Cholinergic Anionic Receptors III

Steric Requirements for Quaternary Ammonium Inhibitors of Acetylcholinesterase II

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The inhibitory activity of 36 structurally related quaternary ammonium compounds was undertaken as a logical extension of our earlier report studying the discrete nature of the anionic receptor of a typical cholinergic receptor, acetylcholinesterase. The results permit some sophistication in the discussion of the several mechanisms apparently operative in this system.

In an earlier report (1) the authors postulated that the inhibition of acetylcholinesterase (AChE) by simple quaternary ammonium compounds could be ascertained as a function of affinity for the enzyme and that certain relationships existed between the ionic volume of the inhibitory ion and its affinity for the enzyme. Our results prompted a logical extension of the compounds under evaluation in order to clarify the role of ion volume in affinity for a cholinergic receptor.

To meet this end 36 quaternary ammonium compounds were prepared and examined enzymologically. The compounds were: *N*-alkyltrimethylammoniums, *N*-alkyltriethylammoniums, and *N*-alkylquinuclidiniums, where the alkyl group was varied from methyl through decyl plus isopropyl; tetrapropylammonium; tetrabutylammonium; and tetrapentylammonium. These compounds include 3 homologous series, many of which have been examined in other cholinergic systems. Several interesting related compounds are included such as the isopropyl derivatives and the tetraalkyl derivatives. The conformational uniqueness of the quinuclidinium compounds was discussed in an earlier paper (1).

The fundamental rationale for studying competitive inhibition of AChE in a study of the receptor site was similarly established in the first report.

EXPERIMENTAL

Chemistry

Properties and recrystallization solvents for all salts are given in Table I.

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Quinuclidine.—The method of Leonard and Elkin (2) as modified in an earlier paper (1) was used. A significant improvement over earlier reports involves an increase in the rate of flow of the vapors of the 4-(2-hydroxyethyl) piperidine by raising the pot temperature to about 200° with the elimination of the air bleed. This reduced the amount of polymer formed in the catalyst bed and the volatile impurities previously obtained in the product. Larger batches could be run before the receiving apparatus became clogged.

N-Alkylquinuclidinium Salts.—One-tenth mole each of the appropriate alkyl halide and quinuclidine were sealed with 100 ml. of absolute ethanol in a citrate bottle for 18 hr. with intermittent shaking. The solvent was then removed by vacuum evaporation, and the salt recrystallized.

N-Alkyltrimethylammonium Salts.—The reaction described for *N*-alkylquinuclidinium salts was used for the preparation of *N*-alkyltrimethylammonium compounds by utilizing water as the solvent and lengthening the reaction time to 1 week.

N-Alkyltriethylammonium Salts.—All *N*-alkyltriethylammonium compounds except *N*-isopropyl[1] were prepared by refluxing 0.1 mole of the appropriate alkyl halide with 0.1 mole of triethylamine in 100 ml. absolute ethanol for 18 hr. The solvent was removed and the solid purified as previously described.

N-Isopropyltriethylammonium Bromide.—Diethylisopropylamine was synthesized according to the procedure of Caspe (3). This compound was subsequently reacted with ethyl bromide according to Robinson (4).

Tetrapentylammonium Bromide.—Tripentylamine, 0.1 mole, and 1-pentyl bromide, 0.1 mole, were reacted together as described for the synthesis of 1-alkylquinuclidinium salts.

