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Conformational Aspects of Systems Related to Acetylcholine. 3. Base-Catalyzed and Acetylcholinesterase-Catalyzed Hydrolysis of the Isomeric *dl*-3-Trimethylammonium-2-acetoxy-*trans*-decalin Halides and the Isomeric *dl*-1-Methyl-3-acetoxy-*trans*-decahydroquinoline Methiodides

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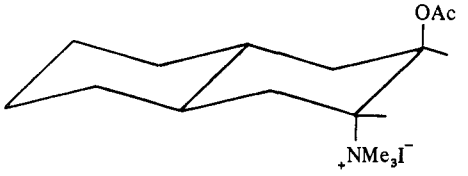
The catalytic rate constants for the base-catalyzed and AChE-catalyzed hydrolysis of 6 conformationally rigid ACh model substrates relative to the rate constants for ACh are reported as a function of solid state torsion angle τ (O-C-C-N^+). The acceleration of the enzymatic reaction over the nonenzymatic reaction relative to the acceleration for ACh (100%), (Rel k_{cat}'/K_m')/(Rel k_{OH}), is 17.5% when $\tau = 147°$, 9.12% when $\tau = 169°$, and <0.24% when $\tau = 60$–$74°$, demonstrating a preference by the enzyme for a transition-state geometry when $\tau \cong 150°$.

As part of an effort to determine the steric requirements of ACh receptor sites, 6 model compounds, in which the groups presumed responsible for attachment to the receptor site are conformationally rigid, *i.e.*, the isomeric *dl*-3-trimethylammonium-2-acetoxy-*trans*-decalin halides and the isomeric *dl*-1-methyl-3-acetoxy-*trans*-decahydroquinoline methiodides, have been synthesized and subjected to preliminary testing as previously reported.[1,2] Relative rates of hydrolysis of ACh in the presence of eel acetylcholinesterase (AChE) showed the trans diaxial isomer **1** to be the best substrate in the trans decalin series and the equatorial acetate **5** to be the better substrate in the trans decahydroquinoline series. This report presents the results of a more detailed investigation into the AChE-catalyzed hydrolysis as well as the nonenzymatic, base-catalyzed hydrolysis of ACh and the 6 model compounds **1-6**. Hydrolysis rates were measured by following the production of acid at 25° using a pH stat. The enzymatic hydrolyses were studied at pH 7.2; the base-catalyzed hydrolyses were studied at constant pH values ranging from 10 to 11.



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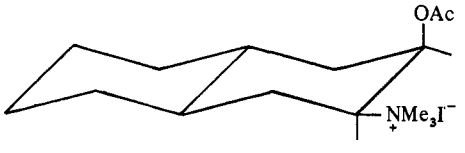

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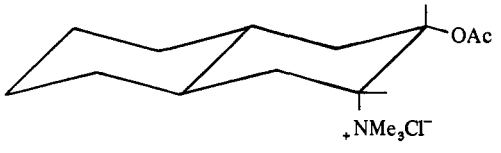

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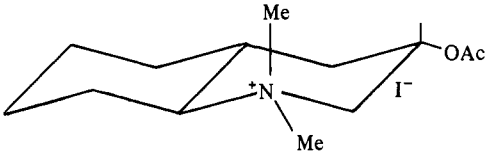

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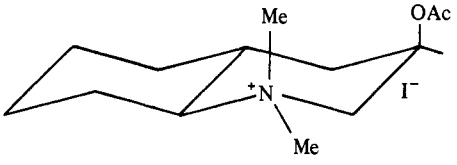

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Results

The observed or estimated solid state torsion angles, τ, the Michaelis-Menten parameters, K_m and V_{max}, for the enzymatic hydrolyses, the second-order rate constants, k_{OH}, for the base-catalyzed hydrolyses, rate constants for the 2 reactions relative to ACh, as well as data and calculations used to determine the relative enzymatic acceleration of the nonenzymatic hydrolyses are given in Table I.

X-ray diffraction data show that the O-C-C-N^+ torsion angles (τ) of **1**, **5**, and **6** are 147°,[3] 169°,† and 74°,† respectively. Deviation from the "expected" angle of 180° for **1** can be assumed to arise from 1,3-diaxial interactions between the bulky Me_3N^+ group and the axial protons.[3] Deviation from the angle of 60° expected in **6** presumably arises from skew interaction of the functional groups as well as 1,3-interaction between the axial bridgehead proton and the axial AcO function. Compds **2** and **3** in which skew interactions of the functional groups and 1,3-diaxial

†E. Shefter, private communication.

