ride (1.4 g, 0.01 mol) during a period of 10 min. The mixture was refluxed for 1 hr and was stirred at room temperature overnight. After evaporation of the solvent, H_2O (100 ml) was added and the product collected by filtration and washed thoroughly with water. Recrystallization of the crude product from 50% ethanol gave the desired compound.

Amino Acids (Table II). The procedure of Kazmirowski, *et al.*,[21] was followed. A mixture of cyano acid in methanol–concentrated ammonium hydroxide (2:1) and W-2 Raney nickel was hydrogenated at room temperature at an initial pressure of 45 psi for 5 hr. The filtrate of the reaction mixture was concentrated to dryness and the residue was recrystallized from water to yield the corresponding amino acid.

Amidino Esters (Table IV). The procedure of Dox[22] was employed.

Ethyl 4-Amidinocinnamate (41). Into a solution of ethyl 4-cyanocinnamate (1.02 g, 5 mmol) in 20 ml of absolute ethanol cooled to 0–5° was passed dry HCl gas for 5 min. The solution was stirred overnight. Evaporation of the ethanol and hydrogen chloride *in vacuo* at room temperature gave a white solid which was collected and washed with ether. The product, an imino ether, was kept in a desiccator over alkali for several days to remove excess HCl. The imino ether was then treated with 1 equiv of alcoholic ammonia solution with stirring overnight. Evaporation of the excess of alcoholic ammonia gave the product, ethyl 4-amidinocinnamate hydrochloride. Recrystallization of the product from ethanol–ether yielded the pure compound.

Ethyl 4-Hydroxyamidinophenoxyacetate (45). To a solution of $NH_2OH \cdot HCl$ (1.05 g, 0.015 mol) and K_2CO_3 (1.04 g, 0.0075 mol) in H_2O (9 ml) was added a solution of ethyl 4-cyanophenoxyacetate (2.05 g, 0.01 mol) in ethanol (40 ml). The mixture was stirred at reflux for 3 hr. After the mixture was cooled to room temperature the solvent was stripped off *in vacuo*. Water (100 ml) was added to the residue. The solids were filtered and washed with water. The crude crystals were recrystallized from 50% EtOH to give ethyl 4-hydroxyamidinophenoxyacetate, which was dissolved in an excess of absolute ethanol saturated with HCl gas. After evaporation of the solvent the residue was recrystallized from EtOH–Et_2O to give compound **45:** mp 159–161° (1.85 g, 67%).

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References and Notes

(1) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240,** 1579 (1965).
(2) F. Markwardt, H. Landmann, and P. Walsmann, *Eur. J. Biochem.*, **6,** 502 (1968).
(3) J. D. Geratz, *Arch. Biochem.*, **118,** 90 (1967).
(4) J. D. Geratz, *Experientia*, **25,** 1254 (1969).
(5) J. D. Geratz, "Pulmonary Emphysema and Proteolysis," C. Mittman, Ed., Academic Press, New York, N.Y., 1972, p 325.
(6) G. E. Davies and J. S. Lowe, *Advan. Exp. Med.*, **8,** 453 (1970).
(7) J. D. Geratz, A. C. Whitemore, M. C.-F. Cheng, and C. Piantadosi, *J. Med. Chem.*, **16,** 970 (1973).
(8) J. D. Geratz and W. P. Webster, *Arch. Int. Pharmacodyn. Ther.*, **194,** 359 (1971).
(9) J. D. Geratz, unpublished observation.
(10) G. Glover, C. C. Wang, and E. Shaw, *J. Med. Chem.*, **16,** 62 (1973).
(11) S. J. Singer, *Advan. Protein Chem.*, **22,** 1 (1967).
(12) K. Tanizawa, S. Ishii, and Y. Kanaoka, *Chem. Pharm. Bull.*, **18,** 2247 (1970).
(13) T. Inagami, *J. Biol. Chem.*, **239,** 787 (1964).
(14) F. Markwardt, H.-P. Klocking, and G. Nowak, *Experientia*, **27,** 812 (1971).
(15) S. W. Nye, T. B. Graham, K. M. Brinkhous, *Amer. J. Med. Sci.*, **243,** 279 (1962).
(16) S. S. Berg and G. Newberg, *J. Chem. Soc.*, 642 (1949).
(17) French Patent Specification 1,375,311 (patent assigned to May and Baker Ltd.) (The Patent Office, Paris, 1965); *Chem. Abstr.*, **62,** 3982*h* (1965).
(18) R. H. Wiley and N. R. Smith, *J. Amer. Chem. Soc.*, **70,** 1560 (1948).
(19) (a) N. Moses, *Chem. Ber.*, **33,** 2623 (1900); (b) H. Rapport, A. R. Williams, O. G. Lorve, and W. W. Spooner, *J. Chem. Soc.*, 1125 (1953).
(20) N. V. Hages and G. E. K. Branch, *J. Amer. Chem. Soc.*, **65,** 1555 (1943).
(21) P. N. Kazmirowski, H. Landmann, and F. Markwardt, *Pharmazie*, **22,** 465 (1967).
(22) A. W. Dox, "Organic Syntheses," Collect. Vol. I, Wiley, New York, N.Y., 1932, p 5.
(23) IBA Ltd., French Patent 1,517,896 (March 22, 1968); *Chem. Abstr.*, **72,** 12413m (1970).
(24) G. Wagner, Ch. Garbe, and P. Richter, *Pharmazie*, **28,** 724 (1973).
(25) P. Walsmann, F. Markwardt, P. Richter, J. Sturzebicher, G. Wagner, and H. Landmann, *Pharmazie*, **29,** 333 (1974).
(26) M. Mares-Guia, E. Shaw, and W. Cohen, *J. Biol. Chem.*, **242,** 5777 (1967).

Base-Catalyzed and Cholinesterase-Catalyzed Hydrolysis of Acetylcholine and Optically Active Analogs

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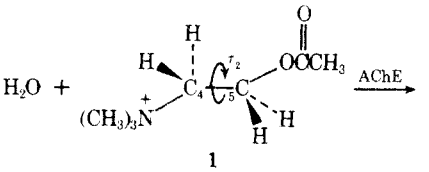
The base- and cholinesterase-catalyzed hydrolyses of the following optically active analogs of acetylcholine were studied: 3(a)-trimethylammonium-2(a)-acetoxy-*trans*-decalin iodide, *threo*- and *erythro*-α,β-dimethylacetylcholine iodide, α-methylacetylcholine, and β-methylacetylcholine. Evidence that the optimum dihedral ^+N-C-C-O angle in the transition state for acetylcholinesterase hydrolysis of acetylcholine analogs is positive and anticlinal is given. The data obtained suggest that acetylcholine undergoes a geometrically flexible mode of attachment to the enzyme.

Acetylcholine [ACh (1)] is well known as the chemical transmitter of nerve impulses in cholinergic neural systems.[1] Once it has accomplished its function at a receptor site it is rapidly destroyed in a hydrolytic reaction catalyzed by the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) as given in eq 1.

