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The Conformation of Acetylcholine and Related Compounds in Aqueous Solution as Studied by Nuclear Magnetic Resonance Spectroscopy

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SUMMARY

The conformations of acetylcholine and 22 related compounds in aqueous solution have been determined by analysis of their NMR spectra. *Gauche* conformations depend on the presence of the onium group and the partial negative charge on the β-oxygen. Good agreement is obtained with crystal structures, except for carbachol, which in solution has a *gauche* O/N^+ conformation and in the crystal structure has a *trans* conformation.

There is no simple correlation between the predominant conformations and the potency of action of the drugs at either the nicotinic or muscarinic receptor.

INTRODUCTION

Acetylcholine is a synaptic transmitter that interacts with at least three distinct receptor types as well as being a substrate for the hydrolytic enzymes acetylcholinesterase and cholinesterase. Because it is a molecule which can exist potentially in several rotameric states, the question of which rotamer predominates in solution enters into discussions of structure-activity relationships. In the past few years extensive X-ray diffraction studies on the crystal structures of acetylcholine and related molecules have been reported. These studies raise the important question of whether the unique conformation present in the crystal is the only conformation present in solution or whether significant populations of other conformations are also present. This problem can be tackled by measurements of vicinal H—H spin coupling constants by high-resolution nuclear magnetic resonance spectroscopy. The principle of the method is that the coupling of the spins of protons on adjacent carbon atoms depends on the dihedral angle ϕ. It was established by Karplus on theoretical grounds that the relationship should be proportional to $\cos^2\phi$. Extensive work on compounds of defined geometry has established the reliability of this relationship, especially when the constants are based on a series of closely related compounds and due allowance is made for the effects on the coupling constants of the electronegativity of the substituents. The method thus provides a basis for determining the relative populations of the conformations produced by rotation about C—C bonds. For the series of compounds examined here one can also derive conformational information from the coupling between ^{14}N nuclei and the protons, which depends on the N—C—C—H dihedral angle. In most molecules containing ^{14}N and ^{1}H, spin coupling between these nuclei is not observed because of the rapid relaxation of the ^{14}N nucleus which results from the strong interaction between its quadrupole moment and the fluctuating electric field

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gradients within the molecule. However, in some quaternary ammonium compounds, the high orbital symmetry around the nitrogen leads to small electric field gradients, and low quadrupolar relaxation rates and hence the N—H couplings become observable.

Culvenor and Ham (1, 2) first pointed out that the sum (N) and an approximation to the difference (L) of the vicinal coupling constants, which are directly observable in the $AA'BB'$ spectrum of acetylcholine, suggest that it is present in the *gauche* conformation. They subsequently showed that the N and L values for acetylthiocholine were quite different from those in acetylcholine, and indicated a *trans* conformation. Cushley and Mautner (3) carried out a fuller analysis of the spectra of acetylcholine, acetylthiocholine, and acetylselenocholine, which solidified these conclusions. Casy, Hassan, and Wu (4) have carried out more extensive studies on the same lines, and also examined α- and β-substituted acetylcholines. The first use of ^{14}N—^{1}H couplings to elucidate the conformation of α- and β-substituted acetylcholines was by Inch, Chittenden, and Dean (5).

In the present work full spectral analysis of a series of acetylcholine congeners has been undertaken to put the calculation of the relative population of rotamers on a more quantitative basis by considering the observed vicinal coupling constants to be weighted averages of the component coupling constants in the individual rotamers.

