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A DIRECT APPROACH TO THE DETERMINATION OF THE ROTATIONAL BARRIERS OF THE ACETYLCHOLINE AND CHOLINE MOLECULES *

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Abstract—The temperature dependence of the N.M.R. spectra of choline and acetylcholine as well as those of their β-dideutero analogs was investigated. While broadening of the β-methylene signals was observed at −90°, the coalescence temperature was still not reached at −110°. From this information an upper limit for the rotational barriers around the central methylene carbons of 7.9 kcal could be calculated.

Acetylcholine (ACh) is known to play an essential role in the transmission of the nerve impulse. Upon attachment to the receptor biopolymer, ACh presumably induces a conformational change which, in turn, alters the membrane permeability to cations [1–4]. The conformational considerations involved in the neurotransmitter–receptor interactions have prompted extensive studies, theoretical and experimental, on the conformations of ACh and its analogs. X-ray [5–7] and n.m.r. studies [8–10] have shown the —OCCN— grouping of ACh and choline to exist predominantly in the *gauche* conformation. Furthermore, the *gauche* conformation was found to be a common feature in a large number of 2-trimethylammoniumethyl or 2-dimethylammoniumethyl esters with cholinergic or local anesthetic activity [11, 12]. On the basis of these findings, it was postulated that the *gauche* conformation is fundamental for cholinergic activity [6, 7, 13, 14] or for local anesthetic activity [11]. This hypothesis, however, was disputed when the conformations of a group of cholinergic compounds and a group of local anesthetics structurally related to ACh were studied in the crystal and in solution [10, 15–17]. In these compounds, it was found that replacement of the acyloxy oxygen in the —NCCO— grouping with nitrogen, sulfur or selenium resulted in major conformational changes around the central carbon–carbon bond. No direct correlation could be found between biological activity and conformation [17–19].

Conformational studies of flexible molecules in the crystal or in solution appear to provide little information about the conformation which such molecules might assume upon attachment to receptor sites. As in the well-known example of *N*-acetyl glucosamine which is distorted to a strained half-chair conformation when bound to the active site of lysozyme [20], the possibility must be considered that cholinergic ligands or local anesthetics might undergo conformational changes in the environment of the membranes to which they are attached. Such conformational alterations might require that both biopolymer and ligand be flexible in order to maintain a productive interaction throughout the conformational change. The old postulate of Schueler [21] that ACh in a "cisoid" conformation might stimulate nicotinic and in a "transoid" conformation muscarinic receptors provides another reason why the investigation of the rotational barriers for the interconversion of *gauche* and *trans* conformers of cholinergic and related molecules is of considerable importance.

A number of theoretical, empirical, and quantum mechanical calculations have been conducted to determine the ground state energy differences between the various conformers of choline and ACh and have dealt with the rotational barriers around the central carbon—carbon bond. With few exceptions only [22, 23], these calculations predict the *gauche* as the most stable conformer but vary widely in their estimation of energy differences between conformers and the energy barriers for conformer interconversion [24–28]. Two such methods [23, 24] have placed the energy barriers at values above 10 kcal, while another has found it to be below 1 kcal [28].

The present study represents the first attempt to determine the rotational barriers of ACh and of choline by a direct method. Microwave spectroscopy, theoretically the best procedure for measuring rotational barriers below 5 kcal/mole, cannot be used because of the extremely low volatility of trimethylammonium compounds. ^{13}C and ^{1}H relaxation measurements have been carried out by Behr and Lehn [29] and by Makriyannis [30] respectively, and served to define the lower limits for the rotational barriers around the central —C—C— bond of choline and ACh. For these determi-

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nations an indirect approach was used in which activation parameters were obtained from the temperature dependence of the spin-lattice relaxation times (T_1).

In the present approach, n.m.r. spectra are obtained at increasingly lower temperatures at which the rate of conformational inversion is reduced gradually. Ideally, it is desirable to cool the sample solutions until the distinct spectra of the conformers can be observed. In practice, such spectra can be obtained only when the barriers for interconversion of the rotamers exceed 6 kcal. A gradual lowering of the sample temperature is accompanied by a progressive broadening of the spectral lines, due to slower rates of rotation. A detailed analysis of the spectral line shapes in the intermediate temperature ranges can then serve to determine the rate constant for the rate of conformer interconversion. Interpretation of such information must take into account changes in the spin–spin relaxation rates which occur at lower temperatures, so that line-broadening due to slow exchange can be distinguished from that due to decreased spin–spin relaxation times (T_2).