The work reported herein is part of a continuing investigation[2-7] into the structural requirements for cholines-



1

$$(CH_3)_3\overset{+}{N}CH_2CH_2OH + CH_3COOH \quad (1)$$

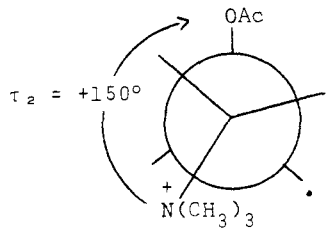
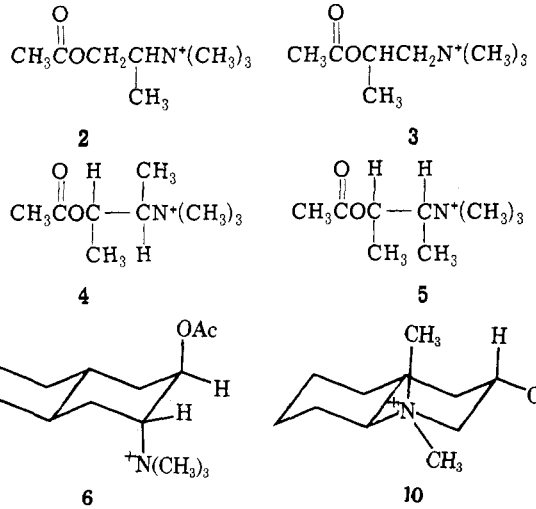


Figure 1.

terase action; of particular interest has been the conformation about the ACh C_4–C_5 bond, that is, the optimum dihedral angle, τ_2, at the transition state of the reaction. Correlation of crystal structure data[8-10] and relative rates[11] of enzymatic hydrolysis of open-chain, conformationally flexible ACh analogs of known absolute configuration led Chothia and Pauling[8] to conclude that the optimum torsion angle in question was +150°† as illustrated in Figure 1. Since there is no necessary correlation between solid state (or even solution-state ground-state structure) and *transition state* conformation of a flexible substrate at an enzyme active site (see discussion in ref 13d), these conclusions required further experimental verification. Nevertheless, numerous studies[2-4,13] of AChE activity toward conformationally *rigid* ACh analogs, *i.e.*, those with restricted rotation about the C_4–C_5 bond (fixed τ_2), where solid-state and transition-state geometry should be little changed, generally supported the Chothia and Pauling contention; conformationally rigid ACh analogs with τ_2 in the synperiplanar† to synclinal range did not hydrolyze in the presence of AChE; analogs with τ_2 in the anticlinal (ac) to antiperiplanar (ap) range did hydrolyze. As recently reported from these laboratories[4] after studying the AChE-catalyzed and nonenzymatic hydrolysis of compounds **6** and **10**, an angle of 147° is preferred to

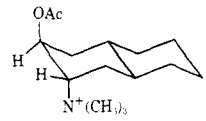
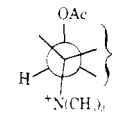
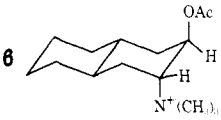
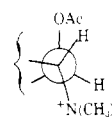


one of 169°, clearly demonstrating a preference on the part of the enzyme for an ac transition state angle over an ap angle. Since the compounds studied were racemic, no conclusions were drawn regarding the sign of the preferred angle. (Contrary to some reports, no pharmacological or kinetics studies on the optically active decalin compounds **6** have been carried out prior to this report; see ref 14 and 15.)

The present work describes the resolution and kinetics

†Conventions for giving the sign of τ_2 (positive when the angle is measured clockwise from the front substituent A to the rear substituent B in a Newman projection for a system A–C–C–B) and the terms synperiplanar (sp, 0 ± 30°), synclinal (sc, 60 ± 30°), anticlinal (ac, 120 ± 30°), and antiperiplanar (ap, 180 ± 30°) are those of Klyne and Prelog.[12]

Table I. Absolute Configuration and Physical Constants for (+)- and (−)-2(a)-Acetoxy-3(a)-trimethylammonium-*trans*-decalin Iodide

Compd	Absolute confign	Mp, °C	$[\alpha]$,[a] deg	τ_2(+N–C–C–O)	Newman projection
(*SS*)-(+)-**6**		218	+10.9	+148°	
(*RR*)-(−)-**6**		218	−10.8	−148°	

[a] $[\alpha]^{30}D$ in 90% v/v EtOH.

of the enzymatic [eel AChE and horse serum butyrylcholinesterase (ChE)] and nonenzymatic, basic hydrolysis of the rigid ACh analog **6** and the four conformationally flexible analogs **2**–**5**.

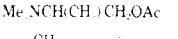
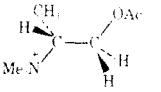
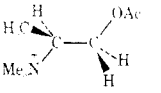
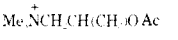
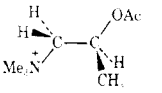
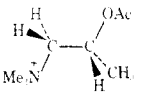
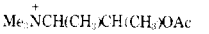
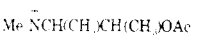
Results

Resolution of the five ACh analogs **2**–**6** into their optical isomers was accomplished according to previously reported[11a,16] or otherwise standard procedures. The absolute configurations of the isomers of **6** were established by X-ray crystallography[7] and are shown in Table I together with the observed value for the dihedral angle ^+N–C_4–C_5–O. The structures, absolute configurations (where known), and specific rotations for compounds **2**–**5** are given in Table II.

All hydrolysis rates were measured by following the production of acid at 25° using the pH-stat method. The enzymatic hydrolyses were studied at pH 7.2, each of the base-catalyzed reactions at four different pH's from 10 to 11. The enzyme concentration varied from 0.1 to 10 μM units/ml depending on the substrate activity; six concentrations were used for each substrate from 0.3 to 1 mM.

The results of the kinetics studies are given in Table III. Column 2 lists the second-order rate constants, k_{OH}, for the base-catalyzed hydrolyses and column 3 gives these values relative to that for ACh. Column 5 tabulates values for the Michaelis-Menten parameter K_m. After dividing the experimentally determined V_{max} values by the enzyme concentration (E_0, in μM units/ml) a normalized value for V_{max} is obtained. This value is a first-order catalytic rate constant (since $V_{max} = k_{cat}E_0$) for the reaction (in units of μmol unit^{-1} min^{-1}) and is designated here as $k'_{cat.}$. Values for $k'_{cat.}$ appear in column 6 of Table III and are related to that for ACh in column 7. Division of these relative $k'_{cat.}$ values by K_m gives a relative second-order rate constant for the enzyme-catalyzed hydrolysis (column 9) which may be compared with that for nonenzymatic hydrolysis (k_{OH}, column 3) to give in column 10 the observed accelerations of the enzymatic reaction over the nonenzymatic reaction for each substrate relative to the acceleration for ACh (arbitrarily set at 100); these values may be used to assess the preference of an enzyme for a particular transition-state geometry. It is important to note that in most previously reported work it is simply relative V_{max}, that is, $k'_{cat.}$, quantities, such as those in column 7 of Table III, that are used to assess the enzymic geometric preference. However, since different substrates have intrinsically different rates of hydrolysis even in the absence of enzyme (refer to columns 2 and 3 of Table III), the nonenzymatic hydrolysis rate constant

Table II. Structure and Specific Rotations (29°; 90% v/v EtOH) for Conformationally Flexible Optically Active Analogs of ACh[a]

Compd	Name	$[\alpha]^{29}D$, deg	Structure
2	(±)-α-Methylacetylcholine		
(*R*)-(+)-**2**	(*R*)-(+)-α-Methylacetylcholine	+8.62	
(*S*)-(−)-**2**	(*S*)-(−)-α-Methylacetylcholine	−8.41	
3	(±)-β-Methylacetylcholine		
(*S*)-(+)-**3**	(*S*)-(+)-β-Methylacetylcholine	+26.4	
(*R*)-(−)-**3**	(*R*)-(−)-β-Methylacetylcholine	−27.8	
4	(±)-*threo*-α,β-Dimethylacetylcholine		
(+)-**4**	(+)-*threo*-α,β-Dimethylacetylcholine	+3.4	
(−)-**4**	(−)-*threo*-α,β-Dimethylacetylcholine	−3.3	
5	(±)-*erythro*-α,β-Dimethylacetylcholine		
(+)-**5**	(+)-*erythro*-α,β-Dimethylacetylcholine	+10.5	
(−)-**5**	(−)-*erythro*-α,β-Dimethylacetylcholine	−10.9	

[a] All compounds are iodides.

must be divided out (as in column 10) before a truly reliable comparison of relative enzymatic activity can be made. Frequently the order of reactivity is unchanged but occasionally an important reversal is seen [see especially ref 4 and note compound (*R*)-(+)-**2** in Table III]. Unfortunately, for some of the discussion below it has been necessary to compare relative V_{max} data as reported in the literature rather than relative enzymatic accelerations since the nonenzymatic rate constants were unavailable.

