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NON-AQUEOUS TITRATIONS AS A TOOL IN THE STUDY OF MOLECULAR RECOGNITION PHENOMENA. USES IN DISTINGUISHING HYDROGEN BONDING FROM PROTON TRANSFER, THE MEASUREMENT OF COMPLEX INDUCED pK_a SHIFTS, AND THE ABILITY TO DISTINGUISH THE CATALYTIC ROLES OF GENERAL ACIDS AND BASES

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Whenever hydrogen bonding is involved in molecular recognition, the possibility of a proton transfer from the donor to the acceptor arises. In most cases the pK_a of the donor is far enough above the pK_a of the conjugate acid of the acceptor for it to be clear that no proton transfer will occur. However, as the difference between the donor and acceptor pK_as decreases, it can become difficult to predict whether a proton transfer will occur. Since most hydrogen bond-driven molecular recognition is studied in low dielectric solvents, non-aqueous titrations can be used to measure the pK_as and therefore predict proton transfers. In this paper three studies which involved non-aqueous titrations are summarized. The first deals with distinguishing simple proton transfer from host–guest complex formation. The second involves measuring pK_a shifts upon host–guest complex formation. The last is a study of the catalysis of a phosphoryl transfer. In all three scenarios the non-aqueous titration method gave results which would have been difficult to obtain by other means, and which proved crucial for a complete understanding of the molecular recognition process. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Molecular recognition using synthetic receptors has primarily focused upon solvophobic binding[1] and hydrogen bond-driven complexation.[2] Although the hydrophobic effect can be used to discriminate guests due to electron donor and acceptor properties,[3] using hydrogen bonding has the most promise for developing selective receptors. The problem with hydrogen bond-driven molecular recognition is that hydrogen bonds are typically weak, and therefore numerous hydrogen bonds are needed to create a receptor which has a significant binding constant for its guest.[4]

Biological systems often use charged hydrogen bonds. Fersht *et al.*[5] have shown that charged donors or acceptors pairing with neutral acceptors or donors, respectively, can significantly increase the strength of hydrogen bonding compared with hydrogen bonding between neutral donors and acceptors. Further, when both the donor and acceptor are charged, the hydrogen bonding interactions also benefits from a strong electrostatic attraction above and beyond the electrostatics associated with normal hydrogen bonding.

To order to construct receptors and catalysts, chemists have explored a range of hydrogen bonding motifs.[6] In general, as found in biological systems, the presence of charges on the donor and acceptor increases the binding strength compared with completely neutral hydrogen bonding motifs. Complementary charges on the donors and acceptors give the largest driving force for complexation, and can result in hydrogen bonding recognition in competitive solvents such as DMSO and water.[7] However, when using cationic hydrogen bond donors, a potential complication arises: proton transfer from donor to acceptor.[8] In fact, this complication exists for any hydrogen bonding scenario,

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but it is particularly relevant when charged donors are used. Usually it is obvious if the pK_a of the donor is lower than that of the conjugate acid of the acceptor, and thereby a proton transfer should occur. However, in many cases it is not obvious, especially when these pK_as are similar. Furthermore, since hydrogen bonding molecular recognition is typically studied in non-aqueous media, the pK_as are difficult to predict. This complication to studying hydrogen bonding molecular recognition can in part be solved by performing non-aqueous titrations.