EXPERIMENTS AND RESULTS

The ^{1}H resonance spectra were measured at 100 MHz and 220 MHz, using Varian

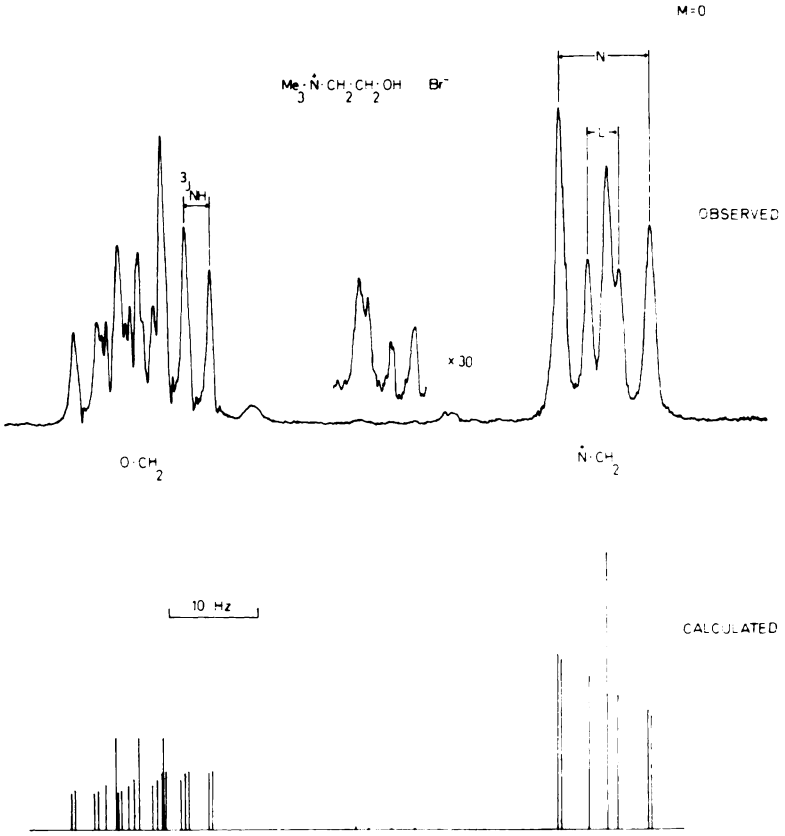


FIG. 1. *Proton NMR spectrum of choline bromide in D_2O at 100 MHz*

The N—H coupling is shown, as are the L and N values used for trial calculations in the LAOCOON program. The weak central lines are shown at a 30-fold increased gain. The lower section shows frequencies and intensities of the transitions calculated for the best fit to the observed spectrum.

XL100-15 and HR 220 spectrometers. All compounds were examined as approximately 1 M solutions in deuterium oxide at 30° [except for acetyl-α-methylcholine (0.25 M)]. Some were also examined in water, but no differences due to the solvent were observed. Samples which showed line widths greater than those expected from the resolving power of the spectrometer were also examined at 90°C to reduce the ^{14}N relaxation rate, so that sharper triplet splitting could be observed on the β-CH_2 proton signals. In some cases dilute solutions of lower viscosity were also used to reduce the ^{14}N relaxation rate and so to reveal the N—H coupling.

Most of the compounds were available from commercial sources. *N*-Benzyldimethylethanolammonium, choline methyl ether, choline phenyl ether, *N*,*N*-dimethylmorpholinium, phenylethyltrimethylammonium, cyclohexylethyltrimethylammonium, and β-aminoethyltrimethylammonium were synthesized by standard methods. β-Methylthioethyltrimethylammonium was a gift from Dr. K. S. Scott; 3,3-dimethylbutan-1-ol, a gift from Dr. V. Whittaker; and acetyl-α-methylcholine, a gift from Dr. E. Lesser.

Analysis of Spectra

ABX type spectra. The CH_2—CH fragments of the α- and β-substituted cholines gave spectra which were analyzed as *ABX* spin systems by the subspectral method (6).

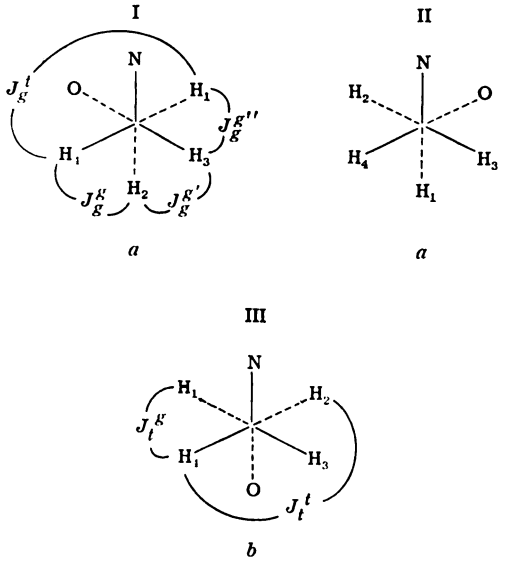
AA'BB' and AA'XX' type spectra. Initially analysis was performed by assuming that the spectrum was approximately *AA'XX'*, and subsequently the parameters were refined using a modified LAOCOON computer program (7). Figure 1 shows the methylene region of the ^1H spectrum of choline bromide recorded at 100 MHz, together with the calculated spectrum obtained by this method. In this case the chemical shift and the vicinal and geminal coupling constants could all be extracted from the spectrum. The geminal couplings could be obtained because the weak central transitions were detectable. The relative signs of the geminal and vicinal couplings could not be determined, because the largest effect of change of sign on a transition frequency was approximately 0.17 Hz, which is comparable with the precision of the line frequency measurements. However, in other substituted ethanes, the relative signs of the geminal and vicinal coupling constants are well documented (8) and are always opposite; the magnitude of the geminal coupling was 13.5 Hz.

