When polyethylene glycol ointment had been used as the vehicle, the color was not only more intense but also presented additional distribution in the corium.

Although exhaustive attempts were made to demonstrate the presence of iodochlorhydroxyquin visually, no satisfactory method could be found.

The levels of resorcinol attained in the skin from the experimental base were highly significant ($p < 0.001$), while the concentrations reached from petrolatum and the polyethylene glycol base were not significantly different from control samples ($0.2 > p > 0.1$ and $p > 0.9$, respectively). Similarly, the levels of salicylate in the skin from the experimental base were very significantly higher than those from the petrolatum base ($p < 0.001$). The significance of the difference between tissue levels of salicylate from the polyethylene glycol base and petrolatum, although less marked, was nevertheless still significant ($0.01 > p > 0.001$). The difference in tissue levels of sulfadiazine attained from petrolatum and the experimental base would not appear to be significant ($0.05 > p > 0.02$), although the concentration reached from the polyethylene glycol was very significantly higher ($p < 0.001$). Likewise, the absorption of iodochlorhydroxyquin from the petrolatum and experimental ointments by the skin were found to be not significantly different ($0.05 > p > 0.02$), whereas a significantly higher level was attained from the polyethylene glycol base ($p < 0.001$).

In conclusion, it appears that dermatologic vehicles based upon the experimental formulas, such as those presented here, present a promising innovation in the formulation of topical medication.

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Cholinergic Anionic Receptors I

Steric Requirements for Quaternary Ammonium Inhibitors of Acetylcholinesterase

By JAMES C. KELLETT, Jr., and CHARLES W. HITE*

As an initial effort to discern some of the fine features of cholinergic anionic receptors, a study was made on inhibitors of acetylcholinesterase which used inhibitors that are relatively free from conformational variation. The inhibitors used were quaternized 1-azabicyclo(2.2.2)octanes. The bicyclic parent amine was prepared by several conventional procedures. The salts were evaluated as competitive inhibitors of acetylcholinesterase by a titrimetric procedure. The affinity of these compounds for the anionic site of this enzyme was compared to the affinity of a series of related salts subject to conformational variation. These comparisons suggest that there are stereochemical requirements for the anionic site of this cholinergic receptor more specific than heretofore suspected.

THE AUTHORS are engaged in a study of cholinergic receptors with the aim of further defining the chemical requirements of receptor substances activated by acetylcholine (ACh).

The active site of acetylcholinesterase (AChE) has been the object of a great many investigations. [See Koelle (1) and Krupka (2) for leading reviews.]

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It is almost universally accepted that the active site of AChE consists of an anionic site (which is responsible for the first contact between the enzyme and ACh) and an esteratic site which, in turn, consists of a serine hydroxyl, an acid site, and a basic site. These last three functional groups act in an integrated fashion to effect hydrolysis of the substrate. Little or no information is available about the details of the anionic site. All expressions for describing the enzyme's activity involve the formation of a Michaelis complex. It is generally accepted that at least a significant portion of the binding in the AChE–ACh complex is an electrostatic attraction for the cationic head of ACh and an anionic site of the enzyme; this point has been

challenged, however (3). In either case, there is an attraction for the cationic head of ACh with some portion of the active site of AChE.

Supporting this idea is the fact that quaternary ammonium ions competitively inhibit the hydrolysis of ACh by AChE (4). Although these simple compounds form weak complexes with AChE (K_i on the order of 10^{-2}–10^{-4}) when compared to a more complex competitive inhibitor such as eserine (K_i about 10^{-8}), their function is more subject to analysis due to the paucity of functional groups. Therefore, they are compounds of choice in studying the anionic requirements of various receptors.

Structure-activity correlations of most molecules are hindered by conformational variation possible in flexible molecules. For example, while the conformation of ACh in the solid state or in aqueous solution has been well established (5), there is no evidence that indicates that this same conformation is complementary to the active site of any receptor. In the reported work here, the authors have largely eliminated conformational variation in simple quaternary ammonium compounds by using compounds containing the 1-azabicyclo(2.2.2)octane (quinuclidine) fragment.

EXPERIMENTAL

Chemistry

Figure 1 summarizes the two general routes by which quinuclidine was synthesized.

Methyl Isonipecotate.—This compound was prepared from methyl isonicotinate according to the procedure of Freifelder and Stone (6).

1 - Carbethoxymethyl - 4 - carbomethoxypiperidine.[1]—This compound was prepared from methyl isonipecotate and ethyl bromoacetate according to the procedure of Grob and Renk (7).

