Synthesis and Enzymatic Evaluation of Pyridostigmine Analogs Used to Probe the Active Sites of Acetylcholinesterase and Butyrylcholinesterase,†,‡

O. Elmo Millner, Jr., James W. Stanley, and William P. Purcell*

Department of Molecular and Quantum Biology, College of Pharmacy, University of Tennessee Medical Units, Memphis, Tennessee 38163. *Received August 31, 1973*

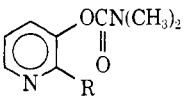
2-Substituted analogs of the drug pyridostigmine were synthesized and evaluated as inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Progressive inhibition was observed in both enzyme systems. AChE inhibition, however, reached a steady state whereas BuChE inhibition progressed until all enzymatic activity stopped. I_{50} values determined in both enzyme systems are compatible with the interpretations that, in the system studied, simple ionic attraction is more important to the inhibition of BuChE than AChE. The results also indicate that steric requirements are more important in the inhibition of AChE.

Studies designed to elucidate the nature of the active centers of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) have revealed certain differences in the active centers of the two enzymes.[1,2] The phenomenon of excess substrate inhibition of AChE and of the absence of substrate inhibition of BuChE has led some researchers to conclude that AChE has two anionic sites for every esteratic site while BuChE has one anionic site for each esteratic site.[3] The simultaneous complexation of the onium groups of two acetylcholine (ACh) molecules with two anionic sites near the esteratic site could prevent the ACh molecule from attaining the proper alignment with the esteratic site necessary for hydrolysis to occur. Others, however, do not consider simultaneous interaction of two ACh molecules with two anionic sites to be the cause of substrate inhibition of AChE. They attribute excess substrate inhibition to the complexing of a second molecule of ACh with the acetylated esteratic site, thereby hindering deacetylation, the final step in the hydrolysis mechanism.[4] Krupka and Laidler postulated that the carbonyl oxygen atom of the second molecule of ACh forms a hydrogen bond with an acidic group in the esteratic site, whereas the carbonyl carbon of the first ACh molecule interacts with the proposed basic group of the esteratic site.[4] This theory of inhibition by excess substrate explains why halogenoacetates do exhibit excess substrate inhibition while other neutral substrates do not.[2] The electronegative halogen may hydrogen bond to the proposed acidic group in the esteratic site of AChE.[2] This theory does not explain the absence of substrate inhibition of BuChE, however, without assuming that the proposed acidic group in the esteratic site of AChE does not have a counterpart in the esteratic site of BuChE.

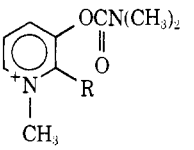
Augustinsson has compared the active centers of electric eel AChE with human BuChE by using (a) a series of carbinol acetates of pyridine and *N*-methylpyridine as substrates of AChE and BuChE and (b) a series of pyridylcarbinols and *N*-methylpyridinium carbinols as inhibitors of AChE and BuChE.[5] By observing the effects that quaternization of the pyridine nitrogen has on activity in the two enzyme systems, Augustinsson concluded that the nonesteratic site of BuChE differs from the anionic site of AChE.[5] The nonesteratic site of BuChE was shown not to possess the anionic nature of the corresponding site in AChE; Coulombic attraction seemed more important to AChE inhibition than to BuChE inhibition.[5] In addition, Augustinsson found that the electronegative pyridine nitrogen of pyridinecarbinol acetates seemed to play a more important role in complex formation with AChE than with BuChE.[5] Consistent with the findings of Krupka and Laidler,[4] Augustinsson concluded that the pyridine nitrogen could hydrogen bond to a postulated acidic group in the esteratic site of AChE.[5]

While considerable research has been directed toward elucidating the nature of the active centers of AChE and BuChE, few *comparative* studies of AChE and BuChE have been conducted.[5-8] In view of the questions that have been raised regarding the nature of the nonesteratic site of BuChE compared with the anionic site of AChE[2,3,5] and in view of the suggestion that the esteratic sites of AChE and BuChE may differ,[5] a comparative study of the active centers of AChE and BuChe is warranted. For this purpose a structurally related series of compounds (Chart I) was synthesized and evaluated for their inhibitory potencies against AChE and BuChE. Aromatic carbamates were chosen as probes of the active site since they are known to react covalently with the active site of cholinesterases yielding a carbamylated esteratic site.[9] The pyridine moiety was incorporated into the inhibitor molecules to see if the observed inhibitory potencies toward AChE and BuChE might lend support to Augustinsson's evidence[5] that the electronegative pyridine nitrogen of pyridinecarbinol acetates plays a more important role in complex formation with AChE than with BuChE. Another reason for selecting the pyridine moiety was that inclusion of the nitrogen in a conjugated system should make the charge on the nitrogen more sensitive to changes in the ring substituents. (Calculations by Clayton[10] have shown that the charge on the quaternary nitrogen of anilinium compounds, in which the nitrogen atom is outside the conjugated ring, does not vary with changes in the ortho and meta substituents.) The pyridinium derivatives II were studied to ascertain the relative importance of Coulombic forces in the attraction of positively charged inhib-

