MOLECULAR PHARMACOLOGY, 9, 144–151

Nuclear Magnetic Resonance Studies of the Conformation and Electron Distributions in Nicotine and in Acetylcholine

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(Received July 28, 1972)

SUMMARY

CHYNOWETH, K. R., TERNAI, B., SIMERAL, L. S., AND MACIEL, G. E.: Nuclear magnetic resonance studies of the conformation and electron distributions in nicotine and in acetylcholine. *Mol. Pharmacol.* **9**, 144–151 (1973).

By the application of intramolecular Overhauser effect and of ^{13}C magnetic resonance spectroscopy, the equilibrium conformation and partial electronic structure of nicotine and acetylcholine have been investigated. The results give experimental support to the theory that acetylcholine acts in one preferred conformation at the different receptor sites.

INTRODUCTION

Many investigations into the conformational and electronic properties of acetylcholine have been performed. The crystal structure has been reported (1, 2) and discussed (3–6). In aqueous solution the conformation of acetylcholine has been studied by NMR spectroscopy (7, 8). In addition, a variety of quantum mechanical calculations have been carried out on acetylcholine (9–11). Local minima in calculated potential energy surfaces have been variously identified with crystal structure, the geometries implicated in cholinergic neural transmission at nicotinic and muscarinic receptor sites, and the geometry favorable for hydrolysis by acetylcholinesterase.

The conformation and electronic structure of nicotine have been the subject of a number of studies. The crystal structure has been reported (12) and further discussed by Chothia and Pauling (4). Simpson, Craig, and Kumler (13) inferred the conformation of nicotine and of the nicotinium ion from the proton magnetic resonance shifts of nicotine and some of its derivatives. Calculations of the preferred conformation and the electronic structure of nicotine using an extended Hückel molecular orbital treatment have been performed by Kier (14). In these calculations it was assumed that the pyridyl ring and the *N*-methyl group were *trans*. The results indicated a negligible energy difference between conformations I and II (Fig. 1).

These studies were supported in part by Grant NSO 9932-01 from the National Institute of Neurological Diseases and Stroke, United States Public Health Service.

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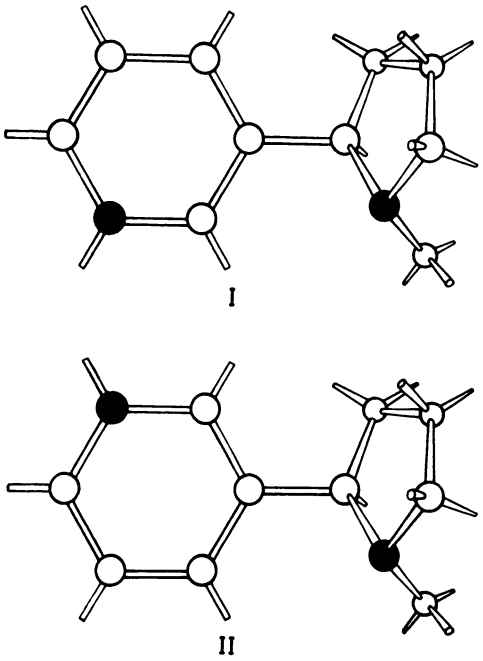


FIG. 1. *The two conformations of nicotine for which calculations have been performed (14)*

There is negligible energy difference between I and II. ●, carbon; ○, nitrogen; hydrogen atoms are not indicated.

More recent calculations by Pullman, Courrière, and Coubeils (11), utilizing a more sophisticated approach (perturbative configuration interaction using localized orbitals, PCILO), also considered the two additional conformational possibilities provided by the steric requirements of a nitrogen lone pair vs. an *N*-methyl group. The results indicated that the *trans* conformation of the *N*-methyl group was more stable than the *cis*. Furthermore, the two calculated preferred conformations of the *trans* form were not of equal energy. The distributions of electronic charge in nicotine and acetylcholine were also calculated and compared by these authors. The results indicated similar charge distributions for nicotine and acetylcholine.

Considering the uncertainties of projecting the results of solid-state studies, or the results of calculations performed on molecules in vacuum, to dilute solutions, we undertook further experimental investigations of nicotine and acetylcholine in dilute solution.

