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Studies on the Conformational Requirements of Substrate and Inhibitor on Acetylcholinesterase

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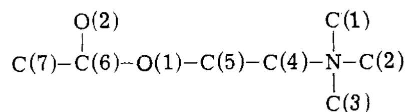
The substrate and inhibitory activity of 2-, 4-, and 6-methyl-3-trimethylammonium phenols and their acetates, propionates, and methyl ethers on AChE (*E. Electricus*) shows that there is a preferred conformation for hydrolysis of the ester group and that the mode of binding for inhibition of AChE by these compounds is not identical with that for substrate activity. A comparison of inhibitory activity of the 2- and 6-methyl-3-trimethylammonium phenols and acetates on substrates such as acetylcholine, acetylthiocholine, and phenyl acetate was used to indicate that these compounds acted on the free enzyme. The data and its interpretation are consistent with an earlier proposal that the imidazole group of histidine is closer than the serine hydroxyl to the anionic site in the AChE active center.

The most probable explanation for the hydrolysis of acetylcholine (ACh, 1) by acetylcholinesterase (AChE) at the esteratic site is by a base-catalyzed mechanism[1] and involves an imidazole group of histidine activating the OH group of serine. The activated serine residue then initiates the hydrolytic mechanism by nucleophilic attack on the carbonyl group of ACh. While considerable effort has been made to study the $N^+ \rightarrow O$ steric parameters[2-6] of the choline portion of ACh and the kinetic aspects of the reaction,[7-16] not as much attention has been given to the conformation of the acetyl group in ACh required for hydrolysis. Recently Belveridge, *et al.*,[17,18] have calculated conformation energy profiles for ACh and a number of analogous molecules using INDO molecular orbital calculations. ACh, (*R*)- and (*S*)-acetyl-α-methylcholine, and *erythro*-acetyl-$\alpha(S),\beta(R)$-dimethylcholine have low conformational energies when the torsion angle that corresponds to C(6)–O(1)–C(5)–C(4) in 1 for these molecules is 100, 120, 120, and 120°, respectively. The rates of hydrolysis of these molecules by AChE are not the same. ACh and the acetyl-α-methylcholine enantiomers have relatively high rates of hydrolysis. However, *erythro*-acetyl-$\alpha(S),\beta(R)$-dimethylcholine is not hydrolyzed by the enzyme while (*S*)-acetyl-β-methylcholine is hydrolyzed 54% as fast as ACh but has an energy minimum when the torsion angle has a value of 30°. Crystallographic studies[5,6] have also shown that the torsion angle that corresponds to C(7)–C(6)–O(1)–C(5) in ACh (1) and many of its derivatives, *e.g.*, (*R*)-(+)-acetyl-α-methylcholine, (*S*)-(+)-acetyl-β-methylcholine, lactoylcholine, and *trans*-(1*S*,2*S*)-(+)-acetoxycyclopropyltrimethylammonium iodide, is approximately 180° for all molecules. However, the torsion angle that corresponds to C(6)–O(1)–C(5)–C(4) in 1 varies and has been measured as +79° in ACh bromide, −147° for (*S*)-(+)-acetyl-β-methylcholine iodide, and −170° for one of the crystal forms of (*R*)-(+)-acetyl-α-methylcholine iodide. These differences in molecular electronic structure and crystal structure only emphasize the dangers of attempting to extend certain parameters to the *in vivo* or *in vitro* or solution conditions.

Wilson and Quan[19] have studied the molecular comple-



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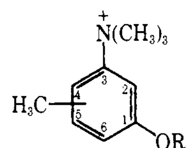
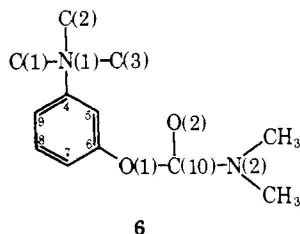
mentarity of a number of trimethylammonium phenols from their strength of bonding to AChE and concluded that there was an apparent preferred conformation of 3-trimethylammonium phenol which reacted with the active center of the enzyme. From studies of the cis-trans isomers of 2-dimethylaminocyclohexyl acetate methiodide[20] on AChE Krupka and Laidler[21] proposed a structure of the active center which places an acid site at a distance of 2.5 Å from the anionic site, the basic group of imidazole some 5 Å away, and the serine hydroxyl further from the anionic site than the basic group. This proposed arrangement of functional groups at the active center of AChE is consistent with the findings of Wilson and Quan.[19] Subsequently, however, Kay, *et al.*,[22] reported contrasting results on the original hydrolysis rates of *cis*-2-dimethylaminocyclohexyl acetate methiodide bringing doubt to the conclusions of Krupka and Laidler.[21] The proposed arrangement of functional groups at the active center of AChE by Krupka and Laidler[21] has not been adhered to in the recent literature. In fact, diagrams arbitrarily show the serine residue closer to the anionic site than the imidazole group of histidine. However, there has been no evidence in the literature to support this assumption.