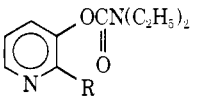
Chart I. Substituted Pyridine Carbamate AChE and BuChE Inhibitors Studied



I, R = H, Cl, Br, I, NO_2, $CH_2N(CH_3)_2$, $CH_2N^+(CH_3)_3$



II, R = H, Br, I



III, R = H, Cl, Br

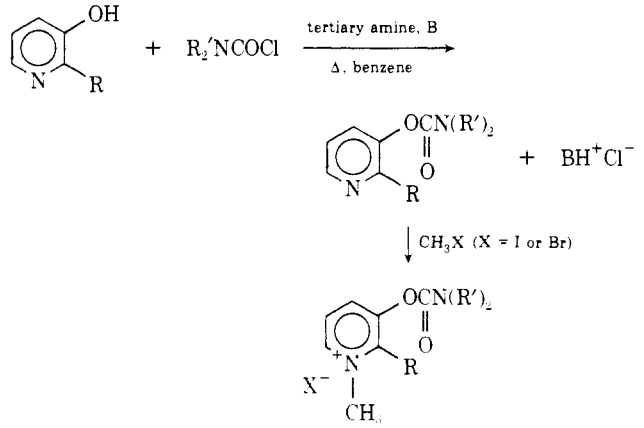
†This is dedicated to Professor Alfred Burger who was particularly helpful to a physical chemist, William P. Purcell, who entered the field of medicinal chemistry in 1963. His encouragement, personal warmth, and sound advice accelerated our research in quantitative structure-activity relationships. For this and Dr. Burger's tremendous contributions to medicinal chemistry, we are deeply grateful.

‡The work reported in this paper constitutes a segment of the dissertation submitted by O. Elmo Millner, Jr., to the Graduate School—Medical Sciences of the University of Tennessee in partial fulfillment of the degree Doctor of Philosophy.

itors to the active centers of AChE and BuChE. Finally, ring substituents of varying electronegativities were chosen to effect a variation in the electron density of the ring nitrogen.

Chemistry. The synthetic pathway, in general, followed Scheme I. The physical data for the synthesized compounds are presented in Table I. The ease of replacement of NO_2, Cl^-, and Br^- at the 2 position by I^- prohibited the quaternization of the corresponding carbamates by methyl iodide. Compound **9** was synthesized by quaternizing compound **4** with methyl bromide. Methyl bromide was found to be an unsuitable quaternizing agent for compound **3** because Br^- replaced Cl^- at the 2 position. Suitable quaternizing agents for compounds **2** and **3** were not found.

Scheme I



Enzymology. The mechanism by which carbamates react with AChE has been studied extensively,[9,11-13] and it is widely accepted to proceed by the reaction sequence shown in eq 1. EH represents free enzyme, CX is the carbamate, EHCX is a reversible enzyme–carbamate complex, X is the leaving group (2-substituted 3-pyridinol in this study), and EC is the carbamylated enzyme.

$$EH + CX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EHCX \xrightarrow{k_2} EC + HX \xrightarrow{k_3} EH + \text{carbamic acid} \quad (1)$$

Carbamates are known to be progressive inhibitors of cholinesterase; *i.e.*, the degree of inhibition by a fixed concentration of carbamate increases as the time of incubation of enzyme with carbamate is increased.[11-13] Owing to difficulties encountered in measuring the affinity constants and carbamylation constants (k_1/k_{-1} and k_2 in eq 1),[9,11,14] all inhibitory activities which are given here are reported as I_{50} values and were determined at equal incubation times.

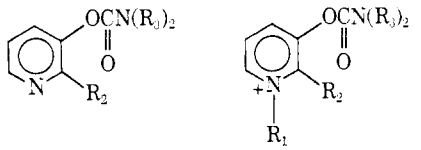
Experimental Section

Synthesis. The synthesis of the reported compounds followed the method of Wuest and Sakal.[15] The carbamates (series I and III) were prepared by refluxing the respective 2-substituted 3-hydroxypyridines with dimethylcarbamyl chloride or diethylcarbamyl chloride in the presence of a tertiary base such as triethylamine. Purification was accomplished by successive recrystallization from diethyl ether or by vacuum distillation if the products were liquids. The quaternary derivatives of the carbamates were prepared by alkylating the purified carbamate with methyl iodide (or methyl bromide where R = Br) in a nonreactive solvent, either benzene or dimethoxyethane. Purification of the quaternary salt was accomplished by repeated recrystallization from absolute ethanol.

The synthesized compounds possessed the requisite ir and nmr spectra for the proposed structures. All melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. As an additional check for purity, thin-layer chromatographs were determined on precoated alumina plates containing a fluorescent indicator. The solvent system used consisted of benzene–methanol (95:5).