Several immediate observations can be made upon examination of Table III. (A) The rate constants for the various analogs **2**–**6** differ from one another and from ACh itself. Thus the rate-limiting process for these compounds cannot be deacylation of the common acetyl enzyme intermediate and the data must pertain to the transition state for a prior (acylation) step. (B) ACh is the best substrate for both enzymatic and nonenzymatic hydrolysis. The relative rates fall off for the open-chain compounds in the order **2** > **3** > **4** > **5** for both types of reaction although the falloff is more pronounced with the enzyme reactions, particularly with the ChE which does not catalyze the hydrolysis of compounds **4**, **5**, or **6** at all. The results are in qualitative agreement with those reported for bovine erythrocytes AChE.[11a,16] (C) The effect of α-methylation (compounds **2**) is to lower the base-catalyzed hydrolysis slightly (to 95% that of ACh) and the enzyme-catalyzed rate somewhat more. For both enzymes studied the hydrolysis rates are in the order (*S*)-(−)-**2** > (±)-**2** > (*R*)-(+)-**2**, that is, the racemate hydrolyzes at a rate in between that of the (+) and (−) isomers. (D) The effect of a β-methyl group (as in compounds **3**), *i.e.*, substitution closer to the reaction center, is predictably to decrease markedly both enzymatic and nonenzymatic hydrolysis. Again the effect is more pronounced with enzyme catalysis and again one of the enantiomers is a much better substrate than the other with the racemate showing intermediate reactivity: (*S*)-(+)-**3** > (±)-**3** ≫ (*R*)-(−)-**3**. (E) The presence of two methyl substituents in the substrate as in the *threo*- and *erythro*-α,β-dimethylacetylcholine (compounds **4** and **5**, respectively) serves to depress the hydrolysis rate relative to ACh still further. In the presence of eel AChE, the erythro series showed extremely slight hydrolytic activity with a nevertheless observable difference between enantiomers; the threo series, although hydrolyzing at an observable rate, showed no significant difference in rate between the racemic or either of the optically active modifications. (F) Compound **6**, although stereochemically analogous to the threo compound **4**, exhibits very different hydrolytic behavior. While its nonenzymatic hydrolysis rate is much less than that of the threo compound, its enzymatic hydrolysis rate is 5–6 times that of **4**. Further, and more significantly, the AChE-catalyzed hydrolysis rate is very sensitive to the absolute configuration of **6**; the (+) isomer hydrolyzes at a rate 25% that of ACh and greater than that for the (±) modification whereas the (−) isomer undergoes no observable reaction.

Discussion

As has been noted above and as can be seen from Tables I and III, the *SS*-(+) isomer of compound **6** with dihedral $^{+}$N–C–C–O angle, τ_2, of +148° is a substrate for eel AChE while the *RR*-(−) isomer with τ_2 of −148° has no substrate activity. These results clearly and for the first time unambiguously demonstrate that the lowest energy transition-state $^{+}$N–C–C–O dihedral angle for AChE-catalyzed hydrolysis of ACh analogs is *positive* and *anticlinal* (+ac) and as represented in Figure 1.

Indeed, correlation of relative activation energies for various rigid ACh analogs with their $^{+}$N–C–C–O torsion angles as presented in Table IV and in Figure 2 suggests

Table III. Kinetic Data for Base- and Esterase-Catalyzed Hydrolysis of ACh and Analogs at 25°

Substrate	k_{OH}, M^{-1} min^{-1}	Rel k_{OH}	Enzyme	K_m, mM	V_{max}[a] = $k'_{cat.}$, μmol unit^{-1} min^{-1}	Rel $k'_{cat.}$	$k'_{cat.}/K_m$,[b] unit^{-1} ml min^{-1}	Rel k'_{cat}/K_m	10^2 rel $k'_{cat.}/K_m$ / rel k_{OH}
1	114 ± 5[c]	100.0	AChE (electric eel)	0.11 ± 0.02[d]	1.1 ± 0.1[d]	100.0	10.00	100.0	100.0
2	108 ± 4	94.8		0.13 ± 0.02	0.95 ± 0.1	87	7.31	73.1	77
(*R*)-(+)-**2**				0.28 ± 0.02	0.92 ± 0.1	84	3.28	32.8	35
(*S*)-(−)-**2**				0.16 ± 0.03	1.22 ± 0.1	110	7.63	76.3	80
3	40 ± 3	35.0		0.56 ± 0.04	0.22 ± 0.02	20	0.39	3.9	11
(*S*)-(+)-**3**				0.57 ± 0.05	0.35 ± 0.03	32	0.61	6.1	17
(*R*)-(−)-**3**					0[e]	0	0	0	0
4	33.7 ± 0.9	29.6		0.69 ± 0.07	0.09 ± 0.01	8	0.11	1.1	4.4
(+)-**4**				0.69 ± 0.07	0.07 ± 0.01	6	0.11	1.1	3.4
(−)-**4**				0.70 ± 0.08	0.08 ± 0.01	7	0.11	1.1	3.7
5	28.1 ± 0.5	25.5		0.43 ± 0.09	0.003 ± 0.001	0.3	0.007	0.07	0.03
(+)-**5**				0.86 ± 0.3	0.014 ± 0.002	1.3	0.016	0.16	0.6
(−)-**5**				0.52 ± 0.21	0.015 ± 0.008	1.4	0.029	0.29	1.1
6	21.7 ± 0.2	19.0		0.43 ± 0.04	0.16 ± 0.06	15	0.37	3.7	19.5
(*SS*)-(+)-**6**				0.84 ± 0.20	0.40 ± 0.05	36	0.48	4.8	25
(*RR*)-(−)-**6**					0	0	0	0	0
1		100.0	ChE (horse serum)	0.51 ± 0.09	0.63 ± 0.07[f]	100	1.23	100.0	100.0
2		94.8		0.70 ± 0.06	0.43	68	0.6	50	52
(*R*)-(+)-**2**				1.34 ± 0.11	0.49	78	0.4	30	32
(*S*)-(−)-**2**				1.40 ± 0.40	1.03	163	0.7	59	63
3		35.0		3.78 ± 0.39	0.01	1.4	0.002	0.002	0
(*S*)-(+)-**3**				0.66 ± 0.07	0.005	0.8	0.007	0.006	0
(*R*)-(−)-**3**					0	0	0	0	0

[a] For comparison purposes, experimental V_{max} values ($V_{max} = k_{cat.}E_0$) have been divided by the enzyme concentration (E_0 in micromolar units/ml). The result gives a value for the catalytic rate constant, $k_{cat.}$, in units of mM min^{-1} unit^{-1} ml or μmol unit^{-1} min^{-1}. (If the enzyme concentration 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] This compares with a value of 120.5 M^{-1} min^{-1} calculated for our conditions of ionic strength from data presented by M. Robson-Wright, *J. Chem. Soc. B*, 545 (1968). [d] Average of 35 runs. These and all other error limits are standard deviations. [e] Smaller velocities cannot be measured accurately by our methods. [f] Average of 25 runs.

that the optimum angle may be significantly less than +148° and possibly as low as +120°. [Values for G^*_{es} were obtained from the relationship $G^*_{es} = -2.3RT \log (6/\text{rel } k'_{cat.})$ where 6 is the approximate factor by which the rate constant for the deacylation step must be multiplied to give the acylation rate constant for the ACh/AChE reaction.] Definitive conclusions must await comparison of relative enzymatic accelerations possible only when the nonenzymatic rate constants for the compounds in question are known.

