for the enzymic reactivity (28). The reactivity of a limited series of acetates has been interpreted quantitatively in terms of their hydrophobicity and their intrinsic hydrolytic reactivity (16).

Despite the extensive existing literature it appeared of interest to reassess the contributions to binding and reactivity of the positive charge and the methyl groups by study of substrates of structure $RCH_2CH_2OCOCH_3$, in which the β-R substituents are positively charged, uncharged electronegative, and uncharged hydrocarbon residues. The results, reported below, lead us to the view that the "anionic" subsite may not have a negative charge, and, thus, may be more specifically explored with uncharged reagents.

MATERIALS AND METHODS

Materials—Acetylcholinesterase (EC 3.1.1.7) from electric organ of *Electrophorus electricus*, was obtained from Worthington Biochemical and from Aldrich. Liquid ester substrates were obtained commercially or prepared by esterification with acetic acid and redistilled: 3,3-dimethylbutyl acetate, b.p. 150–151°C; 3-methyl-1-butyl acetate (Aldrich), b.p. 140–141°C; *n*-butyl acetate (Aldrich), b.p. 125–126°C; *n*-propyl acetate, b.p. 101°C; ethyl acetate (Fisher), b.p. 76°C; 2-chloroethyl acetate (Aldrich), b.p. 84–85°C (100 mm); 2-bromoethyl acetate, b.p. 160°C; 2-methoxyethyl acetate (Aldrich) b.p., 144–145°C; 2-hydroxyethyl acetate (Aldrich), b.p. 187–188°C; 2-cyanoethyl acetate, b.p. 43–45°C (0.1 mm). Acetylcholine iodide (Aldrich) was recrystallized from ethanol, m.p. 160–161°C.

2-Aminoethyl acetate, 2-*N*-methylaminoethyl acetate, and 2-*N*,*N*-dimethylaminoethyl acetate were prepared,[1] as their hydrochlorides, from the corresponding aminoethanols (Aldrich). Solutions of the aminoethanols in excess acetic acid were saturated with hydrogen chloride and, after 20 h, freed of excess hydrogen chloride by a stream of air, concentrated in vacuum, and diluted with acetone. 2-Aminoethyl acetate hydrochloride was crystallized from methanol/acetone, m.p. 130–131°C (literature (31), m.p. 130°C). 2-*N*-Methylaminoethyl acetate hydrochloride and 2-*N*,*N*-dimethylaminoethyl acetate hydrochloride were crystallized from acetone/hexane, m.p. 119°C (literature (32), 110°C) and 125–126°C (literature (33), 126–128°C), respectively. Satisfactory elementary analyses and nmr spectra were obtained for these compounds.

Kinetic Studies—The enzyme, 10^{-5} to 10^{-7} M, was dissolved in 0.05 M phosphate, pH 7.0, and stored at 5°C. It was assayed before each run by a Lineweaver-Burk (34) plot of hydrolysis of acetylcholine, with 1.6×10^4 s^{-1} as k_{cat} (35). Hydrolysis of substrates was studied in 0.18 M NaCl at pH 7.8, 25°C, with a Radiometer Titrator TTT2, Servograph recorder 51 and Autoburette ABU 12, delivering 0.1 N NaOH. The jacketed, thermostatted reaction vessel was flushed with argon and kept under a slow stream of carbon dioxide-free nitrogen. In a typical run 2.0 ml of 0.0024 M acetylcholine iodide, 2.0 ml of 1.8 M NaCl, and 15.9 ml of triply distilled water was stirred mechanically and brought to pH 7.8. The spontaneous hydrolysis rate was followed for 10 min, the chart speed was increased, 0.1 ml of enzyme solution was injected, and the consumption of alkali was recorded. The initial linear rates of 2 to 15% reaction were obtained in 0.5 to 5 min, depending on reactivity, in 5 to 25 runs for each substrate, and were corrected for nonenzymic hydrolysis. Slopes and intercepts of plots of reciprocal of velocity against reciprocal of concentration were obtained by least squares analysis, leading to values of K_m, V_{max} and k_{cat}. Second order rate constants for hydrolysis of substrates by alkali, $k_{(OH)}$, were obtained from rates of hydrolysis at pH 10 in the pH-stat. Alkyl acetates without polar substituent showed low reactivity, with average value of $k_{(OH)} = 0.11 \pm 0.03$ M^{-1} s^{-1}. Acetylcholine and 2-dimethylaminoethyl acetate were reactive, $k_{(OH)} = 2.8$ and 2.7 M^{-1} s^{-1}, respectively. 2-Methylaminoethyl and 2-aminoethyl acetates could not be examined readily in this way since rearrangement to the hydroxyethyl amides generated acid rapidly (36). A study in which the hydrolysis of aminoethyl acetate was separated from the rearrangement led to a value of ~3 M^{-1} s^{-1} for $k_{(OH)}$ for hydrolysis of the ammonium form (36). Rearrangement of the aminoethyl and methylaminoethyl acetates during the enzymic hydrolysis led to larger corrections for nonenzymic generation of acid, ~10%, than for the other substrates.

Apparent Molal Volumes—Values of $\bar{V}^0_{25}$ of the substrates and the β substituents were calculated from the reported values of related compounds. Values of $\bar{V}^0_{25}$ of homologous normal alcohols (37, 38), amines (39), and carboxylic acids (40, 41) lead to 16.0 ml/mol as the value for the methylene group, $\bar{V}^0_{25}$ (—CH_2—), in agreement with the original result of Traube (42). This, combined with values for homologous alcohols and α,ω-diols (37, 43) leads to values for —CH_3, H—(C), and OH. The value for 1,2-dimethoxyethane (43), 95.7 ml/mol leads to that for —OCH_3. Values for methyl bromide (44), 53.0 ml/mol, methyl chloride (44), 46.2 ml/mol, and acetonitrile (45), 48.0 ml/mol, lead to values for —Br, —Cl, and —CN. The value for ethyl acetate (43), 88.8 ml/mol, and that for H— (C) leads to that for —$CH_2CH_2OCOCH_3$, 78.4 ml/mol. Comparison of values for isobutyl and *n*-propyl alcohol (43) indicates that 16 ml/mol also applies for methylene at this extent of branching and this value is used in the calculation of $\bar{V}^0_{25}$ for $(CH_3)_2CH$—. The value for neopentyl alcohol, 86.7 ml/mol (43) indicates 15.2 ml/mol to be an appropriate increment for $(CH_3)_3C$—. The value for $-NH_3^+$ may be calculated from those for methylene, 1,4-diaminobutane (46), 93.6 ml/mol, and ΔV of protonation of ethylamine (36, 39), −5.4 ml/mol. Similar results are obtained from (i) the values of hydroxyethylammonium chloride (47), 70.9 ml/mol, Cl^- (48), 17.8 ml/mol, and hydroxyl and from (ii) methoxyethylammonium chloride (47), 91.5 ml/mol. The difference between $\bar{V}^0_{25}$ values of methyl- and dimethylammonium chlorides (38, 49, 50), 18.2 ml/mol, leads to the values for $-\overset{+}{N}H_2CH_3$. The values for dimethyl- and trimethylammonium chlorides (49, 50) lead to 18.4 ml/mol as the increment for $-\overset{+}{N}H(CH_3)$. The $\bar{V}^0_{25}$ values of methyl- and tetramethylammonium ions (48), 36.1 and 89.6 ml/mol, respectively, indicate that a final increment of 16.9 ml/mol may lead to the value for $-\overset{+}{N}(CH_3)_3$. The method is supported by the reported value for choline chloride (51), 124.9 ml/mol, which leads to the same value for acetylcholine as the calculation.

