small increase in potency from desmethyl- to (+)-α-prodine.

Recently, data have been obtained[16-18] on the potencies of 3-allyl- and 3-propylprodine derivatives which confirm this idea. The potency of the (+)-α isomers is little affected by chain lengthening but become 40 times more potent by the presence of the allyl group. However, the potency of the (+)-β isomer is severely diminished when allyl or n-propyl is substituted for the methyl. These results are indicative of a "tighter" fit to a methyl group in the β rather than α position. Given the importance and inflexibility of τ_2, changes of the 3-substituent could also easily affect the minimum energy conformations of τ_2 and hence cause variations in the relative potencies, in addition to the effect of changes of τ_1 and of the substituent itself. Work is in progress investigating conformational effects and electronic distribution in these compounds.

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Supplementary Material Available. Figures 8–15 will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.50 for photocopy or $2.50 for microfiche, referring to code number JMED-75-1051.

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A Hydrophobic Binding Site in Acetylcholinesterase

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The dissociation constants have been determined and compared for a series of reversible, noncovalent inhibitors of eel acetylcholinesterase that are structurally related to the very potent inhibitor, 1,2,3,4-tetrahydro-9-aminoacridine (THA). It is concluded that there exists on the enzyme protein, closely adjacent to the anionic subsite, a conformationally flexible, hydrophobic area which tends readily to assume a near planar form. The dimensions of this area are unknown, but it is adequate in size to fully accommodate THA. It is this area, acting conjointly with the adjacent anionic subsite, which provides the attraction for THA and related inhibitors. Uv absorbance maxima and pK_a values are reported for many of the compounds.

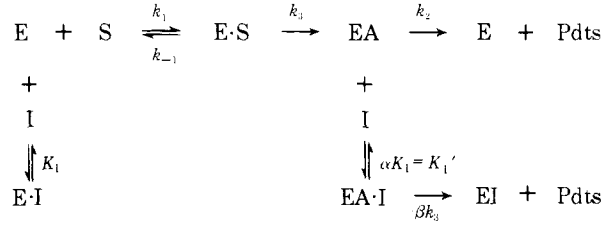
To rationally design inhibitors, reactivators, affinity probes, and other perturbing agents for acetylcholinesterase, one requires a knowledge of the surface of the enzyme in the vicinity of its active site. In charting the surface of a macromolecule it is basically assumed that a probing small molecule exhibits a degree of complementarity to its recognition site on the macromolecule. Hence, correlatable changes in small molecule structure with physical or chemi-

cal properties of the macromolecule will provide information that can be interpreted in terms of specific topography. While most studies of this sort are nonrigorous because of the somewhat simplistic nature of this assumption, probing small molecules have provided useful information concerning the general characteristics of protein binding sites. The results obtained in this study reflect interactions between the enzyme and a rather large and diverse group of compounds. The patterns are reasonably self-consistent. We believe that they provide information concerning the characteristics of a limited area on the surface of the enzyme that includes the anionic subsite.

AChE† is subject to reversible (noncovalent) inhibition by large numbers of organic compounds.[1] Most that have been reported are quaternary ammonium compounds or amine salts. Among the most effective inhibitors are bisquaternary compounds, where binding to the enzyme varies considerably with the distance between the charged groups and to a somewhat lesser extent with the nature of the substituents.[2-6] According to existing evidence bisquaternary compounds bind, in chelate-like fashion, at two negatively charged centers on the enzyme protein.[7] Compounds of an entirely different type which are also powerful reversible inhibitors of AChE are the charged fused ring heterocyclic amine (salt) THA[8] and the related *N*-methylacridinium iodide.[7] Their very low dissociation constants suggest a great deal of specificity in binding to the enzyme. By relating modifications in structure of heterocyclic amines with affinity for the enzyme, we have sought information concerning the properties of this binding site. From other studies there was available a series of such compounds. Their examination represents the substance of this report.