Results obtained for the open-chain analogs **2**–**5** can be tentatively interpreted assuming the requirement (or preference) on the part of AChE for a +ac $^+$N-C-C-O transition-state conformation. Shown in Table V are the Newman projections about the C_4-C_5 bond and the relative enzymatic rate accelerations (from column 10, Table III). Nonbonded interactions and steric strain are expected to be small for ACh and for (*S*)-(−)-α-methylacetylcholine and larger for the (*R*)-(+)-α-methyl isomer. The predicted increases in transition-state energy as the molecule tries to adopt the preferred +ac conformation are reflected in the lowering of hydrolytic reactivity (100% to 80% to 35%) in the presence of AChE. Substitution of a methyl group in the β position (compounds **3**) results in a decrease in substrate activity. Of the two isomers of **3**, the *R*-(−) enantiomer should have a severe restriction to attainment of a +ac conformation and, in fact, this enantiomer is not a substrate for AChE. Results observed with the disubstituted ACh analogs **4** and **5** are less definitive. These are barely reactive substrates and any differences between

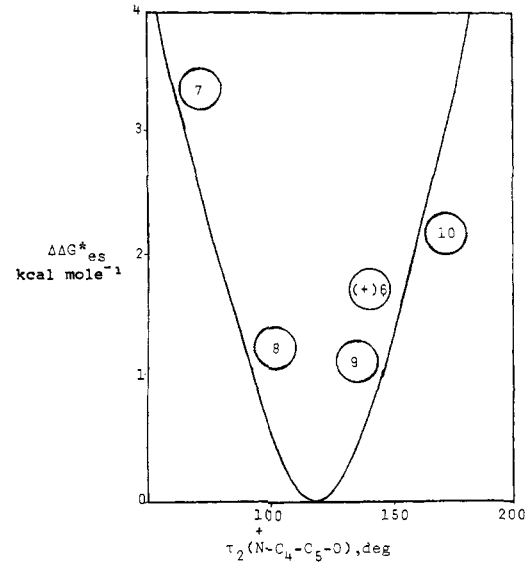
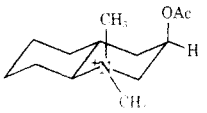
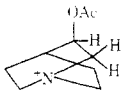
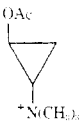
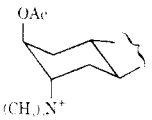
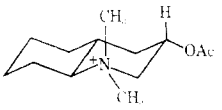


Figure 2. Relative free energy of activation *vs.* dihedral $^+$N-C_4-C_5-O angle for rigid analogs of acetylcholine (see Table IV).

Table IV. Solid-State Torsion Angles ($^+$N-C-C-O) for Rigid ACh Analogs and Relative $k'_{cat.}$ Values for Their AChE-Catalyzed Hydrolyses at 25°

Compd	No.	τ_2 (N–C–C–O), deg	Enzyme	Rel $k'_{cat.}$	Ref
(±) 	7	+74	Eel AChE	~0.1[a]	4
R-(–) 	8	+106	Bovine erythrocytes AChE	83[b]	13d,e
SS-(+) 	9	+137	?	96	13a
SS-(+) 	6	+148	Eel AChE	36	This paper
(±) 	10	+169	Eel AChE	~4.5[a]	4

[a] Compounds 7 and 10 were studied as the racemates; the experimental relative $k'_{cat.}$ values were divided by 2 to give the approximate values given in this table. [b] The AChE used in this study was from bovine erythrocytes. Since relative $k'_{cat.}$ values obtained from this enzyme are somewhat different (see ref 11a and 16) than for eel AChE, the value of 83% given here should be considered approximate.

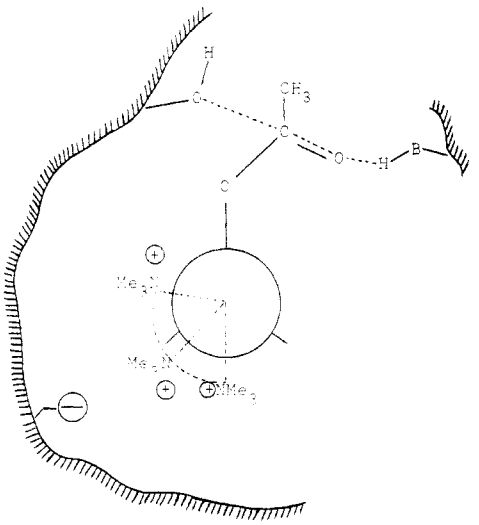


Figure 3. Proposed structure of the AChE active site showing flexibility in binding to the anionic region.

isomers are less likely to be significant. It is, however, difficult to explain the observed reactivity, although slight, of the *RR*-threo isomer if a +ac transition-state angle is required. Such a conformation would be extremely difficult to achieve.

It is important to note that so far in this paper and in earlier work (see, for example, ref 8), it has been attempted to establish (for flexible ACh analogs) a particular transition-state dihedral angle, assumed to be the same for all compounds, and to ascribe any rate differences to difficulties in achieving that particular conformation. It is possible, however, to explain the data in another way.

It is suggested that the dihedral angle between the acetoxy group (which group is bound to the enzyme at the transition state) and the enzyme anionic binding site is +ac (data from Table IV and Figure 2 suggest it may be ~+120°) as illustrated in Figure 3. A molecule whose quaternary ammonium cationic function can achieve that conformation, *e.g.*, a suitably designed rigid molecule or a flexible molecule with minimal barriers to rotation about the C_4-C_5 bond, will result in a maximum electrostatic attraction, a consequent lowering of transition-state energy, and a fast reaction, as observed for compounds **9**, **8**, (*S*)-(–)-**2**, and (*SS*)-(+)-**6**. Furthermore, it is proposed that the ammonium group of flexible analogs will adopt *any* +ac conformation (dihedral angles from ~+90° to +170°) that will result in maximum electrostatic attraction and minimum steric nonbonded repulsions, *i.e.*, a minimization of electrostatic and torsional strain energies.

Figure 4 is a plot of log relative $k'_{cat.}$ *vs.* free energy of activation (relative to ACh) for conformations giving the minimum such energy for four of the eight flexible ACh analogs studied. The activation energy plotted, $\Delta\Delta G^*_{conf}$, was assumed to be the sum of the electrostatic energy (resulting from the purely electrostatic attraction between the anionic site and the quaternary ammonium group) and the rotational barrier energy (resulting from nonbonded interactions encountered upon rotation about the C_4-C_5 bond)

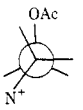
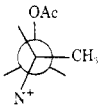
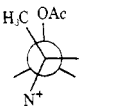
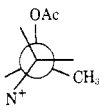
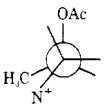
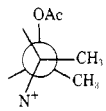
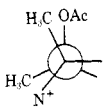
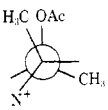
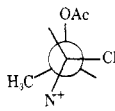
$$\Delta\Delta G^*_{conf} = \Delta\Delta G^*_{es} + \Delta G^*_{ster} + C \qquad (2)$$

where C is a constant. Estimates of $\Delta\Delta G^*_{es}$ at various angles τ_2 can be obtained from Figure 2; the equation for the parabola is

$$\Delta\Delta G^*_{es} = 0.0013(\tau_2 - 120)^2 \qquad (3)$$

Estimates of ΔG^*_{ster}, rotational barriers at various angles τ_2, can be obtained from quantum theoretical studies. Data which were recently reported by Radna, *et al.*,[17] for the same flexible ACh analogs that have been of interest

