crude adenine derivative was converted to the oxalate salt in *i*-PrOH containing 1% oxalic acid: yield 46%; mp 208–210°. *Anal.* ($C_{14}H_{23}N_5O \cdot 0.5C_2H_2O_4$) C, H, N.

3-Amino-2-dodecanone hydrochloride was prepared by method C from 2-aminoundecanoic acid: yield 90.6%; mp 114–117° (THF). *Anal.* ($C_{12}H_{26}ClNO$) C, H, Cl, N.

erythro-3-Amino-2-dodecanol oxalate (4e) was prepared by method D from 3-amino-2-dodecanone hydrochloride and KBH_4: yield 78.9%; mp 103–110° (*i*-PrOH containing 1% oxalic acid). *Anal.* ($C_{12}H_{27}NO \cdot C_2H_2O_4$) C, H, N.

erythro-9-(2-Hydroxy-3-dodecyl)adenine oxalate (10e) was prepared by a combination of methods A and B from 1 and 4e. The crude adenine derivative was converted into the oxalate salt: yield 23%; mp 183–185° (*i*-PrOH containing 1% oxalic acid). *Anal.* ($C_{17}H_{29}N_5O \cdot 0.5C_2H_2O_4$) C, H, N.

Reagents and Assay Procedures. Adenosine deaminase (Type I, calf intestinal mucosa) was purchased from Sigma Chemical Co. The procedure for the assay of reversible inhibitors has previously been described[1-4] and is a modification of the method of Kaplan[16] based on the work of Kalckar.[17]

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Synthesis and Enzymatic Evaluation of Some *N*-Alkyl Branched Chain Piperidine Salts and *N*-Alkyl-3-(*N,N*-diethylcarbamoyl)piperidine Salts as Inhibitors of Acetyl- and Butyrylcholinesterase†

James W. Stanley,‡ Ian W. Mathison,* and James G. Beasley

Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, Tennessee 38103.
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A kinetic investigation of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) using acetylcholine (ACh) as substrate and *N*-alkyl-3-(*N,N*-diethylcarbamoyl)piperidine (I) and *N*-alkyl branched chain piperidine (II) salts as inhibitors was conducted. Conventional inhibitor binding constants (K_i values) were determined for both series of inhibitors, as well as their effects on the carbamylation of AChE and BuChE by dimethylcarbamyl fluoride (DMCF). Acetylcholinesterase was shown to be more sensitive in terms of affinity, to the transition of a tertiary amine to a quaternary ammonium salt in series II, while the affinity of series I increased with the length of the alkyl chain (C_3–C_{12}) in both enzyme systems. The carbamylation studies revealed a high sensitivity of AChE toward carbamylation by DMCF in the presence of series II, in contrast to BuChE. Affinity correlated with rate enhancement in BuChE, while there was little correlation in AChE. The results were interpreted in terms of the relationship between the anionic and esteratic subsites of these two enzymes, which appears to be of a more dynamic nature in AChE. Examples are presented which demonstrate that affinity data alone are insufficient to characterize binding modes and serve as a basis for structure–activity models.

As part of a kinetic investigation of the binding properties of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), various piperidine analogs, demonstrating varying degrees of hydrophobicity, were synthesized and studied as reversible inhibitors of these enzymes. The principal regions of interest were the anionic sites of these two enzymes and their adjacent hydrophobic areas, since it has been postulated that these areas constitute the major difference between the two enzymes.[1-7] Two series of substituted piperidines were synthesized: series I, *n*-alkyl-3-(*N,N*-diethylcarbamoyl)piperdine hydrobromide salts, and series II, 1-butylpiperidine salts, possessing the butyl grouping in all its isomeric forms. Both the hydrobromide salts and the corresponding methyl quaternary ammonium salts of series II were investigated. These compounds can be expected to bind to the anionic sites of the two cholinesterases and to reflect the topography of the area immediately surrounding these sites. Series I provides continuity with earlier work from these laboratories on BuChE[8-10] and provides a comparison of the importance of hydrophobic interactions in the two enzymes.

Also of interest was to establish the relative binding modes of these two series as well as some other 3-substituted piperidine derivatives.[11] In particular, we were interested in establishing whether or not these various groupings in the 3 position of the ring were binding at the esteratic sites of these enzymes. This latter question was investigated by studying the effects of these reversible inhibitors on the rate of carbamylation of AChE and BuChE by dimethylcarbamyl fluoride (DMCF).