RESULTS

Results of kinetic study of hydrolysis by acetylcholinesterase of substrates $RCH_2CH_2OCOCH_3$ are summarized in Table I. The value of the deacylation rate constant, $k_3 = 1.9 \times 10^4$ s^{-1}, common to all the substrates, was calculated from Equation 1 (52):

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (1)$$

For acetylcholine the acylation rate constant k_2 is 6 times k_3 (9, 18) and $k_{cat} = 1.6 \times 10^4$ s^{-1} (35). Values of k_2 for the other substrates were calculated from their values of k_{cat} and k_3 and from Equation 1. For substrates other than acetylcholine, Equation 2 (52)

$$k_{cat}/K_m = k_2/K_s \quad (2)$$

may be applied. Values of k_2 and K_s are similar to values of k_{cat} and K_m. Values of k_2, normalized for the reactivity of the substrates toward hydroxide, $k_2(n)$, were obtained by multiplying k_2 by the ratio of the bimolecular rate constant for hydrolysis of acetylcholine by hydroxide to that for hydrolysis of the substrate by hydroxide, $k_{(OH)}$. Normalized bimolecular

[1] β-Dimethylaminoethyl acetate, Compound III, and β-methylaminoethyl acetate, Compound V, were prepared by Jerome L. Elkind, Brandeis University.

ents. The results are in the last column of Table I. The values for the substrates may be obtained by addition of 78.4 ml/mol to those for the substituents and may be used equally well in the discussion below.

DISCUSSION

In each of the four pairs, Compounds I to VIII, values of k_{cat} and k_2 are greater for the charged compound, but normalization for intrinsic reactivity raises $k_2(n)$ for the uncharged compounds to values similar to or greater than k_2 for the charged analogues. Values of K_s are also similar, differing only by a factor of ~2. Compounds XI to XIV, with uncharged electronegative β substituents, also have values of $k_2(n)$ and $k_2(n)/K_s$ equal to or higher than those of the charged analogues, V and VII. Only Compound X, with its small β-hydroxyl, has lower $k_2(n)$ and $k_2(n)/K_s$ than Compounds V and VII. Thus, neither from binding nor from normalized reactivity of substrates is evidence found for a negative charge in an "anionic" subsite which may interact with positively charged substrates.

The normalization is essentially that used in studies of chymotrypsin (53), based on formation of an acyl enzyme-serine intermediate (52), which was in turn suggested by earlier studies on acetylcholinesterase (3). If acylation is rate-limiting, as it is where values are normalized in this study, the relative reactivities of the substrates toward nucleophiles, such as hydroxide, will be reflected in the values of k_2 for their reaction with the serine hydroxyl (52). This procedure has also been extended to systems with deacylation rate-limiting: the relative reactivities to hydroxide of ethyl esters (53) and even of *p*-nitrophenyl esters of analogues of substrates (54) were considered to be reflected in the rates of hydrolysis of the chymotrypsin-serine esters. The $k_{(OH)}$ normalization with acetylcholinesterase is a most direct one, and has been applied previously (55). The related normalization based on σ^* values has been applied to carbamate (56) and phosphate (57) inhibitors, and, in a multiparameter expression, to acetate ester substrates, (Equation 4 (16, 58)).

$$\log(k_{cat}/K_m) = \log(k_2/K_s) = \rho^*\sigma^* + \phi\pi + C \qquad (4)$$

The terms σ^* and ρ^* have their usual physical-organic meaning (59), reflecting intrinsic hydrolytic reactivity, π is the hydrophobicity constant, derived from 2-octanol/water distribution (60), and ϕ is a positive factor. This expression was applied successfully to certain alkyl esters, including our Compounds II, IV, VI, and VIII ($\sigma^* \sim 0$), and to some slightly polar compounds, allyl, propargyl, ethylthiomethyl, 2-chloroethyl, and 2-nitroethyl acetates. For the latter ρ^* was ~3, indicating a high sensitivity to polar substituents, possibly due to low dielectric constant of the active site. Positively charged Compounds I, III, V, and VII are hydrophilic and Equation 4 would predict low reactivity. Their reactivity was not correlated with the others, but separately, against π, with π for acetylcholine set arbitrarily at 0 and with $\rho^*\sigma^*$ constant (16). Subsequently an additional term θ was added to Equation 4 to compensate for the unobserved decreased reactivity attributed to hydrophilicity (61). This increase, θ, was taken as a measure of the effect of the negative charge of the anionic site, attracting the positive charge and extracting these hydrophilic substrates from water into the enzyme (58).

Values of σ^* were determined originally from effects of substituents in the acyl portion of esters (59) and a similar treatment was applied to substituents in the alkyl parts (62). The values are derived from effects of substituents on acid- and base-catalyzed hydrolyses, and, with steric effects being negligible in β-substituted ethyl acetates (16), the acid-catalyzed rates become irrelevant, and normalization by $k_{(OH)}$ is equivalent to the σ^* treatment and more direct. For uncharged substrates Equation 5

$$\log(k_2(n)/K_s) = C + \phi\pi \qquad (5)$$

becomes equivalent to Equation 4. However, a plot of log $(k_2(n)/K_s)$ against π, Fig. 1, for all the substrates in Table I except the positively charged ones, shows that correlation with π works partially but fails in an instructive way. Values of log $(k_2(n)/K_s)$ for the seven β-H, β-alkyl, and β-halo substrates are linear with π (Fig. 1, *Line A*) (slope, 1.78 ± 0.11; correlation, 0.97), supporting the normalization procedure. However, Substrates X, XI, and XIV, with hydrophilic β substituents HO—, CH_3O—, and —CN, are much too reactive for correlation with hydrophobicity and appear to be on a separate line (Fig. 1, *Line B*). This is reminiscent of the high reactivity of the hydrophilic positively charged substrates (58), but compensation by a coulombic effect (θ) cannot reasonably be applied for these compounds. Further, examination of Line A (Fig. 1) indicates that the π values of the substituents parallel their volumes, consistent with the additivity of component π values (60). Similarly, in Line B (Fig. 1), values for log $(k_2(n)/K_s)$, rising with decreasing hydrophilicity, are also rising with increasing volume. Further, the log $(k_2(n)/K_s)$ values of substituents in Line B (Fig. 1) approximate the values for the β substituents of apparently similar volume in Line A (Fig. 1), CH_3O— intermediate between CH_3— and CH_3CH_2—, —CN little less than that of —CH_3, and HO— similar to H—.

This prompted a quantitative assessment of the relation of normalized reactivity to substrate volume or to volume of the β substituent since the remaining part is the same in all. Values of log $(k_2(n)/K_s)$ for all the substrates (Table I) are plotted against the apparent molal volumes, $\bar{V}^0_{25}$, of the β substituents (Fig. 2). A progressive increase in reactivity is seen from lowest, similar reactivity corresponding to the smallest similar volumes of β-H, β-OH, β-$\overset{+}{N}H_3$, through intermediate reactivities corresponding to intermediate volumes, to the highest, nearly equivalent reactivities caused by the largest substituents of similar volume, $(CH_3)_3\overset{+}{N}$— and $(CH_3)_3C$—. Least squares analysis leads to intercept 4.26 ± 0.15, the log of normalized reactivity that may be ascribed to the —$CH_2CH_2OCOCH_3$ group (slope, 0.049 ± 0.004 ml/mol; correlation, 0.92). Higher reactivity for Compound V, which would correlate it better with the least squares line, has been reported (9), but we did not observe it. The correlation of reactivity with volume is a good one. While hydrophobicity may contribute to reactivity, the reported correlation (16) largely reflects the relation of this property to the more general one, volume.