In light of the above, a series of methyl-3-trimethylammonium phenols **2**, methyl ethers **3**, acetates **4**, and propionates **5** was studied for their activity on AChE to determine if there is a preferred conformation for hydrolysis of the ester group and to study the relative positions of the histidine and serine residues in relation to the anionic site. Space-filling models show that 2- and 6-methyl-3-trimethylammonium phenol derivatives can produce different preferred conformations of groups bound to the phenolic oxygen by rotation of bond τ_1. Acetyl and propionyl esters can have additional conformations by rotation of



2, R = H-
3, R = CH_3-
4, R = $CH_3C(=O)$-
5, R = $CH_3CH_2C(=O)$-

bond τ_2. In each instance, free rotation of the group attached to the oxygen substituent is hindered and located out of the plane of the aromatic ring (Figure 1). In the 2-methyl derivative the substituent would have a preferred conformation in which the substituent (*e.g.*, acetyl) is anticlinal to the aromatic carbons C(1) and C(2). A methyl group at C(6) would cause the oxygen substituent to be synclinal to the C(1), C(2) aromatic carbon atoms. Crystal structure studies on neostigmine bromide[23] (**6**) show that the equivalent group of atoms makes a torsion angle C(10)-O(1)-C(6)-C(5) of 148.2° supporting the out-of-plane location of the phenolic oxygen substituents. The N(2)-C(10)-O(1)-C(6) torsion angle in **6** was found to be antiperiplanar (−174°) and corresponds to the torsion



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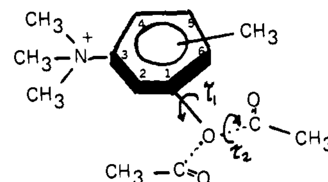
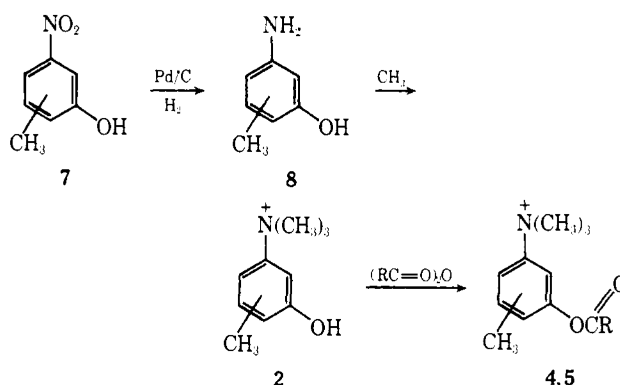


Figure 1. Preferred conformation of 2- and 6-methyl-3-trimethylammonium phenols and derivatives.

angle of C(7)-C(6)-O(1)-C(5) in ACh (**1**). Space-filling models show that rotation around the τ_2 bond is also restricted by a methyl group in the C(2) or C(6) position of the aromatic ring in compounds **4** and **5**. In each case an antiperiplanar conformation of the ester group is not sterically hindered and appears to be one of the more probable conformations for the acetyl and propionyl groups in these molecules. A methyl group at C-4 of the aromatic ring is not in a position to influence the conformation of the substituent on the phenolic oxygen.

The quaternary phenols and their esters were synthesized beginning with the nitrophenol **7**. The nitrophenol