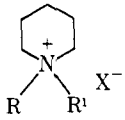
Results and Discussion

It is apparent from the studies with the 1-butylpiperidinium salts that AChE demonstrated a marked response to quaternization of the heterocyclic nitrogen (Table I).

†Dedicated to Professor Alfred Burger.

‡The work reported constitutes a segment of the dissertation submitted by J. W. S. to the University of Tennessee Medical Units in partial fulfillment of the Doctor of Philosophy degree requirements in Medicinal Chemistry. American Foundation for Pharmaceutical Education Fellow, 1970–1972.

Table I. Inhibition and Effects on Carbamylation of AChE and BuChE by 1-Butylpiperidinium Salts



R	R^1	X^-	10^5K_i, AChE	10^5K_i, BuChE	$10^6\alpha$, AChE[a]	$10^5\alpha$, BuChE
n-Butyl	H	Br	33.3	3.00	2.1	3.0
n-Butyl	CH_3	I	3.98	6.53	7.3	3.6
Isobutyl	H	Br	7.53	2.06	2.7	2.0
Isobutyl	CH_3	I	3.43	5.38	2.7	4.0
sec-Butyl[b]	H	Br	5.26	6.06	6.3	7.0
sec-Butyl[b]	CH_3	I	0.31	6.24	0.41	5.0
tert-Butyl	H	Br	25.2	28.1		
tert-Butyl	CH_3	I	11.8	29.2		
$-CH(C{\equiv}N)CH_2CH_3$[b]	CH_3	I	1.88			

[a] *tert*-Butylpiperidinium methiodide accelerates the carbamylation rate of AChE by a factor of 1.6 at 3.1×10^{-5} *M*. [b] Racemic mixture. α = concentration (mol/l.) of inhibitor necessary to accelerate the carbamylation rate by a factor of 2.

Whereas every member of the butyl series demonstrated increased affinity toward AChE upon quaternization, the affinity of these same compounds was either unaltered or below those of the tertiary amine analogs in BuChE. The presence of a single optimally oriented methyl group can determine activity for substrates and some irreversible inhibitors of AChE, for example, ACh and neostigmine *vs.* their desmethyl analogs. As evidenced in this study, it can also result in very marked changes in affinity. It would be of great interest, of course, to know the origins of this general increase in affinity for members of the quaternary series (in AChE) and especially that of the *sec*-butyl analog which gained in affinity by a factor of 16.8 upon quaternization. The high affinity of 1-*sec*-butylpiperidinium methiodide is diminished when the α-methyl group is replaced by a nitrile moiety; however, the cyano compound still maintains a higher affinity for AChE than the remaining butyl isomers (Table I). Since AChE and BuChE have been shown to possess similar steric requirements in the anionic site(s) region, these results may reflect some basic differences in the ability of these enzymes to accommodate methyl groups. This brings up the question of affinity *vs.* reactivity. It is well known that affinity and reactivity do not always correlate in enzyme–substrate relationships; ACh and AChE is the most pertinent example. On the other hand, in some enzymes, such as α-chymotrypsin, the binding specificities and reactivities seem to arise from a nonpolar region on the enzyme which, incidentally, contains the functional groupings necessary for catalyzing the subsequent reaction.[12,13] The fact that the former enzymes (*i.e.*, AChE) invariably have higher turnover numbers (*i.e.*, efficiency) and have also been implicated with the concepts of induced fit and conformational change would suggest a relationship between these two phenomena. Thus, if conformational change is intimately related to catalytic activity in AChE, dramatic changes in affinity are likely to be a consequence of small changes in structure, especially when these changes are made around the moiety to which the enzyme is uniquely sensitive. It follows, therefore, that BuChE would be less likely to evoke these responses if it were less susceptible to conformational perturbation.

