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NMR STUDIES ON THE CONFORMATION OF ACETYLCHOLINE ISOLOGUES*

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Abstract—The high resolution NMR spectra of several acetylcholine analogues were studied. Iterative computer calculations of the AA'BB' spectra of the methylene groups gave $J_{AB} = 11{\cdot}59$ Hz, $J_{AB'} = 5{\cdot}00$ Hz for acetylthiolcholine and $J_{AB} = 12{\cdot}62$ Hz, $J_{AB'} = 4{\cdot}83$ Hz for acetylselenolcholine. The magnitude of the vicinal coupling constants and the negative value for the spectral parameter "L", showed that the compounds exist almost exclusively in the *anti-periplanar* conformation in solution. Previous studies have shown that the *syn-clinal* conformation of acetylcholine predominates in solution. Thus, it seems likely that the free energy differences between the various conformers are greater than had been expected on the basis of previous calculations. Possible explanations for these differences are discussed.

INTRODUCTION

IN VIEW of the importance of the roles played by acetylcholine, I (Fig 1), in the transmission of the nerve impulse[1] and the simplicity of its structure, a large number of analogues of this ester have been prepared and studied.[2]

Due to the stereospecificity of the action of certain cholinergic compounds, numerous optically active compounds related to I have been synthesized, resolved, and their biological actions and conformations investigated. However, only recently has much attention been centered on studies of the conformation of I and of optically inactive compounds related to it.

It had been assumed that I, being a flexible molecule, could readily exist in either an extended or a quasi-cyclic conformation. The postulate was made [3] that conformational isomerism was of importance in the biological actions of I, and that this molecule exerted muscarinic activity with its —N̈—C—C—O— grouping in the *gauche* or *syn-clinal* (*sc*) conformation, and nicotinic activity with this grouping in the *trans* or *anti-periplanar* (*ap*) conformation. This postulate was supported by theoretical calculations which predicted that the potential energies of the *sc* and *ap* conformations of I should be very similar.[4,5]

Studies of the conformation of I in the crystal[6] (x-ray) and in solution[7] (NMR) showed that the conformation of the —N—C—C—O— chain was maintained in either environment. X-ray diffraction studies showed that the *sc* conformation predominates in most of the esters of β-aminosubstituted alcohols hitherto investigated.[8-12] On the other hand, x-ray studies showed that replacement of the alcoholic oxygen of I by sulfur or by selenium greatly modifies the conformation of this molecule in the solid,

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the —N—C—C—B—(B = S, Se) grouping in acetylthiolcholine (II) and acetylselenolcholine (III) being in the extended *ap* conformation.[13, 14] Since II and III and, to an even greater extent, their hydrolysis products exhibit considerable biological activity in a variety of preparations,[15–17] it seemed of interest to determine whether these compounds had conformations different from that of acetylcholine in solution as well as in the crystal. High resolution NMR spectroscopy was used to determine the conformations of these molecules in D_2O solution.

MATERIALS AND METHODS

Choline, cholinethiol, methoxycholine, methylthiocholine, acetylcholine, acetylthiolcholine and acetylselenolcholine were purified by being recrystallized at least three times.

NMR spectra were recorded at 90 MHz using a Bruker HFX-3 spectrometer fitted with a Hewlett-Packard 5216A frequency counter, and at 100 MHz using a Varian HA100 spectrometer. Nitrogen-14 spin-decoupling was accomplished with an NMR specialties SD-60 heteronuclear spin decoupler. Spectra were run in approximately 10% solution in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. Measured line positions are reproducible to $\pm 0{\cdot}1$ Hz.