Apparent molal volumes, $\bar{V}^0_{25}$, are values at infinite dilution in water and reflect the varying interactions of compounds with water. Reordering of water around organic solutes usually

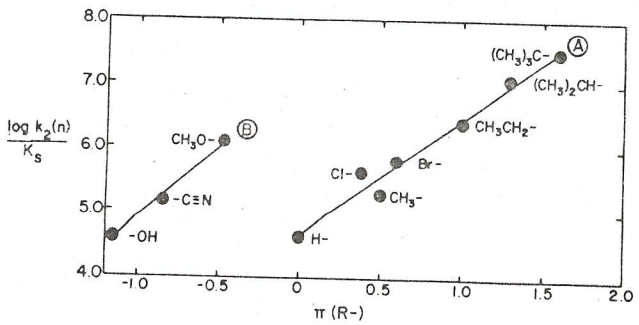


FIG. 1. **Normalized reactivity of $RCH_2CH_2OCOCH_3$ and hydrophobicity, log $(k_2(n)/K_s$ *versus* π (R−).**

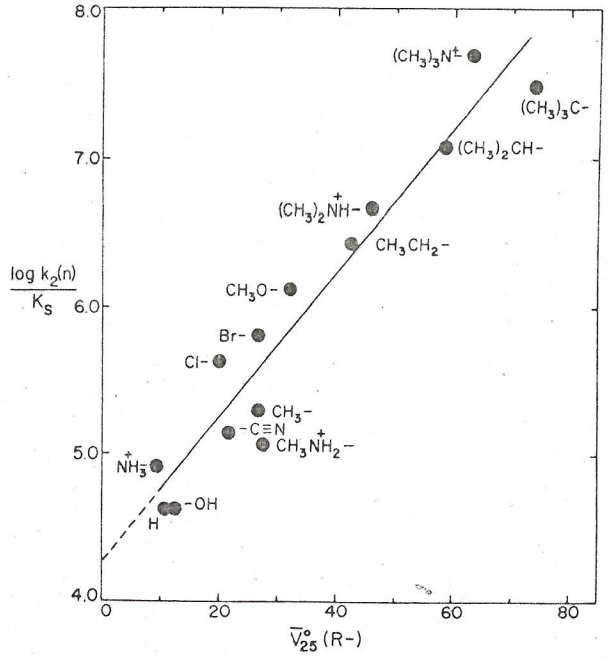


FIG. 2. **Normalized reactivity of $RCH_2CH_2OCOCH_3$ and apparent molal volume, log $(k_2(n)/K_s$ *versus* $\bar{V}^0_{25}$ (R−).**

appears as shrinkage, leading to $\bar{V}^0$ values less than those obtained simply from molecular weight and density. However, $\bar{V}^0$ values are determined under the same conditions for all compounds, the reordering effect is fairly constant for nonionic solutes, group volumes are additive, and consistent sets of values of $\bar{V}^0$ are obtained. The correction, $-\Delta V$, for positive charge, which appears in the $\bar{V}^0$ values of Compounds I, III, V, and VII may arise from electrostriction of water, but the standard calculation assigns it to the solute. Electron and x-ray diffraction studies of dimethyl- and trimethylamine (63) and acetylcholine chloride (64) indicate that the C—N bond length is not shortened by the positive charge. Compound I may have a somewhat greater volume than indicated by $\bar{V}^0$, which would fit it better to the least squares line in Fig. 2. However, refractivities, which yield values which are related to polarizability and volumes enclosed by the valence and nonbonding electrons (65) and may be related to volumes defined by the dispersion forces involved in interactions in the binding site, also show $-\Delta V$ due to positive charge, apparently as it affects polarizability. We have calculated the refraction volumes and find their correlation with log $(k_2(n)/K_s)$ to be similar to that of $\bar{V}^0_{25}$ values. It may be noted, too, that $\bar{V}^0_{25}$ values of quaternary ammonium salts have been correlated with their activity and affinity at acetylcholine receptors (66).

The correlation of normalized reactivity with volume (Fig. 2) is restricted to chain and branching related to choline, but it includes, on a single scale, the positively charged groups of varying hydrophilicity, nonpolar alkyl groups, and uncharged electronegative hydrophobic and hydrophilic groups. This observed correlation with as unspecific a property as volume indicates that two qualities of the alkoxyl group predominantly determine the reactivity of acetate substrates toward acetylcholinesterase: (i) the effect on the intrinsic hydrolytic reactivity as indicated by $k_{(OH)}$, and (ii) the shape and volume, within the above restriction, as they affect the capacity to fill the subsite at which the β substituent binds.

Positive charge and electronegativity of substituent increase intrinsic and enzymic reactivity as they facilitate attack by hydroxide and by the serine hydroxyl on the acyl group and make the alkoxyl a better leaving group, in reactions of acetates, and also of carbamates (56) and phosphates (57). This, rather than interaction with an anionic charge, suffices to account for the effects of k_2, and there is also no substantial effect of charge on K_s. There is no cogent evidence for a negative charge in the subsite which binds the alkoxyl moiety of substrates, and it is better considered an uncharged, nonpolar *Trimethyl* site than an "anionic" site. The dependence of reactivity on volume rather than hydrophobicity makes unnecessary the introduction of an antihydrophilicity parameter, θ. All this is not to deny the presence, at pH 7 to 8, in an enzyme of isoelectric point ~5 (67), of peripheral anionic charges, at which positive groups may associate and, thereby, affect the reactivity of the enzyme.

There is another line of reasoning indicating that the positive charge of Compound I is not a specificity endowing feature. High reactivity of a serine hydrolase requires that both acylation and deacylation be rapid. In substrates for chymotrypsin and trypsin, major specificity lies in the aromatic and positively charged acyl groups respectively, which are present to enhance reactivity in both steps. It need not be belabored that the acetyl groups serves this purpose in substrates of an enzyme named acetylcholinesterase. The acetyl group is a major contributor to specificity in acylation: butyryl choline shows low reactivity (13). Then alone, with no cationic group present, it leads to very rapid deacetylation, $k_3 = 2 \times 10^4$ s^{-1} (18), which is less rapid than acylation by a small factor, for only a very few most reactive substrates. This high reactivity is characteristic of this enzyme. The acetylserine itself in an enzyme is not intrinsically reactive: acetylchymotrypsin is hydrolyzed nearly 7 orders of magnitude less rapidly, $k_3 = 6.8 \times 10^{-3}$ s^{-1} (53). In acetylcholinesterase there appears to be a site of restricted volume, the *Methyl* site, in which the methyl of the acetyl group fits. This, combined with fit of a proper alkoxyl moiety in the *Trimethyl* site, restricts substrate mobility and enhances attack by the imidazole-coordinated (68) serine-hydroxyl in acylation, and, combined with the covalent linkage to serine, restricts mobility for attack by imidazole-coordinated water in deacylation. The alkoxyl moiety also contributes specificity in acylation, as shown by the range of reactivities in Table I, but excessive importance and function have been attributed to the positive charge, leading to misleading focus on a purported anionic site. Schematic representations of the active site are given in Figs. 3 and 4.