Enzymatic Evaluation. The method used to determine the activity of the selected compounds as inhibitors of both AChE and BuChE utilized a pH-stat (Radiometer Type TTT2 automatic ti-

Table I. Physical Data of Selected Carbamates



No.	R_1[a]	R_2	R_3	Mp or bp (mm), °C[e]	Formula	Analyses	% yield
1		H	CH_3	89–90 (3.5) [90 (0.25)][f]	$C_7H_{10}N_2O_2$	C, H, N	71
2		NO_2	CH_3	91–92 [89–92][f]	$C_8H_9N_3O_4$	C, H, N	85
3		Cl	CH	99–99.5 [99–100][f]	$C_8H_9ClN_2O_2$	C, H, Cl	70
4		Br	CH_3	85.5–86.5	$C_8H_9BrN_2O_2$	C, H, Br	50
5		I	CH_3	115 (3.5) [114 (0.15)][f]	$C_8H_9IN_2O_2$	C, H, I	70
6		$-CH_2N-(CH_3)_2$	CH_3	101 (3.5)	$C_{11}H_{17}N_3O_2$	C, H	98
7		$-CH_2N^+-(CH_3)_3$	CH_3	171–172	$C_{12}H_{20}IN_3O_2$	C, H, N	97
8[b]	CH_3	H	CH_3				
9[c]	CH_3	Br	CH_3	150.5–151.5	$C_9H_{12}Br_2N_2O_2$	C, H	35
10[d]	CH_3	I	CH_3	176–177 [171–172][f]	$C_9H_{12}I_2N_2O_2$	C, H, I	55
11		H	C_2H_5	91–93 (3.5)	$C_{10}H_{14}N_2O_2$	C, H	96
12		Cl	C_2H_5	100 (3.5)	$C_{10}H_{13}ClN_2O_2$	C, H	70
13		Br	C_2H_5	130 (3.5)	$C_{10}H_{13}BrN_2O_2$	C, H	68

[a] The absence of an R_1 substituent indicates the free base. The presence of a CH_3 indicates the quaternary nitrogen compound. [b] Pyridostigmine, obtained gratis from Hoffmann-La Roche Co. as the bromide. [c] Bromide salt. [d] Iodide salt. [e] Melting points and boiling points are uncorrected. [f] Literature values taken from ref 15.

trator equipped with a Type SBR2c recorder and a Type SBU1a syringe buret containing a 0.5-ml syringe). Reactions were conducted at pH 7.40 ± 0.05 at 25.0 ± 0.05°. Exclusion of CO_2 from the reaction solution was ensured by maintaining a flow of purified nitrogen over the surface of the assay solution.

The AChE stock solutions were prepared by dissolving 1 mg of AChE (Worthington Biochemical Corp.; Electrophorus electricus 3.1.1.7; 1000 units/mg) in 10 ml of a 2.0×10^{-3} *M* phosphate buffer, pH 7.4, containing 0.15 *M* sodium chloride (Fisher Scientific, biological grade) and 0.10% gelatin (biological grade). Aliquots (0.05 ml) of this stock solution were transferred to vials and frozen. The AChE stock solution for each day was prepared by adding 10 ml of the previously described buffered, gelatin-saline solution to one of the thawed 0.05-ml portions.

Stock solutions of BuChE were prepared by dissolving 35 mg of BuChE (Worthington Biochemical Corp.; horse serum 3.1.1.8; 5.9 units/mg) in 10 ml of 0.15 *M* sodium chloride solution.

Stock solutions of the substrate were prepared fresh every 2 days with acetylcholine bromide (Matheson Coleman and Bell) that had been recrystallized from absolute ethanol.

Stock solutions of inhibitors were prepared the same day that they were assayed. Each compound was checked for nonenzymatic hydrolysis.

The general procedures used to determine the AChE inhibitory activities and the BuChE inhibitory activities were identical. The concentrations, however, were different for the two enzyme systems. The AChE assay solutions contained 0.1 *M* magnesium chloride, 1.40×10^{-3} *M* acetylcholine bromide, 0.10 ml of daily stock AChE, varying volumes of inhibitor stock solution, and enough doubly distilled water to make a total volume of 25 ml. The procedure consisted of (1) adding the magnesium chloride, water, and inhibitor solution to the reaction vessel, (2) adjusting the pH to approximately 7.4 after placing the reaction vessel on the instrument, (3) adding substrate, (4) adjusting pH to exactly 7.4, and (5) adding enzyme solution so that the instrument starts titrating as the reaction is initiated by the addition of the enzyme solution. The time of the addition of enzyme is recorded and designated as zero time, t_0. The titrant used in the AChE assays was 0.0025 *N* sodium hydroxide (Vaughn, Inc.).

The BuChE assays were conducted in the same manner. The substrate concentration was 2.50×10^{-3} *M* in the BuChE assays, however.