As a matter of convenience phenyl acetate was chosen as the substrate rather than the natural substrate acetylcholine (ACh) so that the reactions could be monitored spectrophotometrically. In 1966, Krupka[9] showed that phenyl acetate and acetylcholine are hydrolyzed at the same site on the enzyme and our interpretations are based upon this premise. Krupka[10] showed that most inhibitors of AChE act in a mixed fashion (with k_3 rate-limiting substrates; i.e., acetylcholine and phenyl acetate) (Scheme I), having both

Scheme I



a competitive component which reflects binding to the native enzyme and a noncompetitive component which results from binding at one or more steps in the hydrolysis sequence beyond that of enzyme–substrate interaction. In Scheme I,[10] there are two modifier constants, α and β. The former relates the inhibitor dissociation constants of native and acetyl enzyme and the latter provides a measure of the completeness of the inhibition produced by the binding of the inhibitor, I, to the acetyl enzyme, EA. Such an analysis requires a considerable quantity of highly precise data. For our purposes a more simple analysis was adequate. The data were analyzed to yield the value of K_I, the dissociation constant for competitive inhibition, and α', which is numerically equal to K_I'/K_I ($=\alpha$) when $\beta = 0$. Although α' does not provide a precise value for K_I', since the value of β often lies between 0 and 1,‡ it gives a useful estimate of its order of magnitude. In this work, it provides information relating to the question of whether these very bulky and powerful inhibitors bind over the full active site; i.e., at both the anionic and esteratic subsites, or simply at a nearby adjacent area.

Materials. All compounds were purchased commercially or prepared as previously reported.[11,12] The AChE was electric eel, purified, obtained from Worthington, Code ECHP. Ten thousand units were dissolved in 1.6 ml of a solution containing 0.225 *M* KCl, 0.25% gelatin, and 0.02% NaN_3 (KCl/gel) to yield a concentrate of approximately 2×10^{-5} *M* in active sites.[13] It was stored at −20°. As required, it was further diluted with KCl/gel. The diluted solutions were stored at 4°. Both concentrated and diluted samples are quite stable. No change in activity was observed over a period of at least 6 months.

Methods. Inhibition. All measurements were made on a Zeiss PMQ spectrophotometer, fitted with a thermostated cell compartment and recorded on a Beckman log-linear recorder, Model 1005. To a cuvette containing 2 ml of a solution of 1 μl. of phenyl acetate per milliliter (concentration, 8.0×10^{-3} *M*) in 0.1 *M* MES, pH 6.52, preequilibrated in the cell holder at 25° for 10–15 min, there were added, with rapid mixing, solutions of inhibitor and then enzyme in microliter quantities. A full record (progress curve) was made of the absorbance change at 284.0 nm. Sufficient enzyme was used to obtain complete reaction in 6–10 min. Each daily series of runs included two controls (without inhibitor) and two to four measurements with graded concentrations of each inhibitor. Analysis of the data was made in a manner similar to that of Balcom and Fitch.[14] At any point in the progress curve, the absorbance corresponds to the percentage of substrate hydrolyzed, and the slope corresponds to the reaction velocity. For computation of the former, we need know only the absorbance of the ester and of the hydrolysis products. These are measured directly in each run, i.e., absorbance before addition of enzyme and at the end point, respectively. To estimate the velocity, we took the slope of the chord joining the two points on either side of the point in question. Generally, there was a minimum of 20 data points taken for each analysis. These were chosen at equal time intervals over the first 60–70% of reaction. Beyond this point, the absorbance differences became small and produced much scatter in the subsequent analyses. Lineweaver–Burk plots ($1/v$ vs. $1/s$) gave straight lines in several runs. The values of V_m and K_m were calculated using the procedure of Wilkinson.[15] From these values, K_I and α' were computed from eq 2 and 4, which were derived from Webb's[16] equation for mixed inhibition.

From Webb[16] (Figure 5-6B)

$$(K_m^0 E_T^0/V_m^0)(1 + I/K_I) = K_m^I E_T^I/V_m^I \qquad (1)$$

where V_m^0 and V_m^I are maximum velocities in the absence and presence of inhibitor, respectively, and K_m^0 and K_m^I are the corresponding Michaelis constants. In some runs, a higher concentration of enzyme was required in the presence of inhibitor than in its absence; E_T^0 and E_T^I reflect these concentrations. Algebraic manipulation of eq 1 gives eq 2.

$$K_I = I\left(\frac{K_m^0 E_T^0}{V_m^0 E_T^I}\right) \Big/ \left(\frac{K_m^I}{V_m^I} - \frac{K_m^0 E_T^0}{V_m^0 E_T^I}\right) \qquad (2)$$

†Abbreviations: acetylcholinesterase, AChE, acetylcholine acetylhydrolase, 3.1.1.7; MES, 2-(*N*-morpholino)ethanesulfonic acid; TMA, tetramethylammonium iodide; THA, 9-amino-1,2,3,4-tetrahydroacridine; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; gallamine, 1,2,3-tris(2-triethylammonioethoxy)benzene triiodide.