Table V. Structure and Reactivity of Various Nonrigid Analogs of ACh

Compd	C_4-C_5 Newman projection	10^2 rel $\frac{k'_{cat.}/K_m}{\text{rel } k_{OH}}$
1		100
(*S*)-(–)-**2**		80
(*R*)-(+)-**2**		35
(*S*)-(+)-**3**		17
(*R*)-(–)-**3**		0
(*SS*)-(+)-**4**[a]		3
(*RR*)-(–)-**4**[a]		3
(*RS*)-(+)-**5**[a]		0.6
(*SR*)-(–)-**5**[a]		0

[a] The absolute configuration of these compounds is not known. The structures given are believed to be the most likely.

in these studies were used. (It is recognized that different quantum theoretical methods and different initial assumptions will on occasion give quite different energy/angle relationships; see, for example, discussion in ref 18). From their data the differences in energy from the minimum energy for various τ_2 ($^+$N-C-C-O) angles (with angle C-C-O-C fixed at 180°) were calculated and were plotted *vs.* τ_2 in Figure 5. Since the relationship is essentially linear in the region where τ_2 is ~+90 to +150° an expression for ΔG^*_{ster} (eq 4) was easily obtained. (It is

$$\Delta G^*_{ster} = m(\tau_2 - 150) + \Delta G^*_{150} \quad (4)$$

assumed that the energy differences calculated for ground-state conformations can be carried over to obtain the steric contribution to the activation energy $\Delta\Delta G^*_{conf}$.) The derivative for the evaluated expression given in eq 2 is then set to zero and solved for τ_2 (the dihedral angle of minimum energy). This value of τ_2 is then used to solve for $\Delta\Delta G^*_{conf}$ in order to construct the plot shown in Figure 4. Figure 4 shows that there is a correlation between transition-state conformational energy at the AChE active site and reaction rate. It further suggests

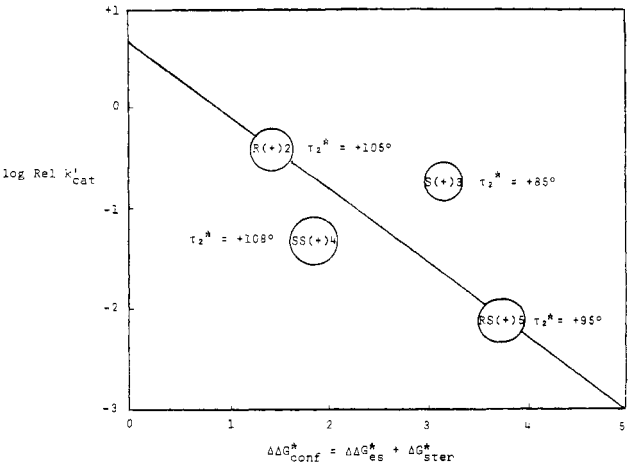


Figure 4. Plot of log rel $k'_{cat.}$ *vs.* minimum relative activation energy for the AChE-catalyzed hydrolysis of four flexible ACh analogs.

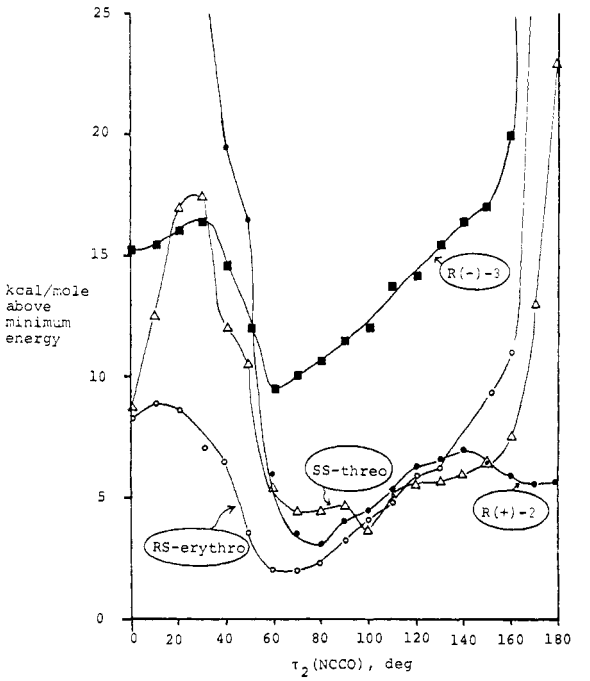


Figure 5. Data obtained from the INDO theoretical study.[17]

that the optimum $^+$N-C-C-O angle is different for the various flexible compounds with values as given. Interesting confirmation for our hypothesis for geometrically flexible substrate conformations arises from recently measured secondary deuterium isotope effects for the ACh/AChE system.[19] Within experimental error the isotope effect for the enzyme-catalyzed hydrolysis of each of four different ACh's deuterated in each of the four different hydrogenic sites, $(L_3C)_3N^+-CL_2-CL_2-OCOCL_3$ was 1.00, an experimentally and mechanistically significant result providing evidence for a rather loose enzyme–substrate interaction. The authors conclude that the rate-determining step for events preceding deacetylation of AChE occurs prior to the acylation process and propose a rate-determining enzyme conformation charge. Their suggestion is entirely consistent with our data.

Conclusion

This work has demonstrated that the optimum dihedral $^+$N-C-C-O angle in the transition state for the acylation step of AChE-catalyzed hydrolysis of ACh analogs is posi-

tive and anticlinal, possibly as low as +120°. The data suggest that flexible ACh analogs will adopt any +ac conformation so as to minimize rotational barriers and maximize attraction between the anionic binding site and the quaternary ammonium cation, thus implying a geometrically flexible mode of attachment to the enzyme.

Experimental Section

Materials. Water. Carbon dioxide free water was prepared by passing distilled water through a Corning LD-3 demineralizer equipped with a cartridge of "ultrahigh purity" resin No. 350A immediately before use.

Magnesium Chloride Stock Solution. Because of the hygroscopic nature of $MgCl_2$ the following method was adopted to prepare a standard stock solution. A saturated aqueous solution of $MgCl_2$ was prepared from reagent grade $MgCl_2 \cdot 6H_2O$ at 20°; 10 ml of this solution 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 concentration of the final standard solution is 190.4 mg/ml at 20°.

Salt Solution. The assay solution was 0.16 and 0.002 *M* in NaCl and $MgCl_2$, respectively, and contained 50 mg of bovine serum albumin per liter.

Enzyme Solution. Immediately prior to use, enough Sigma Chemical Co. electric eel (*Electrophorus electricus*) Type III AChE is dissolved in the enzyme salt assay solution 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 *ca.* 700–2000 μM units/mg of protein.) A 1-μM unit will hydrolyze 1 μmol of ACh per minute at pH 8.0 and 37°. The frozen aqueous solution contains approximately 5 mg of $(NH_4)_2SO_4$/mg of protein. ChE from horse serum (Sigma Type IV; 6 μM units/mg) was dissolved in salt solution to give a final enzyme solution containing from 0.330 to 0.340 μM units/ml.

Acetylcholine Chloride Solution. Because of the extremely hygroscopic nature of AChCl, stock solutions were prepared as follows. About 1 g of AChCl (crystalline, "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 solution is obtained by adding to one of the bottles an amount of H_2O weighing 1.8013 times the number of grams of AChCl. The density of such a solution 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* solution; 8 ml of this solution is removed and diluted to 10 ml giving 0.1613 *M* solution. Repeating this dilution technique four times gives four more solutions: 0.1291, 0.1033, 0.0826, and 0.0661 *M*.

