2,3-Diphenylbutane:[15] crystallization from dilute ethanol, mp 124 °C. 1,4-Diphenylbutane:[16] bp 140–2 °C (15 mmHg). 2-(α-Phenethyl)-p-toluidine:[11] yellow oil, bp 178–181 °C (15 mmHg); picrate from benzene, mp 165–7 °C; acetyl derivative, mp 141–2 °C. 4-(α-Phenethyl)-o-toluidine: prepared as described for 2-(α-phenethyl)-p-toluidine;[11] crystallization from petroleum ether (40–60 °C) as colorless needles, mp 55–6 °C;picrate from ethanol, mp 174–6 °C. Anal. Calcd for $C_{15}H_{17}N$: C, 85.30; H, 8.06; N, 6.64. Found: C, 85.59; H, 7.90; N, 6.86. Its IR absorption spectrum shows two sharp bands due to NH_2 stretching vibrations at 3522 and 3435 cm^{-1} and NH bending vibration at 1620 cm^{-1}. Its mass spectrum shows a molecular ion at m/e 211.

(15) J. B. Conant and A. H. Blatt, *J. Am. Chem. Soc.*, **50**, 551 (1928).
(16) R. Kuhn and A. Winterstein, *Helv. Chem. Acta*, 11, 127 (1928).

Registry No. β-Phenylpropionanilide, 19341-03-8; (o-methyl-β-phenyl)propionanilide, 70660-35-4; (p-methyl-β-phenyl)propionanilide, 70660-36-5; ethylbenzene, 100-41-4; 1,4-diphenylbutane, 1083-56-3; 2,3-diphenylbutane, 5789-35-5; aniline, 62-53-3; o-toluidine, 95-53-4; p-toluidine, 106-49-0; o-(α-phenethyl)aniline, 28534-06-7; p-(α-phenethyl)aniline, 7476-71-3; 4-(α-phenethyl)-o-toluidine, 70660-37-6; 2-(α-phenethyl)-p-toluidine, 70660-38-7; α-phenylacetanilide, 621-06-7; β-naphthol, 135-19-3; p-(α-phenethyl)aniline picrate, 70692-41-0; (4-amino-3-methylphenyl)phenylmethane, 62441-37-6; (4-amino-3-methylphenyl)phenylmethane picrate, 70660-39-8; 2,2′-dinaphthol, 602-09-5; 2,2′-dinaphthol dibenzoate, 53545-44-1; 1-(α-phenethyl)isoquinoline, 70660-40-1; α-phenethyl bromide, 585-71-7; isoquinoline, 119-65-3; 1-benzylisoquinoline, 6907-59-1; 2-(α-phenethyl)-p-toluidine picrate, 70660-41-2; acetyl 2-(α-phenethyl)-p-toluidine, 70660-42-3; 4-(α-phenethyl)-o-toluidine picrate, 70660-43-4; benzoic acid, 65-85-0; acetylaniline, 103-84-4; benzoyl o-toluidine, 584-70-3; benzoyl p-toluidine, 582-78-5.

Molecular Associations of Acetylcholine with Aromatic Molecules in Water. Nuclear Magnetic Resonance Spectral Evidence

M. J. Minch,*[1] John P. Sevenair, and C. Henling

Department of Chemistry, University of the Pacific, Stockton, California 95211

Received January 3, 1979

When the sodium salt of an aromatic carboxylate ion is added to a dilute D_2O solution of acetylcholine chloride, the signals of the *N*-methyl and *N*-methylene protons of acetylcholine are shifted upfield relative to the other signals, indicating preferential interaction between the quaternary ammonium head group of acetylcholine and the aromatic ring of the anion. These spectral changes can be related to apparent association equilibrium constants for 1:1 complexation and association constants for acetylcholine complexes with several aromatic anions and phenols are reported. The equilibrium constants are largest for aromatic anions with hydrophobic or electron-donating groups on the ring, especially methoxy groups. Multiring aromatic anions like indole-3-acetate cause very large spectral changes whereas aromatic anions with low electron density in the ring, e.g. *p*-nitrobenzoate ion, do not cause an upfield shift. These observations suggest that electrostatic, water structure, and dispersion forces are all involved in stabilizing the complex, with dispersion (i.e., van der Waals') forces between the aromatic π system and the protons of the quaternary ammonium group predominating in many cases. The effect of added electrolytes or urea is consistent with this view. Complexation with arenesulfonate ions reduces the rate of hydrolysis of acetylcholine in alkaline solution.

There have been many suggestions that aromatic compounds interact strongly with both cationic micelles and unmicellized tetraalkylammonium ions.[2-6] Larsen[7] has demonstrated that the enthalpies of transfer of aromatic compounds from water to hexadecyltrimethylammonium bromide (CTABr) micelles are larger than those for similar size aliphatic compounds and Bunton[8,9] has shown that arenesulfonate and similar anions interact strongly with the cationic head groups in CTABr micelles. Bunton[9] also demonstrated that aromatic anions associate with nonmicellized *n*-butyltrimethylammonium ion in aqueous solution.