The binding energies are all rather low, indicating that part may be used in desolving the reacting groups. Esters (and amides) have hybrid structures, which may be stabilized by

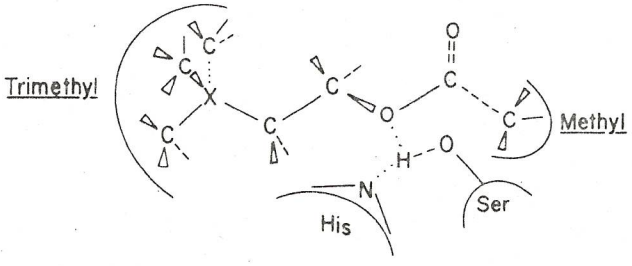


FIG. 3. **Substrate and the *Trimethyl* and *Methyl* subsites.** *X* is N^+, acetylcholine I; *X* is C, neohexyl acetate II.

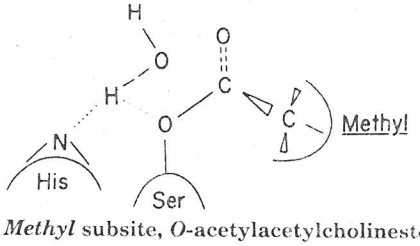
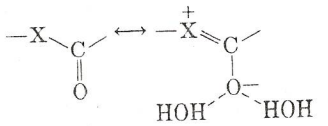


FIG. 4. ***Methyl* subsite, *O*-acetylacetylcholinesterase.**

water against nucleophilic attack at the carbonyl, in solution, Formula XV.



Removal of the ester from water, desolvation of the enzyme substrate interface, and hydrogen bonding at the ether/oxygen (Fig. 3) may raise the reactivity toward that of a true carbonyl and facilitate both attack of the serine/oxygen and ejection of the alcohol (69). Restricted mobility of substrate, precise fit, and proper juxtaposition of reacting groups may suffice for rapid hydrolysis by this enzyme. This is indicated by its very favorable entropy of activation, ~ +16 cal/degree (9) as compared with −30 cal/degree for ester hydrolysis by aqueous alkali (70). Both processes have low similar activation energies, and the aqueous reaction may be slow because of the low concentration in solution of the higher energy more reactive species, hydrogen-bonded at the ether/oxygen, as in the active site.

All the β substituents improve binding as compared with ethyl acetate (Table I), indicating that they enter the *Trimethyl* site. The smaller substituents may not fully occupy and desolvate the site, and weaker binding, less restriction of motion, and lower reactivity ensue, as compared with Compounds I to IV. The values of K_s due to the smaller substituents are similar despite great differences in polarity, charge, and hydration. This may indicate that their environment is relatively unchanged on binding and that their first neighbors are water, in the site as in solution. Similarly, the large difference in free energies of hydration, 12 kcal/mol, attendant on replacement of three hydrogens by three methyls in ammonium ions (12) is not reflected in the difference in binding of Compounds I and VII or in the K_I values of $(CH_3)NH_3^+$ and $(CH_3)_4\overset{+}{N}$ ions (6). The tetraalkylammonium groups are not strongly solvated in solution, and the smaller groups need not be desolvated in the binding site. The trimethyl substituted Compounds I and II may fully occupy the *Trimethyl* site and lead to high reactivity. However, the electronic character, despite apparently proper size and shape may also affect binding at this site. The reported failure of the *N*-oxide of Compound III to be hydrolyzed by acetylcholinesterase (71), despite the successful hydrolysis of β-nitroethyl acetate, in which all three methyls of Compound I are replaced by oxygen, indicates the need for further study of the *Trimethyl* site.

Binding of the $(CH_3)_3\overset{+}{N}$— group of acetylcholine (Compound I) to an uncharged *Trimethyl* site, in contrast to an anionic site, increases the positive charge on the enzyme surface. This introduces possibilities for effecting conformational changes, as by attracting anionic or repelling cationic peptide side chains. While it is generally considered that the sites on the enzyme and receptor at which the $(CH_3)_3\overset{+}{N}$— group binds are similar, our conclusion that the site on the enzyme is uncharged raises the question of, but is not informative about, charge in the binding site of receptor. The uncharged Compound II leads to total response like that of Compound I on the muscarinic receptor, but is less efficient, with equimolar potency ratio ~ 3000 (19). Binding of Compound I to an uncharged site on the receptor could, as indicated above, have a quite different effect from binding of Compound II.

Our analysis is based on the premise that the charged and uncharged substrates, particularly Compounds I and II, bind at the same site. This indicates that the site may be explored more specifically by reagents containing the tertiary butyl group. Such compounds, when not charged, will not interact at peripheral anionic sites, and probably also not at external hydrophobic areas, at which less branched aliphatic and planar aromatic groups may associate. These views are supported by studies, to be reported later, of such compounds, which act on acetylcholinesterase as effective inhibitors, both reversible competitive and irreversible, and do so with the same effectiveness with respect to the hydrolysis of Compounds I and II.

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Cationic and Uncharged Substrates and Reversible Inhibitors in Hydrolysis by Acetylcholinesterase (EC 3.1.1.7)

THE TRIMETHYL SUBSITE*

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Structurally related cationic and uncharged compounds have been studied as inhibitors of hydrolysis by acetylcholinesterase of acetylcholine and its uncharged carbon analog, 3,3-dimethylbutyl acetate. Similar effects of the inhibitors on hydrolysis of the two substrates indicate that the quaternary ammonium group of acetylcholine and the neopentyl group of 3,3-dimethylbutyl acetate bind at the same subsite. Comparison of $(CH_3)_3\overset{+}{N}CH_2CH_2CH_2COCH_3$ (Compound I), K_i = 0.02 mM, and its tertiary homologue, $(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2COCH_3$ (Compound V), K_i = 0.75 mM, with a secondary isomer of Compound I, 3-oxo-(*N-tert*-butyl)-butanaminium, $(CH_3)_3C\overset{+}{N}H_2CH_2CH_2COCH_3$ (Compound II), K_i = 0.15 mM, and its lower homologue, $(CH_3)_2CH\overset{+}{N}H_2CH_2CH_2COCH_3$ (Compound IX), K_i = 2 mM, attests to the importance of the branched trimethyl structure and the smaller effect of hydrophobicity of the quaternary ammonium structure. This is supported by competitive inhibition by *tert*-butyl ammonium, $(CH_3)_3C\overset{+}{N}H_3$ (Compound IV), K_i = 0.45 mM, compared with mixed inhibition by its quaternary isomer, $(CH_3)_4\overset{+}{N}$ (Compound VII), K_i = 1.5 mM, and choline (Compound VI), K_i = 1.0 mM. Uncharged analogues of Compound II, 4-*tert*-butylthio-2-butanone, $(CH_3)_3CSCH_2CH_2COCH_3$ (Compound III), K_i = 0.4 mM, and 4-*tert*-butoxy-2-butanone, $(CH_3)_3COCH_2CH_2COCH_3$ (Compound VIII), K_i = 1.6 mM, and of Compound VI, 3,3-dimethylbutanol (Compound XI), K_i = 7.5 mM, indicate that positive charge contributes factors of 3 to 10 to binding. This may be attributed to peripheral negative charges, present at pH 7–8 in the enzyme of isoelectric point ~5, indicating that the binding subsite may be explored more specifically by *tert*-butyl than by charged reagents.