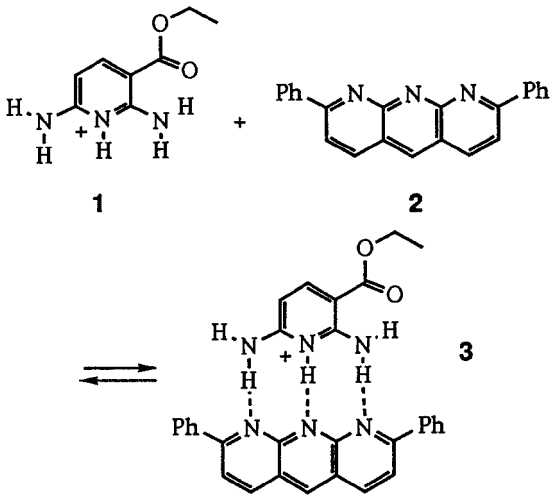
The measurements of pK_as via non-aqueous titration is an old technique.[9] In brief, it involves measuring the potential (mV) of a solution of an acid or base in a low dielectric solvent as a function of an increase in a base or acid, respectively. If the pK_as for two different acids are known in this solvent, a linear relationship between mV and pK_a can be established, thus giving the pK_a of any unknown acid. To measure the potential of the solution, a single-junction proton-sensing electrode is used, along with a reference electrode, commonly $Ag/AgNO_3$. The pK_a scale in a non-aqaueous solvent (HA) is typically larger than that in water since there is often a larger separation between the pK_a of the specific acid (H_2A^+) and the solvent itself (HA). These two pK_as set the low and high ranges for accessible pK_as due to the leveling effect. Further, the values for pK_as are larger than in water since it is more difficult to perform an ionization in a lower dielectric solvent. In fact, relative pK_as can switch in a low dielectric solvent compared with water. For example, the relative pK_a of the conjugate acid of an anion and that of a cationic acid may be such that in water a proton transfer will not occur, but in a lower dielectric solvent the proton transfer between the acid and base will occur because charge neutralization results. This is particularly relevant to the study of hydrogen bonding molecular recognition between cationic donors and anionic acceptors in non-aqueous solvents.

In this paper, three studies in which the method of non-aqueous titration was used to gain critical binding or mechanistic information are summarized. Acetonitrile was used as the solvent in all the studies. The scale of pK_as for acids which can be measured in this solvent is fairly large.[10] Furthermore, acetonitrile is not a strong hydrogen bonding solvent, and therefore is only a moderate competitor for the hydrogen binding sites on the hosts and guests. We discuss our use of non-aqueous titrations for the study of secondary hydrogen bonding and enolate recognition. In addition, we examine the use of this technique to give mechanistic insight into the catalysis of transesterification of a phosphodiester. In all the examples, non-aqueous titrations proved crucial for a complete understanding of the molecular recognition process.

SECONDARY HYDROGEN BONDING ARRAYS

In the study of molecular recognition in low dielectric media, the use of multiple parallel or near parallel hydrogen bonds is a common motif.[6, 11] The binding strength of the complexes is dependent not only upon the number of hydrogen bonds, but also upon the arrangement of the hydrogen bonds. For example, arranging either hydrogen bond donors or acceptors on the same molecule results in stronger binding than if the donors and acceptors are intermingled on the same molecule. The attractive or repulsive forces which depend upon the arrangement of donor and acceptors within a host–guest complex are known as secondary hydrogen bonds. This effect was first recognized by Jorgensen and Pranata,[12] and was experimentally verified by Murray and Zimmerman.[13] Since these early studies, Murray *et al.*[11] have analyzed a variety of hydrogen bonding patterns, which in general verify the concept. We were interested in further probing secondary hydrogen bonding by forming a host–guest complex that possesses a cationic hydrogen bond donor.[14] We expected this combination to result in one of the largest association constants yet reported for a host–guest complex with only three hydrogen bonds.

To probe the secondary hydrogen bonding theory, we chose to focus upon a completely cooperative system such that all the donors are on the same molecule and all the acceptors are on the partner molecule. The 'host' or donor, donor, donor (DDD) molecule was ethyl 2,6-diaminonicotinium tetrakis {3,5-[bis(trifluoromethyl)phenyl]borate} (**1**). The 'guest' or acceptor, acceptor, acceptor (AAA) was 2,8-diphenyl-1,9,10-anthyridine (**2**). This complex is shown as **3**. Figure 1 shows the ^{1}H NMR binding isotherm that results from the addition of host to guest ($6{\cdot}4\times10^{-4}$ M). The lack of curvature indicates a strong association constant, $>3\times10^4$ M^{-1}. We needed a method that could be used to monitor the complexation event at a much lower concentration in order to solve for the binding constant. Since NMR spectroscopy was not appropriate to measure an association constant, we switched to UV–visible spectrophotometry.



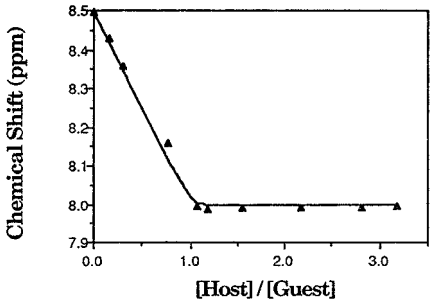


Figure 1. ^{1}H NMR isotherm for the binding between **1** and **2**. The *ortho* protons on the phenyl rings of **2** were followed. The concentration of **2** was kept constant at $6{\cdot}4\times10^{-4}$ M

However, similarly to the NMR experiment, addition of host to guest (now at $5{\cdot}9\times10^{-6}$ M) resulted in saturation at exactly 1 equiv. of host (Figure 2), with no obvious curvature to the plot. This indicated a binding constant of $>5\times10^{5}$ M^{-1}.