The signals from the *O*-methylene protons exhibit the characteristic N—H triplets, and the coupling constant can be measured directly from the spectrum.

In some cases a small (less than 0.05-Hz) unresolved coupling between the methyl protons and the α-methylene protons was observed. This could be removed by double resonance spin-decoupling of the methyl protons to improve the resolution of the α-methylene group.

Calculation of Rotamer Populations

1,2-Disubstituted ethanes. Abraham and co-workers (9) have studied extensively the use of vicinal H—H coupling constants for calculating rotamer populations for 1,2-disubstituted ethanes. Using their nomenclature, we can describe the three most likely minimum energy conformations as I, II, and III, with the component vicinal coupling constants as indicated below.



If the fractional populations of rotamers I, II, and III are *a*, *a*, and *b*, respectively, such that

$$2a + b = 1 \qquad (1)$$

TABLE 1

Vicinal H—H coupling constants in 1,2-disubstituted ethanes

E_x and E_y are substituent electronegativities (Huggins). Spragg's values (10) for morpholine are $J_g{}^t = 12.79$, $J_g{}^{g'} = 1.05$, $J_g{}^g$, $J_g{}^{g''} = 2.82, 1.31$ (not assigned). Errors in $\frac{1}{2}(J_g{}^g + J_g{}^{g''})$ are ±0.3 Hz.

Substituent	$E_x + E_y$	$\frac{1}{2}(J_g{}^g + J_g{}^{g''})$	$J_t{}^t$	$J_t{}^g$	$J_g{}^t + J_g{}^{g'}$
		Hz	*Hz*	*Hz*	*Hz*
O/N	6.55	2.42[a]	12.31	5.48	13.62
Cl/N	6.20	3.14	12.62	5.25	14.33
C/N, S/N	5.65	3.63	13.11	4.90	15.45
N/N	6.1	3.21	12.70	5.19	14.54

[a] Mean value obtained from those of Abraham and Gatti (9) and Spragg (10).

then the observed vicinal coupling constants may be expressed as the population-weighted average of the values in the three possible rotamers. Hence

$$J_{14} = aJ_g{}^t + aJ_g{}^{g'} + bJ_t{}^g \quad (2)$$

$$J_{13} = a(J_g{}^g + J_g{}^{g''}) + bJ_t{}^t \quad (3)$$

From an investigation of a wide series of 1,2-disubstituted ethanes, Abraham and Gatti (9) were able to deduce empirical relationships between the vicinal H—H coupling constants and the electronegativities of the substituents of the form

$$J = K_1 + K_2(E_x + E_y) \quad (4)$$

By combining their results with those of Spragg (10) from the low-temperature proton spectrum of morpholine, we have compiled Table 1, which contains the various vicinal H—H coupling constants for the rotamers of interest in this investigation. Using these values, it was possible to calculate the fractional populations in Table 2. Equation 2 was not used for this purpose because of its insensitivity to changes in a and b resulting from $J_t{}^g$ being similar in magnitude to $\frac{1}{2}(J_g{}^t + J_g{}^{g'})$. However, the results obtained by using the more sensitive Eq. 3 were checked for consistency with Eq. 2.[1]

An alternative method of calculating the fractional populations is provided by the observed ^{14}N—H coupling constants in the fragment N—C—C—H, which can be treated similarly to the vicinal H—H coupling constants. This method is attractive since it does not require full analysis of the spectrum to obtain J_{NH} values; these may be measured as first-order splittings from the spectrum. Figure 2 shows the different N—H coupling constants which feature in the spectra of (a) choline bromide, J_{NH} = 2.7 Hz, indicating a predominant O/N$^+$ *gauche* conformation; (b) β-chloroethyltrimethylammonium, J_{NH} = 1.8 Hz, indicating a mixed *gauche/trans* conformation; and (c) β-methylthioethyltrimethylammonium, J_{NH} = 0.75 Hz, indicating a predominant S/N$^+$ *trans* conformation.