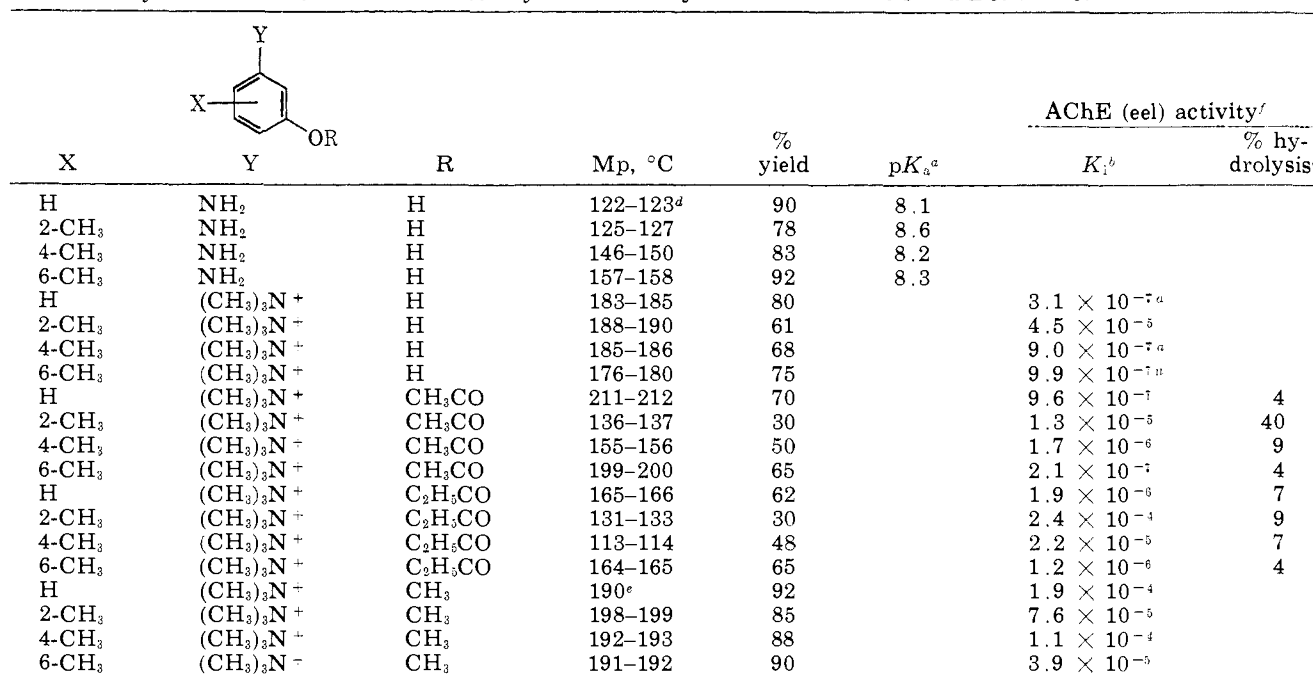


was reduced catalytically to the aniline derivative **8**. The quaternary phenol **2** was prepared by treatment of **8** with methyl iodide. The esterification of **2** by acid anhydrides produced the esters **4** and **5**. The methyl ethers **3** were prepared from the corresponding anisidine derivatives.

Results and Discussion

Table I summarizes the substrate and inhibitory activity of a series of 2-, 4-, or 6-methyl-substituted 3-trimethylammonium phenol acetate and propionate esters on AChE (*E. electricus*). The substrate activity of the esters was followed for a 6-min period and was linear. The K_i values were determined by following the hydrolysis of ACh in the presence of inhibitor. The K_i values determined for 3-trimethylammonium phenol (2.9×10^{-7}) and the 4-methyl (7.5×10^{-7}) and 6-methyl (1.0×10^{-7}) derivatives compared favorably with the values reported by Wilson and Quan.[19] The K_i for 3-trimethylammonium phenol methyl ether (1.9×10^{-4}) did not compare with the value of 7.5×10^{-6} reported.[19] All the compounds were found to be competitive inhibitors of AChE. The 6-methyl derivative is the best inhibitor and the poorest substrate in each series of esters. In contrast, the 2-methyl derivative is the best substrate in each series. Specificity of AChE for acetyl esters limits the 2-methyl acetate as the only bona fide substrate. 2-Methyl-3-trimethylammonium phenol acetate iodide was hydrolyzed as fast as ACh at a concentration of 2 m*M*. Hydrolysis of the 2-methyl derivative at 1.0×10^{-5} *M* was negligible. However, this compound was a weak competitive inhibitor of AChE ($K_i = 1.3 \times 10^{-5}$). The maximum velocity of 2-methyl-3-tri-

Table I. Physical Constants and AChE Activity of 3-Trimethylammonium Phenols and Derivatives



X	Y	R	Mp, °C	% yield	pK_a[a]	AChE (eel) activity[f] K_i[b]	AChE (eel) activity[f] % hydrolysis[c]
H	NH_2	H	122–123[d]	90	8.1		
2-CH_3	NH_2	H	125–127	78	8.6		
4-CH_3	NH_2	H	146–150	83	8.2		
6-CH_3	NH_2	H	157–158	92	8.3		
H	$(CH_3)_3N^+$	H	183–185	80		3.1×10^{-7}[a]	
2-CH_3	$(CH_3)_3N^+$	H	188–190	61		4.5×10^{-5}	
4-CH_3	$(CH_3)_3N^+$	H	185–186	68		9.0×10^{-7}[a]	
6-CH_3	$(CH_3)_3N^+$	H	176–180	75		9.9×10^{-7}[a]	
H	$(CH_3)_3N^+$	CH_3CO	211–212	70		9.6×10^{-7}	4
2-CH_3	$(CH_3)_3N^+$	CH_3CO	136–137	30		1.3×10^{-5}	40
4-CH_3	$(CH_3)_3N^+$	CH_3CO	155–156	50		1.7×10^{-6}	9
6-CH_3	$(CH_3)_3N^+$	CH_3CO	199–200	65		2.1×10^{-7}	4
H	$(CH_3)_3N^+$	C_2H_5CO	165–166	62		1.9×10^{-6}	7
2-CH_3	$(CH_3)_3N^+$	C_2H_5CO	131–133	30		2.4×10^{-4}	9
4-CH_3	$(CH_3)_3N^+$	C_2H_5CO	113–114	48		2.2×10^{-5}	7
6-CH_3	$(CH_3)_3N^+$	C_2H_5CO	164–165	65		1.2×10^{-6}	4
H	$(CH_3)_3N^+$	CH_3	190[e]	92		1.9×10^{-4}	
2-CH_3	$(CH_3)_3N^+$	CH_3	198–199	85		7.6×10^{-5}	
4-CH_3	$(CH_3)_3N^+$	CH_3	192–193	88		1.1×10^{-4}	
6-CH_3	$(CH_3)_3N^+$	CH_3	191–192	90		3.9×10^{-5}	

[a]See ref 19. [b]K_m for ACh was 2.2×10^{-4} *M*. [c]2 m*M* solution of ACh = 100%. [d]Merck Index, 8th ed. [e]A. Funke, J. Bagot, and F. Depriere, *C. R. Acad. Sci.*, **239**, 329 (1954). [f]At pH 7.5 and 25°.