Table I. Solid State O-C-C-N^+ Torsion Angles and Kinetic Data for Base- and AChE-Catalyzed Hydrolysis of ACh and Conformationally Rigid Models at 25°

Substrate	τ O-C-C-N^+, deg	k_{OH}[a] M^{-1} min^{-1}	Rel k_{OH}	K_m, mM	V_{max}[a] = k_{cat}', mM min^{-1} $unit^{-1}$ ml	Rel k_{cat}'	k_{cat}'/K_m, unit ml min^{-1}[b]	Rel k_{cat}/K_m	10^2 Rel k_{cat}/K_m / Rel k_{OH}
ACh	77[c]	114 ± 5[e]	100.0	0.11 ± 0.03[f]	1.1 ± 0.1[f]	100.0	10.000	100.00	100.00
1	147	21.7 ± 0.2	18.8	0.43 ± 0.04	0.16 ± 0.06	14.6	0.37	3.70	19.700
2	~60[d]	14.8 ± 0.3	13.0		<0.0008[g]	~0	~0	~0	~0
3	~60[d]	12.0 ± 0.2	10.5	1.4 ± 0.6	0.0022 ± 0.0006	0.2	0.0016	0.016	0.152
4	~60[d]	19.1 ± 0.5	16.8	1.6 ± 0.5	0.0021 ± 0.0005	0.2	0.0013	0.013	0.077
5	169	62.5 ± 1.0	54.8	0.49 ± 0.03	0.247 ± 0.007	22.5	0.50	5.00	9.120
6	74	33.9 ± 0.3	29.7	0.56 ± 0.05	0.0039 ± 0.0002	0.4	0.0070	0.070	0.236

[a] For comparison purposes, experimental V_{max} values ($V_{max} = k_{cat}E_0$) have been divided by the enzyme conc (E_0) in μmolar units/ml. The result gives a value for the catalytic rate constant, k_{cat}, in units of mM min^{-1} $unit^{-1}$ ml or μmole $unit^{-1}$ min^{-1}. (If the enzyme conc were known in mM, this first-order rate constant would have units of min^{-1}). These normalized V_{max} quantities are designated henceforth as k_{cat}'. [b] This quantity has units of a second-order rate constant. [c] Reference 4. Presumably not the conformation reactive toward AChE. See ref 5. [d] Estimated. [e] This compares with a value of 120.5 M^{-1} min^{-1} calc for our condns of ionic strength from data presented by Robson-Wright.[6] [f] Average of 25 runs. These and all other error limits are standard deviations. [g] Smaller velocities cannot be measured accurately by our methods.

interaction of axial protons and functional groups oppose each other are estimated to have angles less than 74° and close to 60°. The angle for **4** may be somewhat larger than that for **2** and **3** owing to functional group skew interaction.

In the absence of enzyme, **1-6** in aqueous base have reactivities of 18.8, 13.0, 10.5, 16.8, 54.8, and 29.7% that of ACh, respectively. The anomalous[7] inverse ratio of rate constants, k_e/k_a, observed for **1** and **3**, where the axial ester function is hydrolyzed more rapidly than the equatorial, is of some interest. Further study is currently in progress.

Compds **1** and **5** (each as the racemate), with O-C-C-N^+ torsion angles of 147° and 169°, respectively, possess activities toward eel AChE of 14.6% and 22.5% that of the biological substrate ACh, respectively. Each of the remaining compounds, with torsion angles between 60° and 74°, in comparison has almost negligible reactivity.

Discussion

The above data appear to indicate that substrate activity increases with O-C-C-N^+ torsion angle and that an approximate trans (antiplanar‡) arrangement of the quaternary N and AcO functions is preferred by the enzyme over any other arrangement. It is, however, inaccurate to draw conclusions simply from the observed relative reactivities of the model compounds in the presence of the enzyme since the reactivities in the absence of the enzyme also differ among themselves.

In order to assess the preference of AChE for a particular transition-state geometry correctly it is necessary to determine how much the enzyme accelerates the nonenzymatic reaction. This is most simply done by calculating the ratio of second-order rate constants k_{OH} (for base hydrolysis) and k_{cat}'/K_m (for enzyme hydrolysis). The largest ratio $[(k_{cat}'/K_m)/k_{OH}]$ corresponds to the reaction in which the enzyme is contributing most to a lowering of the free energy of activation. Since the enzyme concentration is not known in molar units, the 2 second-order rate constants, k_{OH} and k_{cat}'/K_m, have different units and their ratio is not a direct measure of the enzymatic acceleration. The *relative* rate constants Rel k_{OH} and Rel k_{cat}'/K_m of Table I can, however, be compared. These provide a value (Rel k_{cat}'/K_m)/(Rel k_{OH}) for the observed acceleration of the enzymatic reaction over the nonenzymatic reaction for any compound relative to the acceleration for ACh. (On this relative scale the factor by which the base-catalyzed hydrolysis of ACh is accelerated by AChE is arbitrarily set at 100.) Examination of this relative scale shows AChE accelerates the hydrolysis of **1** (τ = 147°) by 19.7%, **5** (τ = 169°) by only 9.12%, and **2**, **3**, **4**, and **6** (τ = 60-74°) by less than 0.24% that of ACh.