Although the spectral changes were indicative of the formation of a host–guest complex, they could also be interpreted to result from a proton transfer from the host to the guest. In fact, the spectral change used to construct Figure 2 was similar to that seen by direct protonation of **2**. One might expect the spectral change of **2** caused by host–guest complex formation to resemble that of protonation since the complex involves the sharing of a cationic hydrogen bond. Therefore, a method was needed to distinguish simple proton transfer with no host–guest complex from complex formation. Non-aqueous titrations proved invaluable to distinguish the two possibilities.

Before exploring the pK_as of **1** and **2** in low dielectric media, a few comparison compounds were required as a means to demonstrate full proton transfer and partial proton transfers. Given the assumption that 2,6-lutidine and pyridine would not form strong complexes with **1**, and instead primarily acid–base chemistry would occur, UV–visible titrations of **1** with lutidine and pyridine were performed. The resulting isotherm from the lutidine titration was essentially identical in shape with that shown in Figure 2 for **2**. This confirmed our notion that the titration isotherm

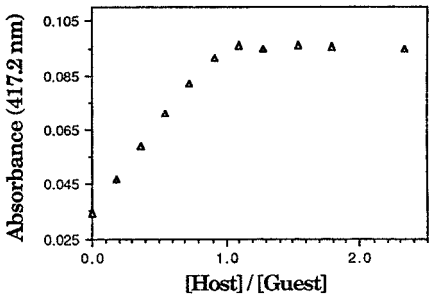


Figure 2. UV–visible isotherm for the binding between **1** and **2**

Table 1. pK_a of 'hosts' and 'guests' used to study secondary hydrogen bonding:conjugate acids in acetonitrile

Compound	pK_a
2,6-Lutidine	14·2
Ethyl 2,6-diaminonicotinate (**1**)	12·6
Pyridine	12·5
2,8-Diphenyl-1,9,10-anthyridine (**2**)	12·2

found for **1** and **2** could have resulted from proton transfer. In contrast, the titration of **1** with pyridine did not show saturation even after the addition of 70 equiv. of pyridine. Therefore, lutidine resulted in a complete proton transfer whereas pyridine gave only a partial proton transfer. Importantly, these results demonstrated that bases with similar or weaker basicity to pyridine would give an isotherm from UV–visible titration of **1** that would not saturate even after large additions of base. In contrast, compounds with a base strength similar to or greater than that of lutidine would result in titration curves that saturate near 1 equiv. of base.

Non-aqueous titrations of lutidine, pyridine, free-base **1** and **2** were performed in acetonitrile as solvent with the addition of 70% perchoric acid. The pK_as determined are given in Table 1. The pK_a of the conjugate acid of **2** was found to be lower than that of host **1**, as was the pK_a of the pyridinium. Further, **2** was found to be even less basic than pyridine. This result confirms the hypothesis that the lack of curvature in the binding isotherm of **1** with **2** (Figure 2) was due to the formation of a strong host–guest complex, since pyridine with a higher basicity did not saturate in the binding isotherm, yet compound **2** with an even lower basicity did show saturation. Had the observed change in the UV–visible spectra resulted from a proton transfer, the binding isotherm with **2** would have not saturated, as was found for pyridine.

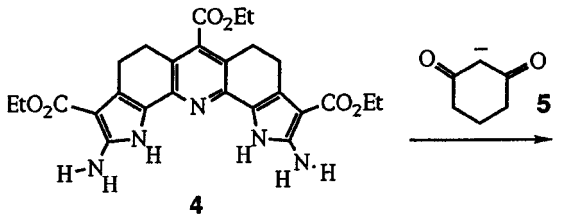
In summary, non-aqueous titrations were used to resolve an ambiguous experiment in host–guest chemistry. The ability to determine the relative acidities and basicities of hosts and guests in low dielectric media afforded the means by which a proton transfer could be distinguished from complex formation. In the next section, we show how non-aqueous titrations can be used to understand binding strength as a function of molecular shape and basicity, and to measure shifts in pK_as upon host–guest complex formation.