The active site of acetylcholinesterase is generally described as comprising (1–4) (*i*) a serine-containing esteratic site, and (*ii*) an anionic site at which positively charged groups related to the quaternary ammonium group of acetylcholine associate, thereby increasing the binding and reactivity of cationic substrates and the binding of cationic inhibitors (5). However, doubt has been expressed about the importance and even the presence of a negative charge in the "anionic" subsite (6a, 6b), and it has been proposed that it be considered a trimethyl subsite, complementary to the tertiary-butyl structure (7). This proposal was based on study of hydrolysis by acetylcholinesterase of 14 acetate esters, $RCH_2CH_2OCOCH_3$, in which the β-substituents, R, included: the four charged ammonium groups containing zero to three *N*-methyl groups; the four isosteric uncharged alkyl groups, methyl, ethyl, isopropyl and tertiary-butyl; three hydrophilic electronegative groups, HO—, CH_3O—, and N≡C—; two hydrophobic electronegative groups, Cl and Br; and the unsubstituted parent compound, ethyl acetate. The substituents affect the intrinsic reactivity of the esters, as indicated by the rate constants of their hydrolysis by alkali, $k_{(OH)}$. When acylation rate constants, k_2, for the uncharged substrates were normalized for intrinsic reactivities, a linear relationship was found between the log of normalized enzymic reactivity, $k_2(n)/K_s$, and apparent molal volume, $\bar{V}^0_{25}$. Effects of positive charge on K_s values for the four isosteric pairs were small.

Attempted correlations of reactivity with hydrophobicity had first used a separate arbitrary scale (8), and later, an antihydrophilicity correction parameter (9) for cationic substrates. Furthermore, the uncharged substrates were limited in range of hydrophobicity (8). The reactivities of the somewhat more hydrophilic β-HO, β-CH_3O, and β-CN substrates were found not to correlate with hydrophobicity (7). The proportionality of normalized reactivity to volume applied to all 14 substrates, with their β-substituents of varied charge and hydrophobicity. It was proposed that the limited apparent relationship to hydrophobicity largely reflected a fundamental dependence upon molal volume (7). Thus, reactivity of acetate esters toward acetylcholinesterase appeared to be largely determined by (*i*) their intrinsic reactivity and (*ii*) by the capacity of the β-substituent to fill the trimethyl subsite. Positive charge would increase intrinsic reactivity of substrates, and its binding at an uncharged trimethyl site might more effectively cause conformational change.

It is implicit in this that the cationic trimethyl ammonium group of acetylcholine and the *tert*-butyl group of the isosteric uncharged carbon analog, 3,3-dimethylbutyl acetate, bind at the one trimethyl subsite (7). It had been proposed previously that uncharged and cationic substrates bind at the same site (10), but the contrary has been inferred from the differing

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characteristics of the irreversible inhibition by aziridinium compounds of the hydrolysis of cationic and neutral substrates (11, 12). Also, it has been suggested that choline and 3,3-dimethylbutyl alcohol, isosteric cationic and neutral inhibitors, bind at different regions (13). It may be noted that the enzyme, with isoelectric point ~5 (14, 15), may well have free carboxylate (and hydrophobic) areas near the active site, and association of cationic (and hydrophobic) groups at peripheral sites may affect conformation and reactivity of the enzyme (16–20). We now report a study of new neutral and cationic reversible inhibitors, addressing the question as to whether uncharged and cationic substrates and related uncharged and cationic inhibitors bind at the same active site in acetylcholinesterase.

MATERIALS AND METHODS AND RESULTS[1, 2]

DISCUSSION

The Active Site—The effects of the competitive inhibitors on hydrolysis of the two substrates (Table I) demonstrate that the positively charged and uncharged substrates, acetylcholine and DMBAc, are hydrolyzed at the same active site and that the related positive and uncharged competitive inhibitors also act by binding at this site. A competitive inhibitor interferes with the binding of a substrate at the active site, and, in principle, it may do so by binding at the active site or at another position. However, when the competitive inhibitor is very closely related in structure to the substrate, it may be concluded that it does act by binding at the same site. Thus the quaternary ammonium compound, Compound I, the most effective inhibitor in this series, which differs from acetylcholine only in substitution of CH_2 for the alkoxy oxygen, and has been described as a possible transition-state analogue (21), may be assumed to occupy the same site as acetylcholine, and do so in the same way. That it has the same efficiency in retarding the hydrolysis of acetylcholine and DMBAc strongly indicates that the latter uncharged compound is also hydrolyzed at this site. This supports the conclusion, reached earlier from consideration of substrate reactivities, which is basic to our proposals about the active site and hydrolysis by this enzyme (7). The similar effects on the hydrolysis of the two substrates by each of the competitive inhibitors (Table I), charged and uncharged, of the same chain length as acetylcholine and containing terminal acetyl and branched groups, Compounds II, III, V, VIII, IX, and X, indicate that they also bind at the one site, in the same fashion. If the uncharged substrate, DMBAc, were hydrolyzed at a different site from acetylcholine, then the uncharged isosteric inhibitor, Compound VIII, would presumably bind at that site and have little effect on hydrolysis of acetylcholine. But this is not observed. It may be noted that inhibitor, Compound VIII, binds somewhat more effectively than its isomer, the substrate DMBAc (K_m = 3.1 mM (7)). This may indicate that a ketone interacts more favorably than an ester at the esteratic subsite.

Substitution on N—Comparison of the potency of isomers ranging from primary to quaternary ammonium compounds indicates that the degree of substitution on nitrogen has relatively small effect on binding. Compound II, a secondary ammonium isomer of quaternary Compound I, is an effective inhibitor, with only ~1.1 kcal/mol less binding energy. This appears to be less by an order of magnitude than the difference in hydration energy caused removal of two alkyl groups in ammonium ions (22), and supports the proposal, based on hydrolysis of substrates, that an alkyl-ammonium group need not be fully desolvated as it fits into the active site (7). The small difference, 0.5 kcal/mol, in binding of the isomeric tertiary and secondary aminoketones, Compounds V and IX, leads to the same conclusion. The slightly better binding of *tert*-butylammonium ion, Compound IV, with a hydrated primary $-NH_3^+$ group, compared with that of its essentially hydrophobic quaternary isomer, tetramethylammonium ion, Compound VII, and that of choline, Compound VI, is also noteworthy in this connection. Compound IV has normal basicity, pK_a = 10.8 (23), indicating that the *tert*-butyl group does not decrease solvation of the cation. It inhibits substantially more effectively than *n*-alkyl primary amines of varying chain length (24), supporting the importance of the fit and interactions of the branched *tert*-butyl or *neo*-structure, with the trimethyl site.