Table II. Comparison of K_i Values of 2- and 6-Methyl-3-trimethylammonium Phenol and Acetate Iodides on AChE[a] with Different Substrates at pH 7.5

Substrate	K_i values at 25°: 2-Methyl-3-trimethylammonium phenol	2-Methyl-3-trimethylammonium phenol acetate	6-Methyl-3-trimethylammonium phenol	6-Methyl-3-trimethylammonium phenol acetate
Acetylcholine[b]	4.5×10^{-5}	1.3×10^{-5}	1.0×10^{-7}	2.1×10^{-7}
Acetylthiocholine[c]	4.5×10^{-5}	3.8×10^{-5}	2.1×10^{-7}	4.9×10^{-7}
Phenyl acetate[d]	8.0×10^{-5}	1.4×10^{-5}	2.9×10^{-7}	5.6×10^{-7}

[a]Worthington Biochemical Corp., Freehold, N. J. [b]$K_m = 1.92 \times 10^{-4}$; $V_{max} = 1.50 \times 10^{-6}$ *M*/min. [c]$K_m = 1.86 \times 10^{-4}$; $V_{max} = 1.66 \times 10^{-6}$ *M*/min. [d]$K_m = 1.58 \times 10^{-3}$; $V_{max} = 2.02 \times 10^{-6}$ *M*/min.

methylammonium phenol acetate was 0.477×10^{-6} mol/min compared to a maximum velocity of 1.50×10^{-6} mol/min for ACh. Since the K_i value for the 2-methyl derivative shows that it binds about ten times more than ACh, its affinity for the enzyme cannot be used to explain the low hydrolysis rate. The lower V_{max} can be attributed to a slower acetylation rate, hence, a lower concentration of acetylated enzyme. Under these conditions the formation of acetylated enzyme becomes rate limiting.[10] The 2-methyl derivatives are approximately 100 times weaker inhibitors than the corresponding 6-methyl analogs. The difference in activity toward AChE by the acetyl and propionyl esters can be attributed to a preferred deployment of the ester group by rotation around bond τ_1 in Figure 1 caused by the aromatic methyl substituent at position 6. This is shown in Figure 2. The activity of the 4-methyl substituent does not differ significantly from the activity of the esters without the aromatic methyl substituent.

The inhibitory activity of the 3-trimethylammonium phenol acetates and propionates can be attributed to the binding of the quaternary ammonium group and the bonding interactions of the aromatic ring. The K_i for phenyltrimethylammonium iodide[19] is 3.8×10^{-5} and is of the same magnitude as the weaker inhibitors. The greater potency of the 6-methylacetyl derivative ($K_i = 1.1 \times 10^{-7}$) indicates additional binding modes involving the ester function. Binding of the ester group is not hydrophobic in nature. The AChE activity of a series of methyl-substituted 3-trimethylammonium phenol methyl ethers **5** shows very little change in potency between the 2- and 6-methyl derivatives (Table I). The difference in binding between the 2-methyl- ($K_i = 1.3 \times 10^{-5}$) and 6-methyl- ($K_i = 2.1 \times 10^{-7}$) 3-trimethylammonium phenol acetates must be due to the bonding interactions of the carbonyl group of the acetate. While the 2-methyl group induces a conformation which causes hydrolysis of the ester function, the 6-methyl group produces a conformation which binds reversibly to the enzyme without causing hydrolysis of the acetyl group. It can be seen from a comparison of the K_i and hydrolysis data that the position of the methyl substituent on the aromatic ring can influence substrate and inhibitory activity.

Figures 2a and 2b indicate how the different conformations of the acetyl group induced by methyl aromatic substitution can produce hydrolysis of the 3-trimethylammonium phenol acetate or inhibition of ACh hydrolysis by interacting with different groups at the esteratic site. In Figure 2b the 2-methyl substituent deploys the acetyl group away from the imidazole group in the esteratic site. Activation of the serine residue by the general base-catalyzed action of the imidazole group of the histidine residue results in a nucleophilic attack by the serine residue on the acetyl group of the 3-trimethylammonium phenol acetate to initiate hydrolysis. Rotational hindrance about

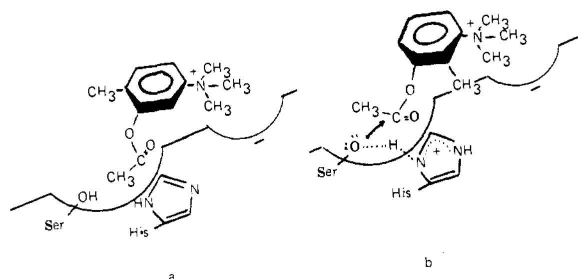


Figure 2. (a) Binding of 6-methyl-3-trimethylammonium phenol acetate to the AChE active site to cause inhibition. (b) Binding of 2-methyl-3-trimethylammonium phenol acetate to the active site of AChE to produce hydrolysis.