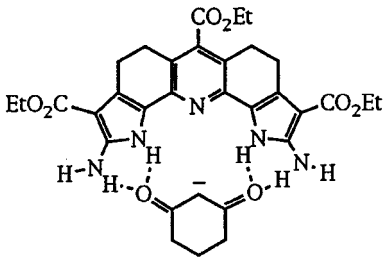
ENOLATE BINDING

Stabilization of an enolate anion and the consequential increase in the acidity of the α-carbon hydrogens is the strategy used by enolase and racemase enzymes.[15] The deprotonation of the α-carbon has both a thermodynamic and kinetic barrier that is overcome by the enzymes due to stabilization of the enolate and the transition state leading to it.[16] Signifcant stabilization is required since a typical α-

hydrogen has a pK_a of 16–20, but this number needs to be lowered to match typical enzyme functional groups (pK_as of 5–9) in order for deprotonation to occur. The stabilization can be imparted by a number of factors including hydrogen bonding, electrostatic attraction, metal coordinations and possibly low-barrier hydrogen bonds.[17] In order to probe the potential to lower the acidities of α-hydrogens by common interactions such as hydrogen bonding and metal interactions, we studied host–guest complexes which bind enolates.[18] Non-aqueous titrations proved useful in understanding the extent to which simple hydrogen bonds can lower the pK_as of carbonyl guests.

As a preliminary experiment to measuring the change in pK_as upon host–guest complexation, the binding constants of a series of enolates to a hydrogen bond-donating host (**4**) were determined. Table 2 shows the binding constants determined in acetonitrile for the series of enolates and **4**. Cyclohexane-1,3-dionate (**5**) proved to have the largest binding constant with **4**. This was interpreted to be due to the obvious better complementarity of this guest for **4** compared with the other enolates. However, it is well known that the greater the basicity of the hydrogen bond acceptor, the greater is the expected binding constant. Therefore, we determined the pK_as of the conjugate acids of all the enolates to discover if a trend existed between binding constants and basicity. The pK_as are given in Table 2, and Figure 3(A) shows a plot of binding constant versus pK_a. There appears to be no obvious trend indicating that the most basic enolates do not result in the largest binding constants. In fact, the most basic anion, that from malonitrile, has the lowest binding constant. The complementarity of the enolates for the host seem also to be of paramount importance. If one plots the binding constants of guests with similar structures (i.e. the first three compounds in Table 1) against their pK_as, a clear trend is found [Figure 3(B)]. The binding constants increase in a linear fashion with basicity.





In conclusion, non-aqueous titrations proved useful for deciphering trends in binding constants as a function of basicity and molecular shape.

Once it was clear that cyclohexane-1,3-dionates were the best guests for **4**, we turned our attention to discovering the extent to which **4** could lower the pK_a of carbonyl guests. It is well accepted that stabilization of an enolate would be expected to cause the conjugate acid to become more acidic. However, it was not known to what extent simple hydrogen bonding could be used to stabilize the enolate and increase the α-hydrogen acidity. Once again, non-aqueous titrations proved to be the experimental tool of choice.

Figure 4 shows the resulting titration curves for **5** alone

Table 2. pK_as and binding constants for **4** and various enolates

Guest	Binding constant (M^{-1})	Guest pK_1
	$1{\cdot}35\times10^{4}$	20·7
	$7{\cdot}90\times10^{2}$	19·2
	$1{\cdot}48\times10^{3}$	19·5
	$8{\cdot}12\times10^{3}$	20·4
	$5{\cdot}88\times10^{3}$	19·2
	$3{\cdot}93\times10^{3}$	16·9
	$6{\cdot}70\times10^{2}$	15·9
	$1{\cdot}75\times10^{2}$	21·2

[a] The binding constant was determined by ^{1}H NMR titrations of enolates with constant concentrations of **4**. The resonances of the NH_2 groups of **4** were followed.