Two conclusions may now be offered. In doing so, it is assumed that the transition-state O-C-C-N^+ angles for the reactions in aqueous solution at 25° are approximated by those observed (or estimated) for the solid state. (1) The enzyme AChE clearly prefers O-C-C-N^+ angles of >74°. The suggestion[8],§ that the activity of the trans diaxial **2** is attributable to a fraction of the agonist existing in a half-boat or twist conformation requiring a rearrangement of the functional groups from an antiplanar to a synclinal orientation is thus inconsistent with the low level of activity of the synclinal isomers **2**, **3**, **4**, and **6**. (2) The enzyme prefers the geometry of **1** (147°) over that of **5** (169°). These results support the conclusion of Chothia and Pauling[5] that the conformation of ACh reactive toward AChE has τ O-C-C-N^+ equal to 150°.

Experimental Section

Materials. Water. CO_2-free H_2O was prepared by passing distilled H_2O through a Corning LD-3 demineralizer equipped with a cartridge of "ultrahigh purity" resin No. 350A immediately before use or by redistn once form $KMnO_4$ (1 g/l.)-H_2SO_4 (1.5 ml/l.) followed by distn from NaOH (10 g/l.) and storage in a polyethylene bottle fitted with a CaO tube.

$MgCl_2$ Stock Soln. Because of the hygroscopic nature of $MgCl_2$ the following method was adopted to prepare a standard stock soln. A saturated soln of $MgCl_2$ in H_2O was prepared from reagent grade $MgCl_2 \cdot 6H_2O$ at 20°; 10 ml of this soln was transferred with a volumetric pipet to a flask containing 18.47 g (18.5 ml) of H_2O. Since the density of H_2O at 20° is 0.9982 g/ml and the solubility of $MgCl_2$ at 20° is 54.25 g/100 ml, the concn of the final stock soln is 190.4 mg/ml at 20°.

Salt Soln. Following the suggestions of Higman,# the assay soln was 0.16 M and 0.002 M in NaCl and $MgCl_2$, and contained 50 mg of bovine serum albumin per l.

Enzyme Soln. Immediately prior to use enough Sigma Chemical Co. electric eel (*Electrophorus electricus*) Type III AchE is dissolved in the enzyme salt assay soln described above to give a convenient rate of reaction (from 0.1 to 10 μM units/ml) and kept under a stream of N_2. (The activity of the enzyme as supplied from Sigma is described as approx 700-2000 μM units/mg of protein. A 1 μM unit will hydrolyze 1 μmole of Ach per min at

‡The terms synplanar, synclinal, anticlinal, and antiplanar describe torsion angles of 0°, 60°, 120°, and 180°, respectively.

§R. Finnegan, personal communication, 1969.

#H. Higman, The University of Pittsburg, Pittsburg, Pa., personal communication, 1969.

pH 8.0 and 37°. The frozen aqueous soln contains approx 5 mg of $(NH_4)_2SO_4$/mg of protein.)

ACh Chloride Soln. Because of the extremely hygroscopic nature of AChCl, stock solutions were prepared as follows. About 1 g of AChCl (cryst, "99%," Sigma Chemical Co) was transferred under N_2 in a glove box to each of several weighing bottles. These bottles were stoppered, weighed, and stored in a freezer until needed. Just prior to a kinetic run a 2.015 *M* stock soln is obtained by adding to one of the bottles an amount of H_2O weighing 1.8013 times the number of g of AChCl. The density of such a soln was previously determined to be 1.0268 g/ml at 20°; 1.026 g (1 ml) of stock is removed and diluted to 10 ml giving a 0.2015 *M* soln; 8 ml of this soln is removed and diluted to 10 ml giving a 0.1613 *M* soln. Repeating this dilution technique 4 times gives 4 more solutions: 0.1291 *M*, 0.1033 *M*, 0.0826 *M*, and 0.0661 *M*.

Isomeric *dl*-3-Trimethylammonium-2-acetoxy-*trans*-decalin Halides. The preparation and properties of these compds have been described previously.[1]

Isomeric-*dl*-1-Methyl-3-acetoxy-*trans*-decahydroquinoline Methiodides. A description of these compds has likewise appeared previously.[2]

Analog Substrate Soln. A stock soln (2 ml) (approx 0.2 *M*) of each of the 6 compds was prepd. Five successive dilutions were made by removing 0.8 ml of the previous soln and diluting to 1 ml with distilled H_2O. Six solns for kinetic study are thus obtained.

