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Binding of Acetylcholine to a Cyclophane Host. Influence of Water and Reliability of NMR Measurements of Small Association Constants

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A detailed analysis of the association of acetylcholine (ACh) and tetramethylammonium (TMA) picrates with the cyclophane tetraester 1b in $CDCl_3$ at $T = 296$ K was undertaken to assess the reliability of measurements of small association constants by NMR shift titrations. Results showed that, with simple expedients and careful treatment of data, binding constants of 13 and 29 M^{-1} for ACh and TMA respectively can be measured through standard nonlinear regression procedures with ±1% accuracy. Systematic errors caused by the interference of water at low host concentration were shown to be the major source of inaccuracy. The plot of residuals of experimental data points from the regression curve was used to monitor systematic deviations while the shift of the water signal was used as a probe of processes occurring in solution.

Keywords: Acetylcholine, cyclophanes, NMR Titrations, binding constants, quaternary ammonium, host-guest complexes

INTRODUCTION

NMR techniques have increasingly been used in recent years for the assessment of receptor binding properties because of the wealth of information they can provide [1,2]. Among these techniques, NMR shift titrations are the most widespread for the measurement of binding constants of fast exchanging complexes on the NMR time scale. However, the narrow range of K_a values accessible, commonly assumed to be $10 \div 10^4\ M^{-1}$ for bimolecular associations, and the somewhat limited precision achieved have been regarded as intrinsic limitations of the NMR technique. While simultaneous use of several signal shifts and a more sophisticated mathematical treatment of data have been reported to significantly improve the precision of nonlinear regression procedures [3], limitations originating from various sources, such as chemical phenomena, may still hold and be particularly severe in the investigation of weak interactions, in which binding constant values lie at the lower end of the range.

In the course of an investigation to quantitatively assess the entity of the primary interaction

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that is established between quaternary ammonium cations and aromatic rings, known as the cation-π interaction [4], we found that small but appreciable associations could be detected in $CDCl_3$ with "adaptive" cyclophane hosts, in the absence of ion-pairing and hydrophobic contributions and even of a preorganized structure of the host [5]. Given the small K_a values observed, an assessment of the reliability of measurements was required for a meaningful analysis of the interaction. We report here the results of a detailed analysis of the association of acetylcholine and tetramethylammonium picrates with a cyclophane tetraester, showing not only that using simple skills in the titration procedure the obtainable precision can be higher than believed, but also that accurate measurements and careful treatment of data may reveal chemical phenomena occurring beside complexation and may disclose the way to avoid interferences, providing reliable K_a values even in the lower limit of the accessible range.

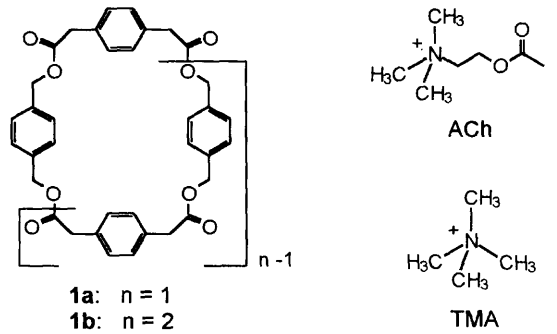

CHART 1

RESULTS AND DISCUSSION

A preliminary screening by ^1H NMR titrations in $CDCl_3$ at $T = 296$ K revealed that acetylcholine (ACh) and tetramethylammonium (TMA) picrates (P) were bound to cyclophane **1b** with association constants K_a of 20 and 38 M^{-1} respectively [5]. NMR spectra were in agreement with fast exchanging complexation, showing time-averaged signals for the free and the complexed species. According to standard methods [1], stock solutions of the guest (G) were titrated with increasing amounts of host (H), following the shift of the N-methyl signal (and of other signals when present) of the guest. Experimental points correctly fitted the equation of the standard binding isotherm for a 1 : 1 association (Eq. (1)), giving the corresponding association constants (K_a), the chemical shift of the free guest (δ_0) and the limiting upfield shift values of the fully saturated guest relative to the free guest ($\Delta\delta_\infty$) through standard nonlinear least-squares regression procedures.

$$\Delta\delta = \delta_{obs} - \delta_0 = K_a[H]\Delta\delta_\infty/(1 + K_a[H]) \quad (1)$$

The large upfield $\Delta\delta_\infty$ values obtained for complexed ACh and TMA, -1.27 and -1.25 respectively, supported beyond doubt the inclusion of the cation into a cavity, where it experiences a strong shielding by the aromatic ring current. In contrast cyclophane **1a**, incapable of binding because of its much too narrow cavity, gave for the guest a negligible shift up to $[H] = 0.1$ M, thus serving as a blank test for measurements.