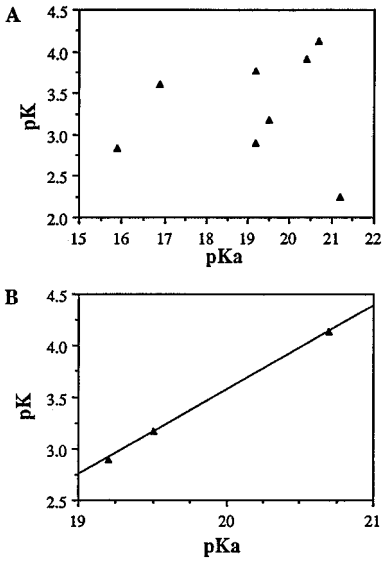


Figure 3. (A) Plot of pK_a versus binding constants with **4** for the guests listed in Table 1. (B) Plot of pK_a versus binding constants with **4** for cyclohexanedionate-like guests

and the complex between **4** and **5**. A 1·0±0·3 pK_a unit shift was found. This shift of 1.0 pK_a unit represents the minimum pK_a shift induced by **4**. Such a small shift, even in a moderately low dielectric medium such as acetronitrile, shows that amide-like NH groups do not impart large stabilizations to enolate anions, and that only small changes in acidities can be expected at enzyme active sites by such interactions. Therefore, one is left to explore charged hydrogen bonding groups and metals as the vehicles by which large pK_a shifts are induced at enzyme active sites.

It is likely that non-aqueous titration is the only method by which the above-discussed information could have been

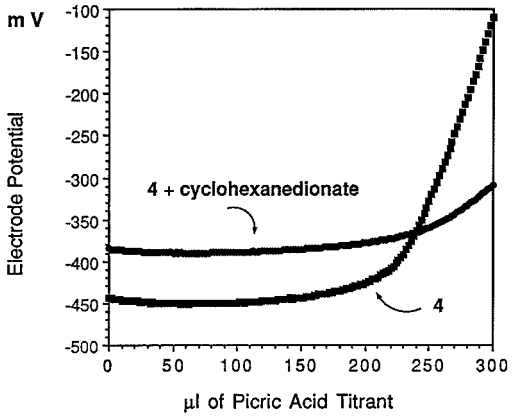
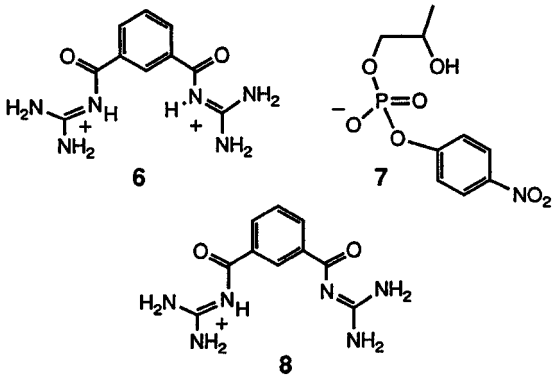


Figure 4. Titration curves for cyclohexanedionate alone and in the presence of **4**



provided. The method allowed us to tie together several pieces of information relating to thermodynamics of binding and thermodynamics of proton transfers, thereby giving an insight into enzyme action using a model system.

PHOSPHODIESTER TRANSESTERIFICATION

Catalysis of phosphoryl transfer reactions is a current goal in bioorganic chemistry owing to potential applications in RNA and DNA manipulations.[19] Bis-guanidinium receptors have been found to be successful catalysts for the transesterification of *p*-nitrophenyl-activated phosphodiesters,[20] the ring opening of strained cyclic phosphodiesters[21] and the cleavage of RNA under neutral conditions.[22] A thorough mechanistic understanding of the catalysis imparted in these systems is necessary for the development of future catalysts which can cleave both RNA and DNA. One facet of the mechanistic understanding is the difference between the pK_as of the guanidinium groups before and after a substrate is bound, and the proper balance between the pK_as of the guanidinium general acids and the added general base required for delivery of the nucleophile.

Jubian *et al.*[20] have demonstrated that receptor **6** in the presence of lutidine in acetonitrile can catalyze the transesterification of **7** with a rate enhancement approaching a factor of 10^3 over lutidine alone. Lutidine was used as the general base to deliver the intramolecular OH nucleophile. The use of acylguanidiniums as in **6** gave larger rate enhancements than alkylguanidiniums. However, when using alkyl guanidiniums, the same group has shown that attachment of an intramolecular general base can further increase the rate enhancement.[23] Acylguanidiniums have pK_as near 7·0 in water, whereas alkylguanidiniums have pK_as near 13 in water. Therefore, the greater activity of the acylguanidiniums probably results from their greater acidity, and therefore increased potential to act as a general acid.