Enzyme Kinetics. The rate of AChE hydrolysis of AChCl and its analogs was followed by measuring the rate of AcOH production by the pH Stat method. The Radiometer Co. TTT 11 Titrator, ABU1 Auto-burette fitted with a 0.25-ml buret, SBR2C Titrograph recorder, PHM26 expanded scale pH meter with Type 202C glass electrode and Type K 401 calomel electrodes, and a TTA3 Titration Assembly equipped with a constant temperature anaerobic assay chamber and motor driven stirrer were used.

Measurements were made under N_2 at pH 7.2 ± 0.1 at 24.90 ± 0.05° using enzyme strengths of 0.1 to 10 μM units/ml and 6 different concns (ranging from 3×10^{-4} *M* to 10×10^{-4} *M*) of substrate. At least 3 runs were performed for each of the 6 concns of each of the 7 substrates (AChCl and 6 analogs). The titrant was 0.0100 *N* NaOH. In a typical run 10 ml of freshly prepared enzyme soln (kept under N_2) is removed to the thermostated assay chamber with a volumetric pipet and allowed to reach constant temp. To this soln, under N_2, is added 0.050 ml of substrate soln, and the instrument is activated. The end-point pH is set at 7.3 with a proportional band setting of 0.2.

The raw data consisting of a chart trace of per cent full buret *vs.* centimeters are punched onto cards. Reaction velocities are extrapolated to 0 time to give the initial reaction velocity for each substrate concn. An iterative least-squares fit directly to the Michaelis equation affords values for K_m and V_{max}.

Base-Hydrolysis Kinetics. Rates were studied at 24.90 ± 0.05° using 10.00 ml of reaction soln containing substrate concn of approximately 6×10^{-4} *M*. At least 4 runs were made for each substrate at each of 4 different base concns ranging from pH 10.0 to 11.0. The base concn was kept constant (±0.01 pH unit) by the addition of 0.0500 *N* NaOH as described in the previous section. Electrodes were calibrated for the pH range 9–12 at various temp with commercial buffer soln and standard NaOH soln; corrections at 25° were found not to be required. Blank runs (no base present) gave negligible rates. Using the relationship between rate constant and ionic strength derived by Robson-Wright for ACh hydrolysis, it was calculated that any variations in ionic strength occurring under our conditions either (a) during a run or (b) between runs at different base concns have a negligible effect (within experimental error) on our rate constant values.

A typical determination is carried out as follows. A NaOH soln (10.00 ml) of approx the desired pH (about 0.1 pH unit higher than the preset end point) is added to a thermostated reaction vessel and stirred under N_2 until const temperature is reached. To this soln is added 0.100 ml of a 0.06 *M* substrate stock soln. The reaction is followed until 90% completion. Raw data (in the form of a recorder chart trace of per cent of full buret *vs.* centimeters) are converted to time and concn of titrant added (*i.e.*, of ester reacted) and fitted by a least-squares technique to the first-order rate equation. The resulting observed (pseudo-first-order) rate constants ($k_{obsd} = k_{OH}[OH^-]$) are plotted against base concn to give k_{OH} values.

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Structural Parameters Determining Cholinergic and Anticholinergic Activities in a Series of 1,3-Dioxolanes

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A series of 1,3-dioxolane-4-dimethylaminomethyl methiodides substituted at the 2 position with alkyl and aryl groups have been examined for their agonist and antagonist activities at muscarinic receptors in guinea pig ileal muscle and rat jejunum. Complete lack of stereoselectivity of antagonist activity was found with the enantiomers of the 2,2-diphenyl and the 2-phenyl-2-cyclohexyl derivatives. A reasonable correlation of pA_2 and π was observed. pA_2 values appeared independent of the agonist employed. Selected compounds were examined for their antihistaminic activities: these were 100–500 times less than the anticholinergic activities but were similarly nonstereoselective.

The 1,3-dioxolane nucleus serves as the basic structure for a number of potent agonists and antagonists active at the muscarinic acetylcholine receptors of smooth muscle.[1] 2-Methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide (Table IV, 23) originally described by Fourneau, *et al.*,[2] is a particularly selective and active agonist at muscarinic receptors:[1,3,4] its interaction with the muscarinic receptor is highly stereospecific, the cis isomer being 5–10 times more potent than the trans isomer[3] and the 2*S*,4*R* isomer being 100 times more active than the 2*S*,4*S* isomer.[4,5]