A representative titration curve of a 0.48 mM solution of AChP with **1b** following the shift of the N-methyl signal, extended to sample data points at low concentration of host, is reported in the plot of Figure 1(A). Although the fit appears to be satisfactory, a closer inspection reveals a systematic error of experimental points with respect to the calculated curve, that is more evident at the extremes. Testing the goodness of fit is recommended in least-squares regression, in order to check the adequacy of the adopted model; a plot of the scatter of the experimental points from the regression curve (residuals) *vs.* titrant concentration (the independent variable) is often used as a general test [6]. Obvious trends other than a random scattering of points within a horizontal band about the mean value indicate poor fit. The residuals plot of the above titration

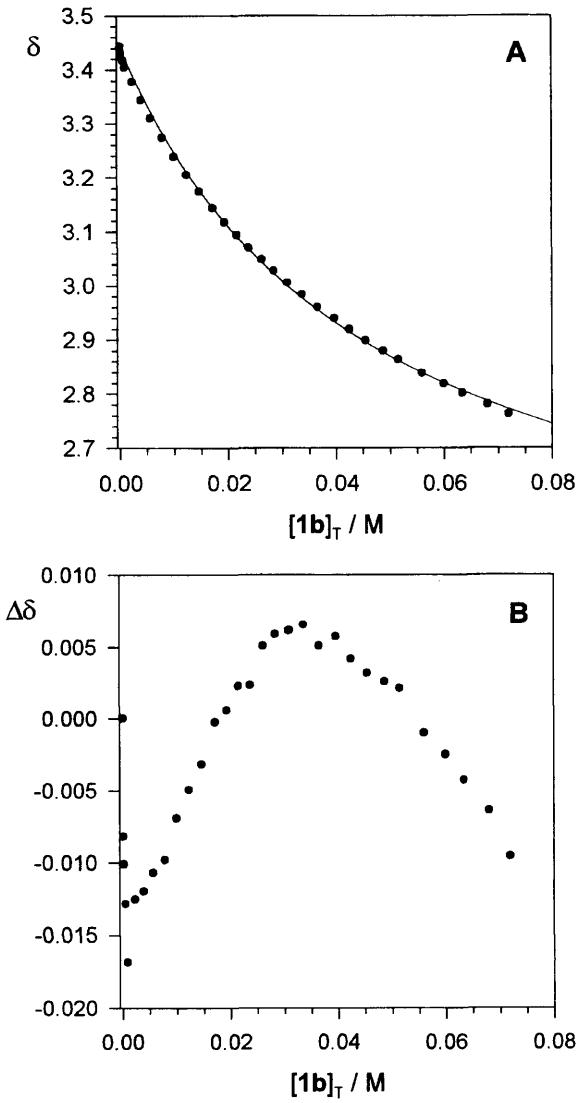

FIGURE 1 (A) ^{1}H NMR titration of 0.48 mM AChP with **1b** in $CDCl_3$ at $T = 296$ K, monitoring the N-Me signal. Symbols are experimental data points; line is best fit curve calculated from Eq. (1) by nonlinear regression. (B) Plot of the scatter of the experimental points from the regression curve (residuals) *vs.* titrant concentration relative to the titration of (A).

(Fig. 1(B)) clearly shows a bell-shaped systematic trend featuring a steep jump of the initial data points, that was consistently reproducible in duplicate experiments. The titration procedure was carefully optimized [7], but increased precision just enhanced the definition of the systematic deviation of data points from the regression curve. Analogous behavior was observed in titrations of TMAP, that showed an even more pronounced deviation at the extremes, with residuals spanning a $\Delta\delta$ range of 0.055 ppm. Occurrence of trivial artifacts or dependence of the phenomenon on the cation could therefore be ruled out.