We have also investigated similar bis-alkylguanidinium receptors, except for aqueous media,[22,24] in which the guanidinium pK_as are significantly above a general base pK_a

of 7·0 required for catalysis at neutral pH. It is important to discover if this large pK_a difference is best for effective catalysis in water, or if a closer match between the pK_as of the general acid and base would be better. However, it is experimentally difficult to follow transesterification of RNA or RNA mimics with general bases whose pK_as approach the high values of guanidiniums, owing to the large specific base catalysis that occurs at high pH.[25] Compound **6**, however, provided a successful catalytic model in acetonitrile with which to test the required pK_a differences of the general acids and bases, since general bases near the acylguanidinium pK_as can be used.

Figure 5 shows mV titrations with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) of lutidinium tetraphenylborate and **6**. The pK_a of lutidinium tetraphenylborate was found to be 14·8, whereas the first and second pK_as of **6** were found to be 13·8 and 16·3, respectively. Under Secondary Hydrogen Bonding Arrays, we report the lutidinium pK_a to be 14·2 (Table 1), whereas under Phosphodiester Transesterification we report the pK_a to be 14·8. Given that the error in our pK_a measurements is typically ±0·2 pK_a units, this difference is real. The experimental difference which led to the pK_a difference is the titrant. In the former section 70% perchloric acid was used, whereas in the latter the system was perfectly anhydrous and DBU was used as the titrant. The introduction of water as in the titration performed in the former section lowers the measured pK_a slightly. We have found this trend to be reproducible.[18] Thus, upon introduction of the general base lutidine to a solution of **6** in acetonitrile, almost complete proton transfer from **6** occurs, implicating **8** as a possible catalytically active form. Figure 6 shows the pseudo-first-order rate constants of the transesterification of **7** catalyzed by **8** alone, and **6** plus lutidine.[20] The rate enhancement imparted by **8** is between 65 and 85% of that reported for **6** and lutidine under comparable conditions. Although **8** is the dominant form of the receptor in a mixture of **6** and lutidine, it is not as efficient.

Enzymes often show significant pK_a changes upon substrate binding.[22] Similarly, the first and second pK_as of **6** with diphenylphosphate bound (Figure 5) shift to 19·5 and 20·4, respectively, nearly 5pK_a units away from lutidine. Thus, when a phosphodiester substrate is bound, the catalyst exists mostly as **6**. The acidic protons are more strongly held between the acylguanidinium and the phosphate than on the solvated acylguanidinium. In confirmation that the role of the lutidine is not just to create catalyst **8** from **6**, Figure 7 shows the pseudo-first-order rate constants of the transesterification of **7** by **6** versus lutidine concentration. If the lutidine were only serving to produce a different form of the catalyst, a leveling of the observed rate when the lutidine exceeds the concentration of **6** would be observed. Instead, a leveling occurs only when a very large excess of lutidine is added. This is most likely due to the lutidine associating with **6** via hydrogen bonding to form a complex.

Figure 8 shows two mechanistic possibilities for the transesterification of RNA or an RNA mimic using a bis-guanidinium receptor. Mechanism A involves the bis-protonated form of the catalyst acting as an electrophilic