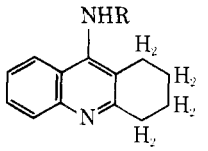
‡See ref 28. Our symbol β corresponds to Krupka's symbol a in the referenced paper.

Table I. Inhibition of Acetylcholinesterase by Reference Compounds

Compd	K_I, M This work	K_I, M Reported
TMA[a]	2.2×10^{-3}	2.85×10^{-3} [b]
Gallamine	2.6×10^{-4}	1.7×10^{-4} [c]

[a]Tetramethylammonium iodide. [b]Reference 28. [c]pH 7.15, 0.225 M KCl, initial slopes method, J. W. Amshey, Jr., unpublished results.

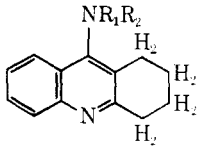
Table II. Inhibition of Acetylcholinesterase by N-Monosubstituted 1,2,3,4-Tetrahydro-9-aminoacridines



Compd no.	R	K_I,[a] $M \times 10^8$	pK_a
1	H (THA)	5	9.85
2	CH_3	8	
3	C_2H_5	20	
4	n-Pr	30	
5	n-Bu	20	
6	n-Pent	7	
7	Allyl	14	9.42
8	Cyclohexyl	13	
9	Benzyl	15	9.26
10	Phenethyl	4	9.27
11	β-Aminoethyl	32	7.40, 9.32[b]

[a]K_I is the competitive component of inhibition, pH 6.52. [b]Titrimetric in 0.04 M KCl; protonation of aliphatic amino group.

Table III. Inhibition of Acetylcholinesterase by N,N-Disubstituted 1,2,3,4-Tetrahydro-9-aminoacridines



Compd no.	R_1	R_2	K_I, $M \times 10^8$	pK_a
1	H	H	5	9.85
12	CH_3	CH_3	57	
13	$-(CH_2)_2O(CH_2)_2-$		114	7.50
14[a]	CH_3	CH_3	170	

[a]10-Methiodide.

From Webb[16] (Figure 5-6B)

$$E_T^I/V_m^I = (E_T^0/V_m^0)(1 + I/\alpha' K_I) \quad (3)$$

Therefore

$$\alpha' = (I/K_I)(V_m^I/[V_m^0(E_T^I/E_T^0) - V_m^I]) \quad (4)$$

In computing K_m and V_m from progress curves, it is necessary to correct for the effects of product if it is an inhibitor or if the reaction is reversible. With AChE the reaction is substantially irreversible. The products of phenyl acetate hydrolysis do inhibit, albeit to a relatively minor extent.[17]

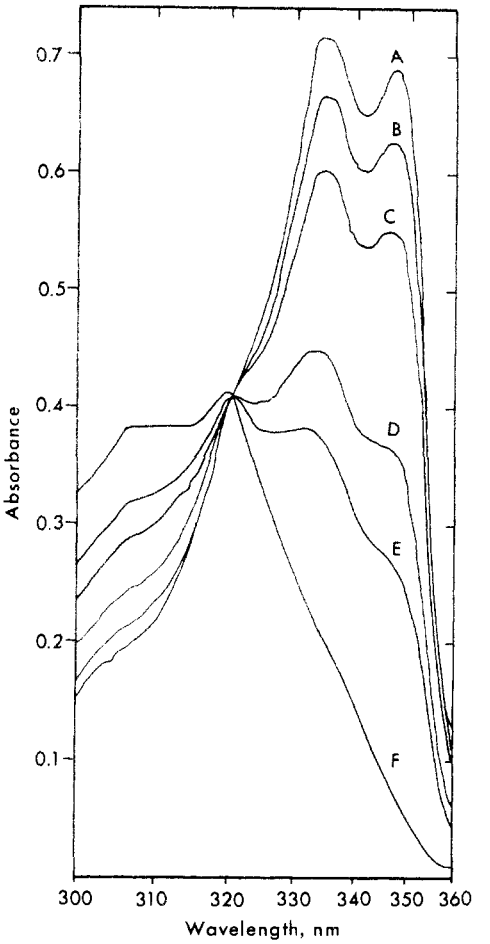


Figure 1. N-Allyl-1,2,3,4-tetrahydro-9-aminoacridine (7): concentration, 5.55×10^{-5} M. A, 0.1 M HCl; B, pH 8.49; C, pH 8.94; D, pH 9.47; E, pH 9.64; F, 0.1 M NaOH.