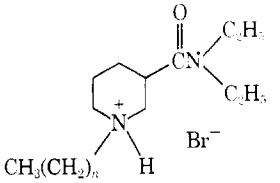
Evidence that this is the case is found in the results concerning the carbamylation of AChE and BuChE by DMCF in the presence of the 1-butylpiperidine salts. As shown by the α values in Table I, the carbamylation of the esteratic site in AChE by DMCF is remarkably sensitive to the presence of these cationic ligands, while BuChE is much less sensitive to these ions. Since these effects are elicited by separate molecules, this may be a reasonable measure of the susceptibility to perturbation or conformational change in the two enzymes. Wilson and later Belleau have shown that this coupling between the subsites is a general property of AChE.[14,15] In addition, Belleau and DiTullio have shown that the ability of a ligand to produce a perturbational response is normally unrelated to its affinity for the macromolecular surface.[16] This is also the case in AChE for the 1-butylpiperidinium salts where affinity as measured by K_i values had little correlation with accelerating ability. (The *sec*-butyl quaternary salt was an exception; this compound has a different binding mode relative to the other compounds in the series as evidenced by its unique effect on decarbamylation: J. W. Stanley, I. W. Mathison, and J. G. Beasley, unpublished results.)

In contrast to AChE, the effects of these compounds on the carbamylation of BuChE were much less pronounced, requiring concentrations very close to the K_i values of the respective compounds (Table I) to achieve a twofold increase in the carbamylation rate (α) relative to the control (DMCF only). The accelerating affect in AChE is apparently not a unique property of quaternary salts as evidenced by the marked potency of some members of the tertiary amine salt series. *n*-Butylpiperidine hydrobromide possesses an α value which is 158 times lower than its corresponding K_i value.

In regard to BuChE, the results indicate that while there is a definite rate enhancement at moderate concentrations of reversible inhibitor, the effect is not dramatic and appears to be related to the affinity of the respective reversible inhibitors. Thus responses of AChE and BuChE were dissimilar in terms of both affinity and rate enhancement in the presence of the butylpiperidinium salts. The source of this difference may lie in the relationship between the anionic and esteratic sites: AChE undergoing conformational changes initiated by cationic moieties with suitable alkyl substituents. It is perhaps of importance that affinity appears to correlate with rate enhancement in BuChE, while having little correlation in AChE.

It is thus understandable why some difficulties have been encountered in distinguishing AChE and BuChE on the basis of affinity constants of reversible inhibitors, since the areas surrounding the catalytic sites of these enzymes are essentially hydrophobic in nature[7] and can therefore be expected to have some measure of affinity for cationic ammonium compounds possessing sufficient hydrophobic character. This is evident from the results obtained on the binding of 1-alkyl-3-(*N,N*-diethylcarbamoyl)-piperidine hydrobromides (series I) to these enzymes (Table II and Figure 1). These compounds are in general

Table II. Inhibition of AChE and BuChE by 1-Alkyl-3-(*N*,*N*-diethylcarbamoyl)piperidine Hydrobromide Salts



No. of C's in alkyl chain	Mp, °C	Formula	Analyses	Purifn solvent	% yield	K_i, AChE[a]	K_i, BuChE[a]
n-Propyl (3)						8.08×10^{-5}	1.31×10^{-4}
n-Pentyl (5)						1.47×10^{-5}	3.92×10^{-5}
n-Octyl (8)	146–147.5	$C_{18}H_{37}N_2OBr$	C, H, N, Br	EtAc	70	1.88×10^{-6}	6.29×10^{-6}
n-Nonyl (9)	155–157	$C_{19}H_{39}N_2OBr$	C, H, N, Br	EtAc	68	9.37×10^{-7}	2.72×10^{-6}
n-Decyl (10)						7.61×10^{-7}	1.39×10^{-6}
n-Undecyl (11)	150–150.5	$C_{21}H_{43}N_2OBr$	C, H, N, Br	EtAc	76	4.89×10^{-7}	6.54×10^{-7}
n-Dodecyl (12)[b]	151.5–153	$C_{22}H_{45}N_2OBr$	C, H, N, Br	EtAc	75	3.75×10^{-7}	2.82×10^{-7}

[a]The values of K_i given above were determined at 25° and are expressed in mol/l. [b]The K_i value for *n*-dodecyltrimethylammonium iodide in AChE is 8.00×10^{-6} *M*.