The paucity of detailed studies of weak intermolecular associations involving small molecules where both the geometry and strength of association have been determined is surprising, especially since the existence of such complexes is taken for granted and the forces involved are invoked to explain a wide variety of phenomena. It is to fulfill the need for an example of how the geometry and strength of association of such a complex depend on systematic changes in the structure of the aromatic component that we examined the weak association complexes between a wide variety of aromatic compounds and acetylcholine cation by NMR spectroscopy. We chose to study acetylcholine because hydrophobic forces are known to play a role[10] in its association with acetylcholinesterase and because it does not self-associate[11,12] at

(1) Support of this work by the Research Corporation and the donors of the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged.
(2) L. S. C. Wan, *J. Pharm. Sci.* **57**, 1903 (1968); H. Takruri and B. Ecanow, *ibid.*, **55**, 979 (1966); G. R. Walter and W. S. Cump, *ibid.*, **51**, 770 (1962).
(3) E. M. Arnett, M. Ho, and L. L. Schaleger, *J. Am. Chem. Soc.*, **92**, 7039 (1970); S. J. Rehfield, *ibid*, **95**, 4489 (1973).
(4) J. E. Desnoyers, G. E. Pelletier, and C. Jolicoer, *Can. J. Chem.*, **43**, 3232 (1965).
(5) E. F. J. Duynstee and E. Grunwald, *Tetrahedron*, **21**, 2401 (1965).
(6) J. Gordon, J. C. Robertson, and R. L. Thorne, *J. Phys. Chem.*, **74**, 957 (1970); J. Gordon and R. L. Thorne, *ibid.*, **73**, 3643, 3652 (1969).
(7) J. W. Larsen and L. J. Magid, *J. Phys. Chem.*, **78**, 834 (1974).
(8) C. A. Bunton, M. Minch, J. Hidalgo, and L. Sepulveda, *J. Am. Chem. Soc.*, **95**, 3262 (1973).
(9) C. A. Bunton and M. J. Minch, *J. Phys. Chem.*, **78**, 1490 (1974).
(10) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).
(11) Even the more hydrophobic salt *n*-hexyltrimethylammonium bromide does not form micelles in water; cf. ref 12.

0022-3263/79/1944-3247$01.00/0

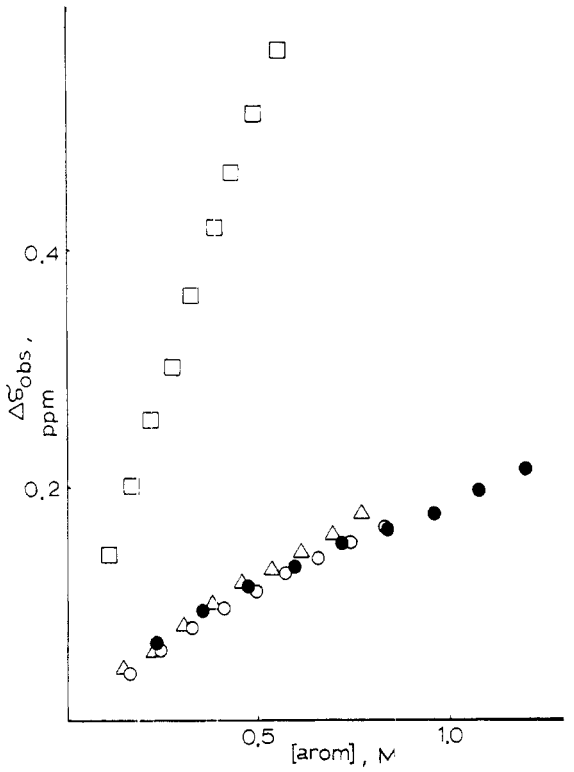

Figure 1. The upfield shift of the *N*-methyl proton signal of acetylcholine with added sodium arenecarboxylates. The points represent the change in the chemical shift difference between the *N*-methyl and *C*-methyl proton signals of 0.032 M acetylcholine chloride in D_2O solutions containing: □ sodium indole-3-acetate; △ phenylacetate; ○ 3-phenylpropionate; and ● benzoate.

the concentrations used in these experiments. The NMR spectrum of acetylcholine in aqueous solution has been the object of considerable study[13] and is well understood. The aromatic compounds represent a wide range of hydrophobicity and permit a partial evaluation of the relative importance of hydrophobic and electrostatic forces in stabilizing these complexes. We also examined the effect of aromatic anions on the rate of saponification of acetylcholine in aqueous buffer solutions.

Results and Discussion

The NMR spectrum of acetylcholine chloride in D_2O is characterized by two singlets at 2.13 and 3.19 ppm for the *N*-methyl and *C*-methyl proton signals and two multiplets centered at 3.71 and 4.54 ppm (downfield from NaDDS) for the *N*- and *O*-methylene proton signals, respectively. The addition of one or more equivalents of the sodium salt of an aromatic carboxylic or sulfonic acid causes an upfield shift in all the signals, the *N*-methyl and *N*-methylene signals shifting most. The upfield shifts of the *N*-methyl proton signals relative to the *C*-methyl proton signals of 0.032 M acetylcholine with various concentrations of several sodium arylcarboxylates are shown in Figure 1. Also the chemical shifts of the *N*-methyl, *N*-methylene, and *O*-methylene proton signals of 0.145 M acetylcholine chloride (measured relative to the *C*-methyl proton signals) for a series of samples containing sodium indole-3-acetate are given in Table I. Since the *C*-methyl proton signal serves as the internal reference, all the *N*-methyl chemical shift changes must reflect changes in the environment of the nitrogen end of the acetylcholine molecule relative to the other end. We take these large upfield shifts to imply