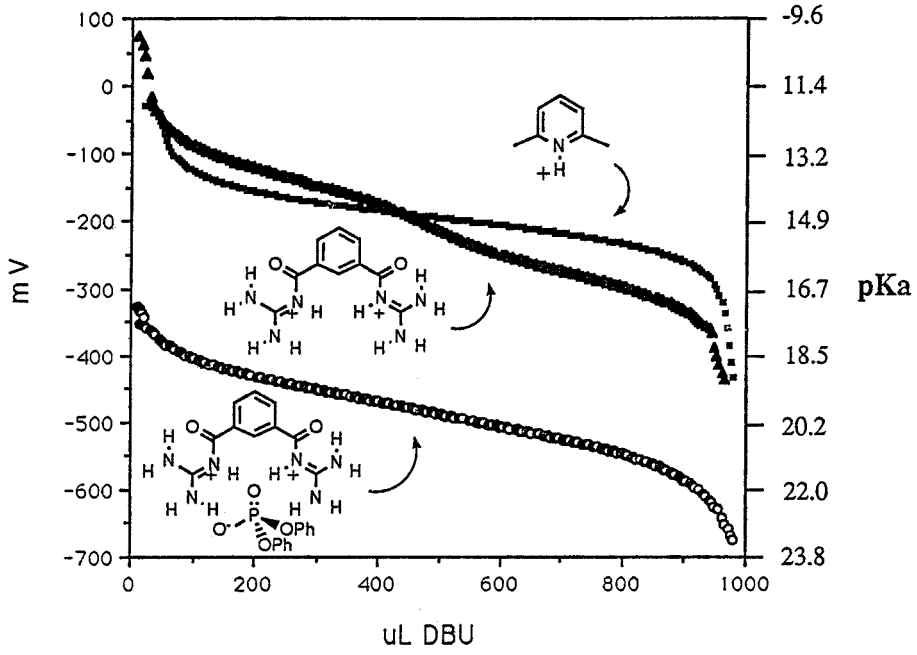


Figure 5. Titration curves for lutidinium, **6**, and **6** in the presence of diphenylphosphate.

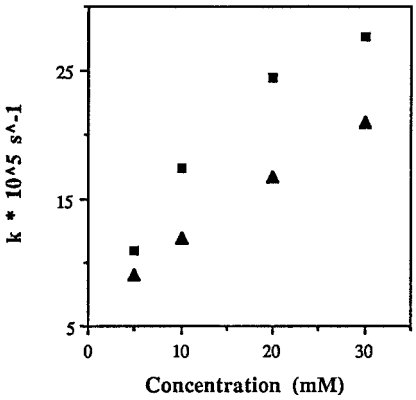


Figure 6. Pseudo-first-order rate constants for the cleavage of **7** catalyzed by **6** (squares) and **8** (triangles) at 25°C. The data shown as squares were taken from ref. 20. The concentration of **7** was 1×10^{-4} M

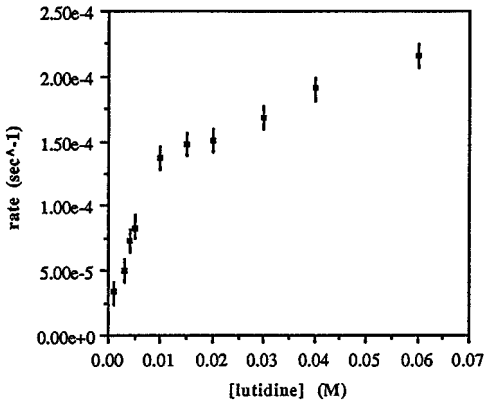


Figure 7. Pseudo-first-order rate constants for the cleavage of **7** catalyzed by **6** as a function of lutidine concentration. The concentration of **6** was 5×10^{-3} M and that of **7** was 1×10^{-4} M

activator of the phosphodiester. Mechanism B involves bifunctional catalysis by a guanidinium and a guanidine. Mechanism A or B will dominate depending upon whether the general base chosen has a pK_a below or above the first pK_a of the receptor–substrate complex, respectively. Thus, it is not necessary to keep the pK_a of the general base below the pK_a of the general acids if the receptor–substrate complex retains a pK_a above that of the general base. Therefore, the large difference between alkylguanidinium pK_as and neutral pH may not be necessary to retain catalytic activity in aqueous media. We are exploring this possibility and these mechanistic alternatives with bis-acyl, bis-phenyl and bis-alkylguanidinium receptors in water.

Given the large pK_a shifts upon substrate binding, it is likely that catalysis by **8** is even more complex than that shown in Figure 8. Once the substrate binds to **8**, the pK_a of the acylguanidiniums shift such that the one acylguanidine of **8** is now more basic than the conjugate base of **8** without **7** bound. Since **8** is in large excess, a proton transfer from free **8** to the complex between **7** and **8** should occur. Hence the catalysis found by adding **8** to acetonitrile may actually involve a complex of **6** with the substrate, and the general base could be the acylguanidine of another molecule of **8**.