It may also be noted that Compound IV appears to inhibit competitively. The other compounds which contain a related branched structure and lack the terminal acetyl group, Compounds VI, VII, and XI, in addition to binding competitively at the trimethyl site of the free enzyme, also bind at this site of the acyl-enzyme (24–28b). A noncompetitive component to the inhibition may arise as the inhibitor may then block access of water and prevent hydrolysis of the acetyl-serine (26). Choline, Compound VI, and DMBAl, Compound XI, may, in addition, be well situated to react with the acetyl-serine and regenerate acetylcholine and DMBAc, respectively, preventing normal hydrolysis to acetic acid (13, 14, 25). The favorable binding of Compound IV to the free enzyme and its size, relative to the binding and size of Compounds VI, VII, and XI, indicate that it too should bind to the trimethyl site of the acetyl-enzyme. However, it may carry water, hydrogen-bonded to the $-\overset{+}{N}H_3$ group, into the site, in position to hydrolyze the acetyl-enzyme, and in this way show little or no noncompetitive component.

TABLE I

Reversible inhibition of hydrolysis by acetylcholinesterase of acetylcholine and 3,3-dimethylbutyl acetate, in 0.18 M NaCl, pH 7.8, at 25 °C

Inhibitor			K_i	
No.	Compound	Concentration	Acetylcholine	DMBAc
		mM	mM[a]	
I[b]	$(CH_3)_3{}^+NCH_2CH_2CH_2COCH_3$ I^-	0.1–0.3	0.022	0.024
II[b]	$(CH_3)_3C^+NH_2CH_2CH_2COCH_3$ Cl^-	0.1–1	0.16	0.14
III[b]	$(CH_3)_3CSCH_2CH_2COCH_3$	1–3	0.40	0.33
IV[b]	$(CH_3)_3C^+NH_3$ Cl^-	0.1–2	0.43	0.44
V[b]	$(CH_3)_2{}^+NHCH_2CH_2CH_2COCH_3$ Cl^-	1–5	0.77	0.71
VI[c]	$(CH_3)_3{}^+NCH_2CH_2OH$ Cl^-	0.1–2	1.0	0.63
VII[c]	$(CH_3)_3{}^+NCH_3$ Cl^-	0.4–2	1.5	1.0
VIII[b]	$(CH_3)_3^{\ddagger}COCH_2CH_2COCH_3$	0.4–3	1.6	1.0
IX[b]	$(CH_3)_2CH^+NH_2CH_2CH_2COCH_3$ Cl^-	1–5	2.0	1.5
X[b]	$(CH_3)_2CHOCH_2CH_2COCH_3$	2–9	4.8	3.1
XI[c]	$(CH_3)_3CCH_2CH_2OH$	2–16	7.5	5.7

[a] ±25%.
[b] Showed competitive inhibition.
[c] Mixed or noncompetitive inhibition.

[1] Portions of this paper (including "Materials and Methods," "Results," and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-2353, cite author(s), and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: AcChE, acetylcholinesterase, AcCh, acetylcholine, DMBAc, 3,3-dimethylbutyl acetate, DMBAl, 3,3-dimethylbutyl alcohol.

Degree of Branching—The effects of a fourth, methyl substituent in pairs of homologues appear to depend upon the efficiency of binding. The most efficient inhibitor, Compound I, shows the greatest effect, being bound 30 times more strongly than its lower homologue, Compound V, corresponding to a difference in binding energy of 2 kcal/mol. In a pair of intermediate effectiveness, the tertiary-butyl aminoketone, Compound II, was bound 12 times more strongly than the isopropylaminoketone, Compound IX; and the least strongly bound pair, the tertiary-butoxy- and isopropoxyketones, Compounds VIII and X, differed by a factor of only ~3. This latter small factor of 3 has also been observed in positively charged pairs of similar, relatively low, binding efficiencies, tetramethylammonium ion, and trimethylammonium ion, and choline and hydroxyethyldimethylammonium ion (27). The effect of the fourth substituent is not merely statistical (29), since the common change from tetra-substituted Compounds, I, II, VI, VII, and VIII to their tri-substituted lower homologues leads to widely different decreases in binding. If the final methyl group can lead to precise fit and immobilization, it has a large effect, as in Compound I, but if the compounds bind less well, the final methyl group has a smaller or marginal effect, in both cationic and uncharged pairs.

Positive Charge—This also has varied effects, but, in contrast to the fourth methyl, effects of charge are greater the less the compounds interact with the entire active site. Thus, the $-\overset{+}{N}H_3$ group is essential for the efficient inhibition by *tert*-butyl amine, Compound IV; we find inhibition by the corresponding neutral compound, *tert*-butyl alcohol, to be only 1/200 as effective, $K_i \sim 130$ mM, a value consistent with its reported potency (28a). However, the positive charge leads to only about an order of magnitude better binding in choline, Compound VI, than in DMBAl, Compound XI, in Compound I than in III, and in II than in VIII. In Compounds II and III, which may be most nearly isosteric and interact with the entire active site, the positive charge improves the K_i by less than a factor of 3.

Cationic inhibitors might bind more strongly than their uncharged analogues, (*i*) because of a specific anionic binding site, or (*ii*) because of excess negative charges at pH 7–8 on the enzyme (7) which has an isoelectric point of ~pH 5 (14, 15). A specific anionic site may lead to large effects, depending upon the distance of approach of the inhibitor. The distance between the center of charge of a $(CH_3)_3\overset{+}{N}$ group and a "contact" anionic charge, O^-, may be 3.25 Å, and at this distance, the interaction would contribute $\Delta F \sim -5$ kcal/mol, and a 3000-fold increase in binding (30). An observed 17-fold effect of charge in binding of inhibitors indicated a distance of approach of ~6 Å (5). This large apparent distance and the smaller effect, a factor of 3 to 10 which we observe for compounds which fit most specifically into the trimethyl and acetyl subsites, offer little evidence for a specific anionic site. The effect may result from the net negative charge of the enzyme (7). Consistent with this, the increase in binding of *N*-methylacridinium ion to acetylcholinesterase, and of hydrolysis of acetylthiocholine with decreasing ionic strength, has recently been interpreted in terms of Debye-Hückel theory to indicate the existence of 6–9 negative charges dispersed in the region of the active site (31).

The view that the enzyme is anionic while the β-substituent-binding site is uncharged and hydrophobic raises a question about reagents that may react with and label that subsite. Aziridinium compounds, such as *N,N*-dimethyl-2-phenylaziridinium (11, 12, 32, 33), have been used to alkylate the anionic site. The modified enzyme showed virtually no reactivity toward cationic substrates and very low sensitivity to cationic inhibitors. However, reactivity toward DMBAc and other neutral esters was much less affected, and this reactivity was destroyed by phosphorylating agents (11, 33). It was suggested that the aziridinium reagents alkylate and block the anionic site, and that uncharged substrates bind at a different "hydrophobic" site, but use the same serine-hydroxyl (11, 12, 32, 33). However, the evidence presented here and in earlier studies is persuasive that cationic and uncharged substrates related to acetylcholine and DMBAc use the same uncharged trimethyl site in the native enzyme (7, 10). The aziridinium compound may alkylate a nearby anionic or uncharged nucleophile and introduce one or two positive charges near the active site. This may prevent binding of cationic substrates and inhibitors and lead to some decrease in reactivity toward neutral substrates without affecting their binding. Our results lead, then, to the view that labeling reagents specific for the active site may be found among compounds containing the *tert*-butyl group. Experiments in progress indicate that such a compound, α-bromopinacolone, $(CH_3)_3CCOCH_2Br$, is an effective irreversible inhibitor for acetylcholinesterase and has the same effect on hydrolysis of acetylcholine and DMBAc.