A source of interference that must be taken into account is the possible aggregation of species in solution. Self-association phenomena have frequently been observed with cyclophane hosts, clearly revealed by shift of NMR signals with concentration [8], and are certainly possible for quaternary ammonium salts in chloroform [9]. A plot of δ values of all signals of **1b** *vs.* concentration, obtained by simple dilution of the host or in the presence of ACh in a titration, just revealed insignificant shifts up to 0.075 M ($\Delta\delta = +0.006$ ppm for the CH_2O signal), so that self-association of the host was excluded. Aggregation phenomena involving the quaternary ammonium cation could also be ruled out in the concentration range used in this work, typically below 1 mM, as shown by the negligible shift exhibited by the *N*-methyl signal of AChP in the range 0.06 – 0.6 mM ($\Delta\delta = -0.002$ ppm). Two independent experiments confirmed the absence of aggregation phenomena for AChP: (a) the osmometric molecular weight measured in $CDCl_3$ at two different concentrations (0.66 and 1.40 mM) corresponded to that of the monomeric ion-pair with excellent agreement (± 5%); (b) positive ESI-MS spectra of solutions of AChP in $CDCl_3$ showed the peak of the ACh cation only, with no evidence of triplets or higher cationic aggregates. In conclusion, no experimental evidence was found to ascribe the systematic deviation of data to aggregation phenomena.

The type of trend exhibited by the residuals plot in Figure 1(B) suggested that deviations may be caused by attempts of fitting the second-order equation for a bimolecular complex formation to experimental data responding to a higher-order equation. Therefore, although as-

sociation equilibria different from 1:1 were unlikely, a check of the association model was mandatory. Due to low solubility of the ammonium salts and to low K_a values, the continuous variation method for stoichiometry determination (Job's plot) [10] was unfeasible. Data were tentatively fitted with the equations for a 1:2 and a 2:1 complex, but neither one improved the fit, giving R values never below 0.5. A different source of discrepancy might be the error on the first data point. Since the standard binding isotherm is a 3-parameter function, *i.e.*, the binding constant and both the chemical shifts of the free and the complexed guest, in a nonlinear regression procedure the number of data points to be sampled can be conveniently reduced and the regression simplified if one parameter can be kept invariant. Such is the case, since the δ_0 value of the free guest can be measured from the stock solution before adding the host. However, errors on this parameter will affect the fit more heavily than errors on other points, because the curve will be forced through this initial point. Therefore, δ_0 was set as a variable parameter in the regression and the first data point was excluded from the fit. As a result, only a minor improvement was obtained, that did not eliminate the systematic deviation. On the contrary, a markedly better fit with the 1:1 binding isotherm, showing well-behaved residuals plots, could be obtained selecting data from narrower host concentration regions. Such an expedient, obviously unacceptable for the actual measure of the association constant, revealed a steep variation of curvature at host concentration in the order of that of the guest and an increase of K_a of more than one order of magnitude along the titration, suggesting the occurrence of chemical interactions rather than experimental artifacts.

The origin of deviations could be understood examining the signal of water, that was used as an independent probe of occurring phenomena [11]. Water's and cation's shifts were strictly related: (a) the water signal exhibited a downfield shift with host's concentration, whose trend closely resembled the titration curve, including variations of curvature (Fig. 2) (b) the larger the water content of the solution, the greater the deviation of guest's titration data points from the regression curve and, correspondingly, the greater the effect on the shift of the water signal. Deviation was prominent in the region where concentration of the host was smaller than that of water, getting steeper with increasing H_2O/H ratio. To confirm such a water dependence, the titration was run in $CDCl_3$ saturated with water. With respect to the titration run in the untreated solvent, deviation was steeper for host concentration below 2 mM, but levelled off at 10 mM. Systematic deviations could thus unequivocally be ascribed to the presence of adventitious water.

In order to ascertain the role played by water, a dilution experiment of host **1b** was run under conditions identical to the above titration in $CDCl_3$ saturated with water. Allowing for a small offset due to a slightly different water content (dilution, 60 mM; titration 55 mM), two perfectly superimposable plots of δ of the water signal were obtained (Fig. 3; ○, dilution; •,

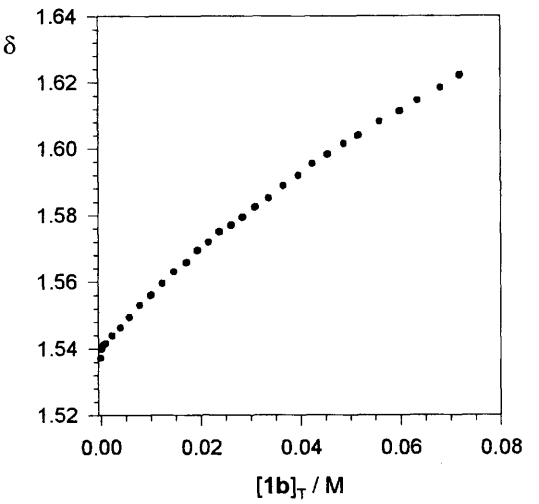

FIGURE 2 Dependence of the ^1H NMR shift of the water signal on the host concentration in the titration of AChP with **1b** of Figure 1(A).