MATERIALS AND METHODS

The nicotine used was of analytical quality (British Drug Houses), $n_D^{20} = 1.528$, and was vacuum-distilled prior to use. Acetylcholine bromide was obtained from J. T. Baker Chemical Company and was not further purified. The proton resonance spectra were recorded as 5% (v/v) solutions in deuteriochloroform and D_2O. A small amount of tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt was added in each case as an internal lock sample. Both samples were thoroughly degassed by at least five freeze-pump-thaw cycles at less than 10^{-4} torr. The experiments were performed at 39° in frequency-sweep mode at 60 MHz. Since the *N*-methyl resonance position is very close to the resonance positions of the pyrrolidine protons, it was necessary to ensure that the H_2 level was perturbing only the *N*-methyl group; otherwise any inferences with respect to methyl group–pyridine ring proton distances would be meaningless. The resonance peaks corresponding to protons 2 and 6 were observed, and the nuclear Overhauser effect was obtained for H_2 levels of 1.0, 2.0, and 3.0 milligauss. These gave respective nuclear Overhauser effect values of 5, 5, and 10%, the large increase for $H_2 = 3.0$ milligauss being taken as due to saturation at higher power levels. For this reason the experiments were carried out at power levels below this value.

It was considered that the concentrations of solutions and quantities of the internal lock species were such that intermolecular relaxation was minimal. At least 10 traces were taken with H_2 both on and off the *N*-methyl peak, and the average of the intensities of the pyridine proton resonances was taken. When the H_2 was off the *N*-methyl peak, it was positioned approximately 100 Hz from any other resonance

position. The results were reproducible to ±1%.

The ^{13}C spectra were recorded on a Bruker HFX-90 spectrometer operating at 22.62 MHz, using 10-mm tubes and natural abundance samples without degassing. The spectrometer was operated in the pulse Fourier transform mode, using a Digilab data system (FTS/NMR-3) and pulse unit. The temperature of the samples was 39.0°. Concentrations were as indicated. Dioxane (2%, v/v) was used as internal standard. The ^{19}F resonance of perfluorobenzene in a 1-mm capillary tube provided the lock signal.

RESULTS AND DISCUSSION

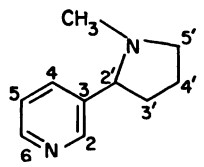
Nuclear Overhauser effects (15) have found wide application in structural and conformational determinations of organic molecules (16–20). The nuclear Overhauser effect, that is, the percentage increase in integrated intensity of the resonance signal of nucleus A when nucleus B is irradiated with a second radio frequency field, is related to the change in spin-lattice relaxation time (T_1) of nucleus A caused by the perturbation of nucleus B, or any nuclei which will affect T_1 of nucleus A. Most often, relaxation takes place mainly through dipole-dipole interactions (21), and in a system such as nicotine the principal contributors to the nuclear Overhauser effect for proton A are then expected to be those other protons within range to contribute to T_1 for proton A. It has been suggested (17) that if a proton is being relaxed only by the protons of a methyl group, the intramolecular nuclear Overhauser effect should be observed for internuclear distances up to approximately 37 nm.

It has been recently pointed out (22) that care must be taken in assigning a conformational preference on the basis of a measured nuclear Overhauser effect at a single temperature in a conformationally mobile system. In view of this warning, it is necessary to comment on the legitimacy of assigning a preferred conformation to nicotine from our nuclear Overhuaser effect data. We must consider what relaxation interactions are present in the absence of irradiation by a second radio frequency field.

Since dipole-dipole interactions are through-space effects, there should be no appreciable mutual contribution to T_1 between protons whose internuclear distance exceeds approximately 40 nm (17). Considering the pyridine protons only (Table 1), proton 2 is too far from any other protons to have its T_1 influenced by other ring protons although the closeness of the pyridine nitrogen atom allows a most efficient relaxation path via the quadrupole relaxation mechanism (20). Proton 4 will be partly relaxed by proton 5; proton 5, by protons 4 and 6; and proton 6, by proton. 5 Irradiation of the pyrrolidine *N*-methyl protons and observation of the nuclear magnetic resonance spectral intensity changes of the pyridine protons form the basis of our argu-

TABLE 1

Observed nuclear Overhauser effect on irradiation of —CH_3 and 2′ protons



These intensity increases were obtained by integration of the relevant resonances of the spectra. Results are averages of 10–15 measurements and are considered to be reproducible to within ±1%.

Proton irradiated	Proton observed	Nuclear Overhauser effect	
		In $CDCl_3$	In D_2O
		% increase	
—NCH_3	2,6	5	4
—NCH_3	4	11	10
—NCH_3	5	3	0
2′	2,6	16	
2′	4	8	

ment concerning the relative proximity of the *N*-methyl group to the pyridine ring.