DISCUSSION

Recently, Culvenor and Ham[7] have reported an NMR study of acetylcholine chloride in D_2O. On the basis of the calculated vicinal coupling constants, 7·0 Hz and 2·5 Hz, and the positive value of the constant "L" in the spectral analysis, they concluded that acetylcholine, I, exists predominantly in the *sc* conformation. The conformation proposed for the disubstituted ethane portion of acetylcholine in solution, therefore, agreed with the *sc* conformation found by x-ray analysis of the crystal structure.[6] In addition, since the 'acylation shift' was normal, the ester function was proposed to exist in the extended form such that the carbonyl group would either bisect the $C\begin{smallmatrix}H\\H\end{smallmatrix}$ plane of the α-methylene group or be slightly twisted to eclipse one of the α-protons. The ester group apparently adopts a different conformation upon going from crystal to solution.[7]

In the present study the high resolution NMR parameters of a number of compounds closely analogous to acetylcholine and its hydrolysis product, choline, were found. The chemical shifts for the compounds studied are summarized in the Table. In all of the compounds except IV the —NMe_3 groups gave a single resonance signal. The N-methyl chemical shifts were very similar although the β-substituents possess quite different anisotropic and field effect properties. The chemical shifts of the acetyl groups in compounds I–III were found to be 2·14, 2·42 and 2·49 ppm, as expected from the electronetativity values of the atoms to which they are attached.

The assignment of the upfield multiplet to methylene protons bonded to nitrogen and downfield multiplet to methylene protons bonded to oxygen in the NMR spectrum of acetylcholine[7] rested on the finding that the NCCH coupling is greater (1·5–2·0 Hz) than the NCH coupling (0·0–0·6 Hz) in quaternary nitrogen compounds.[18, 19] Using the same criterion in the case of the thio (II) and seleno (III) isologues results in the assignment of the upfield signals to the S—CH_2 and Se—CH_2 groups and the

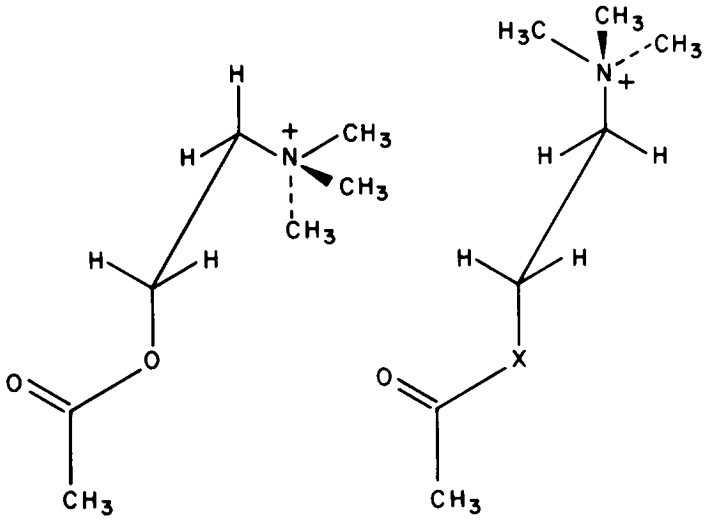
TABLE. PROTON CHEMICAL SHIFTS OF CHOLINE ANALOGUES[a]

	Compounds	CH_2-X	CH_2-N	$(Me_3)N$	Other
I.	Acetylcholine iodide (X = O)	4·55[b]	3·74[b]	3·23	acetyl = 2·14
II.	Acetylthiolcholine bromide (X = S)	3·29[b]	3·48[b]	3·20	acetyl = 2·42
III.	Acetylselenolcholine bromide (X = Se)	3·20[b]	3·52[b]	3·19	acetyl = 2·49
IV.	Choline chloride (X = O)	4·05[b]	3·52[b]	3·20	
V.	Cholinethiol iodide (X = S)	2·96[b]	3·56[b]	3·16	
VI.	Methoxycholine bromide (X = O)	3·91	3·59	3·17	methoxy = 3·40
VII.	Methylthiocholine bromide (X = S)	2·93	3·61	3·17	methylthio = 2·17

[a] Chemical shifts are given in ppm downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate in deuterium oxide.

[b] Values calculated by computer.