Enzymology

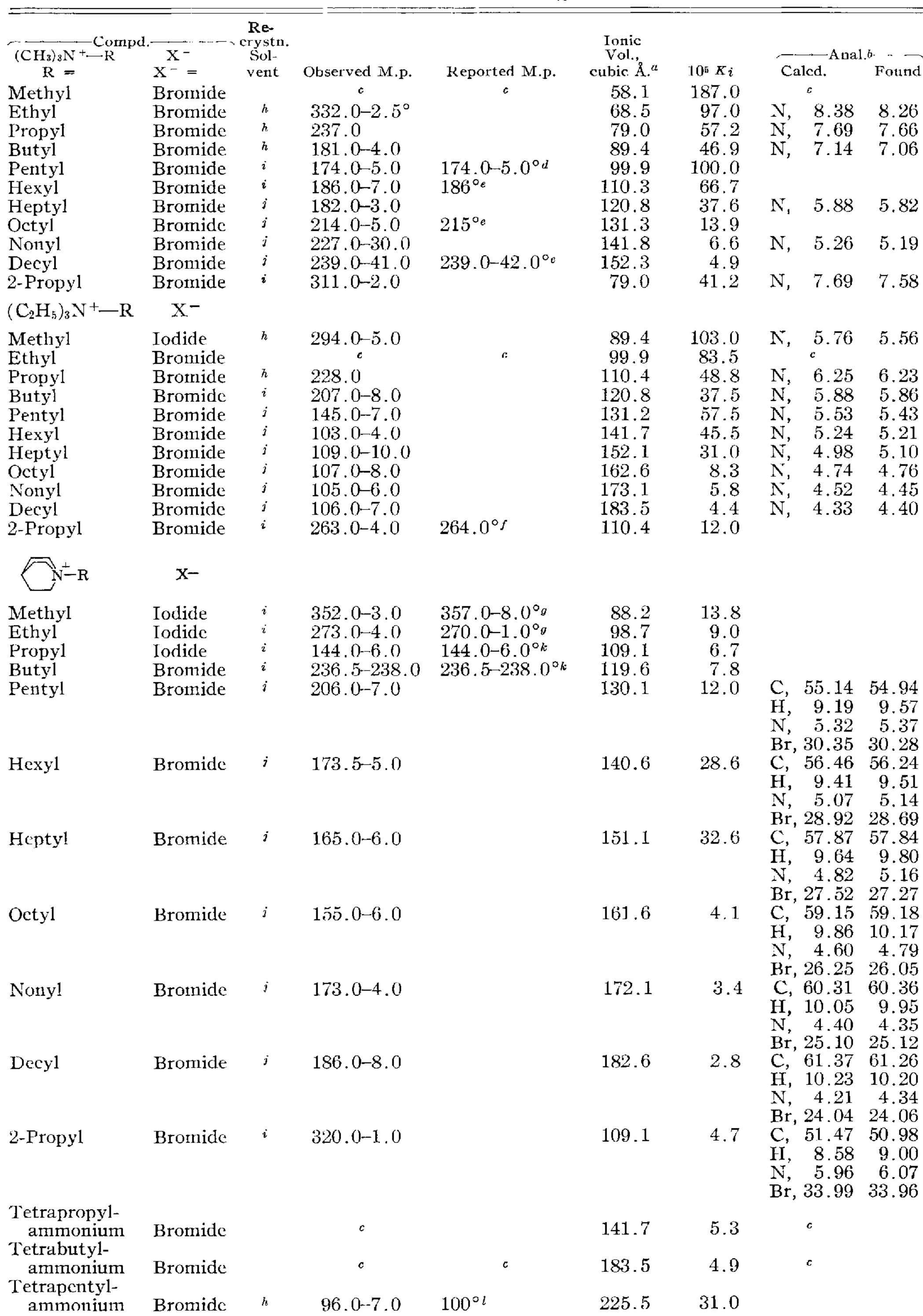
pH-stat titrimetric determinations were made of AChE activity using an apparatus and a procedure described previously (1). The conditions used in these experiments (different from those reported earlier) were: enzyme[2] concentration, 0.01 mg./ml.; NaCl concentration, 0.15 *M;* $MgCl_2$ concentration, 0.05 *M*, and acetylcholine (ACh) concentration, variable. ACh perchlorate was used.

The velocity of the hydrolysis reaction determined over a period of 5 min. (after an initial 2

[1] Only triethylammonium bromide could be isolated from attempts to react triethylamine with isopropyl bromide.

[2] Nutritional Biochemicals (bovine erythrocytes AChE).