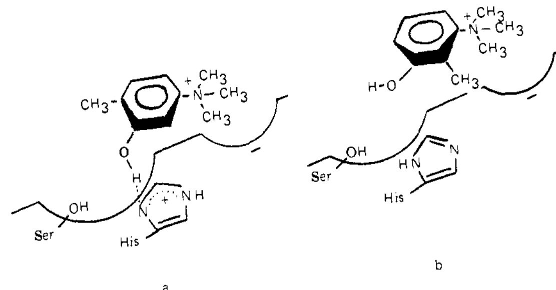
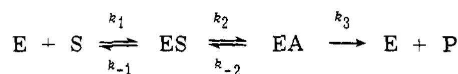


Figure 3. (a) Binding of 6-methyl-3-trimethylammonium phenol to AChE. (b) Binding of 2-methyl-3-trimethylammonium phenol to AChE.

the τ_2 bond shown in Figure 1 may also allow for a favorable orientation of the carbonyl group relative to the serine oxygen. In Figure 2a the 6-methyl substituent deploys the acetyl group toward the imidazole group in the esteratic site. The acetyl group interferes with the activation of the serine residue and can undergo bonding interactions between the nonbonding pair of electrons on the imidazole nitrogen and the electron-deficient carbonyl group of the acetyl ester. These additional interactions may account for the added potency of the 6-methyl esters in general. In a similar fashion the improved potency of the 6-methyl-3-trimethylammonium phenol (K_i = 1.0 × 10^{-7}) over the 2-methyl derivative (K_i = 4.5 × 10^{-5}) can be explained. Figure 3b shows how the 6-methyl aromatic substituent will tend to project the phenolic proton toward the imidazole group in the esteratic site and compete with the serine hydroxyl to protonate the imidazole group. Figure 3a shows the interaction of 2-methyl-3-trimethylammonium phenol with the receptor area where the phenolic proton is projected away from the imidazole group preventing an interaction between the two functional groups. The pK_a values for all phenols do not vary significantly (pK_a = 8.1–8.6) indicating that all were present essentially in the same ratio of dissociated to undissociated species.

The inhibition of the AChE hydrolysis of acetylthiocholine (ACSH) by the 2- and 6-methyl-3-trimethylammonium phenols and acetates was followed according to the method of Ellman, *et al.*[24] The K_i values of the trimethylammonium phenols and derivatives are of the same magnitude (Table II) as those determined from the inhibition of ACh hydrolysis. Since this assay measures the rate of formation of thiocholine and therefore does not depend on the deacetylation rate (k_3) in the scheme below, it can be concluded that the phenols and acetates do not inhibit this step. Moreover, the plot of S/V *vs.* S gave identical slopes supporting a competitive type of inhibition.



$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_3} \mathrm{E} + \mathrm{P}$$

The inhibition of the rate of phenyl acetate hydrolysis by AChE by the trimethylammonium phenols and acetates was also studied. The data are summarized in Table II. These K_i values do not differ significantly from those using ACh as the substrate. However, the rate of AChE-catalyzed hydrolysis of phenyl acetate in the absence or presence of inhibitors is linear and differs in this respect from the nonlinear rates observed with ACh and ACSH. The nonlinear nature of ACh and ACSH hydrolysis by AChE can be explained by a nonequilibrium condition in which the concentration of EA is decreasing in eq 1 due to the formation of choline. The value of ES remains constant because of the high concentration of substrate compared to the amount of enzyme. Carrying out the hydrolysis in the presence of relatively high concentrations of choline (1.0 × 10^{-3}) stabilizes this equilibrium into a steady state and results in a linear hydrolysis rate of both ACh and ACSH when the rate is measured by the formation of acetic acid as in the pH-Stat method. It is not likely that choline inhibits at the deacetylation step to cause the change to a linear rate. Trimethylammonium, a known inhibitor of the deacetylation step[12] (k_3), does not

$$K_2 = k_2/k_{-2} = [\mathrm{EA}][\mathrm{choline}]/[\mathrm{ES}] \quad (1)$$

alter the linearity of the hydrolysis rates of either substrate. The data are summarized in Figures 4 and 5. Further, to attest that the locus of inhibition of choline and trimethylammonium is different in the enzymatic sequence inhibition of ACSH hydrolysis at 3.85 × 10^{-5} M was measured by the Ellman method. Trimethylammonium at a concentration of 3.85 × 10^{-4} M was not an inhibitor while choline at the same concentration inhibited the reaction 100%. Since none of the 2- or 6-methyl-3-trimethylammonium phenols or their acetates alter the linearity of phenyl acetate hydrolysis, it can be assumed that these inhibitors act on the free enzyme competing with each of the three substrates, ACh, ACSH, and phenyl acetate, for bonding interactions on the active site of AChE.