Table I. Effect of Indole Acetate on the NMR Spectrum of Acetylcholine[a]

[arom],[b] M	δ^{N-Me}_{C-Me}, ppm	$\delta^{N-CH_2}_{C-Me^2}$, ppm	$\delta^{O-CH_2}_{C-Me^2}$, ppm
	1.073	1.585	2.425
0.115	0.953	1.426	2.320
0.172	0.903	1.358	2.275
0.229	0.851	1.285	2.228
0.286	0.802	1.221	2.191
0.344	0.751	1.150	2.156
0.401	0.709	1.097	2.213
0.458	0.662	1.035	2.084
0.515	0.615	0.973	2.043
0.573	0.572	0.914	2.011

[a] The chemical shifts of the *N*-methyl, *N*-methylene, and *O*-methylene proton signals of 0.145 M acetylcholine chloride in D_2O solutions containing sodium indole-3-acetate. All chemical shifts are reported (in ppm) relative to the *C*-methyl proton signal of each sample; e.g. δ^{N-Me}_{C-Me} represents the chemical shift difference between the *N*-methyl and *C*-methyl proton signals. [b] [arom] represents the concentration of sodium indole-3-acetate in molarity.

Table II. The Association Constants *K* and Maximum Chemical Shift Changes $\Delta\delta_{max}$ for the Association of Acetylcholine with Aromatic Anions[a]

anion	K,[b] M^{-1}	$\Delta\delta_{max}$, ppm
benzenesulfonate	0.65 ± 0.08	0.31
p-toluenesulfonate	1.13 ± 0.15	0.21
p-ethylbenzenesulfonate	1.8 ± 0.4	0.15
p-isopropylbenzenesulfonate	2.1 ± 0.4[c]	0.14
benzoate	0.32 ± 0.03	0.72
phenylacetate	0.31 ± 0.06	0.87
3-phenylpropionate	0.34 ± 0.04	0.77
p-toluate	0.79 ± 0.04	0.36
o-toluate	0.24 ± 0.07	0.87
3-chlorobenzoate	1.28 ± 0.29	0.10
4-hydroxybenzoate	0.47 ± 0.09	0.50
4-methoxybenzoate	1.16 ± 0.18	0.50
3,4-dimethoxybenzoate	2.8 ± 0.6	0.06
2-naphthoate	0.83 ± 0.03	1.10
phthalate dianion	0.63 ± 0.02	0.30
indole-3-acetate	0.48 ± 0.06	2.70
N-acetyl-DL-tryptophanate	0.41 ± 0.01	1.86
2-nitrobenzoate	*d*	<0.01
4-nitrobenzoate	*d*	<0.01
nicotinate	*d*	<0.01

[a] The *K* and $\Delta\delta_{max}$ values are the average of three determinations in D_2O at 34 °C. [b] The *K* values ± standard deviation. [c] Value obtained for Na salt concentrations ≤0.5 M. [d] These compounds did not give any *N*-methyl proton shift.

that the quaternary ammonium head group of acetylcholine associates preferentially with the aromatic ring of the added anion. When protons are located near the axis of an aromatic ring, their signals are displaced upfield by the aromatic ring current effect.

Plots of the reciprocal of the change in the chemical shift difference between the *N*-methyl and *C*-methyl proton signals, $(\Delta\delta_{obsd})^{-1}$, vs. the reciprocal of the aromatic salt concentration, $[arom]^{-1}$, are generally linear and relate the dependence of the upfield shift on aromatic salt concentration to an apparent association constant *K* for complexation between the acetylcholine cation and the aromatic anion. Nearly identical *K* values are obtained by the Benesi–Hildebrand method[14,15] and the more rigorous computational method of Stockton and Martin[16] and

(12) A. B. Scott and H. V. Tarter, *J. Am. Chem. Soc.*, **65**, 692 (1943).
(13) C. C. J. Culvenor and N. S. Ham, *Chem. Commun.*, 537 (1966).
(14) J. H. Hanna and A. L. Ashbaugh, *J. Phys. Chem.*, **68**, 811 (1964).
(15) I. D. Kuntz and M. D. Johnston, Jr., *J. Am. Chem. Soc.*, **89**, 6008 (1967).

the assumption of 1:1 complex stoichiometry is borne out by the absence of curvature in plots of $(\Delta\delta_{obsd})^{-1}$ vs. $[arom]^{-1}$. By extrapolating to infinite aromatic salt concentration, $[arom]^{-1} = 0$, the maximum upfield shift possible with complete complex formation $\Delta\delta_{max}$ can be estimated. Values of K and $\Delta\delta_{max}$ for the molecular association of acetylcholine cation and various aromatic anions are given in Table II. Although the K values are small they are reproducible; duplicate determinations generally agree within 30% while the difference between K values for different complexes differ by as much as a factor of 10. The $\Delta\delta_{max}$ values are based on observed shift changes $\Delta\delta_{obsd}$ occasionally as large as 0.5 ppm.