For each inhibitor, a control line and progressive inhibition curves for six concentrations of inhibitor were determined. Since the degree of inhibition, *i.e.*, the I_{50} value, produced is a function of time, I_{50} values for all the compounds studied were determined for identical time periods. Inhibition by carbamates has three separate factors, as was seen in eq 1. There is an initial binding contribution, carbamylation, and finally, decarbamylation. Since the decarbamylation rates would be identical for all the dimethyl carbamates and tend to mask differences in activity, I_{50} values were based upon the degree of inhibition occurring exactly 3 min after the initiation of the reaction. Decarbamylation effects should be negligible in this time interval.[11,12]

The method used to obtain hydrolysis rates from the progressive inhibition curves was similar to that of Main.[14] A series of tangents were drawn by intersecting the curve at points equidistant along the time axis on both sides of the point of the intended tangent. The logarithms of the slopes of the tangents, log *V*, were then plotted *vs.* the time corresponding to the point on the curve to which the tangent was drawn. From the six log *V vs.* time plots, the hydrolysis rates at exactly 3 min (t_0 plus 3 min) were determined and plotted *vs.* inhibitor concentration. The inhibitor concentration corresponding to the rate equal to one-half the control rate was taken as the I_{50}.

Experimental error was approximated by repeating the previously described I_{50} determinations four times for compound 2. These repeated determinations were made on different days using different solution preparations.

Decarbamylation Studies. Spontaneous reactivation rates (decarbamylation rates) were determined by (1) incubating enzyme and inhibitor until the enzyme was completely inhibited, (2) quenching the carbamylation process by a large enough dilution (100–200-fold) to reduce the inhibitor to a noninhibiting concentration, and (3) withdrawing samples of the quenched solution at different times for assay. The quenching solution was composed of $MgCl_2$ in water in the same concentration as that used in the I_{50} determinations and was maintained under a nitrogen atmosphere to control the pH.

Reactivation rates in the presence of substrate were determined by injecting a 0.1-ml portion of the enzyme-inhibitor incubation solution into 24.9 ml of the assay solution and following the resulting hydrolysis. A progressive reactivation curve was obtained, and tangents to points on the curve corresponding to various time intervals were drawn according to the method of Main.[14] The concentration of $MgCl_2$ was varied to determine the effect of ionic strength on decarbamylation.

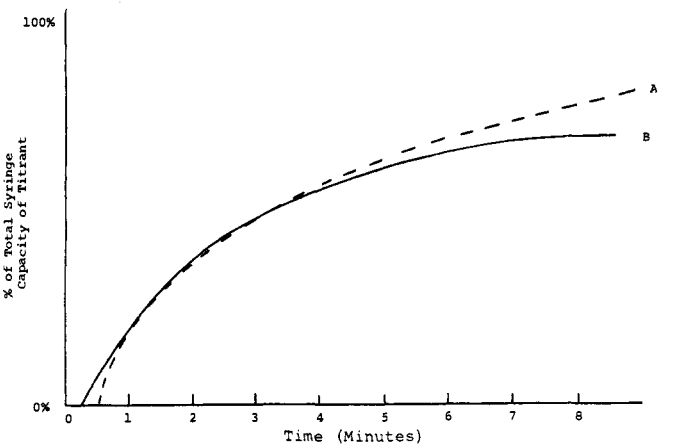


Figure 1. Curve A represents the progressive inhibition curve obtained with AChE inhibited by 1.55×10^{-5} *M* 2-iodo-3-*N*,*N*-dimethylcarbamyloxypyridine (compound **5**, Table II). Curve B represents the progressive inhibition curve obtained with BuChE inhibited by 1.95×10^{-6} *M* 2-iodo-3-*N*,*N*-dimethylcarbamyloxypyridine methiodide (compound **10**, Table II).

Results and Discussion

In comparing the reactions of the selected carbamates with AChE and with BuChE, the shapes of the progressive inhibition curves for the two enzyme systems are found to be different. The progressive inhibition curve for AChE (Figure 1A) became linear after about 7 min to give a constant hydrolysis rate, *i.e.*, steady-state inhibition. The progressive inhibition curve for BuChE (Figure 1B) did not become linear, however, until inhibition was complete, *i.e.*, until all substrate hydrolysis stopped. This can be more dramatically illustrated by Figures 2 and 3 where the logarithms of tangents of curves A and B in Figure 1 are plotted *vs.* the time elapsed since the start of the reaction. One can readily see in Figure 3 that the inhibition of BuChE progresses logarithmically with time as evidenced by the straight line for the log *V vs.* time plot. In Figure 2, the curved line for the log *V vs.* time plot shows that the inhibition of AChE does not continue to progress logarithmically with time. To achieve steady-state inhibition, the rate of decarbamylation, k_3[EC], must equal the rate of carbamylation, k_2[ECX], to maintain a constant amount of free enzyme, E. The observation that the inhibition of BuChE never reaches a steady state while the inhibition of AChE does reach a steady state indicates that k_3 for BuChE is much smaller than k_3 for AChE.

$$\mathrm{E} + \mathrm{CX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ECX} \xrightarrow{k_2} \mathrm{EC} \xrightarrow{k_3} \mathrm{E} + \mathrm{C} \quad (2)$$
$$+$$
$$\mathrm{X}$$