FIG. 1



I Acetylcholine

II Acetylthiolcholine, X = S

III Acetylselenolcholine, X = Se

downfield signals to N—CH_2. This is supported by the fact that the CH_2X chemical shifts of I–III (Table) vary linearly with the Huggins' electronegativities[20] of X.[21]

Since the conformations of compounds II and III were found to be *anti-periplanar* in the crystal state[13, 14] it was of particular interest, especially from a biological point of view, to study the conformations of the two acetylcholine isologues in solution. Based on the calculated vicinal coupling constants elaborated below, both acetylthiol- and acetylselenolcholine chlorides exist to a large extent in the *anti-periplanar* conformation in solution. The two methylene groups of choline compounds form an AA'BB'

spectrum which is further coupled to nitrogen. The N^{14}–H^1 couplings are discernable in some cases because the quadrupolar broadening due to N^{14} is greatly reduced by the near-equivalence of the groups surrounding the tetrahedral N atom.

The NMR spectrum of the methylene region of the thio compound (II) showed two sets of multiplets; one at 3·48 ppm consisting of 8 lines, $J_{N,H} \sim 0$ Hz, and one at 3·29 ppm consisting of 4 broad lines (the remainder of the transitions being obscured by the N-methyl resonance signal), $J_{N,H} < 0{\cdot}7$ Hz. Irradiation with a strong rf at the N^{14} resonance frequency yielded the symmetrical 16 line spectrum shown in Fig 2. Analysis of the spectrum was not hindered by having some transitions buried beneath the large $-\dot{N}Me_3$ peak because of the symmetry. Analysis of the spectrum was accomplished by the iterative computer program, LAOCN 3,[22] on an IBM 7094 computer. Since the program works best when the NMR parameters resemble the actual values, a number of trial spectra were calculated using the program LAOCN 2 to find the best fit. The only assumptions made in the calculations were that J_{gem} is negative and J_{vic} is positive. The results after three iterations yielded: $\delta_{AB} = 18{\cdot}81$ Hz, $J_{AA'} = -13{\cdot}48$ Hz, $J_{BB'} = 13{\cdot}48$ Hz, $J_{AB} = 11{\cdot}59$ Hz, $J_{AB'} = 5{\cdot}00$ Hz with a rms error of 0·06. A plot of the theoretical spectrum is shown in Fig 2, bottom, using the computer plot routine of Wiberg and Barth.[23]

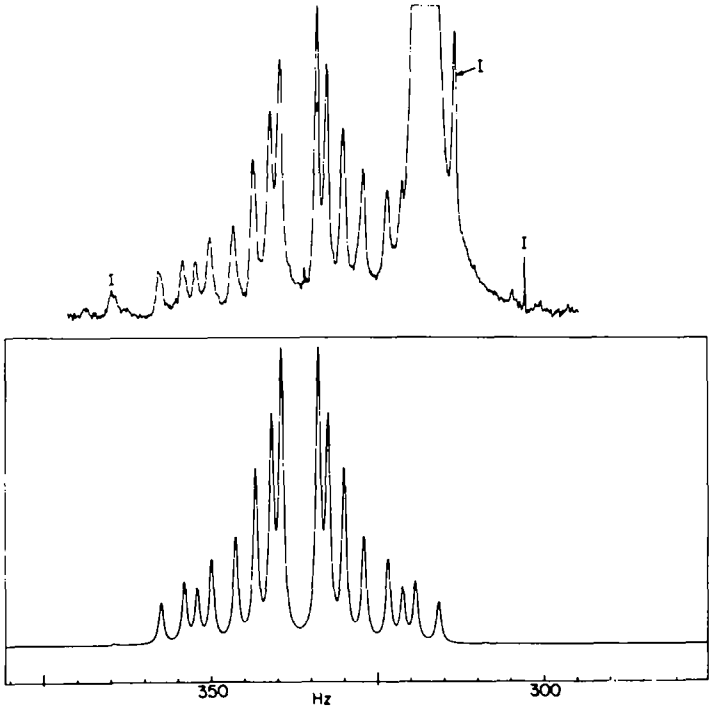


FIG 2. PMR Spectrum (100 MHz) of methylene region of acetylthiolcholine, II, with N^{14} irradiation at 8·420 MHz (top), and theoretical AA'BB' spectrum calculated by computer (Bottom). I = impurity.