Complex Geometry. Because the ring current effect falls off very sharply as a function of the distance from the sixfold axis of an aromatic ring,[17] the large $\Delta\delta_{max}$ values observed in some cases suggest that in these complexes the nitrogen end of the acetylcholine molecule is directly over the ring with an average separation not much greater than the sum of the van der Waals' radii. For example by extrapolating to infinite indole-3-acetate concentration, $\Delta\delta_{max}$ values of 2.7 ± 0.3, 3.6 ± 0.5, and 1.5 ± 0.1 ppm are predicted for the N-methyl and N- and O-methylene signals, respectively. This does not necessarily imply that the N-methylene protons are closer to the ring than any of the N-methyl protons because the smaller N-methyl $\Delta\delta_{max}$ value reflects the possibility that at least one and probably two methyl groups are directed away from the ring on a time average so that the average distance between the N-methyl protons and the indole ring is greater than the distance of closest approach. An exact determination of the time average location of the N-methyl and N-methylene protons would require a detailed knowledge of the lifetime and conformational mobility of the complex and the ring current effect of an indole ring; however the $\Delta\delta_{max}$ values are so large that they compel the conclusion that these protons must be quite close to the ring in the complex. If the ring current effect of indole had the same geometrical requirements as that of benzene, a $\Delta\delta_{max}$ value of 3 ppm would correspond to an average separation of about 2 Å along the axis of the ring.[17,18] A $\Delta\delta_{max}$ value of 2.5 ppm has been reported for the N-methylene protons attached to the thiazolium ion of thiamine in the presence of indole-3-acetate ion and Sable and co-workers[19] estimate that this corresponds to an average separation of 3.7 Å between the carbon and the ring; i.e., the sum of the van der Waals' radii.

None of the NMR signals change shape even with a sixfold excess of sodium indole-3-acetate and the coupling constants determined by the method of Culvenor and Ham[13] from the complicated coupling pattern for the methylene protons are independent of added indole-3-acetate concentration. This indicates that the two methylenes remain in a gauche conformation in the complex. There are many possible orientations of gauche acetylcholine parallel to the ring and we cannot say whether acetylcholine is oriented with the acetyl group directed away from the carboxylate group or if some other orientation is preferred on the time average but it is doubtful such orientations differ much in free energy. On the other hand, an orientation where all three N-methyls are directed toward the ring while the rest of the molecule projects

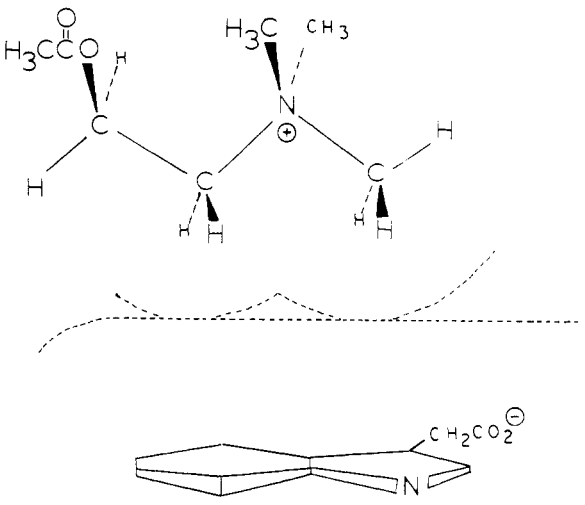

Figure 2. Proposed time-average geometry of the complex between acetylcholine cation and indole-3-acetate anion. The dashed lines represent the van der Waals' radii.

perpendicular to the ring plane is inconsistent with the large NMR shifts observed for the N-methylene protons. These considerations lead us to suggest that the complex between acetylcholine cation and indole-3-acetate anion has an average geometry like that shown in Figure 2. Such a geometry can be shown by space-filling models to involve the most extensive intermolecular contact with maximum van der Waals' and hydrophobic interaction.

Figure 1 indicates that similar sized upfield shifts are caused by sodium benzoate, phenylacetate, and 3-phenylpropionate implying that on a time average the quaternary ammonium group resides over the aromatic ring in all three cases even though equally close interaction with the negatively charged carboxylate group is not possible in each case. With 3-phenylpropionate ion close association between the aromatic ring and the ammonium group prevents interaction between the carboxylate and ammonium groups unless the anion adopts a conformation involving gauche interactions. This result suggests that dispersion forces between ring and ammonium group are at least as strong as electrostatic forces between oppositely charged groups in water.

Complex Stability. The reproducibility of these data is as good as is generally possible with the Benesi–Hildebrand and related methods (for both experimental and theoretical reasons[15,20]) and a cautious discussion of the relationship between anion structure and complex stability (as revealed by K values) is possible. Most importantly we note that most of these K values are too large to be the result of Coulombic forces alone. The observed[21] ion pair association constants for ordinary univalent ions in water are generally less than 0.5 L/mol; i.e. not much larger than that predicted by Fuoss[22] for random encounter between solvated ions. Also we find that phthalate dianion binds acetylcholine no better than many hydrophobic univalent anions, again suggesting that hydrophobic forces are at least as important as electrostatic forces in stabilizing these complexes.