Distinct nuclear Overhauser effect values (Table 1) for protons 2 and 6 could not be obtained because of peak proximity. However, since (a) the *N*-methyl protons are much farther from proton 6 in any conformation than from proton 2, and (b) the T_1 of proton 6 is influenced by other protons besides the *N*-methyl protons as well as the adjacent nitrogen atom, any enhancement in integrated intensity of the 2,6-pair is assumed to be an effect on proton 2 only. Proton 2 is approximately at the limiting distance (17) for the nuclear Overhauser effect. The observed nuclear Overhauser effect of the *N*-methyl group on proton 4 is by far the largest, in spite of the fact that it can be relaxed by interactions with proton 5. Hence it appears that proton 4 is the closest of all the ring protons to the *N*-methyl group, and is certainly closer than the distance, 32.5 nm, indicated by a nuclear Overhauser effect value of 10 (17) for the case of no other relaxation mechanisms. Consistent with this view is the fact that the nuclear Overhauser effect value observed for proton 5 would indicate that it is approximately at the limiting distance, 40 nm. Irradiation of proton 2′ shows a relatively large nuclear Overhauser effect at proton 2 even though this proton is subject to relaxation via the quadrupole relaxation effect of the adjacent nitrogen atom.

The rapid nitrogen inversion in pyrrolidine (23) may be inhibited by the presence of the pyridine ring, the nitrogen lone pair preferring to remain as far as possible from the π-electrons (24). Furthermore, to the extent that the *N*-methyl group is on the side of the pyrrolidine ring away from the pyridine ring, its closest distance of approach to proton 4 of the pyridine ring would be approximately 34 nm. In view of the relatively large nuclear Overhauser effect value of this proton and the contribution of proton 5 to the T_1 of proton 4, it is considered that this distance is too large to produce the observed effect.

It is concluded on the basis of the above arguments that the *N*-methyl group is preferentially on the same side of the pyrrolidine ring as the pyridine ring. In the nicotinium ion there is no nitrogen inversion, but the same argument applies regarding the position of the *N*-methyl group.

On the basis of Dreiding models, if the *N*-methyl group is placed approximately 40 nm from proton 5 (a distance consistent with the observed nuclear Overhauser effect value of proton 5), then protons 4 and 2 are approximately 24 and 40 nm from the *N*-methyl group (17). Proton 2′ is approximately 25 nm away from proton 2. The corresponding nuclear Overhauser effect is larger than the observed value at position 2, but nevertheless the magnitude of enhancement is about 3 times as large at proton 2 as at proton 4, in confirmation of the previous experiments when the *N*-methyl group was irradiated. The observation of a smaller than expected nuclear Overhauser effect value is quite common and in this case is no doubt connected with the quadrupolar relaxation pathway provided by the neighboring pyridine nitrogen atom. The distances obtained from molecular models are quite compatible with the observed nuclear Overhauser effect of each proton, considering the other perturbing effects previously discussed. Although the chemical shift difference between proton 3′ and *N*-methyl protons is only 0.3 ppm (25) and hence accidental irradiation of both resonances might be expected, the argument is unchanged, as irradiation of proton 3′ should increase the nuclear Overhauser effect of proton 4 if the proposed conformation is correct since the proton 3′–proton 4 distance is small while the proton 3′–proton 2 distance is approximately 40 nm. Furthermore, for that conformation, the N—N distance is approximately 47 nm, which is in good agreement with the calculated values of Kier (14) (47.6 nm) and of Pullman

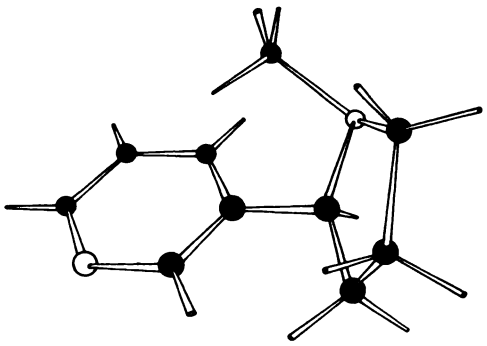


FIG. 2. *Preferred conformation of* (−)-*S-nicotine in aqueous solution*

●, carbon; ○, nitrogen; hydrogen atoms are not indicated.

et al. (11) (43–47 nm). We therefore propose that nicotine has the predominant conformation in dilute solution depicted in Fig. 2. (−)-Nicotine has been assigned the S configuration with respect to L-serine (26).

The stereochemistry of acetylcholine has been studied previously by NMR spectroscopy (2, 7, 8). When a model of this molecule is compared with that of the nicotinium ion (Fig. 3), it appears that there is close similarity between the orientations of the mobile π-system of the acyl group and the mobile π-system of the C_6—N—C_2 part of the pyridine ring, as well as the areas of the sp^2 nitrogen centers.