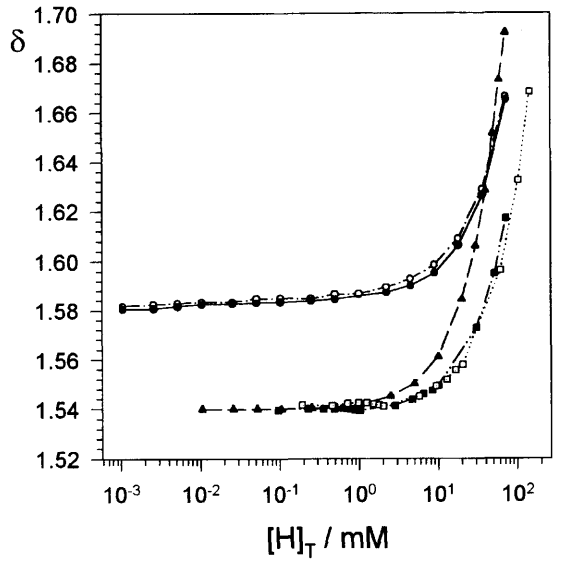

FIGURE 3 Chemical shifts of the ^{1}H NMR signal of water in $CDCl_3$ at $T = 296$ K in titrations of 0.8–0.9 mM AChP with hosts (H) **1a** at $[H_2O] = 5$ mM (□), **1b** at $[H_2O] = 5$ mM (■), **1b** at constant H/H_2O ratio and 5 mM < $[H_2O]$ < 78 mM (▲), **1b** at $[H_2O] = 55$ mM (•), and in dilution of **1b** at $[H_2O] = 60$ mM (○).

titration), indicating that the interference did not depend on the guest. Similarly, titration of AChP with **1a** and **1b** under identical conditions at low water concentration (5 mM) resulted in superimposable plots (Fig. 3; □, **1a**; ■, **1b**), despite the fact that **1a** is incapable of binding [12]. Results led us to conclude that deviations were caused by hydration of the cyclophane [13]. Indeed, a solution of host in $CDCl_3$ dissolved a markedly larger amount of water than neat $CDCl_3$; for example, a 73 mM solution of **1b** dissolved 78 mM of H_2O, while water concentrations not larger than 35–40 mM could be achieved in the neat solvent. Binding of water molecules to the cyclophane, most likely to ester carbonyls rather than to aromatics as inferred from the downfield shift, not responding to a defined stoichiometry, apparently induced a shift on the cation superimposed to that due to complexation.

The 0.04 ppm offset, that can be noted in Figure 3 between the described two sets of experiments, is due to the different water content. It is evident that shifts can be therefore artificially influenced by changing the concentration of water in the course of the measurement. Running a titration at constant H_2O/H ratio, with water concentration varying from 5 to 78 mM, resulted in a steeper plot of the water shift crossing the two previous sets (Fig. 3; ▲). Correspondingly, cation's curve started downfield and ended upfield respect to the titration at constant $[H_2O] = 55$ mM, with a crossing point at $[H] \cong 6$ mM, although lacking steep variations of curvature because of the constant H_2O/H ratio.

The described experiments demonstrated that the curvature of the plot, and consequently the association constant, can be artificially manipulated by the very water content of the solution. A reliable measure of the association constant would thus require titrations in anhydrous medium. Since variable amounts of water are virtually unavoidable during a titration and since saturating the solvent with water to buffer variations introduces larger distortions in the low concentration range, accurate measurements appeared troublesome. Fortunately, it was found that titration curves were essentially unaffected by water at high concentration of host, so that selection of the appropriate concentration range, compatible with the requirements of exploring a wide portion of the binding isotherm and of avoiding non-ideality phenomena, gave reliable K_a values. The residuals trend was taken as the best operative criterion of reliability of data, accepting all points that gave a random scatter, but discarding those for which systematic trends would appear. Accurate measurements of K_a were obtained using host solutions up to 5–6% w/v (up to 0.1 M), for which self-association phenomena were never observed. Under these conditions, titration of AChP with **1b** gave $K_a = 13.0 \pm 0.1\ M^{-1}$ (±1%), a value averaged over 8 independent measurements, with standard deviation $\sigma = 0.4$; the corresponding $\Delta\delta_\infty$ obtained for the complex at saturation was -1.266 ± 0.013 ppm (±1%), with $\sigma = 0.038$. Values obtained for TMAP were

$K_a = 29.0 \pm 0.4\,M^{-1}$ ($\pm 1\%$), $\sigma = 0.8$; $\Delta\delta_\infty = -1.477 \pm 0.008$ ppm ($\pm 0.5\%$), with $\sigma = 0.015$. All titration curves were sampled to an extent of binding larger than 50% for AChP and 70% for TMAP. Comparing the K_a values to those obtained in the preliminary screening, it is easily noted that association constants are significantly affected by the interference of water.