Decarbamylation studies were conducted to elucidate further the relative importance of decarbamylation to the overall inhibition of AChE and BuChE by dimethyl carbamates. The decarbamylation rates of AChE incubated with compound **2**, dimethylcarbamyl fluoride, and neostigmine were found to be equivalent. The half-life of dimethylcarbamylated AChE in the presence of substrate, *i.e.*, the time required for fully inhibited AChE to regain 50% of its normal activity, was found to be 21 min using 0.3 *M* $MgCl_2$. The value obtained for the half-life of carbamylated AChE was found to be influenced by ionic

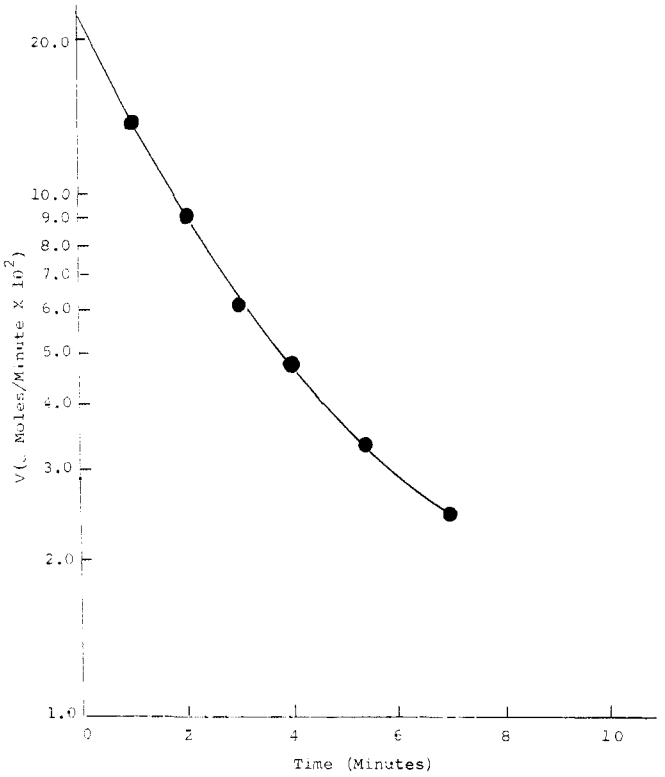


Figure 2. Velocity of substrate hydrolysis by AChE inhibited by 1.55×10^{-5} *M* 2-iodo-3-*N*,*N*-dimethylcarbamyloxypyridine (compound 5, Table II) plotted logarithmically against time elapsed since start of reaction.

strength as evidenced by a half-life of 30 min using 2.8×10^{-3} *M* $MgCl_2$ as opposed to a half-life of approximately 16 min using 0.1 *M* $MgCl_2$. Using both previously described procedures, *i.e.*, decarbamylation in the absence or presence of substrate, no regeneration or reactivation of

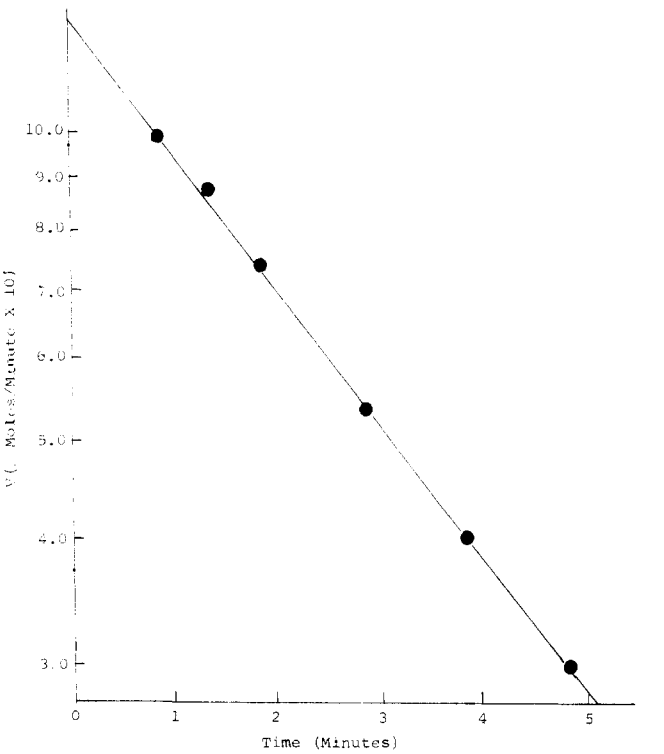


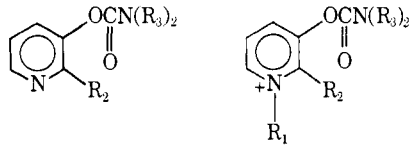
Figure 3. Velocity of substrate hydrolysis by BuChE inhibited by 1.95×10^{-6} *M* 2-iodo-3-*N*,*N*-dimethylcarbamyloxypyridine methiodide (compound 10, Table II) plotted logarithmically against time elapsed since start of reaction.

dimethylcarbamylated BuChE was detected. It must be concluded that although the half-life of dimethylcarbamylated AChE is 15–30 min, depending upon the ionic strength, the half-life of dimethylcarbamylated BuChE must be hours or days, if it decarbamylates at all. In this regard, Reiner has determined the half-life at pH 7.0 of dimethylcarbamylated AChE from electric eel to be 27 min and that of dimethylcarbamylated human serum ChE to be approximately 3.5 hr.[17] *A priori* one might assume that dimethylcarbamylated BuChE from horse serum would have a similar half-life. This has not yet been demonstrated. It is known, however, that the stability of carbamylated cholinesterase is not predictable for cholinesterase from different species. For example, methylcarbamylated AChE from bovine erythrocytes and from housefly heads reactivates more readily than the dimethyl-carbamylated enzymes, while the reverse is true for AChE from electric eel.[17] The absence of detectable decarbamylation of BuChE suggests that a functional group on the enzyme which is responsible for the decarbamylation of AChE may be absent from the active center of BuChE.