TABLE I.—CHEMICAL AND BIOLOGICAL DATA OF SIMPLE QUATERNARY AMMONIUM COMPOUNDS



Compd. $(CH_3)_3N^+$—R, R =	X^- =	Recrystn. Solvent	Observed M.p.	Reported M.p.	Ionic Vol., cubic Å.[a]	$10^5 K_i$	Anal.[b] Calcd.	Anal.[b] Found
Methyl	Bromide		[c]	[c]	58.1	187.0	[c]	
Ethyl	Bromide	[h]	332.0–2.5°		68.5	97.0	N, 8.38	8.26
Propyl	Bromide	[h]	237.0		79.0	57.2	N, 7.69	7.66
Butyl	Bromide	[h]	181.0–4.0		89.4	46.9	N, 7.14	7.06
Pentyl	Bromide	[i]	174.0–5.0	174.0–5.0°[d]	99.9	100.0		
Hexyl	Bromide	[i]	186.0–7.0	186°[e]	110.3	66.7		
Heptyl	Bromide	[j]	182.0–3.0		120.8	37.6	N, 5.88	5.82
Octyl	Bromide	[j]	214.0–5.0	215°[e]	131.3	13.9		
Nonyl	Bromide	[j]	227.0–30.0		141.8	6.6	N, 5.26	5.19
Decyl	Bromide	[j]	239.0–41.0	239.0–42.0°[e]	152.3	4.9		
2-Propyl	Bromide	[i]	311.0–2.0		79.0	41.2	N, 7.69	7.58
$(C_2H_5)_3N^+$—R	X^-							
Methyl	Iodide	[h]	294.0–5.0		89.4	103.0	N, 5.76	5.56
Ethyl	Bromide		[c]	[c]	99.9	83.5	[c]	
Propyl	Bromide	[h]	228.0		110.4	48.8	N, 6.25	6.23
Butyl	Bromide	[i]	207.0–8.0		120.8	37.5	N, 5.88	5.86
Pentyl	Bromide	[j]	145.0–7.0		131.2	57.5	N, 5.53	5.43
Hexyl	Bromide	[j]	103.0–4.0		141.7	45.5	N, 5.24	5.21
Heptyl	Bromide	[j]	109.0–10.0		152.1	31.0	N, 4.98	5.10
Octyl	Bromide	[j]	107.0–8.0		162.6	8.3	N, 4.74	4.76
Nonyl	Bromide	[j]	105.0–6.0		173.1	5.8	N, 4.52	4.45
Decyl	Bromide	[j]	106.0–7.0		183.5	4.4	N, 4.33	4.40
2-Propyl	Bromide	[i]	263.0–4.0	264.0°[f]	110.4	12.0		
(piperidinium) $\overset{+}{N}$—R	X−							
Methyl	Iodide	[i]	352.0–3.0	357.0–8.0°[g]	88.2	13.8		
Ethyl	Iodide	[i]	273.0–4.0	270.0–1.0°[g]	98.7	9.0		
Propyl	Iodide	[i]	144.0–6.0	144.0–6.0°[k]	109.1	6.7		
Butyl	Bromide	[i]	236.5–238.0	236.5–238.0°[k]	119.6	7.8		
Pentyl	Bromide	[j]	206.0–7.0		130.1	12.0	C, 55.14	54.94
							H, 9.19	9.57
							N, 5.32	5.37
							Br, 30.35	30.28
Hexyl	Bromide	[j]	173.5–5.0		140.6	28.6	C, 56.46	56.24
							H, 9.41	9.51
							N, 5.07	5.14
							Br, 28.92	28.69
Heptyl	Bromide	[j]	165.0–6.0		151.1	32.6	C, 57.87	57.84
							H, 9.64	9.80
							N, 4.82	5.16
							Br, 27.52	27.27
Octyl	Bromide	[j]	155.0–6.0		161.6	4.1	C, 59.15	59.18
							H, 9.86	10.17
							N, 4.60	4.79
							Br, 26.25	26.05
Nonyl	Bromide	[j]	173.0–4.0		172.1	3.4	C, 60.31	60.36
							H, 10.05	9.95
							N, 4.40	4.35
							Br, 25.10	25.12
Decyl	Bromide	[j]	186.0–8.0		182.6	2.8	C, 61.37	61.26
							H, 10.23	10.20
							N, 4.21	4.34
							Br, 24.04	24.06
2-Propyl	Bromide	[i]	320.0–1.0		109.1	4.7	C, 51.47	50.98
							H, 8.58	9.00
							N, 5.96	6.07
							Br, 33.99	33.96
Tetrapropylammonium	Bromide		[c]		141.7	5.3	[c]	
Tetrabutylammonium	Bromide		[c]	[c]	183.5	4.9	[c]	
Tetrapentylammonium	Bromide	[h]	96.0–7.0	100°[l]	225.5	31.0		

[a] Calculated according the procedure in *Reference 1.* [b] Microanalyses performed by Alfred Bernhardt, Max Planck Institute, Mulheim, Ruhr, Germany. [c] Obtained commercially. [d] McDowell, M. J., and Kraus, C. A., *J. Am. Chem. Soc.*, **73**, 2170(1951). [e] Kato, T., Morikawa, T., and Suzuki, Y., *J. Pharm. Soc. Japan*, **72**, 117(1952). [f] *Reference 4.* [g] Mosby, W. L., "Heterocyclic Systems with Bridgehead Nitrogen Atoms, Part II," Interscience Publishers, Inc., New York, N. Y., 1961, p. 1339. [h] Isopropyl alcohol–acetone. [i] Ethyl alcohol–ethyl acetate. [j] Methyl ethyl ketone–ethyl acetate. [k] *Reference 1.* [l] *Footnote d* above, p. 3293.

min. of reaction) was determined in the presence of varying concentrations of inhibitor and substrate. The K_m (the Michaelis constant) for this system, determined graphically from Lineweaver-Burk plots, was 1.82×10^{-4}.