The relationship between K and anion structure is complex, depending on the nature of the anionic group and its placement relative to any other substitute. There is no simple relationship between the K values and Hammett

(16) G. W. Stockton and J. S. Martin, *J. Am. Chem. Soc.*, **94**, 6921 (1972).
(17) C. E. Johnson and F. A. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958).
(18) See M. E. Kenney et al., *J. Am. Chem. Soc.*, **91**, 5210 (1969), concerning the ring current effect of some heterocyclic systems.
(19) J. J. Mieyal, J. Suchy, J. E. Biaglow, and H. Z. Sable, *J. Biol. Chem.*, **244**, 4063 (1969).
(20) R. Foster, "Organic Charge-Transfer Complexes", Academic Press, New York, 1969, Chapter 6.
(21) R. L. Kay, *J. Am. Chem. Soc.*, **82**, 2099 (1960).
(22) R. M. Fuoss and K-L. Hsia, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 1550 (1967).

Table III. The Association Constants K and Maximum Chemical Shift Changes $\Delta\delta_{max}$ for the Association of Acetylcholine with Phenols[a]

phenol	K,[b] M^{-1}	$\Delta\delta_{max}$, ppm
phenol	0.12 ± 0.04	1.69
pyrocatechol	0.21 ± 0.02	1.06
pyrogallol	0.27 ± 0.02	0.82
resorcinol	0.17 ± 0.01	1.10
hydroquinone	0.25 ± 0.14	0.97

[a] The K and $\Delta\delta_{max}$ values are the average of three determinations in D_2O at 34 °C. [b] The K values ± standard deviation.

σ or σ^* values. For para-substituted benzoate ions there is a rough correlation between K and anion molecular weight but not with any parameter attributable solely to hydrophobicity such as the π values of Hansch.[23] The variety of anions examined was wide enough to suggest some tendencies.

(1) The structure of the anionic group is important. Arenesulfonates bind acetylcholine better (with larger K values) than structurally similar carboxylate ions.

(2) Increasing the bulk of p-alkyl substituents increases binding.

(3) Large flat aromatic anions like 2-naphthoate and indole-3-acetate give respectable K values along with large $\Delta\delta_{max}$ values but, with smaller aromatic anions, especially ones with bulky substituents, large K values are generally associated with small $\Delta\delta_{max}$ values and vice versa.

Although it is tempting to rationalize each of these observations in terms of a single molecular interaction, the limited structural variation within the series compels us to be more cautious. Effects due to small differences in structure are liable to be small because molecular associations depend upon the sum of a large number of weak physical interactions. The stability and geometry result from a compromise between interrelated electrostatic, van der Waals', and hydrophobic contributions to both enthalpy and entropy and the magnitude of each contribution is not, at present, quantitatively definable. Our results are consistent with the limited conclusion that van der Waals' forces play as large a role as hydrophobic factors[24] and electrostatic forces in maintaining these complexes. The high upfield shifts of the $N-CH_2$ and $N-CH_3$ signals demonstrate that these protons experience the anisotropy effect of the aromatic ring, suggesting a van der Waals' interaction between the polarizable aromatic ring and the partially positive[25] N-methyl protons. However this may not be the only significant interaction. Although π electrons have an exalted polarizability, a molecule containing a π system is no more polarizable than a derived molecule obtained by hydrogenation; the additional H atoms are also polarizable.[26] We observed high K values with bulky p-alkyl substituents and the low $\Delta\delta_{max}$ values in these cases imply that the $N-CH_3$ group may be associated, in part, with the substituent.

Acetylcholine forms weak association complexes with phenols and the data in Table III indicate that association increases with increased hydroxyl group substitution, but the substitution pattern is also important. A similar behavior has been reported for the changes in the NMR spectrum of tetramethylammonium bromide with various phenols.[9] Association with neutral aromatic compounds must involve only van der Waals' and hydrophobic forces, and our observation that association is enhanced by increased hydroxy substitution strongly suggests that the former type of force is more important in these complexes; i.e., the association constant is larger for the more polarizable but less hydrophobic compounds.

Table IV. Effect of Urea on the Association of Acetylcholine with p-Toluate Ion[a]

[urea], M	K, M^{-1}	$\Delta\delta_{max}$, ppm
0.00	0.79 ± 0.04[b]	0.36
1.52	0.88	0.31
3.32	1.27	0.20

[a] The association constant K and maximum chemical shift change $\Delta\delta_{max}$ values are from single determinations in D_2O at 34 °C except where noted. [b] The average of three determinations ± standard deviation.

Effect of Electrolytes and Urea. In this discussion we have assumed the Na^+ and Cl^- counterions play only a limited role in complex formation since the complex is organized to maximize van der Waals' and hydrophobic interactions. This assumption is also supported by our observation that Benesi–Hildebrand plots are linear even for experiments where the ionic strength changes by a factor of 10. There is no difference in the association constants measured for chloride, bromide, and iodide salts of acetylcholine with sodium toluenesulfonate. With sodium toluate, the value of K is decreased by added NaCl. For example with 1.5 M NaCl the association constant and $\Delta\delta_{max}$ value for the complex between acetylcholine chloride and sodium toluate are 0.40 M^{-1} and 0.51 ppm, respectively. We observed that the association constant for toluate complexation is nearly zero if NaCl is in large excess and the use of enough NaCl to maintain a constant ionic strength reduces the differences between the K values of different complexes. At high electrolyte concentrations (1 M), maintaining a constant ionic strength will not keep the complexing ion activities constant unless the added ions are very similar to the complexing ions,[27] in which case competitive complexes may also form. Added NaCl causes negligible change in the NMR spectrum of pure acetylcholine in D_2O. Sable and co-workers[19] reported that the complex between thiamine and indole-3-acetate is sensitive to ionic strength; however this sensitivity is less when the aromatic anion is in large excess,[19] as in our experiments.