Analog Substrate Solution. A stock solution (2 ml, approximately 0.2 *M*) of each of the six compounds was prepared. Five successive dilutions were made by removing 0.8 ml of the previous solutions and diluting to 1 ml with distilled H_2O. Six solutions 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 acetic acid 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 0.1–10 μM units/ml and six different concentrations (ranging from 3×10^{-4} to 10×10^{-4} *M*) of substrate. At least three runs were performed for each of the six concentrations for each substrate. The titrant was 0.0100 *N* NaOH. In a typical run 10 ml of freshly prepared enzyme solution (kept under N_2) is removed to the thermostated assay chamber with a volumetric pipet and allowed to reach constant temperature. To this solution, under N_2, is added 0.050 ml of substrate solution and the instrument 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 concontration. 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 solution containing substrate concentration of approximately 6×10^{-4} *M*. At least four runs were made for each substrate at each of four different base concentrations ranging from pH 10.7 to 11.0. The base concentration 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 temperatures with commercial buffer solution and standard NaOH solution; 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 concentrations have a negligible effect (within experimental error) on our rate constant values.

A typical determination is carried out as follows. A NaOH solution (10.00 ml) of approximately the desired pH (about 0.05 pH unit higher than the preset end point) is added to a thermostated reaction vessel and stirred under N_2 until constant temperature is reached. To this solution is added 0.100 ml of a 0.06 *M* substrate stock solution. 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 concentration 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 concentration to give k_{OH} values.

(*R*)-2-(*N*,*N*-Dimethylamino)propionic Acid. D-Alanine was subjected to reductive alkylation in the presence of formaldehyde and hydrogen using the procedure of Bowman and Stroud:[20] mp 188° (lit.[20] 188°).

(*R*)-2-(*N*,*N*-Dimethylamino)-1-propanol. (*R*)-2-(*N*,*N*-Dimethylamino)propionic acid (10 g, 0.086 mol) was suspended in 300 ml of dry ether. An excess of lithium aluminum hydride in ether was added dropwise with stirring and the mixture stirred for 12 hr. Water was added, the ether layer was separated, dioxane added to the aqueous phase, and the mixture refluxed for 4 hr. The dioxane and ether solutions were combined and dried ($MgSO_4$), and the solvent was removed to give 4 g of crude product.

(*S*)-2-(*N*,*N*-Dimethylamino)propionic Acid. (*S*)-2-Amino-1-propanol (5 g, 0.067 mol; Aldrich Chemical Co.) and formaldehyde (20 g of 40% aqueous solution) were dissolved in 200 ml of 95% ethanol. The mixture was hydrogenated in the presence of palladium-on-carbon catalyst to give the reductively alkylated product. The catalyst was removed by filtration and the solvent and excess formaldehyde were removed by distillation to give the desired product in 60% yield.

α-Methylacetylcholine Iodide [(2-Acetoxy-1-methylethyl)-trimethylammonium Iodide, 2]. Compound 2 was prepared from the amino alcohol, 2-(*N*,*N*-dimethylamino)-1-propanol, prepared by the method of Smissman, *et al.*,[2] in 83% yield: mp 133–134° (lit. mp 131–132°,[21] 137–138°[22]).

β-Methylacetylcholine Iodide [(1-Acetoxy-2-methylethyl)trimethylammonium Iodide, 3]. Compound 3 was prepared from the amino alcohol, 1-(*N*,*N*-dimethylamino)-2-propanol (Eastman practical grade, redistilled bp 124°), as described by Smissman, *et al.*,[2] in 86% yield: mp 135–136° (lit.[21] 131–132°).

(*R*)-(+)-α-Methylacetylcholine [(*R*)-(+)-2]. Compound (*R*)-(+)-2 was prepared in a manner similar to that described previously.[2] Treatment of (*R*)-2-(*N*,*N*-dimethylamino)-1-propanol with methyl iodide in ether solution afforded the white crystalline methiodide (*R*)-(+)-α-methylcholine iodide. *Anal.* ($C_6H_{16}ONI$) C, H, N.

(*R*)-(+)-α-Methylcholine was refluxed in 25 ml of acetic anhydride for 9 hr. The resulting brown solution was cooled and poured into 50 ml of ether to afford 0.46 g (49%) of gray crystals. Recrystallization from absolute ethanol–ether gave 0.15 g of flaky white crystals: mp 109–110° (lit.[21] 107–108°); $[\alpha]^{29}D$ +8.62° (90% ethanol) [lit.[21] $[\alpha]^{29}D$ +8.61° (90% v/v ethanol)]. *Anal.* ($C_8H_{18}O_2NI$) C, H, N.

(*S*)-(−)-α-Methylacetylcholine Iodide [(*S*)-(−)-2]. The *S*-(−) isomer (*S*)-(−)-2 was prepared by treating (*S*)-(2-*N*,*N*-dimethylamino)-1-propanol with methyl iodide as described for the *R*-(+) isomer to give the methiodide (*S*)-(−)-α-methylcholine: mp 328–333°; $[\alpha]^{32}D$ −5.1° (90% ethanol). *Anal.* ($C_6H_{16}ONI$) C, H, N.

Acetylation gave a white crystalline product: mp 108–108.5°; $[\alpha]^{29}D$ −8.41° (90% ethanol) [lit.[21] mp 108–109°; $[\alpha]^{27}D$ −9.07° (90% ethanol)]. *Anal.* ($C_8H_{18}O_2NI$) C, H, N.

Preparation of Resolving Agents. The agents used to resolve the compounds described below were the (+)- and (−)-α-bromo-

camphor-π-sulfonic acids. These were obtained from the corresponding optically active ammonium salts (Aldrich Chemical Co.). To a stirred, ice-cooled suspension of 30 g (0.09 mol) of the α-bromocamphor-π-sulfonic acid, ammonium salt in 900 ml of chloroform, and 450 ml of absolute ethanol was added 100 ml of acetyl chloride dropwise over 45 min. After 3.5 hr the reaction mixture was filtered and the solvent removed. To the remaining yellow oil was added 25 ml of benzene and the solvent removed under reduced pressure. This latter procedure was repeated twice and the remaining oil (29 g) was used without further purification.

Resolution of (±)-1-(*N,N*-Dimethylamino)-2-propanol. Crude (−)-α-bromocamphor-π-sulfonic acid (38.25 g, 0.12 mol) was dissolved in a minimum amount of cold, ethyl acetate (about 60 ml). To this solution was added freshly distilled (±)-1-(*N,N*-dimethylamino)-2-propanol (12.82 g, 0.12 mol) dropwise with cooling. A white salt precipitated and was immediately filtered, rinsed with ethyl acetate, and allowed to dry. The diastereomeric salt was crystallized seven times from a solvent mixture consisting of 5 ml of ethyl acetate and 1 ml of absolute ethanol for each gram of salt according to the procedure of Major and Bonnett.[23] After each crystallization the salt was filtered, rinsed with anhydrous ether, and dried. Optical rotations were obtained using a Perkin-Elmer polarimeter Model 141 using the sodium D line and a micro polarimeter tube at 29° with 90% ethanol as the solvent. The mother liquors were also reworked and the final yield was 9.88 g (39%) of the desired salt of the (−)-amino alcohol and the (−) acid: $[\alpha]^{29}D$ −89.2 ± 2.2°.

To 9.7 g (0.02 mol) of the salt was added 24 ml of 5% sodium hydroxide. The solution was extracted ten times with ether; the extracts were combined, dried (anhydrous K_2CO_3), and filtered. The resulting ether solution containing (−)-1-(*N,N*-dimethylamino)-2-propanol was used without further purification.