3-Quinuclidone.[2]—This compound was prepared from 1-carbethoxymethyl-4-carbomethoxypiperidine according to Mikhlina and Rubtsov (8).

Quinuclidine via 3-Quinuclidone.—This reduction has been reported by Clemo and Metcalf (9), although they did not isolate the free base.

3-Quinuclidone (0.1 mole, 12.5 Gm.) was heated by means of an oil bath with 7.5 ml. of 85% hydrazine, 100 ml. of triethylene glycol, and 10 Gm. of KOH under reflux for 1 hr. After the heating period, the reflux condenser was replaced with a distilling head, and distillation carried out until the liquid temperature reached about 180° (distillation had practically stopped). Heating was continued at this temperature for 3 hr. The cooled reaction mixture was extracted with five 75-ml. portions of ether and the combined extracts dried with K_2CO_3. Distillation of the ether and repeated sublimation of the residue yielded 6.4 Gm. (57.7%) of product, m.p. 156.5–159.0°. [Reported (10) m.p. 158°, 158–159°, 156°, 155–156°.]

Quinuclidine via 4-(2-Hydroxyethyl)piperidine.—A more desirable procedure was adapted from the report of Leonard and Elkin (12) of the dehydration of 4-(2-hydroxyethyl)piperidine[3] in the gas phase over alumina.

The reaction apparatus consisted of (*a*) a 100-ml. distilling flask fitted with an ordinary T-tube of 10-mm. i.d. glass tubing, (*b*) a reaction chamber prepared by coiling nichrome wire (20 gauge) around a 24 × 0.75 in. high-temperature-resistant Pyrex tube so that the wires were spaced about 1 cm. apart and enclosing the wire-covered section with asbestos to a thickness of about 0.5 cm., and (*c*) an ordinary vacuum adapter with a long inner tube extending into a 250-ml. Kjeldahl flask serving as a receiver. A fine glass capillary was fitted to the T-tube so that the tip extended nearly to the bottom of the distilling flask; a pinch clamp was fixed to the exposed end to regulate air flow. The reaction chamber was filled with 8-mesh alumina held in place by wire cups filled with glass wool, and the chamber was attached to the sidearm of the T-tube in a horizontal position. The adapter and receiver were fitted to the opposite end of the reaction chamber. With the receiver and adapter removed, a long (18-in.) probe was imbedded in the catalyst and the temperature of the chamber determined as a function of voltage impressed through the wire by means of a variable transformer attached to the wires with alligator clips.

The distilling flask was charged with 40–50 Gm. of 4-(2-hydroxyethyl)piperidine. The apparatus was attached to an efficient vacuum pump through a series of dry ice–acetone baths, and the receiver was immersed in brine. The reaction chamber was brought to a temperature of 450° and the system evacuated (receiver pressure about 0.05–0.1 mm. Hg). The distilling flask was then heated in an oil bath (temperature about 160°) until vapors passed into the reaction chamber, and the air bleed adjusted so that the material was passed through the catalyst at a rate of not less than 15 Gm./hr. The size of each run was limited by the clogging of traps with solid product. Usually several runs of 40–50 Gm. starting material were combined for workup.

The combined distillates were dissolved in acetone and the solution dried with K_2CO_3. Careful distillation removed acetone and at least two as yet unidentified volatile liquid products; at a temperature of about 100°, solid product begins to collect in the distilling head. Repeated sublimation of the residue resulted in typical yields of 50% of quinuclidine, m.p. 152.0–154.0°.

1-Alkylquinuclidinium Halides.—All compounds were synthesized by refluxing 0.2 mole of quinuclidine with 0.22 mole of the requisite alkyl halide in 50 ml. of ether after the initial reaction upon mixing had subsided. The cooled reaction mixture was then filtered, and the salts recrystallized from ethyl

[1] Sternbach and Kaiser reported the preparation of this compound *via* the hydrogenation of 1-carbethoxymethyl-4-carbomethyoxypyridinium bromide (11). In our hands grossly impure oils only were obtainable; thus, the authors agree with Grob and Renk (7) in their report that catalytic reduction of the pyridinium salt of Sternbach and Kaiser yielded a mixture containing a large fraction of the Δ^1-tetrahydropyridine derivative.

[2] Many variations in the conditions and yields of the Dieckmann condensation forming 3-quinuclidone exist, notably those of Sternbach and Kaiser (11), Grob and Renk (7), and Mikhlina and Rubtsov (8). The latter procedure uses potassium ethoxide in toluene and was found to produce consistently better results than potassium alone, sodium alone, or sodium ethoxide.