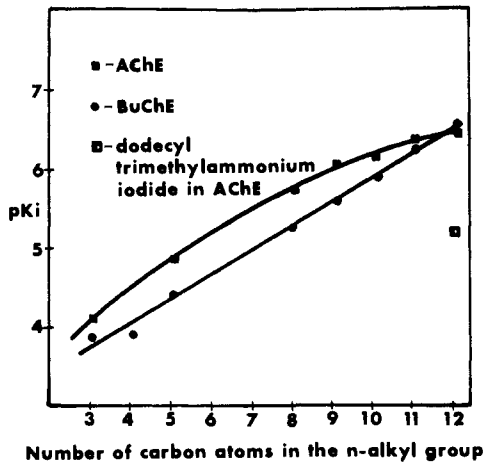


Figure 1. Plot of pK_i *vs.* the number of carbon atoms for 1-alkyl-3-(*N*,*N*-diethylcarbamoyl)piperidine hydrobromides.

somewhat better inhibitors of AChE than BuChE, although the relationship between pK_i and *n* (the number of carbon atoms in the alkyl chain) (Figure 1) is linear for BuChE (this is in disagreement with earlier work although the break observed earlier was not large[8] and the individual K_i values for the two studies are in good agreement) and nonlinear for AChE, with affinity in the latter enzyme plateauing near the C_{12} chain length. While these data would indicate an extensive hydrophobic region on BuChE, and perhaps a less extensive region on AChE, the results are clearly uninformative as to the consequences of these regions in terms of the catalytic differences of these enzymes.

As shown by Belleau[17] and based upon the work of Cohn and Edsall, hydrophobic interactions can readily account for free-energy changes up to 700 cal per methyl group and this value will be approached only when the "solution" of the methyl group follows ideal behavior. The value obtained for the binding of the 1-alkyl-3-(*N*,*N*-diethylcarbamoyl)piperidine hydrobromide salts was 0.41 kcal per methylene group in BuChE and would appear to be adequately explained by this analogy. This proposal is supported by the finding that the free energy per $-CH_2$ group in the octyl to dodecyl sulfate series of solutes to bovine serum albumin (BSA) is approximately 0.55 kcal/mol[18] and the binding of 1-alkyl-3-carbamoylpiperidinium chloride to yeast alcohol dehydrogenase varies in free energy per $-CH_2-$ by 0.37 kcal/mol in the *n*-propyl to *n*-undecyl chain length.[19] Although the results with AChE do not show a linear relationship (Figure 1) a hydrophobic interaction is clearly evident. Many examples could be taken from the literature and while not all are linear, the average variation in affinity per $-CH_2-$ is not great. The linear relationship for pK_i *vs.* the number of carbons in the alkyl moiety (Figure 1) in BuChE is an example of a situation where seemingly precise free-energy data actually represent a very nonspecific binding process.

Further evidence for the essentially nonspecific binding is found by considering the effects of series I on the carbamylation of the esteratic sites of AChE and BuChE. Compounds which interact specifically at the esteratic site of AChE and BuChE can be expected to protect the enzyme from irreversible inhibitors such as DMCF. Thus, ACh and 3-hydroxyphenyltrimethylammonium bromide protect AChE from carbamylation by DMCF. However, the 1-alkyl-3-(*N*,*N*-diethylcarbamoyl)piperidine hydrobromides not only fail to protect against DMCF (Table I) but even accelerate this rate at high concentrations of the reversible inhibitors. The assumption that these compounds are bound to the esteratic site thus appears to be incorrect[20] and prompted us to investigate some other 3-substituted piperidine analogs. In this regard it is interesting to note that 3-acetoxy-1-methylpiperidine is a substrate for AChE. The dimethylcarbamate analog might be expected to be an irreversible inhibitor of AChE; however, we found that neither the C_1 nor C_{10} *N*,*N*-dimethyl[3-(1-alkylpiperidyl)]carbamates[21] demonstrated any progressive inhibition of either AChE or BuChE. Furthermore, the pI_{50} value of *N*,*N*-dimethyl[3-(1-methylpiperidyl)]carbamate has been found to be equal to that of 1-methylpiperidine,[22] showing that the carbamyl grouping contributes virtually nothing to the affinity. In fact, of the three classes of substituted piperidine analogs investigated, namely, 1-alkyl-3-(*N*,*N*-diethylcarbamoyl)piperidine hydrobromides, *N*-[3-(1-alkylpiperidyl)]acetamides,[11] and *N*,*N*-dimethyl-*N*′-[3-(1-alkylpiperidyl)]ureas,[11] only the latter compounds protected AChE and BuChE from carbamylation by DMCF.

These findings illustrate the importance of structural changes on affinity and reactivity. It also points out the need for, and the utility of, additional assay methods which can be used in conjunction with affinity data. Determining that structural changes do not alter the original model can only be successful if the parameter by which both model and synthetic analogs are judged has a common origin. The utility of some alternative assays in the comparison of AChE and BuChE is the topic of a paper to be published elsewhere.