The coupling constants J_{AB} and $J_{AB'}$ are average values of the vicinal coupling constants, J_t, where the protons assume an *anti-periplanar* arrangement, and J_g, where the protons assume $\pm 60°$ *syn-clinal* arrangements, assuming exclusively staggered conformations for the disubstituted ethane.

For a disubstituted ethane:

$J_{AB}=n_tJ_g+\frac{1}{2}n_g(J_t+J_g)$ and

$J_{AB'}=n_tJ_t+n_gJ_g$ where n_g and n_t are the fractions of rotamers present. However, from the calculations one cannot distinguish between J_{AB} and $J_{AB'}$.

Culvenor and Ham[7] determined the sign of the spectral parameter "L" using the relationship due to Abraham and Pachler:[24] $J_{av}=1/3\ (J_t+2\ J_g)=\frac{1}{2}N+1/6\ L=17{\cdot}97-0{\cdot}80\ \Sigma E$, where ΣE is the sum of the Huggins' electronegativity values for all the substituents on the C—C bond, $N=J_{AB}+J_{AB'}$ and $L=J_{AB}-J_{AB'}$. It was shown[24] that if L were positive, the *syn-clinal* conformer would predominate and if L were negative the *anti-periplanar* conformer would predominate.

In the case of acetylthiolcholine (II) the magnitudes of J_{AB} and $J_{AB'}$ are such that II must exist almost exclusively in the conformation wherein the sulfur and nitrogen functions are *anti-periplanar* (i.e. $J_{AB'}\sim J_g$ and $J_{AB}\sim J_t$). This would give L=−6·6 Hz thus leading to the values $J_{av}=7{\cdot}2$ Hz and $\Sigma E=13{\cdot}4$. The actual ΣE for II is 14·45 which is much nearer the value calculated for negative L than the value calculated for positive L ($\Sigma E=8{\cdot}2$). Also, assuming 100% *anti-periplanar* rotamer present, $J_{av}=7{\cdot}2$ Hz, equal to J_{av} calculated above. Finally, the values found for J_{AB} and $J_{AB'}$ agree extremely well with the values $J_g=5$ Hz and $J_t=11$ Hz found previously for 1-chloro-2-bromoethane.[25]

In the case of the seleno isologue, III, the proton NMR spectrum of the methylene region gave two sets of multiplets: N—CH_2 at δ 3·52 consisting of 9 lines, $J_{N.H}\sim 0{\cdot}0$ Hz, and Se—CH_2 at δ 3·20 consisting of 5 broad lines visible beneath the N-methyl resonance signal, $J_{N.H}<0{\cdot}7$ Hz. Upon irradiation of the N^{14} region the spectrum simplified to that shown in Fig 3. Upon inspection, it is seen that the spectral pattern is quite different from that shown in Fig 2. The splitting pattern found for compound III resembles that characteristic of the AA′BB′ case of di-substituted benzene compounds more than it resembles that of di-substituted ethane compounds.[26] Nevertheless, the computer calculated values found for III, after three iterations, were: $\delta_{AB}=32{\cdot}18$ Hz, $J_{AA'}=-12{\cdot}83$ Hz, $J_{BB'}=-12{\cdot}03$ Hz, $J_{AB}=12{\cdot}62$ Hz, and $J_{AB'}=4{\cdot}83$ Hz, with a rms error of 0·07. A computer drawn plot of the theoretical spectrum is shown in Fig 3, bottom. From the magnitude of the two vicinal coupling constants, J_{AB} and $J_{AB'}$, it is apparent that the molecule must exist preponderantly in the *anti-periplanar* conformation. A negative L value would lead to a $\Sigma E=13{\cdot}18$ while a positive value for L would lead to a $\Sigma E=12{\cdot}54$. Since the actual value of $\Sigma E=14{\cdot}40$, a negative value for L is indicated, suggesting that the *anti-periplanar* conformer predominates. A high percentage of *anti-periplanar* conformer is indicated by the fact that, assuming $J_{AB}\sim J_t$ and $J_{AB'}\sim J_c$, $J_{av}=7{\cdot}3$ Hz, which is identical with that calculated above from the Abraham–Pachler equation using a negative value for L.