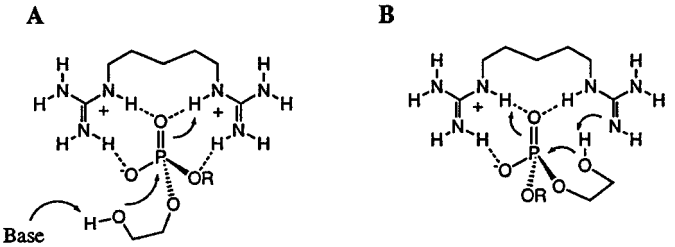
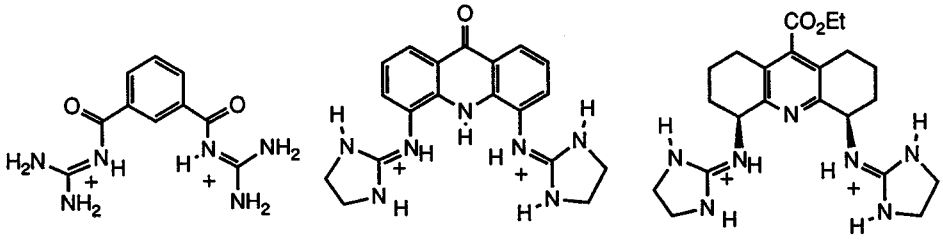


Figure 8. Two possible mechanisms by which bis-guanidinium receptors may enhance the cleavage of RNA and/or RNA mimics. Our results show that a mechanism such as B is not as efficient as that shown in A using catalyst **6**



CONCLUSION

We have shown how non-aqueous titrations can be valuable in deciphering modes of molecular recognition and mechanisms of artificial enzymes. Predicting relative pK_as can distinguish between host–guest complex formation and simple proton transfer. Measuring pK_as can be used to quantitate the ability of host functional groups to influence guest acidities. Further, measuring pK_as in low dielectric media can be used to shed light on mechanistic questions. As molecular recognition continues to use charged hydrogen bond donors and acceptors in non-aqueous media, we predict an increase in the reliance on non-aqueous titration methods.

EXPERIMENTAL

All pK_a measurements were carried out in Omnisolve 'Low water' acetonitrile (11 ppm water), which was used as received. Hamilton gas-tight syringes and glassware were dried and solvent transfers were performed via syringes through a septum or in a glove-bag or dry-box under N_2. Picric acid was recrystallized from acetone and dried under vacuum over P_2O_5. The electrolyte Et_4NClO_4 was purchased from Eastman Kodak and used as received. Tetraethylammonium picrate was prepared and dried according to the method of Coetzee and Padmanabhan.[28b] $DBUH^+Ph_4B^-$ was synthesized as described previously.[18] Standards were run before and after each titration to allow conversion of mV to pH. The two standards used were a 1:1 mixture (pK_a = 11)[28] of picric acid (PA) with tetraethylammonium picrate ($Et_4N^+PA^-$) and a 1:1 mixture (pK_a = 23·9)[29] of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) with its conjugate acid tetraphenylborate salt ($DBUH^+Ph_4B^-$). The electrolyte concentration was equal to that of the anion solution which was being titrated. The electrode was allowed to equilibrate with the solution for 30–40 min until the readings stabilized.

Titrations performed for the first two topics used either picric acid or 70% perchloric acid. Titrations performed for the third topic used DBU as titrant. Readings were taken on an Orion Model 720A pH meter in millivolt mode. Titrant was added using a programmable Harvard Apparatus Syringe Infusion Pump 22. An IBM-compatible computer was used to record mV and volume readings. An Orion Model 91–01 glass pH electrode was used in conjunction with a silver/silver nitrate reference electrode.

The titration cell was constructed of Pyrex with three glass joints at the top. One was an Ace-Thred (Ace Glass) and the other two were female 14/20 standard taper. The Orion glass electrode was then placed through the Ace-Thred bushing. One of the 14/20 joints was sealed with a septum. This position was used to introduce solutions and titrant via a syringe and a nitrogen balloon. The other 14/20 joint was used for the reference electrode, the outer section of which was constructed with a 14/20 male joint. The inner section of the reference electrode was constructed of a rod of silver extending 4·5 cm below where it is sealed with glue into a glass tube. The silver rod was soldered to a silver wire which was then connected to the wire leading to the reference jack of the pH meter via an alligator clip. The reference electrode was separated from the titration solution by a Vycor tip puchased from Bioanalytical Systems. The inner portion was filled with 0·01 M $AgNO_3$ and 0·1 M Et_4NClO_4 in dry acetonitrile.

For a direct comparison between the titration curve and a curve generated by the Henderson–Hasselbach equation, it was necessary to convert the curves from mV readings to pH. A FORTRAN program (available on request) was used to generate the pH values for each point based on the values of two standards described above. The same program included a subroutine for the generation of the theoretical titration curve for any given pK_a value.

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