Since product inhibition is minor and a single substrate concentration was used with all of the inhibitors, product inhibition may be ignored. The results in Table I demonstrate the adequacy of this simplification. Further, inhibitor concentrations were varied over a 4–10-fold range in replicate determinations, yet gave closely agreeing values of K_I (e.g., with compound **7**, [I] = 2.5×10^{-8}–2.5×10^{-7} M, K_I values are 1.08, 1.09, 1.18, 1.20, 1.32, 1.42, and 2.65×10^{-7} M).

pK_a Determination. The pK_a values are calculated from spectrophotometric recordings obtained on a Beckman DK-2 using thermostated cell holders, 25°. Recordings were made over a sufficient spectral range to include the isobestic point to ensure that a single equilibrium was being measured. Measurements were made in 0.1 M buffer, generally BICINE, over a pH range of 1.25–1.5 units and also in 0.1 M HCl and 0.1 M NaOH. A typical example is given in Figure 1. The pK_a values were calculated using eq 5 from each of four spectra which were reasonably distributed between the "acidic" and "basic" extremes and the average was reported

$$pK_a = pH + \log [A_0 - B_T]/[A_T - A_0] \quad (5)$$

where A_T and B_T represent respectively absorbance at the selected wavelength for the acidic and basic solutions and A_0, the absorbance at intermediate pH.

Results

The progress curve procedure for data acquisition is very rapid, permitting several compounds to be evaluated daily.

Results with two reference compounds are given in Table I. Conformity is adequate with those obtained by the more conventional initial slopes method.

Presence and Site of Charge. 4-Aminoquinolines and related compounds are dibasic and accept two protons, one at the ring nitrogen atom and the other at the substituent amino group. These occur in widely separated steps. The first adds to the ring nitrogen in the "near neutral" pH range, whereas the dication forms only in concentrated acid solution.[18] As indicated in Figure 1, with compound **7** which is typical, there is a single isobestic point which shows equilibrium between two species, i.e., $BH^+ \rightleftharpoons B + H^+$. The protonated form, BH^+, absorbs at a longer wavelength than does the unprotonated B, which is consistent with binding of the proton at the ring nitrogen atom.[19,20,§] With the exception of the morpholino derivative, compound **13**, and of the β-aminoethyl derivative, compound **11**, all of the THA related compounds have pK_a values above 9 (Tables II–IV). However, even these less basic compounds are extensively ring protonated (75%) at the pH of this study. The close similarity in spectra between compounds **12** and **14** indicates that the third methyl group in the latter compound is located at the ring nitrogen atom.

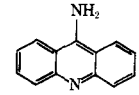
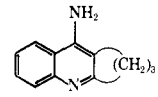
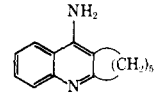
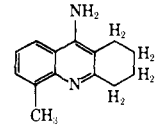
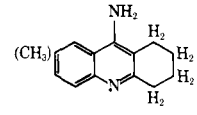
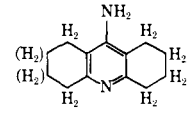
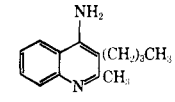
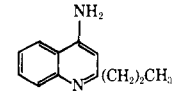
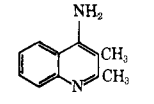
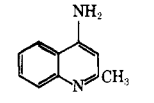
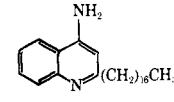
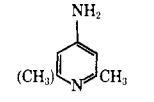
Pyrazine is a very weak base, having a pK_a of 0.65.[21] Hence, the substituted pyrazines (**30–32**) are certainly unprotonated at pH 6.52. Similarly, the amides (**27, 28**) are substantially unprotonated at this pH.[&] Finally, by analogy with pyridine-4-carboxylic acid,[**] compound **27** may be assumed to exist as the zwitterion at pH 6.52 (Table V).