It is interesting to note that the *ap* conformations for the —Ṅ—C—C—X— group in II and III are maintained in both the crystal and in solution, just as the *sc* conformation of I is maintained in both the crystal and solution.

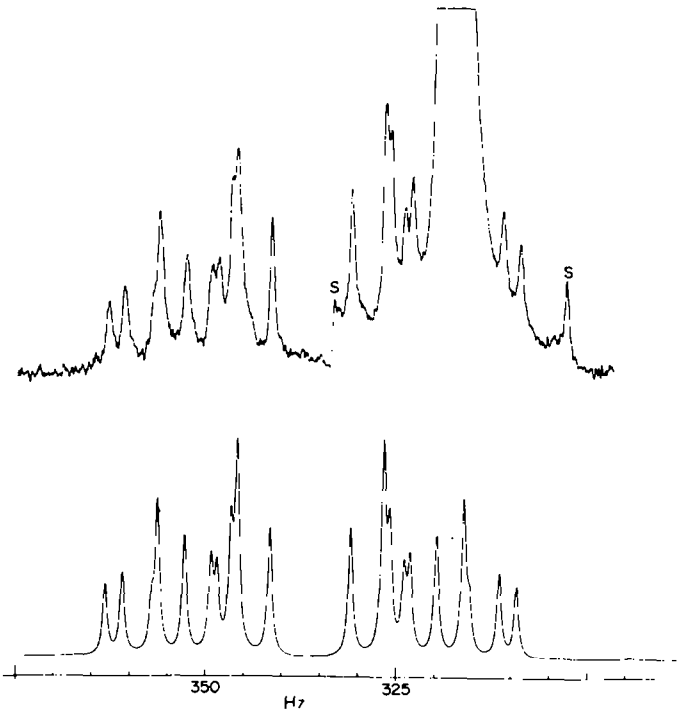


FIG 3. PMR spectrum (100 MHz) of methylene region of acetylselenolcholine, III, with N^{14} irradiation at 8·420 MHz (top), and theoretical AA'BB' spectrum calculated by computer (bottom). S = sideband.

The "Karplus curve",[27] relating the magnitude of the vicinal coupling constants and dihedral angles, allows one to distinguish between a *syn-clinal* and an *anti-periplanar* arrangement. Precise information about the amount of conformer present, or the values of the dihedral angles involved, cannot be elicited from the coupling constant data. In addition to the significant dihedral angle dependence, carbon atom hybridization, carbon–carbon bond length, electronegativity of substituents and other factors contribute to the coupling constant value.[28]

The work of Abraham and Pachler[24] has related the effect on J_{vic} of substituent electronegativity for a large number of disubstituted ethanes. However, their study showed the effect of electronegative substituents on the average value of the coupling constant (J_{av}) and not on the true couplings J_c and J_t.

The conformation of the acetyl moiety for the thio and seleno compounds, II and III, still remains in question because of the paucity of information available on the effect of acylation on the α-carbon atom of thiol and selenol esters.

The NMR spectrum of the methylene region of acetylcholine, upon N^{14} irradiation, gave a simple 10-line spectrum. Using the iterative procedure and the vicinal coupling constant values of 7·0 Hz and 2·5 Hz proposed by Culvenor and Ham[7] led to refined values of 7·21 Hz and 2·51 Hz while the two geminal coupling constants were found to be −28·50 and −28·50. The improbable values for the two geminal coupling

constants were due no doubt to the simplicity of the AA'BB' spectrum and our inability to observe the weak transition lines. The probable errors associated with the geminal coupling constants were large at 2·534 each.