Added urea causes a small increase in the association constant for the complex between acetylcholine chloride and sodium p-toluate; see Table IV. Since urea reduces water structure[28] the increased association observed with increased urea concentration rules out water structure forces as the predominate factor in these complexes. Why the K values increase with increased urea concentration is not clear.

Acetylcholine Hydrolysis Kinetics. The rate of specific base-catalyzed hydrolysis of acetylcholine is reduced by electrolytes like NaCl and several studies have shown that the second-order rate constants for hydrolysis can be related to the ionic strength μ by eq 1.[29,30]

$$\log k_{II,obsd} = \log k^{\circ}_{II} - \frac{1.04\mu^{1/2}}{1 + \mu^{1/2}} \quad (1)$$

(23) C. Hansch, *Acc. Chem. Res.*, **2**, 232 (1969).

(24) The term hydrophobic forces refers to those factors promoting hydrophobic association that stem from the strong cohesive energy of hydrogen-bonded water molecules which forces less polar solute molecules together even if there is little net attractive force between them; cf. G. Nemethy and H. A. Scheraga, *J. Chem. Phys.*, **36**, 3401 (1962).

(25) B. Pullman, P. H. Courriere, and J. L. Coubeils, *Mol. Pharmacol.*, **7**, 397 (1971).

(26) T. C. Bruice in "The Enzymes", Vol. II, student ed, P. D. Boyer, Ed., Academic Press, New York, 1970, pp 258–259.

(27) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions", 2nd ed (revised), Butterworths, London, 1968 pp 491–509.

(28) E. G. Finer, F. Franks, and M. J. Tait, *J. Am. Chem. Soc.*, **94**, 4424 (1972).

Table V. The Effect of Sodium Benzenesulfonate on the Rate of Acetylcholine Saponification[a]

[NaBs],[b] M	μ[c]	$10^4 k_{obsd}$,[d] s^{-1}	k°_{II},[d] $M^{-1} s^{-1}$
	0.06	3.33	2.78
0.05	0.11	2.67	2.52
0.10	0.16	2.62	2.70
0.15	0.21	1.96	2.16
0.25	0.31	1.78	2.17
0.50	0.56	1.53	2.19
0.70	0.76	1.23	1.91
0.80	0.86	1.25	1.98
0.90	0.96	0.92	1.54
1.00	1.01	0.93	1.55
0.05	1.01	2.31	3.90
0.08	1.01	2.30	3.80
0.13	1.01	2.22	3.68
0.25	1.01	2.02	3.36

[a] In 0.05 M NH_3-NH_4Cl buffer (pH 10.28) at 32 °C. [b] The concentration of added sodium benzenesulfonate. [c] Ionic strength. [d] k_{obsd} and k°_{II} denote the first- and second-order rate constants in s^{-1} and $M^{-1} s^{-1}$, respectively. The latter rate constant was corrected for nonspecific electrolyte effects.

We have found that high concentrations of sodium arenesulfonates retard the rate of hydroxide ion attack on the carbonyl group of acetylcholine and that this inhibitory effect is larger than that caused by more hydrophilic salts like NaCl. The effect of sodium benzenesulfonate on the rate of specific base-catalyzed hydrolysis of 0.008 M acetylcholine at 32 °C is shown in Table V. At high concentrations of benzenesulfonate ion the first- and second-order rate constants were decreased even when the ionic strength was maintained at 1.01 M with added NaCl. The second-order rate constants reported in Table V were obtained by dividing the observed first-order constants by the stoichiometric hydroxide concentration (1.9×10^{-4} M, calculated from the measured pH) and then applying the corrections for μ given in eq 1. Although eq 1 is rigorously applicable only at low ionic strength ($\mu < 0.2$),[29] it offers a reasonable estimate of nonspecific electrolyte effects on the second-order rate constants. The fact that the corrected k°_{II} value decreases with increased benzenesulfonate ion concentration strongly suggests that the neutral complex formed from acetylcholine cation and the aromatic anion reacts more slowly with hydroxide ion than free acetylcholine. The initial state is stabilized by complex formation while the transition state for hydroxide ion attack on the neutral complex does not benefit from the charge neutralization that takes place with hydroxide ion attack on uncomplexed acetylcholine cation.

The inhibitory effect of arenesulfonate ions increases with their increased tendency to associate with acetylcholine cation. Although this increase is small it is larger than the range in experimental error. The calculated second-order rate constants for the hydrolysis of acetylcholine chloride with sodium benzenesulfonate, *p*-toluenesulfonate, and *p*-isopropylbenzenesulfonate are given in Figure 3. The rate constants were obtained by dividing the first-order rate constants by the stoichiometric hydroxide concentration and correcting for nonspecific electrolyte effects.[29] All the rate constants were measured at low buffer concentrations where general base catalysis terms are negligible. A plot of the logarithm of the observed first-order rate constant k_{obsd} vs. stoichiometric hydroxide concentration was linear with a slope of 1. The