The interaction of water with the guest also deserves a comment. The described procedure requires a three-parameter nonlinear fit of data. For a sufficiently large concentration range and points/parameters ratio, the curve is well defined and the standard error of the fit can be considerably small. Under these conditions δ_0, *i.e.*, the chemical shift of the free guest, is accurately obtained from the fit and can be compared with the experimental value measured on the stock solution of the salt. We found that the calculated value was consistently upfield from the experimental value, and that both were affected by the water content of the solution. The δ value of the *N*-methyl signal of AChP in $CDCl_3$ was experimentally found to linearly depend on the water concentration, although with low sensitivity ($\Delta\delta_{max} = -0.02$; slope $= -0.26$ ppm L mol^{-1}). *Vice versa*, the downfield shift of the water signal showed a strong, linear dependence on the AChP concentration (slope $= +12.4$ ppm L mol^{-1}). For this reason, titrations were run at constant cation concentration, diluting the host with the stock solution of the guest. Given the modest and linear influence of water on the δ value of the guest, the titration curve was corrected for this contribution by subtracting the δ_0 measured on the stock solution from the observed δ value, *i.e.*, by normalizing the curve to a constant water contribution to the cation shift, and neglecting the effect of fluctuations of the water content during titration. $\Delta\delta$ plots of two titrations of AChP with **1b**, run (a) at $[H_2O] = 6$ mM (•) and (b) using $CDCl_3$ saturated with water (○), are reported in Figure 4. Divergence of plots in the low concentration region shows that the effect of water on the host (and hence on the complex) adds *nonlinearly* to that on the cation, but the effect vanishes with increasing H/H_2O ratio. For a sufficiently large ratio, the offset between curves is constant (inset of Fig. 4), the association constant is unaffected, but the calculated δ_0 includes the upfield contribution of water on both the host and the guest. The apparent enhancement of the binding constant is thus the consequence of the *nonlinear* component of the guest upfield-shift caused by the interaction of water with the host in the region of low H/H_2O ratio.

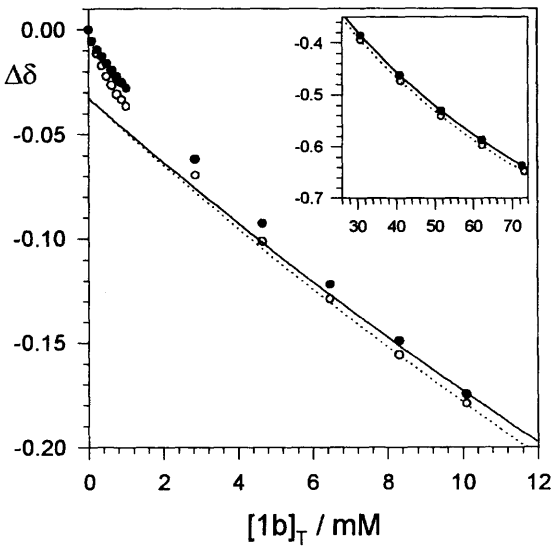

FIGURE 4 ^{1}H NMR titrations of 0.95 mM AChP with host **1b** at $T = 296$ K in $CDCl_3$: (a) at $[H_2O] = 6$ mM (•) and (b) using $CDCl_3$ saturated with water (○). Symbols are experimental data points; lines are best fit curves calculated from Eq. (1) by nonlinear regression. Chemical shift values ($\Delta\delta$) of the N-Me signal are relative to the experimental δ_0 measured on the stock solution of AChP in the two media.