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SUPPLEMENTAL MATERIAL TO

Cationic and Uncharged Substrates and Reversible Inhibitors in Hydrolysis by Acetylcholinesterase (E.C.3.1.1.7). The Trimethyl Subsite

Fariza B. Hasan, Jerome L. Elkind, Saul G. Cohen and Jonathan B. Cohen

This section contains Materials and Methods, Results and Figures.

MATERIALS AND METHODS

Materials. Acetylcholinesterase (E.C.3.1.1.7) from electric eel, was obtained from Worthington Biochemical and from Aldrich. Acetylcholine iodide, choline chloride, VI, tetramethylammonium chloride, VII (Aldrich), were crystallized from absolute ethanol, mp 160-1°, 303-306° dec., and 310-318° dec., respectively. 3,3-Dimethyl-1-butanol, XI, (Aldrich) was distilled, bp 142-3°. 3,3-Dimethyl-1-butyl acetate was available from previous work [7]. tert-Butylamine (Eastman) was distilled under argon, bp 44-46°, dissolved in dry acetone and treated with dry hydrogen chloride, leading to the amine hydrochloride, IV, mp 278-280°, from absolute ethanol.

5-Dimethylamino-2-pentanone (Sapon Laboratories) treated as above, led to the amine hydrochloride, V, mp 99-100°, after treatment with Norit and crystallization from absolute ethanol.

Anal. Calcd for $C_7H_{16}NOCl$: C, 50.76; H, 9.74; N, 8.46
Found: C, 50.21; H, 10.07; N, 8.35.

4-Oxo-N,N,N-trimethylpentanaminium iodide, I, was prepared by treatment of 0.8 g of 5-dimethylamino-2-pentanone with 2 g of methyl iodide in 5 ml of dry acetone at 5° overnight, yield 0.7 g, mp 110-111°, after treatment with Norit and crystallization from absolute ethanol-ether.

Anal. Calcd for $C_8H_{18}NOI$: C, 35.41; H, 6.69; N, 5.17
Found: C, 36.07; H, 6.72; N, 5.01.

3-Oxo-(N-tert-butyl)-butanaminium chloride, II, was prepared by addition of 14 g of methyl vinyl ketone (Aldrich) in 50 ml of anhydrous ether to 29 g of tert-butylamine in 100 ml of ether over a period of 40 minutes at 0-5°, followed by treatment with anhydrous hydrogen chloride, yield 25 g, mp 120-122°, after crystallization from tetrahydrofuran-ether, lit. mp 124-125° [34].

3-Oxo-(N-isopropyl)-butanaminium chloride, IX, was prepared in the same way, from 24 g of isopropyl amine and 14 g of methyl vinyl ketone, yield 24 g, mp 85-90°, from ethanol-ether.

Anal. Found for $C_7H_{16}NOCl$: C, 50.42; H, 9.44.

4-tert-Butoxy-2-butanone. A solution of 22 ml of methyl vinyl ketone in 40 ml of tert-butyl alcohol was added with stirring to a warm mixture of 0.6 ml of tert-butyl alcohol, 0.6 g of mercuric oxide and 0.3 ml of boron trifluoride etherate, [35] boiled for one hour, stirred overnight, neutralized with anhydrous potassium carbonate, concentrated and distilled leading to the product, bp 80° (30 mm) lit. [36] bp 54-57° (15 mm).

4-Isopropoxy-2-butanone was prepared similarly by treatment of 22 ml of methyl vinyl ketone in 41 ml of 2-propanol with 0.75 g of mercuric oxide, 0.5 ml of boron trifluoride etherate and 1 ml of 2-propanol, bp 65°, (25 mm), lit. [36] 72-75° (37 mm).

4-tert-Butylthio-2-butanone. A solution of 23 ml of tert-butyl thiol, 17 ml of methyl vinyl ketone and four drops of pyridine was boiled for one hour and allowed to stand overnight. It was washed with two 20 ml portions of 5% sulfuric acid, distilled at reduced pressure, dissolved in ether, washed with 5 portions of 5% sodium hydroxide, dried and distilled, leading to the product, bp 130° (30 mm), lit. [37] 112° (18 mm), molecular weight 160 by mass spectrometry.

Kinetic studies. The enzyme was dissolved in 0.05 M phosphate buffer, pH 7.0 and stored at 5°. It was assayed before each run by hydrolysis of acetylcholine, [7] with 1.6×10^4 s^{-1} as k_{cat} [38]. Hydrolysis of substrates in the absence and in the presence of inhibitors was studied in 0.18 M NaCl at pH 7.8, with a Radiometer Titrator TTT2, Servograph Recorder 51 and Autoburette ABU 12, delivering 0.1 N NaOH. The jacketed, thermostated reaction vessel was flushed with argon and kept under a slow stream of carbon dioxide free nitrogen. In a typical run 2.0 ml of 0.0020 M acetylcholine iodide, 2.0 ml of 1.8 M NaCl, 2.0 ml of 0.0086 M tetramethylammonium chloride and 13.9 ml of triply distilled water was stirred mechanically and brought to pH 7.8. The spontaneous hydrolysis rate was followed for 10 minutes, 0.1 ml of enzyme was injected and the consumption of alkali was recorded. The initial linear rates over 2-15% reaction were obtained and were corrected for non-enzymic hydrolysis. Concentrations of enzyme were ~ 10^{-9} M. Concentrations of AcCh were 0.04-0.4 mM, of DMBAc, 0.2-2.0 mM. Initial rates were determined with 3-5 concentrations of substrate, in the absence of inhibitor and in the presence of 3-4 concentrations of inhibitor. Linear plots of 1/V vs. 1/S were obtained and slopes and intercepts were calculated by least squares analysis. Where y-ordinate intercepts were found constant at varying concentration of inhibitor, the inhibition was deemed to be competitive. Linear plots of slopes of the 1/V vs. 1/S curves against inhibitor concentration, [I], were obtained, least squares slopes and intercepts were calculated, and the ratio of the y-ordinate intercept to the slope gave K_i, [39].

RESULTS

Cationic and uncharged inhibitors, related in structure to AcCh and with an acetyl group in a position corresponding to that in the substrate, were prepared by addition of selected compounds to methyl vinyl ketone, eq. 1. Addition of tert-butyl amine led to 3-oxo-(N-tert-butyl)-butanaminium,

$$RXH + CH_2{=}CHCOCH_3 \rightarrow RXCH_2CH_2COCH_3 \qquad (1)$$

$(CH_3)_3\overset{+}{C}NH_2CH_2CH_2COCH_3$, II, a secondary amino isomer of the very effective quaternary ammonium inhibitor, 4-oxo-N,N,N-trimethylpentanaminium, $(CH_3)_3\overset{+}{N}CH_2CH_2CH_2COCH_3$, I [21,40]. Isopropylamine led to the lower homologue of II, Compound IX; the analogous lower homologue of I, Compound V, was available. Addition of tert-butyl mercaptan and tert-butyl alcohol led to the uncharged S and O analogues of II, 4-tert-butylthio-2-butanone, $(CH_3)_3CSCH_2CH_2COCH_3$, III, and 4-tert-butoxy-2-butanone, $(CH_3)_3COCH_2CH_2COCH_3$, VIII, an isomer of the substrate DMBAc. Addition of isopropyl alcohol led to the lower homologue of VIII, inhibitor X. The primary ammonium ion from tert-butyl amine, $(CH_3)_3C\overset{+}{N}H_3$, IV, and its quaternary ammonium isomer, tetramethylammonium ion, $(CH_3)_3\overset{+}{N}CH_3$, VII, the quaternary ammonium inhibitor, choline, VI, and its uncharged carbon analogue, 3,3-dimethylbutyl alcohol, DMBAl, XI, were also studied.