[3] Commercially available; Reilly Tar and Chemical Co., Indianapolis, Ind.

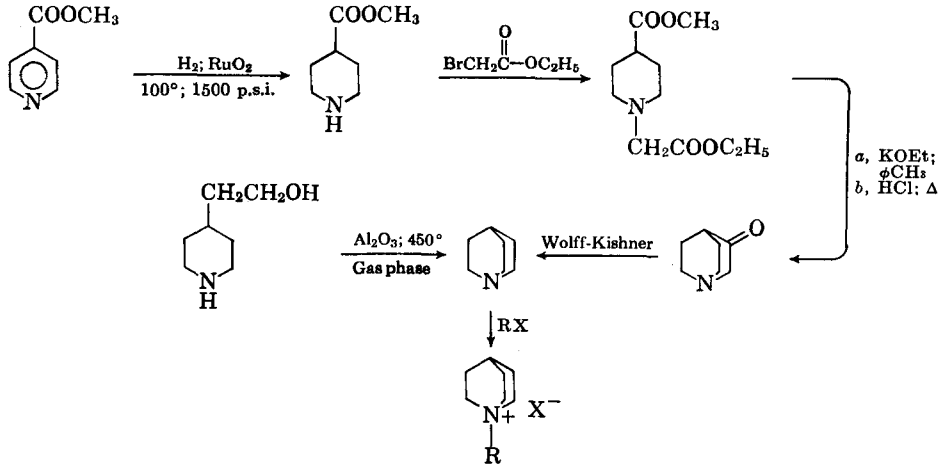
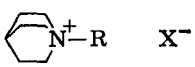


Fig. 1.—Syntheses of *N*-alkylquinuclidinium salts.

TABLE I.—PROPERTIES OF 1-ALKYLQUINUCLIDINIUM HALIDES



R	X	M.p., °C.	Reported	Anal.	
				Calcd.	Found
Methyl	Iodide	352.0–353.0° dec.	357–358°[a]	C, 37.96	37.96
				H, 6.37	6.35
				N, 5.53	5.50
				I, 49.43	49.43
Ethyl	Iodide	273.0–274.0° dec.	270–271°[a]	C, 40.46	40.59
				H, 6.79	6.79
				N, 5.24	5.30
				I, 47.50	47.48
Propyl	Iodide	144.0–146.0°	...	C, 42.71	42.58
				H, 7.17	6.95
				N, 4.98	4.85
				I, 45.14	45.20
Butyl	Bromide	236.5–238.0° dec.	...	C, 53.23	53.27
				H, 8.93	8.90
				N, 5.64	5.46
				Br, 32.19	31.65

[a] *Reference 10.*

acetate–ethanol or isopropyl alcohol–acetone. Table I summarizes the properties of these materials.

Tetraalkylammonium Salts.—These materials were obtained commercially[4] and were recrystallized until analytically pure.

Volume Measurements

Estimates of ionic volume were made by preparing molds of ordinary Stuart-Briegleb atom models using a conventional silicone rubber molding material.[5] Using manufacturer's directions, accurate molds were made of both methyl groups and hydrogen atoms. The tared molds were then filled with water and weighed to 0.05 Gm. From these data the volume of various molecules was calculated. Table II summarizes the volume data on ions used in this work.

[4] Eastman Chemicals, white label.
[5] Dow Corning Silastic RTV Silicone Rubber.

Enzymological Studies

The procedure used was a modification of the pH-stat titrimetric procedure of Smallman and Wolfe (13).

Titrations were carried out using a Coleman microburet in a specially constructed jacketed cell with internal measurements of 2.5 cm. diameter and 7 cm. height. Cell contents were stirred with a microbar over a magnetic stirrer, and pH reading obtained with a Beckman Zeromatic pH meter with miniature calomel and glass electrodes. Velocity determinations were carried out by pipeting volumes of ACh, NaCl, AChE solution, and water (plus inhibitor when used) of such size and concentration as to result in 10 ml. of a mixture of the desired concentrations; temperature was maintained at 33°. All determinations were made in 0.1 *N* NaCl and 0.01 mg./ml. AChE[6]; titrant was 0.01 *N* NaOH.

[6] Bovine erythrocytes acetylcholinesterase; Mann Research Laboratories.