CONCLUSION

The influence of water on hydrogen bond based molecular recognition systems has been demonstrated and quantitatively assessed by Wilcox and coworkers [14]. Far less obvious is the

influence of water on host-guest systems not involving hydrogen bonding as the binding interaction, as in the case of the cation-π interaction observed with neutral cyclophane esters. The present study highlights this aspect and shows that, with the appropriate choice of experimental conditions, the NMR technique can give accurate association constants even when values of the latter are small. It should be remarked that evaluation of binding constants from NMR titrations is commonly carried out under the (unchecked) assumption that the observed shift is caused *exclusively* by complexation. This leads to underestimate the fact that the shift of signals of a species is often subjected to a variety of influences [15], not least to that of the ubiquitous water. It has been shown here that underestimating these interferences may compromise the reliability of measurements. Furthermore, distortions are enhanced when employing small amounts of host, as is often the consequence of low solubility, large K_a values, or various practical reasons. Sampling data in regions of low H/G ratio, where H_2O/H is high, may artificially boost apparent K_a values to a marked extent. Although this phenomenon was observed with cyclophane esters, we believe that it might be of more general occurrence than expected, especially with hosts prone to interact with water.

EXPERIMENTAL

General

^{1}H NMR spectra were acquired at 200 MHz on a Varian GEMINI 2000 and at 300 MHz on a Varian VXR 300, equipped with a variable temperature apparatus. Chemical shifts (δ) in $CDCl_3$ are given in ppm from the $CHCl_3$ signal at δ 7.26. Electrospray ionization mass (ESI-MS) spectra were recorded in the positive-ion mode on a Fisons Instruments VG-Platform benchtop mass spectrometer equipped with a pneumatically assisted electrospray LC/MS interface and a single quadrupole operating at 3.8 kV. Osmometric measurements were performed on a Wescan 233 Molecular Weight apparatus. For the determination of association constants, NMR data were analyzed using the HYPNMR program [16] and the SIGMA Plot software package (Jandel Co). Acetylcholine and tetramethylammonium picrates were obtained from the corresponding iodides by treatment with silver picrate in H_2O/CH_3CN 1:1. Cyclophanes **1a** and **1b** have been described previously [5].

^{1}H NMR Titrations

Deuterochloroform (Merck, 99.8%) stored in the dark over 3A and 13X activated molecular sieves and silver foil to prevent decomposition was used for the NMR measurements. Due to the low solubility of quaternary ammonium salts in $CDCl_3$, stock solutions of the guest were freshly prepared right before use by stirring a small amount of the salt in the solvent at r.t. for 1 h and filtering the suspension. Concentration of the guest (0.1 – 1 mM) was measured by integrating the NMR signals *vs.* that of an internal standard (dimethylsulfone) of comparable intensity. To ensure proper integration, T_1 relaxation times of the involved signals were measured and spectra were acquired with recycle times of $5*T_1$ of the longest relaxing signal and pulse widths PW $\approx 60°$. A weighted amount of host (up to a maximum of 60 mg/mL) was dissolved with the stock solution of the guest in a 5 mm NMR tube, previously calibrated with $CDCl_3$ to 0.700 mL using a Hamilton microsyringe and checking the calibration by weight. After allowing the sample temperature to stabilize in the probe, spectra were acquired with a 0.0005 ppm digital resolution using the carrier frequency as chemical shift reference. The titration was performed directly in the NMR tube by adding with a Hamilton microsyringe known amounts of guest's stock solution and syringing out after mixing the excess solution volume down to the 0.700 mL

calibration. An average of 15–20 spectra were acquired for each titration. Association constants were obtained from experimental shift values by nonlinear regression procedure as described in text.

Acknowledgements

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[7] We found that a major improvement in the precision of the NMR measurements could be achieved by acquiring spectra on samples of constant volume. With this expedient, under fine control of temperature and using the carrier frequency as external reference, δ values were highly reproducible. We experimentally measured an average error of 0.0003 ppm and a maximum error of the same order of the digital resolution used (0.0005 ppm), which were 1-2 orders of magnitude smaller than the observed residuals range (0.025–0.030 ppm).
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[11] A linear downfield shift was observed for the water signal in $CDCl_3$ with increasing water concentration (slope = 0.9 ppm L mol^{-1}) either in the absence or in the presence of a constant amount of cation. Since the water signal has $\delta = 1.5$ in $CDCl_3$ and $\delta = 4.8$ in bulk water, the observed downfield shift indicates that, as expected, water tends to cluster in $CDCl_3$ even in dilute solution, approaching the chemical shift of the bulk. This accounts for the sensitivity of the water signal as a probe of small variations of water content of the solution.
[12] Irregularities observable in the curves are due to inconsistency of water content. As discussed in the previous footnote, the shift of the water signal is very sensitive to variations of water concentration, such that even trivial fluctuations occurring during the titration give rise to observable distortions of the plot.
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