Reference has been made to the relative stimulant activities of nicotine and its conjugate acid, inasmuch as quaternization with a methyl group does not greatly influence the activity of nicotine (27, 28). It can be shown by simple calculation that, at physiological pH and temperature, the more basic pyrrolidine nitrogen atom (pk_a = 7.65) is protonated to the extent that nicotine is present as the conjugate acid to approximately 80%. The principal agonists and antagonists at nicotinic receptors are known to contain a positive nitrogen center (28). It is not unreasonable, therefore, to propose (26) that the stimulant activity of nicotine is, in fact, manifested by the nicotinium ion. It is worthy of note in this context that our results indicate approximately the same conformation for both the nicotinium ion and the free base.

In contrast to the conformational investigations, the electron distributions in nicotine and acetylcholine have not been experimentally investigated. Few techniques for investigation of electron dis-

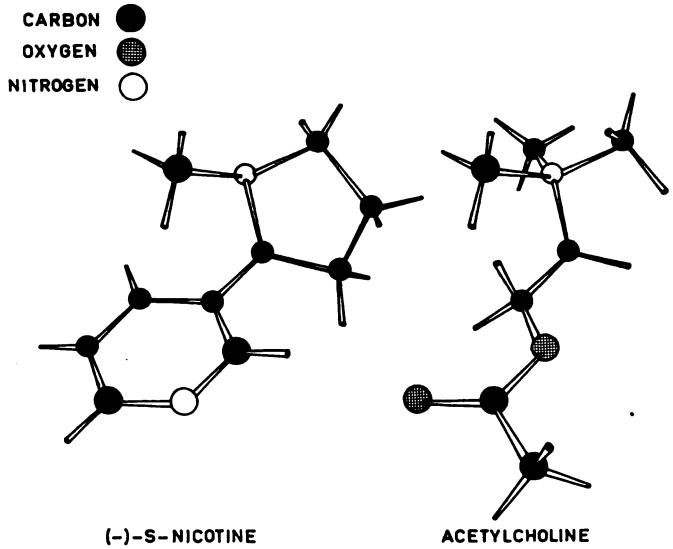


FIG. 3. *Comparison of nicotine in its preferred conformation and of acetylcholine in its "nicotinic" form*

tributions in molecules are amenable to dilute aqueous solutions. A technique which is sensitive to electron distribution in molecules in dilute solution is ^{13}C magnetic resonance (CMR) spectroscopy (29, 30).

A variety of investigations (31–37) have been concerned with the relationship between ^{13}C chemical shifts and electron distributions in molecules. Some such studies have indicated linear relationships between ^{13}C chemical shifts and total electron densities for individual series of related compounds, provided that other perturbing factors such as steric and neighboring group anisotropy were absent or similar. Although the theory of ^{13}C chemical shifts is not yet sufficiently advanced to provide routine and straightforward interpretations of electron distributions, rapid progress is being made in this direction (30, 38, 39). It seems likely that current theoretical developments will soon provide a bridge between experimental ^{13}C data and fundamental electronic interpretations.

The CMR spectrum of nicotine has been reported previously (40). However, the conditions under which the spectrum was obtained (1 M in chloroform) are far from biological conditions. The effects of solvent and concentration upon conformation and chemical shifts in flexible molecules are not well established. We have recorded the spectrum in relatively dilute (0.40 M) aqueous solution. Our data, together with the previous results, are shown in Table 2. The resonances of individual carbon atoms have been assigned previously (40). We con-

TABLE 2

^{13}C chemical shifts of nicotine and acetylcholine

$CH_3(CO)OCH_2CH_2N^+(CH_3)_3$
1 2 3 4 5

All shifts are in parts per million with respect to dioxane (external in the case of ref. 40 and internal for this investigation). More positive values correspond to increasing shielding. Values obtained with respect to carbon disulfide (40) were converted to the dioxane reference by employing the equation

$$\delta CS_2 = (C_4H_8O_2) + 126.0$$

Carbon	Nicotine[a]	Nicotine[b]	Nicotine[c]	Acetylcholine[d]
	ppm	*ppm*	*ppm*	*ppm*
1				+46.07
2	−82.3[e]	−81.88[e]	−81.8[e]	−106.39
3	−71.8	−71.07	−70.9	+8.25
4	−67.5	−69.97	−70.6	+2.20[f]
5	−56.1	−57.82	−58.2	+12.62[g]
6	−81.3[e]	−81.26[e]	−81.3[e]	
2′	−1.2	−1.79	−2.0	
3′	+32.1	+33.21	+33.5	
4′	+44.8	+44.76	+44.8	
5′	+10.7	+10.29	+10.2	
N—CH_3	+27.3	+27.45	+27.6	

[a] Reference 40 (1 M in chloroform).