Spectra. The absorbance spectra of THA, the N-monosubstituted and ring-substituted 1,2,3,4-tetrahydro-9-aminoacridines, and the corresponding quinolines are closely similar (Table VI). With each there is a single maximum for the unprotonated compound which ranges in wavelength from 304 to 326 nm; occasionally, the peak is flattened or split. Upon protonation, the maximum is split and shifted to a somewhat higher wavelength with an increase in absorbance. Disubstitution on the amino nitrogen atom as in compounds **12–14** causes a reduction in intensity or a complete loss of the high-energy acid band and displacement of the second band to longer wavelength. These changes may be ascribed to steric factors which prevent the latter compounds from achieving molecular planarity and, hence, reduce interaction with the heterocyclic rings.[22,23] Compound **20**, octahydro-9-aminoacridine, has the spectrum of a typical alkyl-substituted pyridine such as compound **26**.

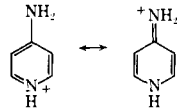
Competitive Inhibition. The effect of amino monoalkylation is seen in Table II. There are noticeable patterns relating structure with activity, such as the increase in dissociation constant upon monoalkylation, the increase to a maximum when R reaches the C_2 to C_4 range (the phenyl and cyclohexyl rings have a length corresponding approximately to C_3), followed by a decrease with further extension, and the increase of the dissociation constant with the addition of a positively charged group (compound **11**) at the end of the chain. However, these are all relatively minor. Despite the very considerable increase in bulk at the amino nitrogen atom, there is only a sixfold variation in inhibitory capacity. Evidently, binding is due principally to interaction between protein and the nearly planar ring system. The comparatively small effects produced by the various alkyl or aralkyl substituents suggest that they probably project away from the protein and have little or no involvement in binding.

Replacement of both amino hydrogen atoms (Table III) produces a somewhat greater degree of change, but, here

Table IV. Inhibition of Acetylcholinesterase by Compounds Related to 1,2,3,4-Tetrahydro-9-aminoacridine. Ring Substitution and Variation

Compd no.	Structure	K_I, $M \times 10^8$	pK_a
15		7	
16		13	9.06
17		2	9.49
18		22	10.07
19		15	9.92
20		11	11.0
21		80	9.57
22		260	9.47
23		32	9.51
24		225	9.62
25		550	
26		6300	9.93

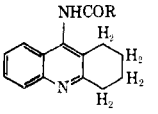
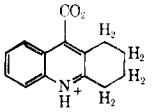
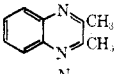
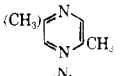
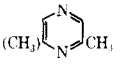
§As was pointed out by one referee, the resulting charge is to some extent delocalized. The pK_a of pyridine is 5.2, whereas for 4-aminopyridine it is 9.2. This is ascribed to resonance stabilization of the cation.



&For the homologous 4-aminopyridine and its acetyl and benzoyl amides, the pK_a values are respectively 9.17, 5.87, and 5.32.[21] Attempts to determine the pK_a of compound **25** spectrophotometrically failed because of difficulties probably caused by its fluorescence. However, the close similarity of its spectra at pH 6.61 and at 9.4 suggests a pK_a value substantially below 6.61.

**The pK_a of the carboxylic proton loss is 4.86.[21]

Table V. Inhibition of Acetylcholinesterase. Miscellaneous Compounds

Compd no.	Structure	K_I, $M \times 10^8$
27 (R = CH_3)		5,000
28 (R = Ph)		5,000
29		15,000
30		5,500
31		29,000
32		23,000
33	Triethylenetetramine	2.4×10^6
34	Poly(*N*-methylethylenimine)	70,000

too, the compounds remain very powerful inhibitors. The reduction in affinity resulting from disubstitution on the amino nitrogen atom parallels the change in the uv absorption spectrum. It, too, is probably due to steric interference with molecular planarity and, hence, interference with the interaction between protein and the nearly planar ring system.

The compounds containing ring substituents and also variants of the polycyclic system are listed in Table IV. Complete aromatization to 9-aminoacridine (**15**), which creates a fully planar three-ring system, has no effect upon binding. Similarly, condensation or enlargement of the unsaturated ring (compounds **16** and **17**) has little effect. Again, pendant methyl groups as in **18** and **19** or further reduction in aromatic character (**20**) have little effect upon binding.

