Cholinergic Anionic Receptors II

Examination of a Conformationally Restricted Analog of Acetylcholine

By ALAN W. SOLTER*

The hydrolysis of acetylcholine consists of a number of stages, including the formation of an enzyme–substrate complex and the subsequent dissociation of this complex yielding the free enzyme, substrate, and new products. In these experiments compounds of structure similar to that of acetylcholine were tested in a cholinesterase system so as to determine their K_m or K_i values. This kinetic study led to useful information concerning the active site of the enzyme and the conformation of acetylcholine at the instant it binds with the enzyme.

ONE OF the factors which determines the duration of action of acetylcholine is its destruction by acetylcholinesterase. The mechanism of this enzyme–substrate reaction appears to consist of an acetylation of the enzyme by the release of choline from the enzyme–substrate complex, and then the subsequent deacetylation, releasing acetic acid (1).

The reaction between enzyme and substrate is not one which is limited to a lock and key fitting of rigid structures. According to Belleau's macromolecular perturbation theory (2), enzymes can exhibit the property of conformational adaptability. Hence, while the acetylcholine molecule is undergoing slight changes to be able to fit into the anionic and esteratic sites of the enzyme, at the same time, the enzyme is also undergoing some variation in structure to accommodate its substrate.

On the basis of infrared spectroscopic results and other data, Fujita and Fellman (3) have presented substantial evidence that acetylcholine in solution assumes a cyclic nature (Fig. 1) due to an electrostatic attraction between the polarized carbonyl group and the quaternary nitrogen.

This report presents the results of a kinetic study used to gain knowledge of the conformational relationship between acetylcholine and acetylcholinesterase. The compounds utilized in this experiment were structural analogs of acetylcholine and choline—quinuclidine derivatives. Because of the rigid structure, quinuclidine compounds were very convenient, for they minimized the conformational variability of the substrate, thereby leaving only one unknown—the conformational change of the enzyme.

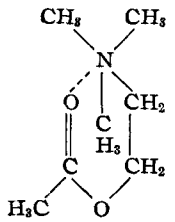

Fig. 1.—Cyclic conformation of acetylcholine.

EXPERIMENTAL

Chemistry

3-Quinuclidone.—This compound was prepared by the method of Mikhlina and Rubstov (4). (See also *Reference 10.*)

3-Quinuclidone Methyl Iodide.—A 25.0-Gm. (0.2-mole) quantity of 3-quinuclidone was reacted with 43.2 Gm. (0.3 mole) of methyl iodide in ether. After the initial reaction had subsided and the mixture had stood at room temperature for 1 hr., the precipitate was filtered and recrystallized from ether–ethanol, yielding 45.2 Gm. (84.7%), m.p. 296–298°. [Reported (5) m.p. 310°.]

3-Quinuclidinol Methyl Iodide.—To 20.0 Gm. (0.075 mole) of 3-quinuclidone methyl iodide was added 200 ml. of water and 0.112 Gm. of platinum oxide. The mixture was reduced for 5 hr. at 800 p.s.i. of hydrogen and allowed to sit for 16 hr. at a pressure of 860 p.s.i. It was then decolorized with charcoal and evaporated to dryness. Recrystallization was accomplished with 95% ethanol and ethyl acetate, yielding 12.5 Gm. (61.8%), m.p. 309.5°–310°.

Anal.—Calcd. for $C_8H_{16}INO$: N, 5.21%. Found: N, 5.16%.

3-Acetoxyquinuclidine Methyl Iodide.—A 6.0-Gm. (0.022 mole) quantity of 3-quinuclidinol methyl iodide was reacted with 45 ml. of acetic anhydride. The mixture was refluxed for 2 hr. and allowed to stand overnight. The residue was filtered and recrystallized with absolute ethanol and ethyl acetate, yielding 4.5 Gm. (65.8%), m.p. 164–165°. [Reported (6) m.p. 165°–166°.]

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Address inquiries and reprint requests to J. C. Kellett, School of Pharmacy, University of North Carolina, Chapel Hill.
* National Science Foundation Undergraduate Research Participant.
Third Place National Winner, *Lunsford Richardson* Undergraduate Research Award, 1965.