In conclusion, the data shown are consistent with the interpretations of Krupka and Laidler[21] placing the imidazole group closer to the anionic site than the serine residue. It is apparent that in the 3-trimethylammonium phenol acetate series described above the conformation of the acetyl group can determine whether or not hydrolysis by AChE can occur. It can be suggested that the normal substrate, ACh, may undergo different binding modes, especially involving the acetyl group in one binding mode in which it may be hydrolyzed and another where it may not.

Experimental Section

The experimental procedure for one compound of each series is described below. The physical constants are summarized in Table I. Melting points were taken on a Mel-Temp apparatus and are uncorrected. Microanalyses were carried out by M-H-W Laboratories, Garden City, Mich.

2-, 4-, or 6-Methyl-3-trimethylammonium Phenol Iodides (2). Methyl-3-nitrophenol (10 g, 0.08 mol) in 100 ml of EtOH was shaken with 2 g of Pd/C (10%) on a low-pressure Parr hydrogenator at 60 lbs psig initial pressure, until it consumed the theoretical amount of hydrogen. The catalyst was removed by filtration and the solvent removed on a rotary evaporator. The residue was recrystallized to give the methyl-3-aminophenol (8). The intermediate could be purified by recrystallization from *i*-PrOH. *Anal.* (C_7H_9O) C, H. Methyl-3-aminophenol (10 g, 0.08 mol) was refluxed with 40 ml of methyl iodide and 8 g of Na_2CO_3 in 50 ml of MeOH overnight. The Na_2CO_3 was filtered and the filtrate removed of its solvent on a rotary evaporator. The residue was refluxed with 40 ml of 47% hydriodic acid for 1 hr and the reaction then evaporated to dryness. The residue was recrystallized from

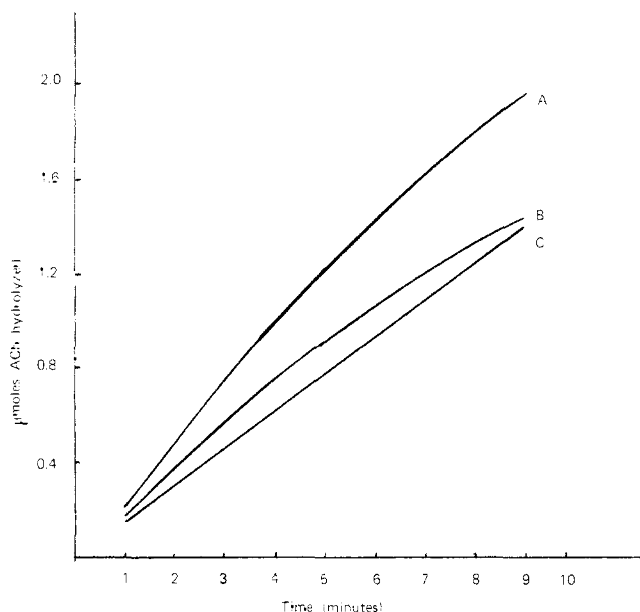


Figure 4. (A) AChE hydrolysis of ACh, 5.0×10^{-4} *M*; (B) AChE hydrolysis of 5.0×10^{-4} *M* ACh + 1.0×10^{-3} *M* trimethylammonium hydrochloride; (C) AChE hydrolysis of 5.0×10^{-4} *M* ACh + 1.0×10^{-3} *M* choline iodide.

i-PrOH to produce pure methyl-3-trimethylammonium phenol iodide (**2**). *Anal.* ($C_{10}H_{16}NOI$) C, H.

2-, 4-, or 6-Methyl-3-trimethylammonium Phenol Acetate Iodides (4). Methyl-3-trimethylammonium phenol iodide (1 g, 3.4 mmol) was dissolved in the least amount of boiling acetonitrile and 5 ml of acetic anhydride added. After keeping the solution warm for 5 min the mixture was allowed to stand at room temperature for 1 hr. Anhydrous ether was added to precipitate the product. The product was purified by recrystallization from *i*-PrOH. *Anal.* ($C_{12}H_{18}NO_2I$) C, H.

2-, 4-, or 6-Methyl-3-trimethylammonium Phenol Propionate Iodides (5). Methyl-3-trimethylammonium phenol iodide (1 g, 3.4 mmol) was dissolved in the least amount of boiling acetonitrile. To this 5 ml of propionic anhydride was added and the mixture refluxed for 1 hr. Anhydrous ether was added to the cooled reaction mixture. The precipitated crude product was purified by recrystallization from *i*-PrOH. *Anal.* ($C_{13}H_{20}NO_2I$) C, H.