Choline and ACh exist overwhelmingly in the *gauche* conformation even at very low temperatures. Since no detectable amount of *trans* conformer could be visualized, cooling experiments had to rely on the increasing magnetic non-equivalence of the $-CH_2-$ protons as a function of cooling. The central ethylene group of choline or ACh yields an AA'BB' multiplet complicated by interaction with the vicinal nitrogen. ^{14}N–H coupling involves primarily the β-methylene proton signals. Therefore, the effects of lowering temperature can be studied most conveniently using the simpler spectrum of the α-methylene protons.

$$(CH_3)_3\overset{+}{N}\underset{\alpha}{-CH_2}-\underset{\beta}{CH_2}-OR \quad R = -H, -\overset{O}{\overset{\|}{C}}CH_3$$

In order to simplify the spectra of choline and ACh and to make the coalescence points more discernible, the β-dideutero analogs were prepared. In these compounds, the α-methylene protons are equivalent at room temperature and appear as an A_2 singlet, while coupling with the deuterium atoms and with the quaternary nitrogen is insignificant and cannot be observed in the spectrum. As the temperature is lowered, the $-CH_2-$ protons would be expected to become magnetically non-equivalent and to give rise to an AB quartet.

The deuterated compounds were synthesized in analogy to the procedure of Dauben and Gee [31]. The yield obtained was considerably higher than that achieved by Hogg and Schowen [32] who prepared β-deutero ACh by a different procedure.

One of the major limitations encountered in this study proved to be the insolubility of the halides of choline and ACh in most solvents used for low temperature studies. The best results were obtained with a methanol-d_4 : trifluoroethanol-d_3 (2 : 1) mixture. By supercooling the solutions, spectra could be obtained at temperatures as low as $-110°$. Spectra for each compound were obtained at 10° intervals over a temperature range of $+30$ to $-110°$. Tetramethylsilane was used as an internal standard to monitor any line broadening due to magnetic inhomogeneity. Simplification of the spectra was also achieved by decoupling one methylene group in non-deuterated ACh through the irradiation of the other methylene. Such decoupling experiments are better performed on a very high resolution instrument which ensures ample separation of the two methylene proton signals. In a CD_3OD solution of ACh at room temperature, irradiation of the OCH_2 protons gave rise to an A_2 singlet due to the NCH_2 protons in a 270 MHz spectrum. On the other hand, irradiation of the NCH_2 protons gave rise to an A_2 triplet due to the OCH_2 protons which are coupled with the ^{14}N nitrogen ($J\ ^{14}N^1H = 2.7$ Hz). At lower temperatures this triplet changes into a broad singlet.

EXPERIMENTAL

Nuclear magnetic resonance spectra were obtained with a Hitachi R-20B and a Bruker WH-270 FT spectrometer operating at 60 MHz and 270 MHz, respectively, and equipped with temperature controllers. Temperature readings were calibrated using the methanol chemical-shift method. 60 MHz Spectra were obtained using 0.5 M solutions with 3% tetramethylsilane (TMS) added while the 270 MHz spectra were measured in 0.1 M solutions with 0.5% TMS.

Elemental analyses were carried out by Dr. Agahigian, Baron Consulting Co., Orange, CT. Melting points were obtained with a Gallenkamp melting point apparatus and are not corrected. $LiAlD_4$ was purchased from the Aldrich Co.

1-Dideutero-2-dimethylaminoethanol methiodide. To a refluxing mixture of $LiAlD_4$ (1.00 g, 23.82 m-moles) in anhydrous Et_2O (5 ml) was added dropwise, over a period of 30 min, a solution of 2-dimethylaminoethyl acetate (0.89 g, 6.78 m-moles) in anhydrous Et_2O (10 ml). After refluxing for an additional 1.5 hr, the mixture was cooled and treated with EtOAc (10 ml) and H_2O (2 ml) and filtered. The filtrate was then dried (Na_2SO_4) and the solvent was removed. The clear colorless residue of 1-dideutero-2-dimethylaminoethanol was dissolved in anhydrous Et_2O (5 ml), treated with methyl iodide (2.3 g, 16.2 m-moles) and allowed to stand for 12 hr at 5°. The white precipitate formed was filtered and recrystallized from an ethanol–ether mixture (1:3) to give colorless crystals (1.03 g, 4.42 m-moles, yield (65%), m.p. 261–262°. Anal. Calc. for $C_5H_{12}D_2NOI$: C, 25.76; H, 6.92; N, 6.01. Found: C, 25.75; H, 6.89; N, 5.99.