Several workers have indicated the possibility of using vicinal N—H coupling constants for this purpose (11). We have attempted to define the values of the appropriate N—H coupling constants in the individual rotamers to permit quantitative calculation of the fractional populations.

As a first approximation, we have assumed that only two coupling constants, J_{NH}^g (*gauche*) and J_{NH}^t (*trans*), need to be defined, to describe the observed averaged coupling constants, and we have neglected electronegativity corrections. Thus, for rotamers I, II, and III, the expression for the observed vicinal N—H coupling constant is

$$J_{NH} = a(J_{NH}^g + J_{NH}^t) + bJ_{NH}^g$$

The values for the component coupling constants were estimated in the following manner. The observed $^3J_{NH}$ coupling constant in the ethyltrimethylammonium ion is 2.1 Hz, and because the conformational state

[1] Because the component coupling constants are estimated by this empirical approach, the actual values will be corrected for any possible distortions of the normally staggered dihedral angles.

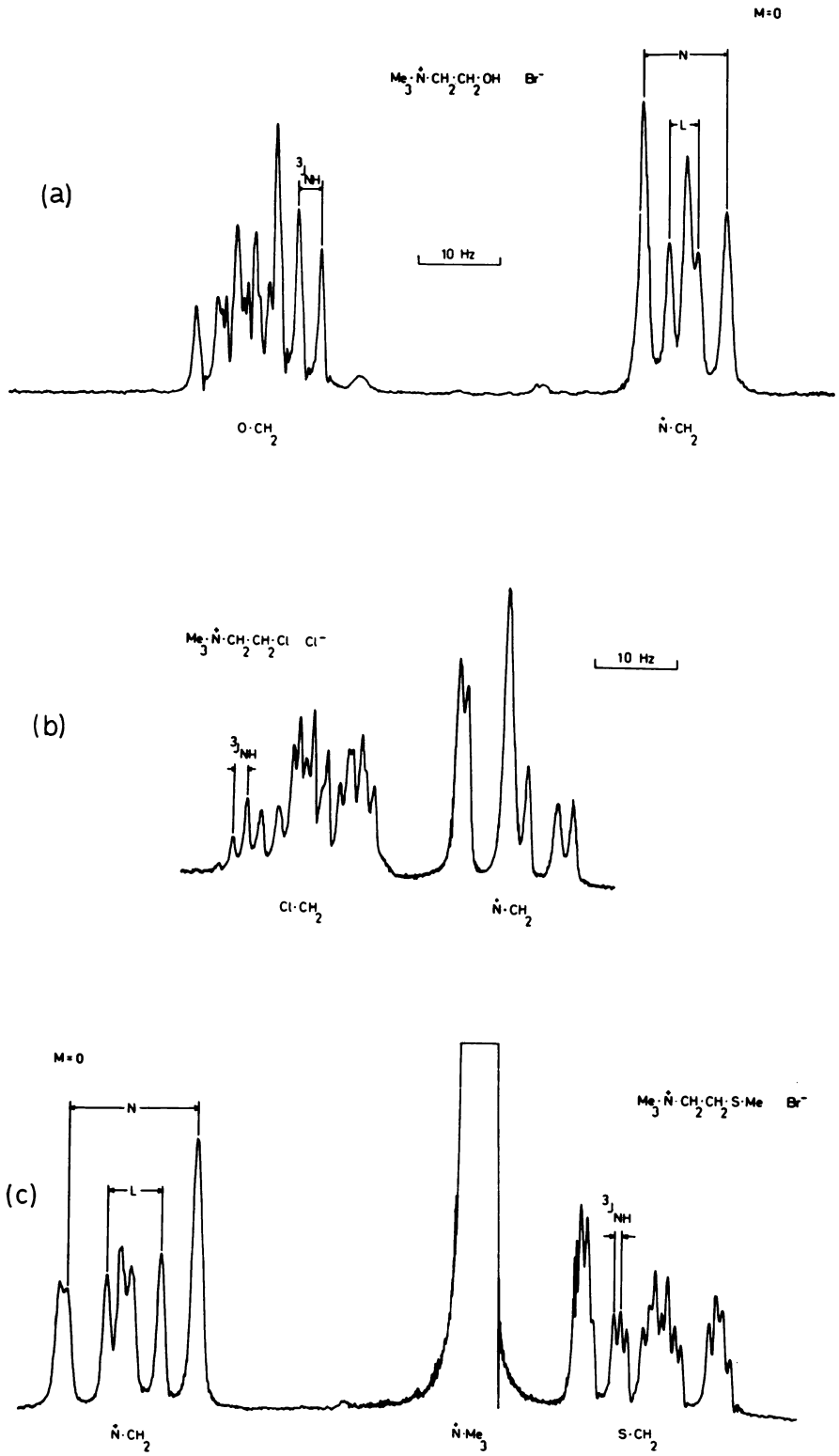


FIG. 2. *Proton NMR spectra of* (a) *choline*, (b) *chlorocholine, and* (c) *β-methylthioethyltrimethylammonium at 100 MHz.*

Notice the large J_{NH} in (a), the small J_{NH} in (c), and the intermediate value in (b). The N—CH_2 part of the spectrum can be readily identified by the lack of N—H couplings.

of an ethyl group is well defined we can state that

$$J_{NH} = \tfrac{1}{3}(J^{t}_{NH} + 2J^{g}_{NH}) = 2.1$$

The smallest value of J_{NH} observed in this work falls in the range ±0.7 Hz, and such cases were shown by the proton coupling constants to be almost exclusively *trans* O/N^+ rotamers. This places a limit on the range of the value of J^{t}_{NH}, being 6.3 ± 1.4 Hz. We have used values of J^{g}_{NH} and J^{t}_{NH} in these ranges to calculate fractional populations, and we obtain the best agreement with the populations obtained from proton coupling constants when the N—H coupling constants have the same sign. The best values are