2-, 4-, or 6-Methyl-3-trimethylammonium Phenol Methyl Ether Iodides (3). Methyl-3-nitroanisole (10 g, 0.06 mol) in 100 ml of EtOH was shaken with 2 g of Pd/C on a low-pressure hydrogenator at 60 psig initial pressure until the theoretical amount of hydrogen was consumed. The crude methyl-*m*-anisidine obtained after removing the catalyst and solvent was dissolved in 80 ml of acetonitrile and mixed with 16 ml of 37% formaldehyde solution and 0.06 mol of sodium cyanoborohydride. Glacial acetic acid (2 ml) was added and stirring continued for 30 min more. The solvent was evaporated and 100 ml of 10% KOH solution was added. The mixture was extracted several times with ether and the crude 3-methyl-*N*,*N*-dimethylaminophenol methyl ether purified by distillation under vacuum: 2-CH_3, bp 57° (0.1 mm); 4-CH_3, 61° (0.15 mm); 6-CH_3, 73° (0.7 mm). The quaternary compound was prepared by dissolving the tertiary amine in anhydrous ether and adding methyl iodide. The compound which precipitated from the ether solution on standing overnight was purified by recrystallization from *i*-PrOH (60% overall yield). *Anal.* ($C_{11}H_{18}NOI$) C, H.

Enzymology. Inhibition of enzyme-catalyzed hydrolysis of acetylcholine perchlorate by the methyl-substituted 3-trimethylammonium phenols and their derivatives was determined at pH 7.5 by titration of the liberated acetic acid with 0.01 *N* NaOH solution at 25° using a Sargent pH-Stat. Substrate concentrations varying from 1.0 to 5.0×10^{-4} were used in a medium consisting of 0.02 *M* $MgCl_2$ and 0.1 *M* NaCl, with 0.1 unit of eel acetylcholinesterase. Inhibitor concentrations were varied using amounts which decreased the velocity of the 5.0×10^{-4} *M* ACh hydrolysis rate by no more than 10%. The reaction rates were measured at 25° for 3 min following addition of AChE, allowing 1 min for equilibration and using the last 2 min for the data. The plots were linear. Following the reaction for a longer period of time or using higher concentrations of enzyme decreased the linearity of the reaction rate. A graphic plot of S/V *vs.* S provided K_m and K_i values. The data were also calculated by the method of least squares on a Digital PDP-8/L laboratory computer. The K_m for 2-methyl-3-trimethylammonium phenol acetate was similarly measured on a pH-Stat for 5 min. The rate plot was linear. Comparisons of hydrolysis rates were carried out at 2×10^{-4} *M* substrate concentration over a 5-min period.

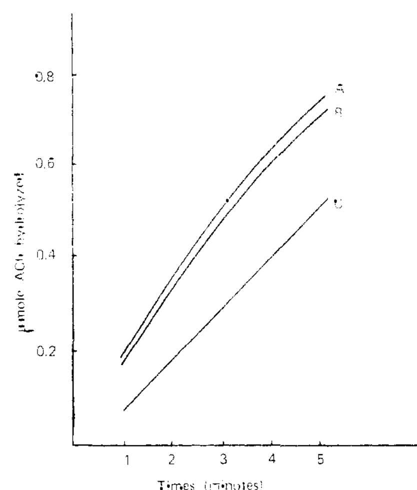


Figure 5. (A) AChE hydrolysis of 1.0×10^{-5} *M* ACSH + 1.0×10^{-3} *M* trimethylammonium hydrochloride; (B) AChE hydrolysis of 1.0×10^{-5} *M* ACSH; (C) AChE hydrolysis of 1.0×10^{-5} *M* ACSH + 1.0×10^{-3} *M* choline iodide.

Inhibition studies using phenyl acetate as a substrate were carried out as above. A 1.0-5.0×10^{-3} *M* solution of phenyl acetate in 2% EtOH was used. An ACh perchlorate solution containing 2% EtOH did not significantly differ in hydrolysis rate from the 100% aqueous substrate solution. Rates over a 5-min period were linear. The K_m and K_i values were determined as above.

The rate of acetylthiocholine iodide hydrolysis at pH 7.5 was determined by the method of Ellman, *et al.*[24] The velocity was followed on a Cary 15 spectrophotometer at 412 nm for 60 sec after allowing 15 sec for stirring and stabilization of the spectrophotometer. The rates were not linear. The acetylthiocholine was dissolved in 0.1 *M* PO_4 buffer, pH 7.5, and concentrations from 0.385 to 1.925×10^{-4} *M* were used in the presence of 1.0 ml of Ellman's reagent and 0.1 unit of AChE in 0.1 *M* PO_4 buffer in a total volume of 2.6 ml in the cuvette.

The linearity of the hydrolysis rates by the pH-Stat method was determined over a period of 9 min. A 5.0×10^{-4} *M* concentration of ACh was measured in the presence or absence of 1.0×10^{-3} *M* concentrations of either choline or trimethylammonium hydrochloride. The acetylthiocholine rate was measured at a concentration of 1.0×10^{-5} *M* for a period of 5 min in the presence or absence of choline and trimethylammonium inhibitor concentrations described.