1-Dideutero-2-dimethylaminoethanol methochloride. A mixture of 1-dideutero-2-dimethylaminoethanol methiodide (0.75 g, 3.22 m-moles) and Ag_2O (1.00 g, 4.32 m-moles) in H_2O (5 ml) was stirred for 12 hr at 25° and filtered. The filtrate was titrated to pH 7.0 using 0.1 N HCl and lyophilized. The white crystalline residue was recrystallized from a methanol–ether mixture (1:5 to give colorless crystals (0.40 g, 2.82 m-moles, yield 88%) m.p. 260–263°. Anal Calc. for $C_5H_{12}D_2NOCl$: C, 42.40; H, 11.38; N, 9.89. Found; C, 42.29; H, 11.22; N, 9.79.

1-Dideutero-2-trimethylammoniumethyl acetate iodide. A mixture of Ac_2O (4 ml) and glacial HOAc (2 ml) and 1-dideutero-2-dimethylaminoethyl methiodide (1.00 g, 4.29 m-moles) was refluxed under N_2 for 4 hr. The solution was then concentrated by distilling off 3 ml HOAc and Ac_2O and cooled overnight at 5°. The colorless crystals formed were filtered and dried over NaOH pellets (1.00 g 3.65 m-moles, yield

85%, m.p. 159–160°. Anal. Calc. for ($C_7H_{14}D_2O_2NI$: C, 30.50 H, 6.50; N, 5.01. Found: C, 30.59; H, 6.48; N, 4.99.

1-Dideutero-2-trimethylammoniumethyl acetate chloride. The procedure used in the preparation of 1-dideutero-choline chloride was followed. Yield: 80%, m.p. 159–161°. Anal. Calc. for $C_7H_{14}D_2O_2NCl$: C, 50.14; H, 10.81; N, 8.35. Found: C, 50.23; H, 10.74; N, 8.21.

RESULTS AND DISCUSSION

The spectra of ACh and of choline as well as those of their dideutero analogs showed broadening of the α-methylene proton signals at −90°. While this effect intensified at lower temperatures, at −110°, the lowest temperature which could be attained, splitting of the singlet could not be seen either with choline or ACh, indicating that the coalescence temperature had not been reached (Fig. 1). For this reason, only the upper limits for the free energies of activation could be estimated from the data available.

Similar results were obtained when the decoupled 270 MHz spectrum of ACh in CD_3OD was measured at temperatures as low as −110° (Fig. 2). The broadening in the NCH_2 singlet of the ACh ^1H spectrum, observed at lower temperatures, was comparable in both 60 MHz and 270 MHz experiments. This is an indication that the line broadening was due mostly to sample magnetic inhomogeneity and/or increased ^1H relaxation rates and less to a decrease in exchange rate between the H_A and H_B protons.

A rough estimate for the rate constant (k_c) for intramolecular exchange for an AB spin system at the coalescence point can be obtained [33] from the relationship:

$$k_c = \frac{\pi\sqrt{[(\nu_A - \nu_B)^2 + 6J_{AB}^2]}}{\sqrt{2}}$$

This rate constant, in turn, can, be used to calculate the free energy of activation, $\Delta G^{\ddagger}$ for molecular rotation by means of the Eyring equation:

$$k_c = \frac{k_B T_c}{h} \cdot \exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right)$$

or

$$\Delta G^{\ddagger} = 4.57 T_c \left(10.32 + \log_{10}\frac{T_c}{}\right)$$

k_B = Boltzmann's constant, h = Planck's constant, R = gas constant, T = absolute temperature, and T_C = coalescence temperature.