[b] Present work [0.40 M in H_2O and 2% (v/v) dioxane as internal reference].

[c] Calculated chemical shifts for the nicotinium ion.

[d] Present work [acetylcholine bromide, 0.40 M in H_2O, plus 2% (v/v) dioxane as internal reference].

[e] Represents carbons with almost identical chemical shifts, where the assignment may possibly be reversed.

[f] ^{14}N—^{13}C coupling constant is 2.89 ± 0.04 Hz.

[g] ^{14}N—^{13}C coupling constant is 1.7 ± 0.1 Hz.

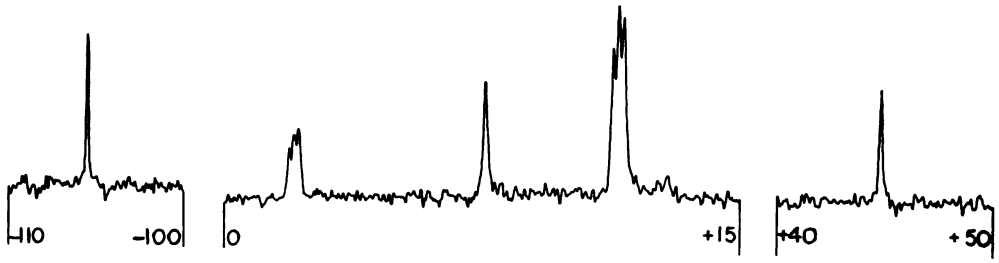


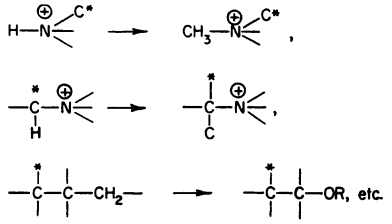
FIG. 4. *^{13}C magnetic resonance spectrum of acetylcholine bromide*
Chemical shifts are referred to internal dioxane.

firmed these assignments by employing off-resonance proton decoupling (41, 42).

The CMR spectrum of acetylcholine has not been reported previously. The chemical shifts obtained in this study are reported in Table 2. The resonances were readily assigned by inspection of chemical shifts, intensities, the observed ^{14}N—^{13}C couplings (Fig. 4), and off-resonance proton decoupling.

It is generally believed that the character of the cationic region is important for nicotinic activity (43, 44). Hence it is of interest to focus attention on the ^{13}C resonances due to the cationic region of acetylcholine and nicotinium ion. Both cases involve an ammonium ion moeity. The data for nicotine in water represent a "weighted average" of the shifts for the nicotinium ion and the free nicotine. Knowing from the pk: value that nicotine in water exists as 80% nicotinium ion and 20% free nicotine, and assuming that nicotine in chloroform exists 100% in the neutral form, one can calculate the chemical shifts of the carbons in nicotinium ion. These are also given in Table 2, and it is seen that they differ little from the raw data on nicotine in water.

Comparison of the ^{13}C chemical shifts of the carbon atoms that are attached directly to the nitrogen atoms (α-carbons) in nicotinium ion and acetylcholine reveals no startling departures from expected patterns. For example, the resonances of all 3 α-carbons in acetylcholine exhibit the decrease in shielding (relative to comparable carbons in the nicotinium ion) that is expected from the β-effect of a CH_3 (attached to nitrogen) compared to H.[2] Thus there appear to be no dramatic differences between the electron distributions in the cationic regions of these two species, and the major differences that do exist are qualitatively similar to the constitutive effects that one expects with simple structural changes such as



(25, 45–52). A more detailed interpretation must await further developments in ^{13}C chemical shift theory.

In conclusion, the nuclear Overhauser effect and CMR spectroscopy provide information that is consistent with the proposal (43) that the activity of a nicotinic agonist is a function of the spatial arrangement of certain functional parts of a particu-

[2] This difference is illustrated by the CMR spectra of the following model systems:

	$HOCH_2CH_2N(CH_3)_2$ + (1 2 3)	$HOCH_2CH_2N(CH_3)_2$ + (1 2 3)
	ppm (with respect to dioxane)	
C_1	+11.22	+10.79
C_2	+7.66	−0.92
C_3	+23.63	+12.57[a]

lar molecule and the corresponding electronic environment.

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