Opening of the saturated ring to give 2- and/or 3-substituted 4-aminoquinolines does result in reduced binding and further cleavage to the single ring in **26** again increases the dissociation constant. In Table V, we see that the uncharged compounds, the amides (**27**, **28**) and pyrazines (**30–32**), and also the zwitterionic carboxylic acid (**29**) have considerably reduced binding, as have the two linear polyamines (**33**, **34**).

Noncompetitive Binding. With virtually all of the compounds, the value of α' lies in the range of 2–10. Thus, all of the compounds show considerable affinity for the acetyl enzyme.

pK_a. No correlation is observed between pK_a and affinity for the enzyme. It is noteworthy that octahydro-9-aminoacridine, compound **20**, has an exceptionally high affinity for the proton. With pK_a equal to 11.0, it is an even stronger base than most aliphatic amines.

Discussion

In treating the observed relationships between chemical structure and K_I, the dissociation constant of the enzyme–inhibitor complex, to postulate topographic features of the enzyme, the following considerations have been applied.

1. It is assumed that the inhibitors bind at (or sufficiently close to) the active site to prevent substrate binding. The alternate possibility of off-site binding, massive conformational change with active site disruption, is discounted.

2. Like most proteins, AChE is probably conformationally adaptable. However, in the absence of positive information suggesting that AChE readily undergoes extensive conformational distortion, we have taken the conservative view and assume that it is minimal.

3. For the above reason, we assume that the conclusions concerning topography refer generally to the native enzyme although the data specifically apply to the protein under conditions of its interaction with inhibitors.

4. Since the effect of charge delocalization,[8] if any, upon small molecule–protein interactions is not known, we have chosen to regard this factor as unimportant.

5. Aromatic ring systems can contribute additionally to intermolecular association through π-orbital bonding. Among the compounds included in this study, π-orbital bonding seems of little importance. Thus, the partially alicyclic compounds THA (**1**), **16**, **17**, and **20** all bind as firmly as does the fully aromatic 9-aminoacridine (**15**).

From the results of this work, we postulate that the site of inhibitor binding is a conformationally flexible, hydrophobic area closely adjacent to the anionic subsite. The

Table VI. Uv Absorbance, Maxima and Isobestic

Compd	0.1 *N* HCl: λ max_a, nm ($\epsilon \times 10^{-4}$)	0.1 *N* HCl: λ max_b, nm ($\epsilon \times 10^{-4}$)	λ max (0.1 *N* NaOH), nm ($\epsilon \times 10^{-3}$)	λ (isobestic), nm ($\epsilon \times 10^{-3}$)
	A. THA and Related Unsubstituted and N-Monoalkylated Compounds			
1	323 (1.1)	335 (0.94)	315 (5.5)	312 (5.0)
2	337 (1.15)	351 (1.13)	326 (7.1)	328 (7.1)
3	337 (1.24)	350 (1.19)	310 (7.4), 322 (7.6)	325 (7.4)
4	338 (1.3)	351 (1.3)	322 (8.5)	326 (7.9)
5	337 (1.1)	351 (1.1)	322 (7.0)	325 (6.7)
6	339 (1.1)	352 (1.1)	322 (6.8)	325 (6.5)
7	334 (1.3)	347 (1.2)	320 (7.4)	320 (7.4)
9	335 (1.7)	347 (1.5)	320 (8.3)	320 (8.8)
10	338 (1.2)	348 (1.26)	320 (7.8)	325 (7.0)
11	336 (1.04)	348 (0.96)	322 (6.6)	322 (6.6)
16	318 (1.2)	330 (1.11)	315 (6.7)	304 (6.9)
17	322 (1.15)	332 (1.06)	306 (6.7)	307 (6.7)
18	326 (1.23)	338 (1.03)	310 (7.5)	313 (7.5)
19	327 (0.93)	338 (0.79)	311 (6.1)	312 (6.1)
21	323 (1.2)	332 (1.04)	308 (6.9)	309 (6.9)
22	319 (1.1)	329 (0.94)	304 (6.7)	304 (6.7)
23	321 (1.14)	330 (1.0)	306 (6.4)	306 (6.4)
24	318 (1.1)	328 (0.92)	304 (6.8)	304 (6.8)
	B. N,N-Dialkylated Tetrahydroacridines			
12	318 (.32)	379 (1.06)	323 (4.4), 352 (3.8)	348 (3.8)
13		372 (0.2)	Below 330	341 (3.9)
14	321 (0.4)	391 (1.2)		
	C. Aminopyridines			
26	261 (1.8)		Below 240	246 (9.7)
20	267 (0.84)		Below 250	256 (4.4)
	D. Amide			
27	326[a] (1.5)		297 (8.0), 309 (8.7), 325 (8.0)	304 (8.0)
	E. Carboxylate			
29	322 (1.3)		288 (5.2), 306 (5.6), 320 (6.4)	292 (5.0)

[a] In 85% methanol.

area, although flexible, tends to assume a near planar form or is easily adapted to such a form. The dimensions of this area are unknown, but it is adequate in size to fully accommodate THA. The basis for these conclusions follows.