Experimental Section

Chemistry. The synthesis of these compounds in general followed established synthetic routes; however, the *tert*-butylpiperidine analog could not be obtained by simple alkylation procedures in satisfactory yields and therefore a method of synthesis for this compound was investigated; a discussion of the mechanism has been described elsewhere.[23] All chemicals used in the synthesis were reagent grade or its equivalent unless otherwise specified. Melting points were determined on a Büchi melting point apparatus and are uncorrected. Infrared spectra (ir) were performed on either a Perkin-Elmer 137 Infracord spectrophotometer or a Beckman Model IR-33 grating spectrophotometer. Nuclear magnetic resonance spectra (nmr) of selected compounds were recorded on a Varian Associates A-60A nuclear magnetic spectrometer at 25°. Elemental analyses were performed on unreported as well as some known compounds by Galbraith Laboratories, Inc., Knoxville, Tenn. Where analyses are indicated, the results were within ±0.3% of the theoretical values.

General Methods. 1-Butylpiperidines (Series II). The *n*-butyl, isobutyl, and *sec*-butyl homologs were obtained by a procedure adapted from Magnesson and Schiery.[24] To a solution of piperidine (0.50 mol) in 50 ml of ethylene glycol dimethyl ether (glyme) was added 0.25 mol of the appropriate alkyl bromides and the solutions were stirred overnight at room temperature. The white precipitate which formed consisted of the hydrobromide salt of the tertiary amine. The precipitate was washed thoroughly with Et_2O and then redissolved in H_2O, which was made strongly basic with 6 *N* NaOH and extracted with Et_2O, and the combined extracts were dried over $MgSO_4$. The Et_2O was removed on a rotary evaporator to yield the crude tertiary amine, which was then distilled at atmospheric pressure. The ir ($CHCl_3$) and nmr ($CDCl_3$) spectra were consistent with the proposed structures and the boiling points were in agreement with the literature.[24-28]

The hydrobromide and methyl quaternary salts were prepared from these purified amines. The HBr salts were obtained by treating an Et_2O solution of the respective amine with a saturated solution of HBr in Et_2O. The quaternary salts were prepared by dissolving the tertiary amine in glyme and adding a solution of methyl iodide in glyme, dropwise at room temperature. The resulting salts precipitated from solution and were recovered by filtration and recrystallized. The melting points of these compounds were in agreement with the literature values.[24-28]

***tert*-Butylpiperidine.** This compound was prepared by reacting the appropriate N,N-disubstituted α-aminonitrile with methyl Grignard reagent, which can be used as a general procedure for preparing sterically hindered amines.[29,30] While α-aminonitriles are normally obtained by the Strecker synthesis,[31] this method is unsuitable for secondary amines and ketones, and the preferred method is through the amine, cyanohydrin, and corresponding ketone.[32] α-Piperidinoisobutyronitrile was prepared according to the procedure described by Stanley, *et al.*[23] To methylmagnesium iodide, formed from magnesium turnings (7.3 g, 0.30 mol) and methyl iodide (38.7 g, 0.28 mol) in 500 ml of anhydrous Et_2O, was added dropwise an ethereal solution of the α-piperidinoisobutyronitrile (21.3 g, 0.140 mol) at 4°. A gray precipitate formed immediately as addition of the α-aminonitrile continued for 1 hr, after which time the mixture was warmed to reflux and stirred vigorously for 4 hr. The adduct was decomposed with 1 *N* HCl and ice. The water layer was made alkaline with NH_4OH and extracted with Et_2O. Distillation under atmospheric pressure results in decomposition of unreacted α-aminonitrile when the temperature exceeds 150°; therefore, only one fraction was obtained which was *tert*-butylpiperidine [bp 166° (lit.[31,33] bp 165–166°)] (3.8 g, 20.0%). The ir ($CHCl_3$) gave two bands, one at 1385 cm^{-1} and a more intense band at 1370 cm^{-1}, characteristic of the *tert*-butyl grouping. The nmr spectra confirmed the presence of the *tert*-butyl grouping: nmr ($CDCl_3$) δ 1.05 (s, 9, CH_3).

1-Alkyl-3-(*N,N*-diethylcarbamoyl)piperidine Hydrobromides (Series I). The chemistry of some of these compounds has been reported and for the unreported compounds, procedures were adopted similar to those described by Lasslo.[34] The physical properties of the new compounds synthesized are included in Table II.