$$J^{g}_{NH} = 0.7 \text{ Hz}$$

$$J^{t}_{NH} = 4.9 \text{ Hz}$$

All the fractional population data are summarized in Table 2.

β-Methacholine, carbamyl-β-methylcholine, and acetyl-α-methylcholine. It is more difficult to obtain quantitative conformational information for these compounds, because the component vicinal coupling constants are known with less certainty. Casy and coworkers (4) have provided reasonable esti-

TABLE 2

Conformation of acetylcholine and related compounds

The references cited below provide X-ray crystallographic data for the compounds indicated.

Compound	$J_{^{14}NH}$	J_{14}	J_{13}	*Gauche*	*Trans*
				%	%
Class I					
Choline (12)	2.7	6.56	3.55	89	11
N-Benzyldimethylethanolammonium	2.8	6.6	3.6	88	12
N-Dimethylethanolammonium		6.68	3.93	85	15
Acetylcholine (13)	2.5	6.93	2.35	100	
Butyrylcholine	2.6	7.19	2.27	100	
Suxamethonium	2.5	7.17	2.29	100	
Carbamylcholine (14)	2.7	6.98	2.20	100	
Benzilylcholine		6.98	2.83	96	4
Choline methyl ether	2.7	6.55	3.57	88	12
Choline phenyl ether	2.8	6.44	2.83	96	4
Choline 2,6-xylyl ether (15)	2.7	6.6	3.5	89	11
N,*N*-Dimethylmorpholinium	2.2	6.57	3.54		
Class II					
Acetylthiocholine (16)[a]	0.75	5.00	11.59	16	84
Acetylselenocholine (17)[a]	<0.7	4.83	12.63	5	95
β-Methylthioethyltrimethylammonium	0.75	5.02	11.43	17	83
Phenylethyltrimethylammonium	0.7	4.68	11.49	17	83
Cyclohexylethyltrimethylammonium		5.00	11.00	22	78
β-Aminoethyltrimethylammonium	<0.7	4.70	11.50	13	87
3,3-Dimethylbutan-1-ol(carbocholine)		5.70	10.06	32	68
4-Hydroxylphenylethyldimethylammonium	0.7	5.00	11.00	22	78
Class III					
β-Chloroethyltrimethylammonium (chlorocholine)	1.8	6.42	6.53	64	36
N-Dimethylethanolamine		6.5	6.5	64	36
Class IV					
Methacholine (18)	1.3	8.8	1.4	77/23[b]	0
Carbamyl-β-methylcholine	1.3	9.4	1.5	79/21[b]	0
Acetyl-α-methylcholine (19)	2.2			80	20

[a] Data of Cushley and Mautner (3).