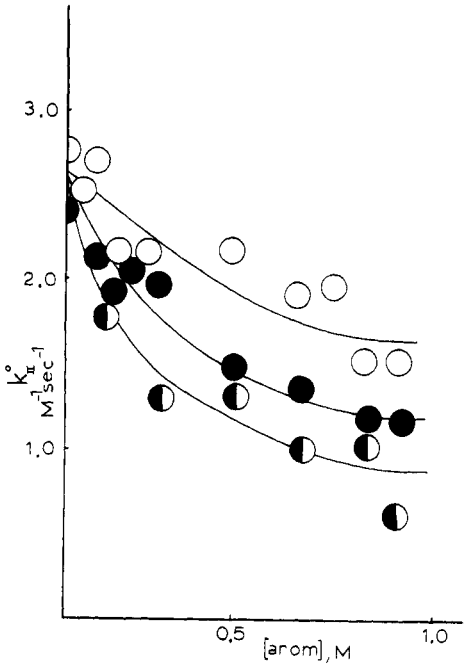

Figure 3. The second-order rate constants ($M^{-1} s^{-1}$) for the hydrolysis of 0.008 M acetylcholine in NH_3–NH_4Cl buffer at 32 °C as a function of the concentration of added sodium arenesulfonates. The symbol ○ represents the rate constants determined at pH 10.28 with added sodium benzenesulfonate. The symbols ● and ◐ represent the rate constants for pH 10.50 with added sodium *p*-toluenesulfonate and *p*-isopropylbenzenesulfonate respectively. All rate constants have been corrected for nonspecific electrolyte effects.

k°_{II} values calculated for pH 10.28 and 10.50 differed (see Figure 3), but the average second-order rate constant observed in the absence of sodium arenesulfonates is 2.6 $M^{-1} s^{-1}$ at 32°, in reasonable agreement with the value of 2.86 $M^{-1} s^{-1}$ reported for a comparable ionic strength (μ = 0.06) at 35.5 °C.[29]

Experimental Section

Materials. Deuterium oxide (Stohler isotope or Mallinckrodt) was of 99.8% isotopic purity and afforded NMR spectra free of extraneous peaks. Purchased samples of acetylcholine chloride (Aldrich), bromide, and iodide (J.T. Baker) were of good quality and were used without purification. Sodium benzoate (J.T. Baker) was purchased and the sodium salts of all other carboxylic acids were made by neutralization with an exact equivalent of sodium hydroxide in aqueous ethanol, followed by evaporation of the solvent. The resulting solids were recrystallized usually from aqueous ethanol. The sodium salts of arenesulfonic acids were made by neutralizing the sulfonation products of the appropriate aromatic hydrocarbon. All salts afforded NMR spectra consistent with the assigned structure with no extraneous peaks. All salts were free from excess NaOH or acid, as indicated by the absence of any acetylcholine hydrolysis over several hours.

NMR Study. Changes in the chemical shift difference between the *N*-methyl and *C*-methyl proton signals of acetylcholine in D_2O were determined as a function of added aromatic salt concentration on a Jeol-JNM-MN-100 NMR spectrometer. Each chemical shift difference was based on four separate measurements of the spectrum of each sample (at 108-Hz sweep width) with the mean deviation never greater than 0.01 ppm. All samples were thermally equilibrated to the probe for 15–20 min prior to the first measurement and all spectra for any one series of samples were recorded as close together in time as possible to avoid temperature fluctuations. During each series the probe temperature was determined with a Varian standard methanol sample. The average probe temperature was 34 ± 1 °C.

The association constants reported herein were determined (in triplicate) from the change in the *N*-methyl proton chemical shift, measured relative to the *C*-methyl proton signal. The triplicate

(29) M. R. Wright, *J. Chem. Soc. B*, 545 (1968).
(30) G. Aksnes and J. E. Prue, *J. Chem. Soc.*, 103 (1959).

determinations involved three acetylcholine concentrations ranging from 0.004 to 0.08 M, each with a large excess (0.1–1.1 M) of aromatic salt. Usually each series involved ten samples. The association constants were independent of acetylcholine concentration and small changes in ionic strength.

The change in the chemical shift difference between the *N*-methyl and *C*-methyl signals, as defined by

$$\Delta\delta_{obsd} = (\delta_{C\text{-}Me}{}^{N\text{-}Me})_0 - (\delta_{C\text{-}Me}{}^{N\text{-}M})_{arom} \quad (2)$$

is related to an apparent association equilibrium constant K by an expression of the Benesi–Hildebrand type.[14,15]

$$(\Delta\delta_{obsd})^{-1} = (\Delta\delta_{max})^{-1} + (\Delta\delta_{max}K[arom])^{-1} \quad (3)$$

The symbols $(\delta_{C\text{-}Me}{}^{N\text{-}Me})_{arom}$ and $(\delta_{C\text{-}Me}{}^{N\text{-}Me})_0$ represent the chemical shift differences between the *N*-methyl and *C*-methyl signals (as positive numbers) in the presence and absence of added aromatic compound.[9] The term [arom] denotes the concentration of added aromatic and $(\Delta\delta_{max})^{-1}$ is the y-axis intercept of a plot of $(\Delta\delta_{obsd})^{-1}$ vs. [arom]. The value of K can be determined as the ratio of intercept to slope of such a plot.

Strictly speaking eq 3 is valid only if the concentration of the aromatic compound is much larger than that of acetylcholine.[14,15] Although our experimental conditions satisfy this requirement we also calculated K values by the iterative method of Stockton and Martin,[16] which is valid even when the two concentrations are comparable. Usually the association constants obtained by both methods agreed within 10%. The data given in Table II were calculated by the latter method using a least-squares computer analysis.