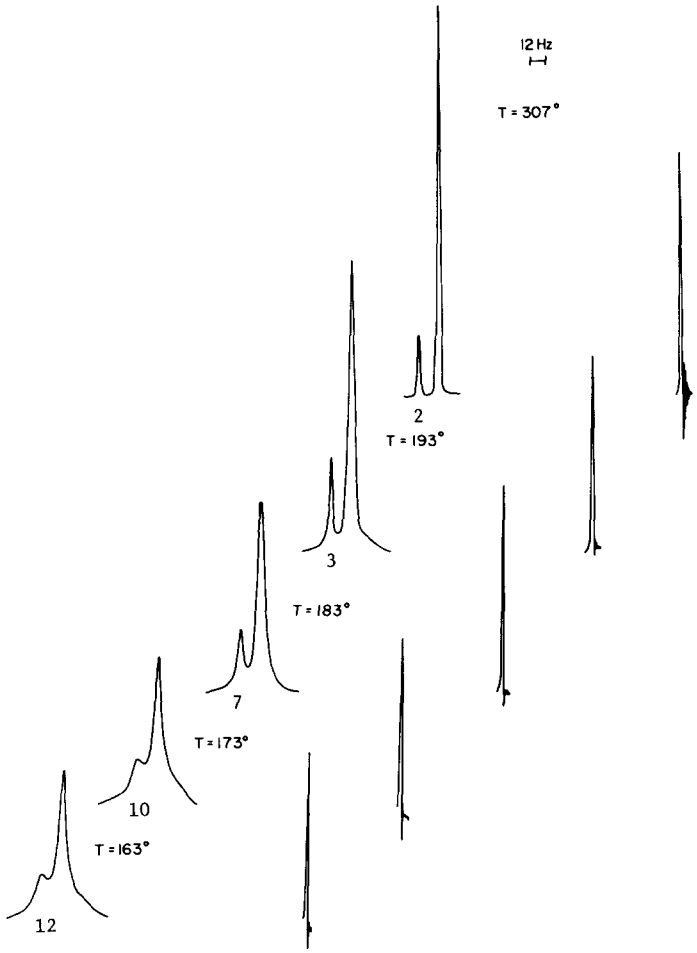


Fig. 1. N—CH_2 and N—CH_3 peaks from the 60 MHz, ^1H spectra of α-dideuterocholine chloride, in a methanol-d_4:trifluoroethanol-d_3 (2:1) mixture at different temperatures. The corresponding widths of the —NCH_2— resonance lines at half height are shown in Hz underneath the peak.

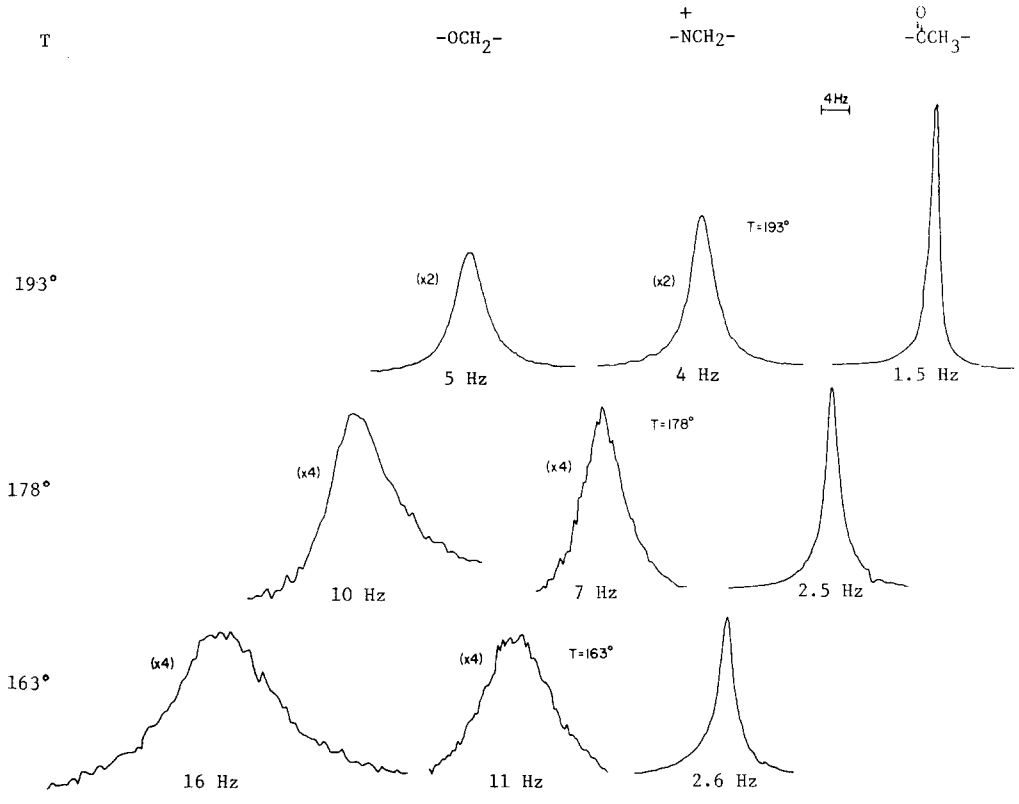


Fig. 2. —OCH_2—, —$\overset{+}{N}CH_2$— and —$COCH_3$ peaks from the 270MHz ^1H spectra of ACh chloride in CD_3OD at different temperatures. The proton-decoupled $\overset{+}{N}CH_2$ peaks were obtained by irradiating the —OCH_2— frequencies in the corresponding spectra, while the proton-decoupled OCH_2 peaks were obtained with —$\overset{+}{N}CH_2$—irradiation. The broader line width of the OCH_2 peaks is due to their strong coupling with the ^{14}N nitrogen. Individual lines in the —OCH_2— triplet cannot be observed at low temperatures. The corresponding widths of the —NCH_2—, —OCH_2—, and —$COCH_3$ proton resonance lines at half height are shown. The line intensities are on different scales; the magnification factor is shown in the figure.