Biochemical Evaluation. Acetylcholine bromide was obtained from Sigma Chemical Co. and recrystallized twice from absolute ethanol. Preparations of acetylcholinesterase (AChE) isolated from Electrophorus electricus (E.C. 3.1.1.7) were obtained from Worthington Biochemical Corp. (code, ECHP) as was butyrylcholinesterase (BuChE) isolated from horse serum (E.C. 3.1.1.8). Both AChE and BuChE were assayed using 0.10 *M* $MgCl_2$ by the pH-Stat method using Radiometer titration equipment. For stability reasons AChE was handled in the following manner. The enzyme (1 mg, 1000 units) was dissolved in 10 ml of 0.15 *M* NaCl containing 1% gelatin and 0.002 *M* sodium phosphate buffer (pH 7.4). From this solution were taken 0.05-ml aliquots and placed in 10-ml vials and frozen. Each day 10 ml of the same saline solution was again added and the vial kept at 4° during the assay period; usually 0.10 ml of enzyme was used per assay. The concentration of ACh in 25 ml of assay solution varied between 1.75 and 7.00×10^{-4} *M*. The kinetics were followed by titrating the acid formed with 0.0025 *N* NaOH in a 0.500-ml syringe Type B101 in a Type SBU1a syringe burette assembly and delivered to the assay vessel through a 4-in. needle with an internal diameter of 0.15 mm (Hamilton Co., Inc., N-728).

BuChE was found to be stable for several months in a saline solution if kept at 4°. Therefore, 35 mg of protein was weighed into a 10-ml vial and dissolved in 10 ml of 0.15 *M* NaCl; usually 0.1 ml of enzyme solution was used per assay. The concentration of ACh in 25 ml of assay solution for this enzyme varied between 4.00 and 25.00×10^{-4} *M*, and the titrant was 0.01 *N* NaOH.

Reactions were conducted at pH 7.40 ± 0.05 in a jacketed glass reaction cell, thermostated at 25 ± 0.05° by circulating water from a Heto (Denmark) ultrathermostat. Prepurified nitrogen (The Matheson Co.) was bubbled through water and passed over the surface of the reaction solution to minimize absorption of carbon dioxide. Additions of enzyme and substrate and in some cases inhibitor were syringe pipetted by means of 250-μl syringes with 4-in. needles (Hamilton Co.), one of which was equipped with a Chaney adapter for repetitive pipettings of enzyme.

General Assay Procedure. A substrate hydrolysis control point was determined periodically throughout the test day to ensure activity remained constant. A K_m determination was also normally determined each day although it was found that K_m values for both AChE (2.30×10^{-4} *M*) and BuChE (1.24×10^{-3} *M*) remained constant (±3%) and control points at high substrate concentrations normally varied by less than ±2% during the day. Individual assays were conducted as follows. Into a reaction vessel was transferred quantitatively 2.50 ml of a 1.0 *M* $MgCl_2$ solution, inhibitor if the experiment necessitated any, and sufficient water to bring the volume to 24.70 ml (in the AChE assays, a small amount of 1 *N* NaOH was added to bring the pH to near 7.4). The vessel was then placed into the thermostated jacket and mounted onto the titration assembly and the pH adjusted to 7.4, if necessary. The enzyme solution (0.10 ml) was syringe pipetted into the vessel, through a hole in the top of the slightly modified electrode holder. The ACh solution (0.20 ml) was then syringe pipetted into the vessel and after an approximate 15-sec equilibration period the reaction was followed for about 40% of the syringe capacity (usually about 3 min or less). Repeating the above procedure using various concentrations of substrates (or inhibitors) yielded straight lines of varying slopes which were used to calculate the initial rates.

Assay Procedure for Carbamylation Studies. The basic difference between this assay and the general assay for reversible inhibitors involves the progressive nature of inhibition by carbamylating agents. Therefore, the observed lines will be curved rather than linear, under appropriate assay conditions. Since the rate of carbamylation constitutes a loss of enzymatic activity as a function of time, this loss of activity is compared to an initial control rate, V_0. This V_0 may be an optimal activity rate, or a slightly inhibited rate, if the concentration of reversible inhibitor necessary to affect carbamylation is sufficiently high. When the latter is the case, it was necessary to determine this inhibited rate and consider it as V_0 (Figure 2, line i).