The results of the I_{50} determinations of AChE and BuChE are presented in Table II. It will be observed that compounds 2–5 are more potent inhibitors of AChE than BuChE, while the relative potencies in each enzyme system are about the same. In contrast, compounds 6 and 7, which are also 2-substituted pyridine carbamates, are more potent inhibitors of BuChE. It is possible that the $CH_2N(CH_3)_2$ and $CH_2N^+(CH_3)_3$ moieties are situated more optimally for interaction with the proposed anionic site of BuChE than that of AChE. One would expect ionic attractions to be almost equally important for compounds 6 and 7 since the pK_a of the tertiary nitrogen of compound 6 is approximately 8.12_1.[18] The observation that compounds 6 and 7 have the same activities against BuChE while having significantly different activities against AChE indicates that simple ionic attraction may be more important in the binding of BuChE than to AChE. On the other hand, the additional methyl group on the quaternary nitrogen of compound 7 affects the activity significantly in the AChE system. This is consistent with the postulate that tetramethylammonium ion induces an activating conformational change in AChE.[19] Simple quaternary ammonium ions have been shown to accelerate the rate of inhibition of AChE by carbamate inhibitors.[20,21] Thus, it seems quite possible that an alkyl quaternary ammonium moiety might accelerate the rate of inhibition by the carbamate of which the quaternary moiety is a part.

The quaternary pyridinium analogs represented by compounds 8–10 exhibit relative potencies which do not coincide with expected ionic attraction. Bromine, being the most electronegative 2-substituent in compounds 8–10 should make the pyridinium nitrogen more positively charged than the corresponding iodine derivative. The positive charge, which affects the strength of an ionic interaction, should give compound 11 a greater affinity for the anionic site of AChE. Compound 9, however, is less active than the iodo analog, compound 10, in both enzyme systems. Also, compound 8, which possesses a quaternary pyridinium nitrogen, is less active in AChE than compounds 4 and 5, which do not contain a positively charged pyridinium nitrogen. These results indicate that the size of the halogen substituents, and not the charge on the ring nitrogen, is the governing factor in the inhibition by the selected carbamates. This is in agreement with the results of Metcalf[9] and Kolbenzen, *et al.*,[22] who have reported that the inhibitory activities of ortho-substituted

Table II. I_{50} Values for Inhibition of Acetylcholinesterase and Butyrylcholinesterase by Selected Carbamates[a]



Compd no.	R_1[b]	R_2	R_3	AChE I_{50} ($\times 10^6$ M)	BuChE I_{50} ($\times 10^6$ M)
1		H	CH_3	*c*	*c*
2		NO_2	CH_3	79 ± 4	190 ± 10
3		Cl	CH_3	61 ± 3	87.5 ± 4.0
4		Br	CH_3	21.5 ± 1.1	60 ± 3
5		I	CH_3	9.00 ± 0.45	19.5 ± 1.2
6		$-CH_2N(CH_3)_2$	CH_3	4.90 ± 0.24	1.44 ± 0.07
7		$-CH_2N^+(CH_3)_3$	CH_3	3.60 ± 0.18	1.35 ± 0.07
8	CH_3	H	CH_3	54.0 ± 2.7	10.0 ± 0.5
9[d]	CH_3	Br	CH_3	0.83 ± 0.04	7.50 ± 0.38
10[e]	CH_3	I	CH_3	0.15 ± 0.01	1.42 ± 0.07
11[c]		H	C_2H_5	*b*	*b*
12		Cl	C_2H_5	1800 ± 90	225 ± 11
13		Br	C_2H_5	1310 ± 60	160 ± 8

[a] I_{50} values ± experimental error determined per description in text. [b] The absence of an R_1 substituent indicates the free base. The presence of a CH_3 indicates the quaternary nitrogen compound. [c] These compounds underwent rapid autohydrolysis, prohibiting their I_{50} measurements. [d] Quaternary bromide salt. [e] Quaternary iodide salt.

halophenyl carbamates increase as the size of the halogen substituent increases.