Enzymology

After the necessary compounds had been synthesized, they were tested in a pH-stat titrimetric system which consisted of 0.10 *M* sodium chloride solution and the enzyme[1] at a concentration of 0.01 mg./ml. The temperature was maintained at 24° ± 0.6°, and the pH was kept at 7.5 ± 0.1.

The K_m of acetylcholine chloride and of its quinuclidine analog, 3-acetoxyquinuclidine methyl iodide, was determined by the customary procedure. (See *Reference 10* for details.) Conventional Lineweaver-Burk plots of $1/v$ *versus* $1/S$ were applied to determine the K_m value graphically.

The K_i values of choline, 3-hydroxyquinuclidine methyl iodide, and 3-quinuclidinone methyl iodide were established using a procedure similar to that used for the K_m determinations (but plotted $[I]$ *versus* v_0/v) except that the inhibitors were placed in the reaction mixture before the substrate was added. In all inhibition experiments, the same initial substrate concentration was used and velocity determinations made with varying inhibitor concentrations.

RESULTS

When working with the ACh–AChE system (and with the analogous ester, for that matter), it was found that, although the rate of hydrolysis normally increased with an increase in substrate concentration, there reached a point at which further addition of substrate actually caused a reduction in the hydrolysis rate. This is due to the phenomenon of substrate inhibition. In the proposed system of double-sited receptors, substrate inhibition could be due to the fact that at high concentrations the substrate can force a single point attachment, thus tying up the enzyme and acting as an antagonist instead of an agonist (7). Another possible explanation of substrate inhibition is that of the supercomplex, in which two substrate molecules are bound to one enzyme molecule, thus rendering the enzyme inactive.

The relative affinities for the acetylcholinesterase exhibited by 3-acetoxyquinuclidine methyl iodide and acetylcholine were obtained by comparing their respective K_m values (assuming that K_m is approximately equal to K_s, the dissociation constant of the enzyme–substrate complex). As the

$$K_m = \frac{k_2 + k_3}{k_1}$$

in the reaction

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow{k_3} EH + P$$

(EH is the concentration of the enzyme; S is the concentration of the substrate; EHS is the concentration of enzyme–substrate complex; P is the concentration of products), it can be seen that a lower K_m value indicates a lower degree of dissociation of the enzyme–substrate complex. This low degree of dissociation of the complex would suggest a stronger affinity existing between its constituents. By plotting the reciprocal of the velocity of the substrate hydrolysis *versus* the reciprocal of the substrate concentration (Fig. 2), it was found that the K_m of the quinuclidine ester was lower than that of acetylcholine (Table I).

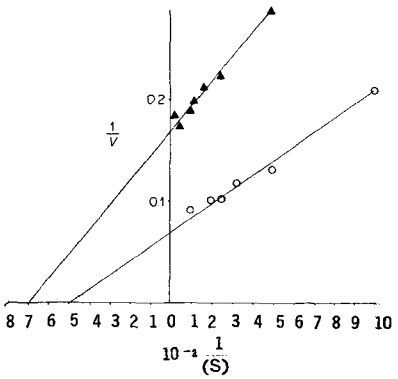

Fig. 2.—Key: ○, acetylcholine chloride; ▲, *dl*-3-acetoxyquinuclidine methyl iodide.

TABLE I.—SUMMARY OF KINETIC DATA

Compd.	K_m	$V_{max.}$
ACh	2.08×10^{-4}	19.27
Q-ester	1.53×10^{-4}	6.13
	K_i	I_{50}
Choline	6.90×10^{-4}	3.99×10^{-3}
Q-one	3.50×10^{-4}	2.61×10^{-3}
Q-ol	1.67×10^{-4}	1.26×10^{-3}

It is also evident from Fig. 2 that the maximum velocity of the hydrolysis was more than three times as great for acetylcholine than for its quinuclidine analog. This might be explained by taking a closer look at the reaction

$$EH + S \rightleftharpoons EHS \begin{cases} \rightarrow \text{E-acetyl} \\ \downarrow \\ \text{choline} \end{cases} \begin{cases} \rightarrow EH \\ \downarrow \\ \text{HOAc} \end{cases}$$

Krupka (8) has proposed that the rate-limiting step of this equation for acetylcholine is the deacetylation. Therefore, the difference in the velocities of hydrolysis of acetylcholine and of its quinuclidine analog is probably due to some phenomenon which occurs before *E*-acetyl is formed. The lower $V_{max.}$ of the quinuclidine ester could be due to its being a substrate (S') which is more complementary to the active site of the enzyme than is acetylcholine itself. Hence EHS' would be more stable than EHS. Another possibility is that in order for hydrolysis to occur, the enzyme might effect a conformational change in the substrate. As the quinuclidine molecule is quite rigid, it would be less apt to undergo this change and would, therefore, be less prone to hydrolysis.