The NMR samples were prepared in NMR tubes by combining various proportions of freshly prepared stock solutions. High accuracy automatic pipets were used and all concentrations were certain within 5% as confirmed by final solution volumes.

Similar techniques were used in the other NMR spectral studies reported here.

Acetylcholine Hydrolysis Kinetics. The rate of hydrolysis of 0.008 M acetylcholine chloride in 0.05 M NH_3–NH_4Cl buffer (pH 10.3–10.5) with added sodium arenesulfonates was studied by the ferric hydroxamate method of Hestrin.[31] The reactions were initiated by adding an aliquot of acetylcholine chloride solution to a buffered sodium arenesulfonate solution in a volumetric flask suspended in a thermostated water bath (32 ± 0.5 °C). Aliquots (1 mL) were removed at intervals, mixed with an equal volume of freshly prepared solution containing the stoichiometric equivalent of 4 N NaOH and 2 M NH_2OH·HCl, and after 15 min diluted to 4 mL with 0.37 M ferric chloride in 0.1 N HCl. The concentration of unhydrolyzed acetylcholine remaining in the volumetric flask at time t (when the aliquot was taken) was estimated by the absorbance A_t at 540 nm measured on a Beckman Model DB spectrophotometer. The absorbance A_∞ for aliquots removed after complete hydrolysis was always near zero. Rate constants were determined from the slopes of plots of log $(A_t - A_\infty)$ vs. t (s); such plots were defined by more than 20 points and were nicely linear for more than three half-lives. The second-order rate constants were determined by dividing the first-order rate constants by the hydroxide concentration, which was estimated from the pH. All pH measurements were made with a Beckman Zeromatic SS-3 pH meter and a Curtin combination electrode (calomel reference). All rates run with the same arenesulfonate corresponded to the same pH.

Acknowledgment. Support of this work by the Research Corporation, the Petroleum Research Fund, administered by the American Chemical Society, and Tulane University is gratefully acknowledged. The helpful insight and encouragement of Dr. C. A. Bunton is also acknowledged.

Registry No. Acetylcholine chloride, 60-31-1; acetylcholine bromide, 66-23-9; acetylcholine iodide, 2260-50-6; sodium indole-3-acetate, 6505-45-9; sodium benzenesulfonate, 515-42-4; sodium *p*-toluenesulfonate, 657-84-1; sodium *p*-ethylbenzenesulfonate, 14995-38-1; sodium *p*-isopropylbenzenesulfonate, 15763-76-5; sodium benzoate, 532-32-1; sodium phenylacetate, 114-70-5; sodium 3-phenylpropionate, 114-84-1; sodium *p*-toluate, 17264-54-9; sodium *o*-toluate, 17264-71-0; sodium 3-chlorobenzoate, 17264-88-9; sodium 4-hydroxybenzoate, 114-63-6; sodium 4-methoxybenzoate, 536-45-8; sodium 3,4-dimethoxybenzoate, 34535-88-1; sodium 2-naphthoate, 17273-79-9; disodium phthalate, 15968-01-1; sodium *N*-acetyl-DL-tryptophanate, 62307-74-8; sodium 2-nitrobenzoate, 17264-82-3; sodium 4-nitrobenzoate, 3847-57-2; sodium nicotinate, 54-86-4; phenol, 108-95-2; pyrocatechol, 120-80-9; pyrogallol, 87-66-1; resorcinol, 108-46-3; hydroquinone, 123-31-9.

(31) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

Spectroscopic Studies of Hydrophobic Association. Merocyanine Dyes in Cationic and Anionic Micelles[1]

M. J. Minch* and S. Sadiq Shah

Department of Chemistry, University of the Pacific, Stockton, California 95211

Received January 3, 1979

The effect of the micelle forming surfactants sodium dodecyl sulfate and hexadecyltrimethylammonium bromide on the visible spectra of merocyanine dyes, 1-alkyl-4-[(oxocyclohexadienylidene)ethylidene]-1,4-dihydropyridines, with various length 1-alkyl chains is reported. In all cases the dye spectra were shifted to the red when incorporated into micelles and the magnitude of this shift increases with more hydrophobic dyes. The dependency of the spectral shift on dye chain length also depended on the nature of the surfactant head group.

The solubility of many sparingly soluble solutes in water can be increased by the addition of a surfactant which forms micelles that incorporate the solute. Lawrence[2] first suggested in 1937 that the site of incorporation of solubilized molecules depends on their relative hydrophobic and hydrophilic tendencies but, even after four decades of work, the details of micellar structure and the mechanism of solubilization remain poorly understood and there is still uncertainty over the average site of incorporation. Water penetrates into a micelle[3,4] so that there is a con-

(1) Support of this work by the donors of the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged.
(2) A. S. C. Lawrence, *Trans. Faraday Soc.*, **33**, 815 (1937).
(3) N. Muller and R. H. Birkhahn, *J. Phys. Chem.*, **71**, 957 (1967).
(4) F. M. Menger, J. M. Jerkunica, and J. C. Johnston, *J. Am. Chem. Soc.*, **100**, 4676 (1978).

0022-3263/79/1944-3252$01.00/0