All values for K_i were determined graphically from Lineweaver-Burk plots utilizing at least a fourfold range in $[S]$.[3] Each velocity determination was made at least twice, and a minimum of 4 points were plotted for each K_i determination. It was observed that several of the compounds tested displayed noncompetitive kinetics when employed in concentrations at or above I_{50}. For uniformity, therefore, the $[I]$ values used fall between I_{20} and I_{30}. Under these conditions, all compounds tested display competitive kinetics in this system. The expression used to determine K_i on the $-1/(S)$ intercept was

$$K_i = \frac{K_p}{K_m[I] - \frac{1}{[I]}}$$

where $-1/K_p$ is the value of $1/[S]$ at the intercept. All data are summarized in Table I.

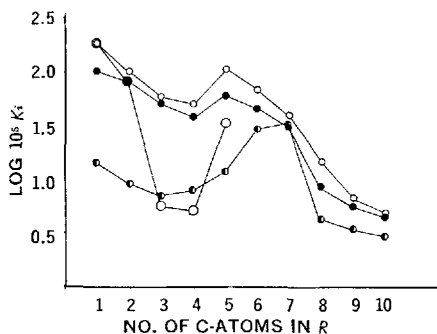


Fig. 1.—The relationship between K_i and length of alkyl chain of ammonium compounds. Key: large circle, R_4N^+; small circle, $(CH_3)_3N^+$—R; ●, $(C_2H_5)_3N^+$—R; ◐, $C_7H_{13}N^+$—R.

RESULTS AND DISCUSSION

Figure 1 displays a comparison between K_i and the chain length in the 3 homologous series and the R groups of the tetraalkylammonium compounds. A significant difference between the data presented here and the first paper merit comment. This difference relates to the minimum in the line for tetraalkylammonium. Earlier it was found to be at tetrapropylammonium; in this work it is at tetrabutylammonium. The authors have established that this difference (as well as some other quantitative differences) arises from the changed conditions of assay. The present procedure is used more commonly. The point remains that correlation of structure to activity in ACh–AChE systems is quite sensitive to the conditions of assay and should be compared accordingly.

Figure 1 reveals an interesting group of parallels. Note particularly the parallelism shown by the methyl, ethyl, and propyl homologs in the trimethylammonium and triethylammonium series; even more striking is the relationship among the octyl, nonyl, and decyl homologs in the trimethylammonium, triethylammonium, and quinuclidinium series. It is obvious that a parallelism exists between the entire series of triethylammonium and trimethylammonium compounds. These parallels are strongly suggestive of mechanistic parallelism.

The bioisosteric quinuclidinium series fails to display such complete similarity to the other 2 homologous series. The first 3 members and the last 3 members are near parallel, but 2 distinct differences are obvious. First, there is a dramatic quantitative difference in affinity between the first 3 quinuclidinium compounds and their trimethylammonium or triethylammonium bioisosteres. Second, the group butyl, pentyl, hexyl, and heptylquinuclidinium actually form a group of reversed slope from the trimethylammonium or triethylammonium series.

Apparently there is a lack of correlation between the tetraalkylammonium series and any of the N-alkyltrialkylammonium series, in terms of the function alkyl group length.

Figure 2 illustrates the relationship between K_i and the volume of the inhibitory ion. From this figure it is apparent that ion volume data do not offer a general explanation for an inhibitory ion's affinity for the enzyme. However, there is a crude generalization evident from the tendency for all of the compounds tested (*except* the lower members of the quinuclidinium series) to scatter around a line terminating in a minimum value for K_i at 170 Å.[3] Suggestions on a mechanism parallel between compounds is not evident from this figure. Indeed, much variation in affinity is obvious within a given volume increment which must be due to structural differences among equivolume ions. There are no minima of K_i as a function of volume common to 2 or more homologous series.