A second control involved determining the carbamylation rate in the absence of the reversible inhibitor, *i.e.*, in the presence of enzyme, substrate, and DMCF (Figure 2, line ii). With these determinations, it is then possible to assess the effects of the reversible inhibitors on the carbamylation rate by assaying the progressive inhibition with both DMCF and the reversible inhibitor present (Figure 2, i + ii). In this assay, enzyme was the final ingredient to be injected (T_0 = time zero) and observations were recorded when titration was equilibrated (T_r = time recording started). Thus, $T_r - T_0$ is the time interval before the actual recording started. The progressive inhibition curve was followed until velocity approached zero. Carbamylation rates were determined by subtending an arc of 1 cm at various times along the progressive inhibition curve.[35]

If no effect was observed at reversible inhibitor concentrations equal to the respective K_i values, a second method of assay was

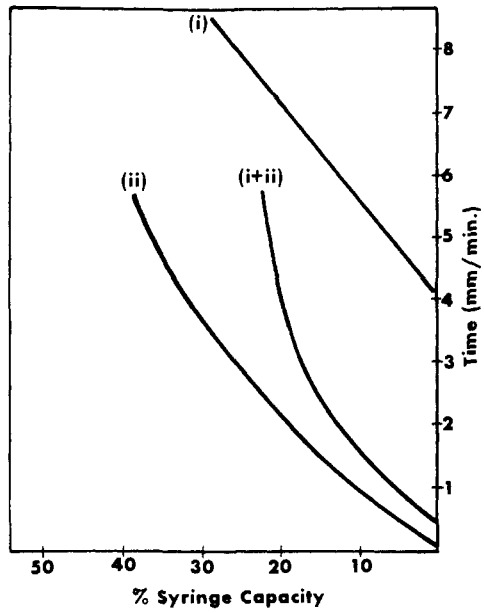


Figure 2. Typical observed carbamylation data: (i) control reversible inhibitor only; (ii) control DMCF only.

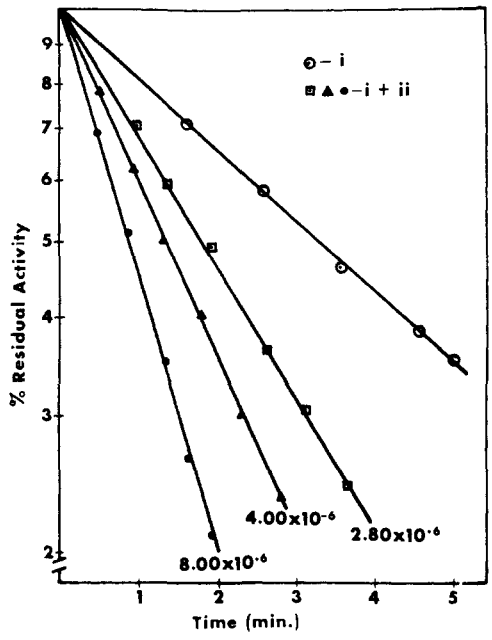
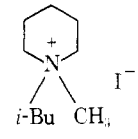


Figure 3. Typical semilog plot of data for carbamylation of AChE by DMCF in the presence of 1-butylpiperidinium salts: (i) control DMCF; (ii)  in concentration indicated.

used. This involved incubating stock solutions of the enzyme and DMCF with and without high concentrations (in this instance a saturating concentration is considered to be 100 times the K_i value) of the reversible inhibitor and then quenching the carbamylation by diluting by a factor of 200, followed by assaying the residual enzyme activity.

Graphical Determinations and Calculations of Kinetic Parameters. The K_m values and inhibitor–enzyme dissociation constants were determined by use of Hofstee plots of v *vs.* $v/[S]$ and confirmed the competitive nature of these inhibitors of series I and II.

The carbamylation results were evaluated quantitatively where effects were apparent at concentrations equal to the K_i values or below and qualitatively when the quenching method was required. Quantitative estimates were made by expressing the observed rates at various times as a per cent of the initial velocity (per cent residual activity) and making semilog plots of these per cents as a function of time.[36] As shown in Figure 3 straight lines were obtained from which the time required for the present residual activity to fall to 50% of the initial velocity can be ascertained. Since inhibitors will vary with respect to the concentration of reversible inhibitor necessary to affect the control carbamylation rate (DMCF alone), this method can be used to elucidate their potencies by calculating a value α, the concentration of reversible inhibitor necessary to accelerate the carbamylation rate relative to the control by a factor of 2.

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