TABLE II.—IONIC VOLUME AND K_i OF SOME BIOLOGICALLY ACTIVE QUATERNARY AMMONIUM SALTS

Ion	Vol., Å.[3]	10^5 K_i
Tetramethylammonium	58.1	187.0
Tetraethylammonium	99.9	104.0
Tetrapropylammonium	141.7	1.5
Tetrabutylammonium	183.5	9.8
1-Methylquinuclidinium	88.2	12.0
1-Ethylquinuclidinium	98.7	6.5
1-Propylquinuclidinium	109.1	2.6
1-Butylquinuclidinium	119.6	5.1
Ethyltrimethylammonium[a]	68.5	68.0[b]
Propyltrimethylammonium	79.0	27.0
Butyltrimethylammonium	89.4	18.0
Pentyltrimethylammonium	99.9	13.5
Hexyltrimethylammonium	110.3	6.2
Heptyltrimethylammonium	120.8	5.4
Tetramethylammonium	58.1	135.0

[a] All compounds of type *N*-alkyltrimethylammonium were prepared and I_{50} values obtained by Bergmann and Segal (15). [b] K_i values for all compounds of type *N*-alkyltrimethylammonium were calculated from reported I_{50} values according to the relation $K_i = 0.09I_{50}$, as suggested by Cohen, J. A., and Oostenbaan, R. A., in Kolle, G. B., subed., "Handbuch der Experimentellen Pharmakologie," vol. XV, Springer-Verlag, Berlin, Germany, 1963, p. 323.

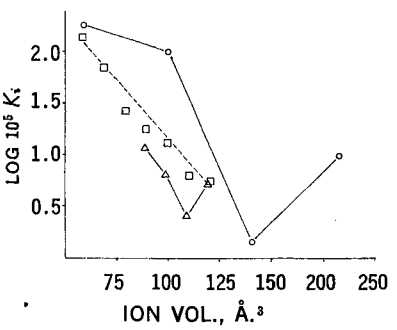


Fig. 2.—Relationship observed between K_i of some inhibitory ions and the ion's volume. Key: ○, R_4N^+; △, *N*-alkylquinuclidinium; □, $Me_3\overset{+}{N}$—R.

Plots of volume titrant *versus* time were linear for periods well in excess of times used to calculate velocity (5 min.). All velocity determinations were made in duplicate and averages used for plots. K_m for the system reported here was determined graphically to be 1.39×10^{-4}. S_{opt} was determined graphically (log *S versus* velocity) to be 1×10^{-3} *M* ACh. Substrate was ACh chloride.

Values for K_i were obtained from Lineweaver-Burke plots using at least a fourfold range of (I). A simplified rate expression was used to obtain K_i as the intercept as

$$K_i\left[1 + \frac{(S)}{K_m}\right]$$

Table II contains the values for K_i of the compounds used in this work as well as some calculated from the literature.

DISCUSSION

In several previous studies of simple quaternary ammonium ions as enzyme inhibitors, the relationship between affinity for the enzyme (as K_i) and the length in carbon atoms of some one or more substituents was observed (14, 15). Other workers have related affinity for the enzyme to the radius of the hydrated ion (4). These two variables of an ion's structure may not be related; therefore, the authors were concerned with determining which stereochemical parameters were significant contributors to enzyme affinity.

Figure 2 illustrates the relationship observed in the experiments reported here between the K_i of some inhibitory ions and the ion's volume. The values given in Table II and in Fig. 2 for ionic volume are obviously accurate in a relative sense only. These numbers were derived from a model system in which certain assumptions were made (for example, shortening the calculated radius) for each atom and therefore these assumptions are carried over into the calculations reported here. Also, no correction for any hydration of the ions was applied; indeed, recent work suggests indirectly that it should not be considered (16). Thus, when we speak of a volume function for a biological receptor, we make all the assumptions of Stuart and Briegleb in formulating their model system.

First, it is evident that there is probably an optimum ionic volume for the anionic site in the region of 110–150 Å.[3]. While it is not definitely clear if this represents a range of equally acceptable volume or a range approaching a single optimum value, later arguments will be developed which suggest that the former situation is more likely. Figure 2 also contains data calculated from Bergmann and Segal (15) to illustrate how compounds of the type $Me_3\overset{+}{N}$—R conform to the postulates suggested by the work in this report.[7]

In the series R_4N^+, the loss in affinity of the tetrabutylammonium compound has been attributed to its size being so large that it does not fit into the "limited space" of the active center (4). This result was discussed in light of the proposal that quaternary ammonium ions were *directed* to the enzyme by electrostatic forces, but that *affinity* was a function of dispersion forces and thus should be intimately related to molecular (ionic) size. Therefore, the general conclusion to be drawn from previous work was that the larger the ion, the better, until some size was reached (in the vicinity of tetrabutylammonium) in which the ion was actually too large for the limited space of the active site.