Inhibitors which contain the acetyl group located as in AcCh appear to show competitive inhibition: Plots of 1/V vs. 1/S at varying concentration of inhibitor had essentially constant y-ordinate intercepts, which did not show regular increase with rising concentration of inhibitor. Of the others, only tert-butyl ammonium ion, IV, appears to show competitive inhibition. Data for the new inhibitors, Compounds II, III, IV and VII are summarized in Figures 1-4 and the Table.

The most effective inhibitor is the quaternary ammonium compound I, K_i = 0.02 mM, a value intermediate between those previously reported for this compound under somewhat different conditions, 0.051 mM [40] and 0.008 mM [21]. Effects of charge, degree of substitution on N and of methyl branching in related structures are observed and are described in the Discussion.

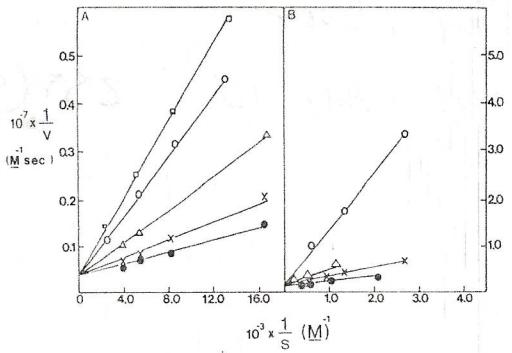

Fig. 1A. Inhibition by $(CH_3)_3\overset{+}{C}NH_2CH_2CH_2COCH_3$, II, of hydrolysis of AcCh, 2.7×10^{-10} M AcChE. ●, No inhibitor, slope 0.0070×10^4 s; X, 0.059 mM II, slope 0.010×10^4 s; Δ, 0.18 mM II, slope 0.019×10^4 s; ○, 0.047 mM II, slope 0.032×10^4 s; □, 0.71 mM II, slope 0.043×10^4 s.

Fig. 1B. Inhibition by $(CH_3)_3\overset{+}{C}NH_2CH_2CH_2COCH_3$, II, of hydrolysis of DMBAc, 5.8×10^{-10} M AcChE. ●, No inhibitor, slope 0.14×10^4 s; X, 0.12 mM II, slope 0.24×10^4 s; Δ, 0.47 mM II, slope 0.50×10^4 s; ○, 1.2 mM II, slope 1.2×10^4 s.

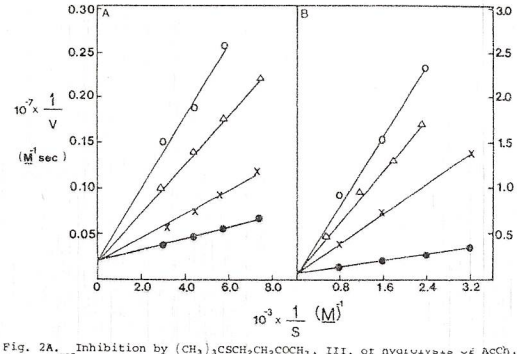

Fig. 2A. Inhibition by $(CH_3)_3CSCH_2CH_2COCH_3$, III, of hydrolysis of AcCh, 3.5×10^{-10} AcChE. ●, No inhibitor, slope 0.0066×10^4 s; X, 0.52 mM III, slope 0.015×10^4 s; Δ, 1.4 mM III, slope 0.028×10^4 s; ○, 2.4 mM III, slope 0.042×10^4 s.

Fig. 2B. Inhibition by $(CH_3)_3CSCH_2CH_2COCH_3$, III, of hydrolysis of DMBAc, 5.0×10^{-10} M AcChE. ●, No inhibitor, slope 0.068×10^4 s; X, 1.0 mM III, slope 0.41×10^4 s; Δ, 2.1 mM III, slope 0.67×10^4 s; Δ, 3.0 mM III, slope 0.92×10^4 s.

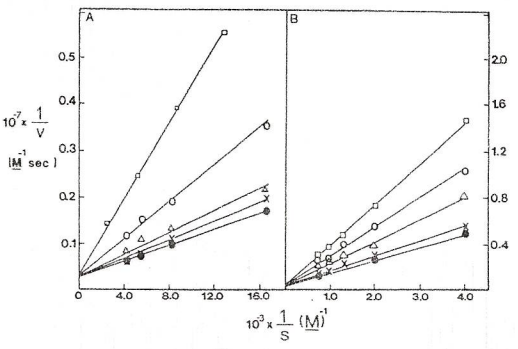

Fig. 3A. Inhibition by $(CH_3)_3C\overset{+}{N}H_3$, IV, of hydrolysis of AcCh, 2.2×10^{-10} M AcChE. ●, No inhibitor, slope 0.0090×10^4 s; X, 0.17 mM IV, slope 0.010×10^4 s; Δ, 0.34 mM IV, slope 0.012×10^4 s; ○, 0.85 mM IV, slope 0.019×10^4 s; □, 2.2 mM IV, slope 0.041×10^4 s.

Fig. 3B. Inhibition by $(CH_3)_3C\overset{+}{N}H_3$, IV, of hydrolysis of DMBAc, 2.1×10^{-10} M AcChE. ●, No inhibitor, slope 0.12×10^4 s; X, 0.085 mM IV, slope 0.13×10^4 s; Δ, 0.17 mM IV, slope 0.19×10^4 s; ○, 0.51 mM IV, slope 0.26×10^4 s; □, 0.85 mM IV, slope 0.36×10^4 s.

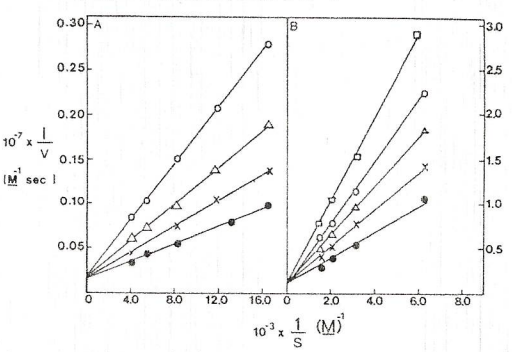

Fig. 4A. Inhibition by $(CH_3)_3COCH_2CH_2COCH_3$, VIII, of hydrolysis of AcCh, 4.0×10^{-10} M AcChE. ●, No inhibitor, slope 0.0048×10^4 s; X, 0.64 mM VIII, slope 0.0073×10^4 s; Δ, 1.6 mM VIII, slope 0.010×10^4 s; ○, 3.4 mM VIII, slope 0.016×10^4 s.

Fig. 4B. Inhibition by $(CH_3)_3COCH_2CH_2COCH_3$, VIII, of hydrolysis of DMBAc, 6.3×10^{-10} M AcChE. ●, No inhibitor, slope 0.16×10^4 s; X, 0.40 mM VIII, slope 0.22×10^4 s; Δ, 0.80 mM VIII, slope 0.29×10^4 s; ○, 1.20 mM VIII, slope 0.35×10^4 s; □, 2.0 mM VIII, slope 0.48×10^4 s.