[b] Rotamer with O, N^+, and Me *gauche* to each other is least populated.

Error in rotamer populations is ±10%.

mates for some of the component coupling constants by examining *N*,*N*-dimethyl-2,6-dimethylmorpholinium iodide and assuming the molecule to be exclusively in the conformation with the methyl groups in equatorial positions (J_{vic} = 11.2 and 2.1 Hz). We have used a combination of these values and those previously used for 1,2-disubstituted ethanes to calculate conformation populations from observed vicinal coupling constants. The best internal consistency was obtained when we employed the same values as used for the 1,2-disubstituted ethanes.

DISCUSSION

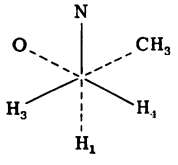
Table 2 contains the vicinal coupling constants for all the compounds studied, together with the calculated fractional populations of the *gauche* and *trans* rotamers. Four classes of results can be defined.

Class I. In these compounds the *gauche* (synclinal) conformation is strongly preferred. The J_{NH} coupling constants fall in the range 2.2–2.8 Hz, and one of the vicinal proton coupling constants, J_{13}, is the narrow range 2.3–3.9 Hz.

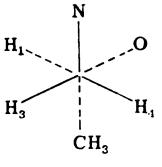
Class II. In these compounds the *trans* (antiplanar) conformation is strongly preferred. The J_{NH} is small, approximately 0.7 Hz, and one of the vicinal proton coupling constants, J_{13}, is large, 11.0–12.6 Hz.

Class III. These two compounds are mixtures of *gauche* and *trans* conformations, as shown by the intermediate value for J_{13} of 6.4–6.5 Hz and the value of J_{NH} for chlorocholine. The J_{NH} was not observable in the tertiary amine *N*-dimethylethanolamine. The distribution of *gauche* and *trans* is close to the 2:1 ratio expected when there is no conformational preference.

Class IV. For methacholine and carbamyl-β-methylcholine, the *gauche* O/N^+ conformations are strongly preferred; however, there are two distinct *gauche* conformations, I and II, of which II is the most populated (approximately 80%).



I



II

From the spectrum of acetyl-α-methylcholine it was possible only to obtain the sum of the vicinal H—H coupling constants, because of the fortuitous equivalence of the β-CH_2 protons. This considerably reduces the conformational information, but from the large observed value of J_{NH} (2.2 Hz) the molecules are obviously at least 75% in the *gauche* O/N^+ forms. Our conclusions are entirely consistent with those of Casy *et al.* (4) and Inch *et al.* (5).

The correspondence of the conformations obtained by NMR with the X-ray crystallographic data is very good. Among the compounds in classes I and II, the only disagreement is for carbamylcholine, which in the crystal is in the *trans* conformation (13). In the case of methacholine, the structure found by X-ray is the *gauche* II structure, which we find to be the most populated conformation, but no trace is seen of the significant population of *gauche* I. In acetyl-α-methylcholine the X-ray shows the unusual feature of twinning of a distorted *trans* conformation with a *gauche*.

The unusual crystal structure of carbachol can be attributed to an intermolecular hydrogen bond, but the otherwise good agreement between the two methods does not mean that discrepancies will not occasionally occur when requirements of packing, or interactions between adjacent molecules, are sufficient to overcome the intrinsic energy distribution of the isolated molecules.

The assessment of the relative populations in class I give an average *gauche* to *trans* ratio of 95:5 (excluding the tertiary amine *N*-dimethylethanolammonium), compared with the ratio 67:33 to be expected if there were no conformational preference. The free energy needed to produce this distribution is at least −1.8 kcal/mole.

Examination of space-filling models, or simplified calculations of van der Waals interactions (including contributions from torsional barriers), shows that a 60-degree *gauche* conformation is a high-energy one and that a *trans* conformation is likely to be preferred. In the group I molecules there is the additional factor of an electrostatic interaction between the positive charge of the onium and the partial negative charge on the ether or alcohol oxygen. This interaction is stronger in the *gauche* form, and therefore

favors it. The strength of the van der Waals repulsion nevertheless prevents the 60-degree torsional angle from being attained, the equilibrium torsional angle being 75–85 degrees in the crystal states, as found in the X-ray studies.