Finally, it will be observed in Table II that the diethyl carbamates (compounds 12 and 13) are much less potent inhibitors of both AChE and BuChE than their corresponding dimethyl analogs. These results contrast with those of Beasley, *et al.*,[23] who found that *N,N*-diethylpiperidine carboxamides were more potent inhibitors of BuChE than their corresponding dimethyl analogs. Since the compounds studied by Beasley, *et al.*,[23] were reversible inhibitors, the increase in potencies observed for the diethyl derivatives is probably due to an increase in the affinity for the enzyme's active center. If one considers the greater hydrophobicity of the diethyl carbamates in our studies, one would conclude that their affinities for the enzyme's surface should also be greater than that of the corresponding dimethyl carbamates. The inhibitory activities of the carbamates (compounds 12 and 13) do not reflect this, however. Furthermore, the diethyl carbamates did not behave primarily as progressive inhibitors. A slight amount of progressive inhibition was detected only when concentrations of inhibitor great enough to inhibit substrate hydrolysis by 50% were employed. The observation that the diethyl carbamates are weaker inhibitors and that they exhibit very little progressive behavior indicates that inhibition is occurring principally through the formation of a reversible complex (ECX) and that k_2 in eq 2 is quite small. These results agree with those of Zahavi, *et al.*,[24] who found the *O,O*-diethyl analog of the organophosphate, malaoxon, to be a less potent inhibitor of spider mite AChE than the corresponding dimethyl analog.

The apparent reduction of k_2 (eq 2) for compounds 12 and 13 may be the result of steric hindrance provided by the larger ethyl groups which prevents intimate interaction of the carbamyl portion of the inhibitors with the esteratic site. The reduction in activity caused by the introduction of the ethyl groups of compounds 12 and 13 was much greater in AChE than in BuChE. Thus, it seems that the esteratic site of BuChE can accommodate larger groups than the corresponding site of AChE. This is to be expected, since BuChE hydrolyzes butyrylcholine (BuCh) more rapidly than ACh.[6] The reduction in inhibition for the diethyl carbamates in BuChE, however, indicates that the esteratic site of BuChE accommodates the $CH_3CH_2CH_2C(=O)O-$ moiety of BuCh but not the $(CH_3CH_2)_2NC(=O)O-$ moiety of the diethylpyridine carbamates.

Conclusion

I_{50} values determined in the AChE and BuChE systems for the nitro, chloro, bromo, and iodo derivatives of series I showed the same relative order of activities against both cholinesterases. Thus, it seems that, since the activity in both enzyme systems was affected to the same extent by an increase in the negative character of the pyridine nitrogen, the importance of the negative pyridine nitrogen to complex formation with AChE and BuChE must be about the same. This is contrary to the hypothesis of Augustinsson that AChE and BuChE differ in this respect and that AChE contains an acidic group which can hydrogen bond to a pyridine nitrogen while BuChE does not.[5]

AChE and BuChE do differ in two aspects. First, dimethyl-carbamylated BuChE does not reactivate spontaneously in aqueous solution as readily as dimethylcarbamylated AChE. Thus, there must be a fundamental difference in the active centers of AChE and BuChE which accounts for decarbamylation of AChE and the absence of detectable decarbamylation of BuChE. If decarbamylation proceeds according to the mechanism for deacetylation proposed by Brestkin and Rozengart,[25] then it seems highly probable that the imidazole which has been implicated in the deacetylation of AChE is either absent from the active center of BuChE or that it is situated differently with respect to the serine residue in the esteratic site. The second distinguishing feature is that the esteratic site of BuChE can accommodate larger groups than that of AChE.

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References

(1) J. A. Cohen and R. A. Oosterbann, "Handbuch der Experimentallen Pharmakologie," Vol. XV, G. B. Koelle, Subed., Springer-Verlag, Berlin, 1963, pp 299-373.
(2) E. Usdin, "International Encyclopedia of Pharmacology and

Therapeutics," Section 13, Vol. I, Pergamon, Oxford, 1970, pp 47-357.
(3) F. Bergmann, *Advan. Catal.*, **10**, 131 (1958).
(4) R. M. Krupka and K. J. Laidler, *J. Amer. Chem. Soc.*, **83**, 1445 (1961).
(5) K. B. Augustinsson, *Biochim. Biophys. Acta*, **128**, 351 (1966).
(6) K. B. Augustinsson, "Handbuch der Experimentellen Pharmakologie," Vol. XV, G. B. Koelle, Subed., Springer-Verlag, Berlin, 1963, pp 89-128.
(7) M. I. Kabachnik, A. P. Brestkin, N. N. Godovikov, M. J. Michelson, E. V. Rozengart, and V. I. Rozengart, *Pharmacol. Rev.*, **22**, 355 (1970).
(8) D. H. Adams and V. P. Whittaker, *Biochim. Biophys. Acta*, **4**, 543 (1950).
(9) R. L. Metcalf, *Bull. W. H. O.*, **44**, 43 (1971).
(10) J. M. Clayton, Ph.D. Thesis, University of Tennessee Medical Units, Memphis, 1971.
(11) R. D. O'Brien, *Mol. Pharmacol.*, **4**, 121 (1968).
(12) F. L. Hastings, A. R. Main, and F. Iverson, *J. Agr. Food Chem.*, **18**, 497 (1970).
(13) R. D. O'Brien, B. D. Hilton, and L. Gilmour, *Mol. Pharmacol.*, **2**, 593 (1966).
(14) A. R. Main and F. Iverson, *Biochem. J.*, **100**, 525 (1966).
(15) H. M. Wuest and E. H. Sakal, *J. Amer. Chem. Soc.*, **73**, 1210 (1951).
(16) A. R. Main, "Proceedings of the International Conference on Structure and Reactions of DFP Sensitive Enzymes," E. Hilbronn, Ed., Swedish Research Institute of National Defense, Stockholm, 1967, pp 129-139.
(17) E. Reiner, *Bull. W. H. O.*, **44**, 109 (1971).
(18) D. D. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solution," Buttersworths, London, 1965.
(19) I. B. Wilson, *Ann. N. Y. Acad. Sci.*, **144**, 664 (1967).
(20) R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **238**, 745 (1963).
(21) H. P. Metzger and I. B. Wilson, *J. Biol. Chem.*, **238**, 3432 (1963).
(22) M. J. Kolvezen, R. L. Metcalf, and T. R. Fukuto, *J. Agr. Food Chem.*, **2**, 864 (1954).
(23) J. G. Beasley, R. P. Quintana, and G. G. Nelms, *J. Med. Chem.*, **7**, 698 (1964).
(24). M. Zahavi, A. S. Tahori, and F. Klimer, *Mol. Pharmacol.*, **7**, 611 (1971).
(25) A. P. Brestkin and E. V. Rozengart, *Nature (London)*, **205**, 388 (1965).