The temperature dependence on $\Delta G^{\ddagger}$ is ignored since, in this unimolecular process, entropic factors would be expected to be negligible [34].

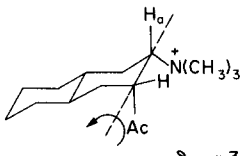
Values for chemical shift differences (ν_A-ν_B) and coupling constants (J_{AB}) were obtained from the literature [10, 35]. In β-dideutero ACh or β-dideutero choline, protons H_A and H_B form dihedral angles of 60° and 180°, respectively, with the acetoxy group and, therefore, should have different chemical shifts.

To estimate such chemical shifts we used, as models, the *trans*-decalin analogs of ACh synthesized by Smissman *et al.* [35]. In (e)-trimethylammonium-2-(e)-acetoxy-*trans*-decalin chloride, the hydrogen H_A and the acetoxy group form a 60° angle. On the other hand, in the isomer 3(e)-trimethylammonium-2(a)-acetoxy-*trans*-decalin, a 180° angle is formed by the hydrogen $H_B$with respect to the acetoxy group (Fig. 3). The ^1H spectra of these systems allowed us to obtain values for the chemical shift differences for the protons in question. These differences (ν_A-ν_B) are equal to *ca.* 5 Hz and 22.5 Hz for the 60 MHz and the 270 MHz spectra respectively. The value used for the geminal coupling constant, J_{AB}, -13.5 Hz, was that obtained from the spectrum of ACh [10] and is identical at both frequencies.

Since at −110° the coalescence temperature for ACh or choline had not been reached, only an upper limit for the rotational barriers for these compounds could be obtained. There was no significant difference between the calculated $\Delta G^{\ddagger}$ values in the 60 MHz and 270 MHz experiments. This calculated upper limit of $\Delta G^{\ddagger} = 7.9$ kcal for the rotational barriers of choline and ACh can be compared to the lower limits obtained, indirectly, from relaxation measurements [29, 30] and found to be 3.0 to 3.5 kcal for choline and 3.5 to 4.0 kcal for ACh.

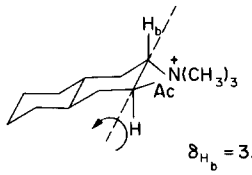
Thus, the rotational barriers around the central methylene carbons of choline and ACh must lie between 3.0 and 7.9 kcal, in agreement with theoretical calculations using the PCILO [26] and INDO [27] methods and in disagreement with the very high values obtained from HMO [24] and *ab initio* [23] calculations as well as with very low values obtained by the application of empirical methods [28].

It has been proposed [16–19] that the *gauche* conformation of the —OCCN— grouping of choline and ACh involves an electrostatic "hard acid–hard base" interaction between the nitrogen and oxygen. This proposal is supported by the observation that in the non-protonated dimethylamino analogs of 2-trimethylammoniumethyl esters, no specific rotamer is favored in solution [17]. The observed limits for the rotational barriers are in agreement with that postulate. It should be added that such interactions should be extremely



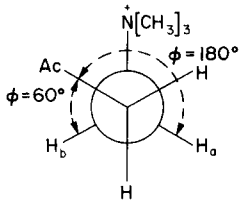
$\delta_{H_a} = 3.80$ ppm

3(e)-dimethylamino-2(a)-acetoxy-trans-decalin methochloride



$\delta_{H_b} = 3.88$ ppm

3(e)-dimethylamino-2(e)-acetoxy-trans-decalin methochloride



acetylcholine

Fig. 3. Trans-decalin model compounds with angle projections corresponding to those of Ach in the *gauche* conformation.

sensitive to the dielectric constant of the medium in which the molecule under observation would be located, raising the possibility that the rotational barriers for the central —CH_2—CH_2— bond of choline and ACh observed in solution might differ from those seen when these molecules are attached to the active sites of their target biopolymers.

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