In the case of the hydrolysis products, choline (IV) and cholinethiol (V), the splitting patterns closely resembled those of the esters. Because of the extremely small two-bond N—H coupling and the symmetry of the methylene portion of the spectrum, IV and V were analyzed by computer without the aid of N^{14} spin-decoupling. Although such results are not as accurate, the vicinal coupling constant values for IV (6·25 Hz and 3·26 Hz) and V (12·10 Hz and 4·99 Hz) indicate that the amounts of each conformer present in IV and V do not vary greatly from that of their esters. Again, the simplicity of the spectrum of IV led to improbably high geminal coupling constants. Cholineselenol could not be included since the compound was oxidized in solution.

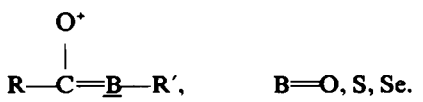
The findings that I and IV and II and V maintain approximately the same conformation argues against intramolecular hydrogen bonding as a major stabilizing force, especially since spectra were run in deuterium oxide. The proposed H-bonded quasi-cyclic structure[6] was ruled out by the fact that in all but one instance the $-\dot{N}Me_3$ group was a singlet. The exception was choline chloride (IV). For the $-\dot{N}Me_3$ group of IV, a poorly resolved triplet ($J \sim 0{\cdot}4$ Hz) was found. When the solution was heated to 85° the triplet, instead of collapsing to a singlet, split further giving six lines while the half-band width remained the same ($J = 1{\cdot}68$ Hz). Under identical heating conditions, the $-\dot{N}Me_3$ peak of acetylcholine (I) remained a singlet whose half-band width was essentially constant. Since the methylene protons bound to N showed no appreciable coupling with N (*vida infra*), N—H coupling with the methyl groups is unlikely. On the basis of NMR and other physical measurements, impurities were also unlikely to be present. Also, upon acetylation of IV, compound I was produced whose NMR spectrum gave a single resonance line for $-\dot{N}Me_3$. Further investigation into the nature of this phenomenon is contemplated.

In considering the non-bonded interactions found in the molecules studied, two of the strongest contributors are steric repulsion of the two large substituents in the disubstituted ethane moiety and dipole–dipole interactions between the C—$\dot{N}$ bond and the C—X bond.

The atomic radii of sulfur and selenium are very similar to one another and greatly exceed that of oxygen. In addition, the effective size of the $-\dot{N}Me_3$ group may be larger than the van der Waals radii suggest due to strong solvation. Hence, the thio and seleno compounds should prefer to adopt an *ap* conformation.

Since the series of compounds studied contains a quaternary amine function, the C—$\dot{N}$ and C—O dipoles in acetylcholine and choline will tend to align as closely as possible—adopting a *syn-clinal* conformation.

A decreased dipole–dipole attraction in the case of the thiol and selenol esters cannot be expected since dipole moment and spectroscopic measurements[29] have shown that replacement of the acyloxy oxygen of esters progressively favors the importance of resonance forms of the type:



The predominance of an *ap* conformation in the S and Se compounds must therefore be due mainly to steric interactions.

The biological actions of acetylthiolcholine and acetylselenolcholine are very different even though the size, shape and conformation of these molecules appear to be very similar in solution. Therefore, it seems reasonable to assume that the differences in the biological actions of these isosteric molecules are associated with differences in electron distribution rather than with differences in their abilities to fit receptor sites. It must be emphasized that although the NMR spectral parameters show that the preponderant conformer in solution appears to be in the *ap* conformation, this is not necessarily the conformer present at the active site.

The present NMR study gives information on the ground state free energies of the various conformers involved. Further work should be done in obtaining more information about the rotational barriers of these flexible molecules. This problem is of great importance since "induced fit" conformational changes in small molecules as the result of being attached to macromolecular receptors may be of importance in interpreting the action of many biologically active molecules.

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