1. The very high affinity of the THA compounds suggests multiple interactions, either by fitting into an appropriate slot or through closeness of fit to an exposed surface.

2. The failure of the variety of appended groups either to markedly reduce or to enhance binding makes the fit into a slot improbable.

3. Linear molecules with closely spaced positive charges (the polyamines, Table V) bind poorly.

4. Uncharged multiring planar molecules bind rather strongly, which suggests fit onto a conformationally corresponding hydrophobic area on the protein.

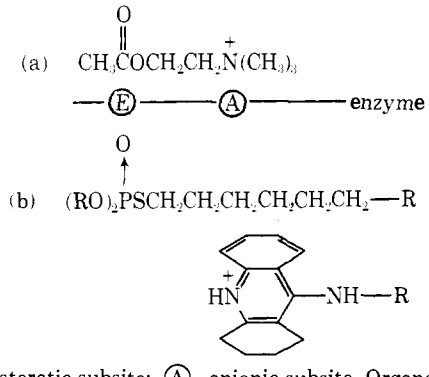
5. Addition of a positive charge to the planar molecules markedly enhances binding.

6. Conformational flexibility in the protein binding area is suggested by the equal affinity of the truly planar 9-aminoacridine (**15**) with that of the tetrahydro- and octahydroaminoacridines (**1**, **20**) and also of compounds **16** and **17**, all of which are twisted out of a perfect plane.

7. The appreciable loss of affinity in the substituted quinolines, compounds **21–25**, points to the need for rigidity in the structure of the inhibitor. The requirement for rigidity further supports the suggestion of flexibility in the hydrophobic patch of the protein.

8. The high affinity of all of the compounds for the acetyl enzyme suggests that the binding site is adjacent to but does not encompass the esteratic subsite, Scheme II. An alternate possibility involving binding over the esteratic subsite in the native enzyme (the distance between charge and unshared nitrogen atom electrons in THA (**1**) can correspond to the distance between charge and unshared carbonyl oxygen electrons in a contracted ACh) and then binding away from this subsite in a conformationally altered acetyl enzyme seems unattractive. In this model one would expect much greater differences in the respective values of K_I and K_I' than were observed.

Scheme II



[a] Ⓔ, esteratic subsite; Ⓐ, anionic subsite. Organophosphorus inhibitors react at Ⓔ; THA compounds bind at Ⓐ.

The existence of hydrophobic binding areas adjacent to the anionic subsite has been suggested earlier from relationships observed between the structure of organophosphorus inhibitors (covalent binding) and their rates of reaction with the enzyme[24,25] and also from the binding of linear long-chain quaternary ammonium salts.[1,26] Both the organophosphorus inhibitors and the quaternary ammonium salts were aliphatic and thus do not provide multidimensional information concerning the binding area. However, as indicated in Scheme II, the results obtained with the organophosphorous compounds are compatible with those reported here. Maximum rate (plateau) was reached at $(CH_2)_6$. If we phosphorylate at the esteratic subsite, the six-atom carbon chain projects to approximately the position of the amino group in THA (**1**). Kabatchnik[25] refers to this part of the protein as A_2. In both cases, further extension with aliphatic carbon chains has little or no effect upon binding to the protein.

Significance with Regard to Poisoning by Anticholinesterase Compounds. The very high affinity of the THA and acridine compounds for the anionic subsite of AChE, particularly as envisioned in Scheme II, suggests that far more rapid AChE-reacting phosphonate and carbamate compounds could be designed which incorporate these structures. That such compounds would necessarily be highly toxic cannot be assumed. Speed of inhibition is not always directly related to high toxicity.[27] However, should such compounds couple a high reaction rate with high toxicity, they could fall into the class of oxime refractory compounds along with Soman.

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