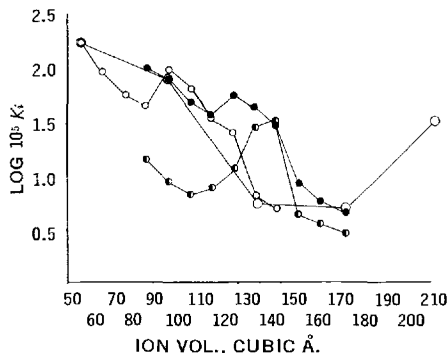


Fig. 2.—The relationship between K_i and ion volume of inhibitory cation. Key: large circle, R_4N^+; small circle, $(CH_3)_3N^+$—R; ●, $(C_2H_5)_3N^+$—R; ◐, $C_7H_{13}N^+$ – R.

Not illustrated in the figures are the data from the isopropyl compounds. Table I reveals that the isopropyl compounds have in every case lower values of K_i than their n-propyl analogs. Simi-

[3] These data are consistent with values obtained from v/v_i *versus* $[I]$ plots.

larly, these compounds represent further equivolume ions with an affinity for AChE which is uniformly enhanced by changing an *n*-propyl group for an isopropyl group. The implications of this observation are several in number, but no conclusion is evident. The fact that adding an alpha-carbon to an ethyl-substituted ammonium ion produces a better inhibitor than adding a terminal methyl group implies that the receptor surface does not contain a simple channel at all but is irregular in nature near the anionic center. The production of a different conformational perturbation by the isopropyl compounds is not deducible from these results, but must be considered. Obviously, the effect of α branching on one or more of these homologous series would constitute an interesting experiment.

According to Belleau (5) the compounds in the *N*-alkyltrimethylammonium series act in inducing 2 different conformational perturbations (with a transitory phase in between) dependent upon the length of the alkyl substituent. The parallelism between the *N*-alkyltrimethylammonium and *N*-alkyltriethylammonium series in this work strongly suggests that the same mechanism is operative for either series. The qualitative similarity between the 3 lowest and 3 highest members of all 3 of the homologous series (as well as the quantitative similarity between the 3 highest members) suggests that a similar mechanism is being invoked in each case. This similarity is independent of ion volume, as adequately demonstrated by Fig. 2.

These suggestions are modified by the lack of parallelism between the "middle region" of the *N*-alkylquinuclidinium series and the other 2 series. This feature, together with the quantitative dissimilarity displayed by the lower members, suggests that an entirely different mechanism is being demonstrated by the quinuclidinium derivatives up to C-7.

It is apparent that several forces are active in determining affinity for AChE. Very closely interdependent are: (*a*) ion volume, (*b*) chain length of longest alkyl chain, (*c*) the conformational possibilities of the ion, and (*d*) the exact mechanism whereby the ion acts (*i.e.*, type of conformational perturbation induced in the receptor).

Specifically excluded are arguments based on relative electron deficiency of the ion. It appears that this area is best thought of in terms of the conformational possibilities of the ion, rather than to attempt to quantify charge. All the compounds are easily dissociated at the concentrations employed. Therefore, the only electrostatic variables anticipated are those arising from the relative availability of the charge center(s). It appears from this approach to the problem that a very significant function of affinity is the shielding of the quaternary nitrogen atom, or the availability of the α-carbon atoms to a plane surface. Some discussion of α plane availability as related to cholinergic activity has been given by Thomas (6, 7).

CONCLUSIONS

No single simple molecular function serves to predict affinity of simple quaternary ammonium ions for the anionic site of AChE. The several features, related above, that obviously affect affinity are interdependent to a high degree. For example, it appears that for optimum affinity, some gross criteria of ion volume must be met; however, if the ion has other unique features enhancing affinity (such as the compact quinuclidinium "head"), the disadvantage conferred by the "wrong" size may be offset. In addition, it is likely that changing one molecular feature changes the mechanism whereby the ion acts. Therefore, the optimum molecular requirements must be stated in terms of a single mechanism to have meaning. The present state of these experiments does not permit this fine a conclusion.

Therefore, it is apparently correct to say that, among simple quaternary ammonium ions (*a*) the affinity of members in a homologous series tends to increase (with significant variations at intermediate positions) to a maximum in the region of 170 Å.^{3} volume of the ion; (*b*) butyl groups (rarely propyl) represent a maximum of affinity (however less than octyl, nonyl, or decyl) within a homologous series; (*c*) restricting the conformational variability of the cationic "head" generally enhances affinity; and (*d*) it is suggested that at least 4 discrete "mechanisms" of action are demonstrated by the compounds in this report. Changes of mechanism refer, in this context, to changes in the conformational perturbation type effected in the protein.

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