Structure–Activity Relationships in Reactivators of Organophosphorus-Inhibited Acetylcholinesterase. 7. 1-Aryl-2-hydroxyiminomethyl-3-methylimidazolium Iodides†,[1]

Palmarisa Franchetti, Mario Grifantini, Sante Martelli,

Institute of Pharmaceutical and Organic Chemistry, University of Camerino, Italy

and Maria L. Stein*,‡

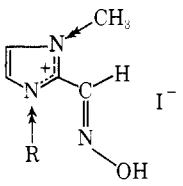
Institute of Pharmaceutical and Toxicological Chemistry, University of Naples, Italy. *Received June 1, 1973*

A series of 1-aryl-2-hydroxyiminomethyl-3-methylimidazolium iodides, where the aryl group is either phenyl or substituted phenyl, was prepared and tested for their reactivating potency on phosphorylated acetylcholinesterase (AChE) and for *anti*-AChE activity. The *in vitro* testing revealed that some of the new compounds are good reactivators. Correlations between their structure and biological activities have been attempted.

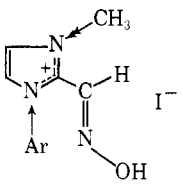
Wilson and coworkers[2] gave some important criteria for reactivation of phosphorylated acetylcholinesterase (AChE) by oximes. The high potency of 2-hydroxyiminomethyl-1-methylpyridinium iodide (2-PAM) was attributed to its capacity for forming a complex with the phosphorylated enzyme in such a way that the nucleophilic oxime group is suitably oriented toward the phosphorus atom.[3,4]

Ashani and Cohen[5] found that at physiological pH, the value of the reactivation rate constant is more a function of the basicity of the reactivator molecule than of its nucleophilic reactivity, reaching an optimum for compounds with pK_a values in the range of 7.6–8.0. Salvador and coworkers[6] supported these observations after studying the activity of some trifluoromethyl ketoximes.

In the course of our research on reactivators of phosphorylated AChE, we found that the methiodides of 1-methyl- and 1-benzyl-2-hydroxyiminomethylimidazole (**1a** and **b**) are only slightly less effective than 2-PAM as reactivators of bovine erythrocyte AChE inhibited by diisopropylphosphorofluoridate (DFP).[7] As these compounds have a pK_a of 8.3, which is thus higher than the optimal values, it was decided to modify the structure of the imidazole oximes by replacing an alkyl group with an aryl group on one of the ring nitrogen atoms. As a consequence of the smaller inductive effect of an aryl group relative to that of an alkyl group, the positive charge delocalized between the nitrogen atoms would be increased and better able to stabilize the oximate anion.



1a, R = CH_3
b, R = $CH_2C_6H_5$



2

We thus prepared a series of methiodides of 1-aryl-2-hydroxyiminomethylimidazoles **2** variously substituted in the aromatic ring so as to modulate the acidity of the oxime function by means of inductive effects.

Chemistry. 2-Hydroxyiminomethyl-1-phenylimidazole was prepared by treating 1-phenylimidazole-2-aldehyde[8,9] with hydroxylamine hydrochloride. The other oximes listed in Table VI were obtained in the same way. The hitherto unknown imidazole aldehydes were prepared as shown in Scheme I.

Marckwald's classical synthesis[10] comprising the first four steps shown in Scheme I was used for the preparation of the 1-arylimidazoles **7**. By hydroxymethylation with formaldehyde, the arylimidazoles were converted into the alcohols **8**, which were oxidized with SeO_2 to give the corresponding aldehydes **9**.

In the reaction between the aldehydes and hydroxyl-

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