Acknowledgment. This work was supported in part by Grant No. NS09088, National Institute of Neurological Diseases and Stroke.

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Inhibition of Mammalian Dihydrofolate Reductase by Selected 2,4-Diaminoquinazolines and Related Compounds

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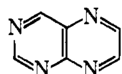
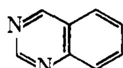
A variety of quinazolines, pyrido[2,3-*d*]pyrimidines, and pteridines were evaluated as inhibitors of dihydrofolate reductase obtained from rat liver and L1210 mouse leukemia cells. Certain of the 2,4-diaminoquinazolines bearing benzylamino or anilinomethyl substituents at position 6 proved to be capable of inhibiting enzyme activity, from both sources, to a degree (ID_{50} 10^{-8}–10^{-9} *M*) comparable to that observed for the antineoplastic agent methotrexate. In the quinazoline series, the presence of primary amino groups at positions 2 and 4 was essential for high inhibitory potency. Neither introduction of halogen, methyl, or hydroxyl substituents anywhere into the phenyl ring of 2,4-diamino-6-benzylaminoquinazoline (1) nor replacement of the phenyl substituent with heteroaromatic functions (pyridyl, furyl, thienyl) markedly affected inhibitory potency. On the other hand, the presence of a chloro substituent at position 5 of 1 was associated with a pronounced increase in inhibitory potency. Interestingly, 2,4-diamino-6-piperidinoquinazoline was found to possess modest activity ($ID_{50} = 2 \times 10^{-6}$ *M*) as an inhibitor of both enzyme systems studied, and insertion of a 2-ethyl function into the piperidine ring of this compound produced an approximately 50-fold increase in inhibitory potency. 2,4-Diaminopyrido[2,3-*d*]pyrimidines investigated were as potent as the analogous quinazolines as inhibitors of dihydrofolate reductase activity from both sources. A series of 2,4-diaminopteridines characterized by a progressive increase in the size of an alicyclic ring fused to positions 6 and 7 of the pteridine nucleus was evaluated against both enzyme systems. A consistent trend of increased inhibitory potency with increasing ring size was observed and is exemplified by the lower ID_{50} observed for the compound bearing a 12-membered ring ($ID_{50} = 2.1 \times 10^{-7}$ *M*) compared with that for the compound bearing a six-membered ring ($ID_{50} = 1.9 \times 10^{-4}$ *M*).

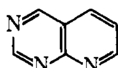
Many compounds possessing the 2,4-diaminopyrimidine nucleus, including a variety of pteridines, can function as inhibitors of dihydrofolate reductase [5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase E.C. 1.5.1.3.] from bacterial,[1-3] protozoal,[4-7] and mammalian sources.[8-10] The types of compounds which have been synthesized as potential inhibitors of this enzyme system include quinazolines and pyrido[2,3-*d*]pyrimidines, which differ from pteridine by, respectively, the absence of two and one nitrogen atoms in the ring fused to the pyrimidine nucleus.



pteridine



quinazoline



pyrido[2,3-*d*]pyrimidine

Such "deaza" pteridines have been found to exhibit a variety of important pharmacological actions which frequently can be ascribed to their interference with folic acid metabolism.[1,11-18] Elslager and his colleagues have reported recently[19-22] on the antiprotozoal and antibacterial activity of a series of selected 2,4-diaminoquinazolines and pyrido[2,3-*d*]pyrimidine analogs. We have been interested, for several years, in investigating the efficacy of a variety of 2,4-diamino heterocyclic compounds as inhibitors of dihydrofolate reductase from different sources in an effort to establish relationships between chemical structure and inhibitory potency. It is hoped that studies of this type may provide guidelines for synthesis of new antifolate agents and may identify compounds which merit examination as antineoplastic, antibacterial, and antiprotozoal agents of potential clinical utility. The present report describes results of studies of the inhibitory potency, evaluated against dihydrofolate reductase obtained from rat liver and L1210 mouse leukemia cells, of a series of 2,4-diaminoquinazolines, some related pyrido[2,3-*d*]pyrimidines, and a series of 2,4-diaminopteridines characterized by the presence of an alicyclic ring fused to positions 6 and 7 of pteridine nucleus.

Experimental Section

Acetone powders prepared from rat liver (Sigma Chemical So., St. Louis, Mo.) and L1210 mouse leukemia cells (kindly provided by Dr. J. A. R. Mead, National Cancer Institute, Bethesda, Md.) were extracted with pH 7.0 phosphate buffer (Na_2HPO_4 and KH_2PO_4 in a molar ratio of 6:4); centrifugation of the mixture yielded a supernatant solution which was used as the enzyme source. The assay system used was a slight modification of that described by Burchall and Hitchings[1] involving measurement of the decrease in absorbance at 340 nm catalyzed by dihydrofolate reductase in a system (1.0 ml total volume) composed of NADPH (1×10^{-4} *M;* PL Biochemicals, Milwaukee, Wis.), 2-mercap-