(−)-1-(*N,N*-Dimethylamino)-2-propanol Methiodide. To the ether solution of the resolved alcohol was added 1.63 ml (2.5 g, 0.0176 mol) of methyl iodide. The solid which formed immediately was rinsed with anhydrous ether, air-dried, and recrystallized from 1:1 absolute ethanol–benzene: mp 176–178° (lit.[21] 175.5–176.5°); $[\alpha]^{30}D$ −28.79° (90% ethanol) [lit.[21] $[\alpha]^{23}D$ −29.04° (90% ethanol)].

(*R*)-(−)-β-Methylacetylcholine Iodide [(*R*)-(−)-3]. To 0.63 g (0.0026 mol) of (−) alcohol was added 7.5 ml of acetic anhydride (8.15 g, 0.08 mol). The mixture was allowed to stir at 100° until all the solid had disappeared (7.5 hr). The yellow solution was filtered and the filtrate poured into 150 ml of anhydrous ether. A white solid precipitated immediately, was filtered, rinsed with anhydrous ether, and dried. The substance was recrystallized from absolute ethanol–ether giving 0.6 g (80%) of (*R*)-(−)-3: mp 172–174°; $[\alpha]^{30}D$ −27.8° (90% ethanol) [lit.[21] mp 176–178°; $[\alpha]^{23}D$ −27.4° (90% ethanol)]. *Anal.* ($C_8H_{18}O_2NI$) C, H, N.

(*S*)-(+)-β-Methylacetylcholine Iodide [(*S*)-(+)-3]. The preparation of this compound was accomplished as described above for the *R*-(−) isomer, utilizing the (+) resolving agent: mp 176.5–177.5°; $[\alpha]^{29}D$ +26.4° (90% ethanol) [lit.[21] mp 177.5–178.5°; $[\alpha]^{23}D$ +27.4° (90% ethanol)].

Resolution of (±)-*threo*-3-(*N,N*-Dimethylamino)-2-butanol. To a cooled, stirred solution of 28 g (0.09 mol) of (−)-α-bromocamphor-π-sulfonic acid in 50 ml of ethyl acetate was added 10.7 g (0.09 mol) of freshly distilled (±)-amino alcohols.[2] On removal of the solvent 43.1 g of a syrup remained. The syrup was dissolved in 170 ml of ethyl acetate and 40 ml of absolute ethanol and filtered, the filtrate was heated to boiling, ether added to the cloud point, and the solution allowed to cool. White crystals (30 g) were recovered and recrystallized to constant specific rotation from ethyl acetate–ether to give 9.0 g (75%): mp 88–92°; $[\alpha]^{29}D$ −76.9° (90% ethanol).

(−)-*threo*-α,β-Dimethylcholine Iodide. To 9.0 g (0.021 mol) of the salt obtained above was added 5% NaOH until the solution was basic to litmus. The solution was saturated with sodium chloride and extracted five times with ether. The ether extracts were combined, dried (K_2CO_3), and concentrated. The addition of 1.7 ml of methyl iodide gave 1.7 g (30%) of methiodide: mp 262–265° dec; $[\alpha]^{29}D$ −3.14°.

(+)-*threo*-α,β-Dimethylacetylcholine Iodide [(+)-4]. The compound (−)-*threo*-α,β-dimethylcholine iodide (1.7 g, 0.006 mol) was heated with 20 ml of acetic anhydride for 5 hr. The cooled solution, to which 25 ml of ether had been added, was placed in the refrigerator for 4 days and gave 0.21 g of a white solid, mp 101–104°. Recrystallization from isopropyl alcohol–ether gave 0.12 g of a white solid. This compound was dried *in vacuo*: mp 107–110°; $[\alpha]^{29}D$ +3.4° (90% ethanol). *Anal.* ($C_9H_{20}O_2NI$) C, H, N.

(−)-*threo*-α,β-Dimethylacetylcholine Iodide [(−)-4]. Compound (−)-4 was prepared in a manner similar to that described for the (+) isomer. The salt of (+)-α-bromocamphor-π-sulfonic acid and (+)-*threo*-3-*N,N*-dimethylamino-2-butanol was obtained in 90% yield after three recrystallizations from ethyl acetate–ethanol to a constant specific rotation $[\alpha]^{29}D$ +75.8°, mp 86–90°. The resolved amino alcohol was obtained from the salt by the addition of a calculated amount of 5% sodium hydroxide and extraction into ether. The ether extract was dried (K_2CO_3), concentrated, and mixed with a 10% molar excess of methyl iodide. After standing in the cold for 2 days the white crystalline methiodide was obtained: mp 265–266.5° dec; $[\alpha]^{29}D$ +3.5° (90% ethanol). Acetylation afforded, after recrystallization from ethyl acetate, very hygroscopic crystals: $[\alpha]^{29}D$ −3.3° (90% ethanol). The ir spectrum was identical in all respects with that of the (+) isomer. *Anal.* ($C_9H_{20}O_2NI$) C, H, N.

(+)-*erythro*-α,β-Dimethylacetylcholine Iodide [(+)-5] and (−)-*erythro*-α,β-Dimethylacetylcholine Iodide [(−)-5]. Compound **5**, (±)-*erythro*-α,β-dimethylacetylcholine iodide, was prepared according to the procedure of Smissman, *et al.*[2] The resolution and the preparation of the optically active erythro compounds (+)-**5** and (−)-**5** were performed as described previously for the threo isomers. Racemic (±)-*erythro*-3-(*N,N*-dimethylamino)-2-butanol when treated with (+)-α-bromocamphor-π-sulfonic acid gave a 50% yield of a salt: mp 176.5–179°; $[\alpha]^{29}D$ +83.6° (90% ethanol). Treatment of the racemic *erythro*-amino alcohol with the (−) resolving agent afforded a 60% yield of a salt: mp 176–179°; $[\alpha]^{29}D$ −85.5° (90% ethanol). Addition of methyl iodide to an ether solution of the resolved amino alcohols produced white crystalline methiodides: mp 250–251° dec, $[\alpha]^{29}D$ +21.3° (90% ethanol); and mp 249–251° dec, $[\alpha]^{29}D$ −21.9° (90% ethanol), respectively. Acetylation and crystallization from ethyl acetate afforded (+)-*erythro*-α,β-dimethylacetylcholine iodide, $[\alpha]^{29}D$ +10.5° (90% ethanol), and (−)-*erythro*-α,β-dimethylacetylcholine iodide, $[\alpha]^{29}D$ −10.9° (90% ethanol), respectively. *Anal.* ($C_9H_{20}O_2NI$, both isomers) C, H, N. The ir spectra of the (+) and (−) isomers were identical.

(±)-2(a)-Acetoxy-3(a)-trimethylammonium-*trans*-decalin Iodide (6). The preparation of **6** has been described in a previous paper.[2]

(+)-2(a)-Acetoxy-3(a)-trimethylammonium-*trans*-decalin Iodide [(+)-6] and (−)-2(a)-Acetoxy-3(a)-trimethylammonium-*trans*-decalin Iodide [(−)-6]. The preparation of these compounds is described in the paper concerning the X-ray crystallographic study of these compounds.[7]

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References and Notes

(1) M. Martin-Smith, G. A. Smail, and J. B. Stenlake, *J. Pharm. Pharmacol.*, **19**, 561 (1967).
(2) E. E. Smissman, W. L. Nelson, J. B. LaPidus, and J. L. Day, *J. Med. Chem.*, **9**, 458 (1966).
(3) E. E. Smissman and G. S. Chappell, *J. Med. Chem.*, **12**, 432 (1969).
(4) W. F. Stephen, Jr., E. E. Smissman, K. B. Schowen, and G. W. Self, *J. Med. Chem.*, **15**, 241 (1972).
(5) E. E. Smissman, R. T. Borchardt, and K. B. Schowen, *J. Med. Chem.*, **15**, 545 (1972).
(6) E. E. Smissman and G. R. Parker, *J. Med. Chem.*, **16**, 23 (1973).
(7) B. K. Lee, E. E. Smissman, K. B. Schowen, M. A. Paske, and R. A. Magarian, manuscript in preparation.
(8) C. Chothia and P. J. Pauling, *Nature (London)*, **223**, 919 (1969).
(9) E. Shefter, H. G. Mautner, and E. E. Smissman, *Acta Crystallogr., Sect. A*, **25**, S201 (1969).
(10) E. Shefter and O. Kennard, *Science*, **153**, 1389 (1966).
(11) (a) A. H. Beckett, N. J. Harper, and J. W. Clitherow, *J. Pharm. Pharmacol.*, **15**, 362 (1963); (b) K. A. Scott and H. G. Mautner, *Biochem. Pharmacol.*, **13**, 907 (1964).
(12) W. Klyne and V. Prelog, *Experientia*, **16**, 521 (1960).