While the results generally confirm this proposal, at least in so far as increasing size tends to increase affinity, an interesting anomaly develops when one observes the shapes of the lines in Fig. 2. The line formed by *N*-alkylquinuclidinium compounds and the line formed by tetraalkylammonium compounds have the same shape; in both series, the *N*-butyl member shows less affinity than the *N*-propyl member. Thus, there appears in both series a maximum affinity in the *N*-propyl member. Such a maximum does *not* appear in the series from Berg-

[7] A recent report (16) indicates that affinity in the *N*-alkyltrimethylammonium series increases well beyond C-7. However, another recent reference (17) indicates that the mechanism of action in the series *changes* at C-7 or C-8. The mechanism of action of *N*-alkyltrimethylammonium compounds higher than C-7 or C-8 is, according to Belleau (17), the induction of a *non*specific conformational perturbation in the receptor. Therefore our work, limited to the mapping of the anionic portions of the cholinergic receptor (conforming to Belleau's specific conformational perturbation), would logically not involve compounds known to act with a receptor nonspecific to ACh.

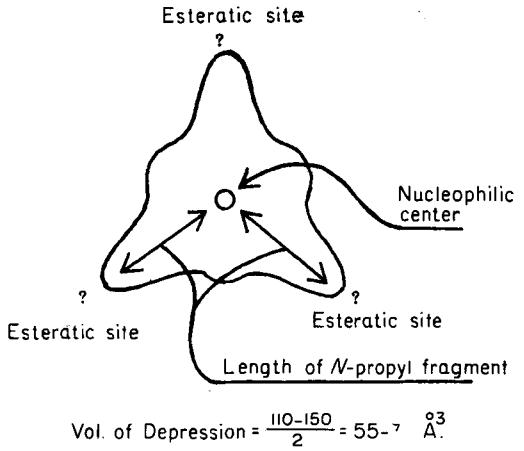


Fig. 3.—A possible configuration of the anionic site of AChE.

mann and Segal for $Me_3\overset{+}{N}$—R ions. However, in this series the *N*-propyl compound has a volume of only 79.0 Å.^{3}, a volume well below that indicated by all three series of compounds to be an overriding requirement for maximum affinity.

The significance of *N*-propyl specificity at the anionic site of AChE is not readily explained. A relatively static model of the active site (2) includes a channel in the esteratic portion of the site to accommodate the multiple functional groups believed to be associated with the hydrolytic mechanism. Of these functional groups, one (an acidic one) is believed to lie relatively close (about 2.5 Å.) to the anionic site. It is difficult, however, to recognize specific binding forces between an acidic function and an alkyl chain of particular length. It is possible that the propyl fragment does become involved through dispersion forces with a channel assumed to exist in the static model.

This suggestion fails to explain the apparent increase in affinity demonstrated by increasing the *number* of propyl fragments, as evidenced by the smaller K_i of Pr_4N^+ than of *N*-propylquinuclidinium. However, the difference in affinity is small (see Table II), and the difference could be due only to the statistically improved chances of Pr_4N^+ extending a N—Pr fragment toward the active site.

Also, it is apparent that a steric factor concerning the compactness and/or symmetry of the ion is very significant to activity, even in ions of less than optimum volume. Note from Fig. 3 that the pair of compounds *N*-ethylquinuclidinium and TEA have very similar volumes, but the rigid quinuclidinium compound has a much smaller K_i than the variable TEA.

CONCLUSIONS

It appears that the affinity of quaternary ammonium ions for the anionic site of AChE depends upon a threefold steric requirement. First, the ion must have a volume of about 110–150 Å.^{3} (within the limitations of defining ionic volume described under *Discussion*). Second, ions possessing the requisite volume display increased affinity when they possess one or more *N*-propyl fragments. Third, ions with relatively high symmetry and of a compact form have enhanced affinity.

These data are consistent with the suggestion that the anionic site of AChE is a cavity of such size as to accommodate ions of about 110–150 Å.^{3}; the cavity contains a point charge so that molecules or ions containing electron-poor centers may best fit when the availability of the cationic center is maximized by compactness and/or symmetry in the ion. The cavity is also irregular to the extent that it is capable of accommodating with enhanced specificity propyl groups, presumably by dispersion forces since there is relatively little change in the nature and magnitude of the cationic charge in the ions. Figure 3 presents a possible configuration of the anionic site.

The recent proposal of dynamic rather than static receptors (16) in AChE (and related receptor systems) is compatible with these proposals. In the dynamic model, one may consider the conformation of the specifically perturbed protein (with the specific perturbation precipitated by the initial binding of ion and receptor) in much the same regard as the static model's configuration.

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