N-Dimethylethanolamine is interesting in this connection; in the protonated form it is predominantly *gauche*, while in the non-protonated form there is an equal distribution of the rotamers. The electrostatic interaction probably stabilizes the *gauche* form, but it is not possible to assess the effect of the potential N—H···O hydrogen bond.

In the other molecule that shows a mixture of rotamers, chlorocholine, the electronegativity of chlorine is lower than that of oxygen, and thus the electrostatic interaction is weaker and insufficient to favor a predominance of the *gauche* conformation. The size of the chlorine atom must also play some part in this, because of the greater van der Waals repulsion. In the thiocholines, since sulfur possesses low electronegativity, no electrostatic interaction can develop, and the basic *trans* preference persists. In 3,3-dimethylbutanol the electrostatic interaction is no longer present, because of the lack of an onium group.

It should be noted that spin coupling data of the kind discussed here give no information about the conformation of either the C—C—O—C or the C—C—N—C dihedral angles, and we are at present reliant on X-ray or theoretical calculations for our knowledge of these angles.

The relationship of the conformation of cholinomimetic drugs to their biological activity has been the subject of much discussion. A comparison of the predominant conformations found in this study does not show any simple correlation with the potency of action of the drugs at either the nicotinic or muscarinic receptor. Beers and Reich (20) have taken the interesting empirical approach of looking for common dimensional features in agonists and antagonists, and adopting conformations that suit these dimensions; they are led to the conclusion that at both receptors acetylcholine is present in the *trans* conformation. Chiou *et al.* (21) have defined the conformation by making the enantiomers of 2-acetoxycyclopropyltrimethylammonium, in which the O—C—C—N dihedral angle is fixed. They found that the (+)-*trans* isomer was as active as acetylcholine at muscarinic receptors, whereas the other three isomers all had very low activity. The torsion angle of the *trans* isomer is 137 degrees (22), which is neither *gauche* nor *trans* but just about equidistant from the two. At the nicotinic receptor this isomer is much less active than acetylcholine, but this is presumably analogous to the low activity of methacholine and must be attributed to steric effects of β-substitution.

The careful study by Hillman and Mautner (23) of the properties of acetylcholine, acetylthiocholine, and acetylselenocholine as substrates for acetylcholinesterase are of interest here. The binding constants for these three substrates form a smoothly increasing set, without any discontinuity between the oxy and thioesters, such as might be expected if the conformational difference dominated the binding differences; a similar smooth series is found in the sensitivity of the electroplax.

These results do not lead to much confidence in attributing maximal biological activity to either *gauche* or *trans* conformations, and indeed are most consistent with an intermediate conformation (anticlinal). Rather similar considerations caused Chothia and Pauling (24) to suggest that the *gauche* conformation had to be opened out before acetylcholinesterase could act on the substrates.

If the conformation binding to the receptor is not the strongly preferred one in solution, a thermodynamic price must be paid, represented approximately by the energy factor $-RT \ln p$, where p is the ratio of the populations within and outside the limits of the torsion angle acceptable to the binding site. Consider first the case in which acetylcholine, which is *gauche* in solution, is required to bind in the *trans* form. Since in solution $p_{trans} < 0.05$, the free energy needed to correct it to the *trans* form for binding is at least 1.8 kcal/mole. Note that this also means that the potency of acetylcholine is less than 5% of that which it would have been if the solution population were all *trans*. If we now consider the anticlinal angle of 135 degrees appropriate to the comparison

with the *trans* cyclopropyl compound, the extra free energy is about 3.3 kcal/mole [from molecular orbital calculations (25)], so that the potency in this case would be reduced by a factor of approximately 200. These arguments point to the very significant factor that considerations of bound conformations introduce into discussions of structure-activity relationships, and show how unclear the situation is at the present time. An illustration of this is the much used comparison of acetylcholine with the uncharged carbon analogue 3,3-dimethylbutylacetate (26). This comparison has been used to evaluate the contribution of an ionic interaction to the binding process, but since the uncharged analogue is *trans*, whereas acetylcholine is *gauche*, there is a conformational free energy component in binding that prevents an unequivocal assessment of the contribution of the ionic interaction to binding.

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