(13) (a) C. Y. Chiou, J. P. Long, J. G. Cannon, and P. D. Armstrong, *J. Pharmacol. Exp. Ther.*, **166**, 243 (1969); (b) W. L. Nelson and R. Wilson, *J. Med. Chem.*, **14**, 169 (1971); (c) N. J. Lewis, K. K. Barker, R. M. Fox, and M. P. Mertes, *ibid.*, **16**, 156 (1973); (d) J. B. Robinson, B. Belleau, and B. Cox, *ibid.*, **12**, 848 (1969); (e) B. Belleau and P. Pauling, *ibid.*, **13**, 737 (1970); (f) J. B. Kay, J. B. Robinson, B. Cox, and D. Polkonjak, *J. Pharm. Pharmacol.*, **22**, 214 (1970).
(14) E. Shefter and D. L. Triggle, *Nature (London)*, **227**, 1354 (1970).
(15) R. W. Baker, C. H. Chothia, P. Pauling, and T. J. Petcher, *Nature (London)*, **230**, 439 (1971).
(16) G. H. Cocolas, E. C. Robinson, and W. L. Dewey, *J. Med. Chem.*, **13**, 299 (1970).
(17) R. J. Radna, D. L. Beveridge, and A. L. Bender, *J. Amer. Chem. Soc.*, **95**, 3831 (1973).
(18) D. W. Genson and R. E. Christoffersen, *J. Amer. Chem. Soc.*, **95**, 362 (1973).
(19) J. L. Hogg, Department of Chemistry, The University of Kansas, unpublished results.
(20) R. E. Bowman and H. H. Stroud, *J. Chem. Soc.*, 1342 (1950).
(21) A. H. Beckett, N. J. Harper, and J. W. Clitherow, *J. Pharm. Pharmacol.*, **15**, 349 (1963).
(22) G. H. Cocolas, E. C. Robinson, and W. L. Dewey, *J. Med. Chem.*, **13**, 299 (1970).
(23) R. T. Major and H. T. Bonnett, *J. Amer. Chem. Soc.*, **57**, 2125 (1935).

Potential Inhibitors of *S*-Adenosylmethionine-Dependent Methyltransferases. 3. Modifications of the Sugar Portion of *S*-Adenosylhomocysteine

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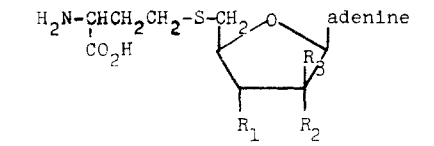
Structural analogs of *S*-adenosyl-L-homocysteine (L-SAH), with modification in the ribose portion of the molecule, have been synthesized and their abilities to inhibit catechol *O*-methyltransferase (COMT), phenylethanolamine *N*-methyltransferase (PNMT), histamine *N*-methyltransferase (HMT), and hydroxyindole *O*-methyltransferase (HIOMT) have been investigated. From these studies it was concluded that, in general, the 2′-hydroxyl and 3′-hydroxyl groups of the ribose moiety of SAH play crucial roles in the binding of this molecule to most methyltransferases. However, several interesting exceptions to this strict structural specificity have been observed. While *S*-3′-deoxyadenosyl-L-homocysteine produced no inhibition of HMT and HIOMT, it produced strong inhibition of the transmethylation catalyzed by PNMT and COMT. Likewise, *S*-2′-deoxyadenosyl-L-homocysteine and *S*-5′-[9-(arabinofuranosyl)adenyl]-L-homocysteine had little or no effect of COMT, HMT, and HIOMT but were potent inhibitors of PNMT. The significance of these data relative to the nature of the SAH binding sites and the potential for *in vivo* differential inhibition of methyltransferases will be discussed.

S-Adenosyl-L-homocysteine (SAH)‡ produces strong product inhibition of most *S*-adenosylmethionine (SAM)-dependent methyltransferases.[1] In the preceding papers of this series,[2,3] we described the synthesis and enzymatic evaluation of SAH analogs with modifications in the homocysteine or base portions of the SAH molecule. From the results of these studies[2,3] it was concluded that there exist at least four functional groups on SAH, which play a primary role in its binding to methyltransferases. These points of attachment appear to be the terminal carboxyl, the terminal amino and the sulfur atom of the homocysteine portion, and the 6-amino group of the adenine moiety. In an effort to further elucidate the nature of the intermolecular forces involved in enzymatic binding of SAH, we have synthesized a series of SAH derivatives with minor modifications in the sugar portion of this molecule (Chart I). Coward and coworkers[4,5] previously prepared analogs in which the ribose moiety of SAH was replaced by a cyclopentyl group, a 2,3-dihydroxycyclopentyl group, or a five-carbon acyclic bridge. However, these analogs were nearly inactive as inhibitors of SAM-dependent methyltransferases. In the present study we have made very minor changes in the ribose moiety of SAH in an effort to elucidate the important structural features of this portion of SAH in its binding to methyltransferases. Using these sugar-modified SAH analogs (Chart I) as probes of the active sites of COMT, PNMT, HMT, and HIOMT, we have delineated the contribution of the ribose moiety in the enzymatic binding of SAH, which is the subject of this paper.

Chart I. Sugar-Modified Analogs of SAH Synthesized to Probe the Binding Sites on COMT, PMNT, HMT, and HIOMT



Compd	R_1	R_2	R_3
SAH	OH	OH	H
2′-deoxy-SAH	OH	H	H
3′-deoxy-SAH	H	OH	H
SAraAH	OH	H	OH

Experimental Section

Melting points were obtained on a calibrated Thomas-Hoover Uni-Melt and were corrected. Microanalyses were conducted on an F & M Model 185 C, H, N analyzer, The University of Kansas, Lawrence, Kan. Unless otherwise stated, the ir, nmr, and uv data were consistent with the assigned structures. Ir data were recorded on a Beckman IR-33 spectrophotometer, nmr data on a Varian Associates Model T-60 spectrophotometer (TMS), and uv data on a Cary Model 14 spectrophotometer. Scintillation counting was done on a Beckman LS-150 scintillation counter. Tlc were run on Analtech silica gel GF (250 μ) or Avicel F (250 μ). Spots were detected by visual examination under uv light and/or ninhydrin for compounds containing amino moieties.

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‡Abbreviations used are SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; 2′-deoxy-SAH, *S*-2′-deoxyadenosyl-L-homocysteine; 3′-deoxy-SAH, *S*-3′-deoxyadenosyl-L-homocysteine; SAraAH, *S*-5′-[9-(arabinofuranosyl)adenyl]-L-homocysteine; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); PNMT, phenylethanolamine *N*-methyltransferase (E.C. 2.1.1); HMT, histamine *N*-methyltransferase (E.C. 2.1.1.8); HIOMT, hydroxyindole *O*-methyltransferase (E.C. 2.1.1.4); K_{is}, inhibition constant for the slope.