The K_i value is the dissociation constant of the enzyme–inhibitor complex in a reversible inhibition system. It is determined by a plot of v_0/v (ratio of velocity of hydrolysis of uninhibited substrate to that of inhibited substrate) *versus* $[I]$ (concentration of inhibitor). According to the equation (9)

$$v_0/v = 1 + \frac{[I]}{K_i[1 + S/K_m]}$$

[1] Nutritional Biochemical bovine erythrocyte acetylcholinesterase.

it follows that the x-intercept of this graph is equal to

$$K_i[1 + S/K_m]$$

A look at the results (Fig. 3) shows that both the quinuclidine alcohol and ketone had a lower K_i than choline.

The results show that the quinuclidine compounds have a greater affinity for acetylcholinesterase than do acetylcholine and choline. The 3-acetoxyquinuclidine methyl iodide–acetylcholinesterase complex was more reluctant to dissociate than the acetylcholine–enzyme complex. The 3-hydroxyquinuclidine methyl iodide (and even the 3-quinuclidone methyl iodide) had a lower K_i than choline, and, therefore, proved to be a better inhibitor than choline. The racemic quinuclidinol methyl iodide mixture was twice as potent an inhibitor as its corresponding ketone, implying that one of the alcoholic antipodes possesses a very strong inhibitory action. (Work is now in progress to demonstrate the activity of each individual antipode.)

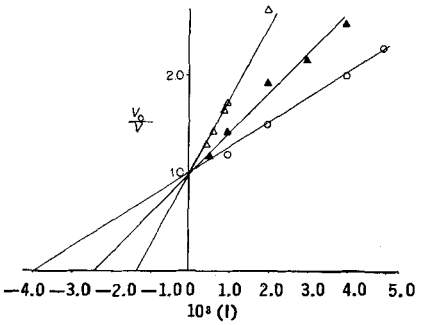

Fig. 3.—Key: ○, choline chloride; ▲, 3-quinuclidone methyl iodide; △, *dl*-3-quinuclidinol methyl iodide.

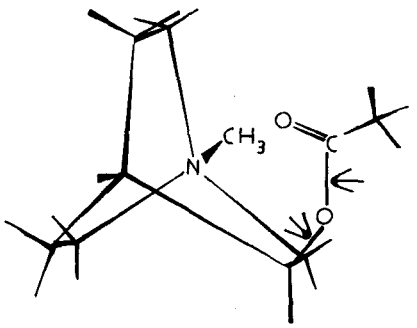

Fig. 4.—3-Acetoxyquinuclidine methyl iodide, in the conformation *nearest* "cisoid," illustrating overall "transoid" character. Arrows indicate only bonds around which rotation is conformationally significant.

As it is evident that these quinuclidine compounds are more strongly attracted to the enzyme than acetylcholine, it is probable that the dimensions of the quinuclidine compounds are closer to those of the enzyme's active site than are the dimensions of the acetylcholine molecule (at least with respect to functional groups).

The functional groups of the quinuclidine compounds are in the relatively transoid conformation (Fig. 4). This fact would imply that the enzyme would favor acetylcholine with its functional groups in a transoid conformation. It was mentioned earlier that Fujita and Fellman (3) have shown that acetylcholine assumes a cyclic structure when in solution. They also stated that the infrared spectra revealed the functional groups of the molecule to be in a cisoid conformation. While this is very possible with the substance in a simple solution, in the biological system set up in this experiment, the acetylcholine molecule is under the influence of acetylcholinesterase, and both molecules undergo some changes to achieve a proper fitting for the reaction to occur. From the results it is apparent that this fitting would favor an acetylcholine molecule with its functional groups in a conformation other than purely cisoid.

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