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Determination of Association Constants (K_a) from Solution NMR Data

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1. Introduction

Molecular recognition is a major part of modern organic chemistry. In view of the importance of association constants (K_a) for the communication of results in this field, it is usually essential that K_a be quantified. This report discusses the methodology behind one of the most widely used techniques for measuring K_a in host–guest chemistry—NMR spectroscopy.

To determine the equilibrium constant for the simple reaction

$$A + B \rightleftharpoons C$$

requires knowledge of the equilibrium concentrations (strictly speaking, thermodynamic activities) of the species

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A, B and C.[1] When A and B are host (H) and guest (G) species that form a complex which is held together by weak intermolecular forces (e.g. hydrogen bonding and van der Waals forces) the equilibrium constant is usually referred to as a binding constant or association constant and the species C may be written as H·G to indicate that the product has chemical characteristics which still strongly resemble the un-associated ('free') molecules.

$$K_a = [H{\cdot}G]/[H][G] \quad (1)$$

The appearance of the NMR spectrum of the mixture represented by (1) would depend on K_a and on the rate of the reaction. This paper is primarily concerned with the case where the rate of reaction is fast on the NMR time scale and only a time averaged spectrum of the guest, (and/or host) and the host–guest complex are observed.[†] In this case any observed chemical shift is the mole fraction weighted average of the shifts observed in the free and complexed molecule.

$$\delta_{obs} = X_G\delta_G + X_{HG}\delta_{HG} \quad (2)$$

and for the formation of a 1:1 complex then,

$$[G] + [HG] = [G]_0 \quad (3)$$

and

$$[H] + [HG] = [H]_0 \quad (4)$$

Eqs. (1)–(4) describe the relationships between defined parameters (the intrinsic, or starting concentrations of species G and H); experiment observables (δ_{obs} and δ_G) and the parameter to be determined (K_a). Note that the relationship between δ_{obs} and K_a is non-linear, and there is another parameter (δ_{HG}) which cannot usually be directly determined. Note also that the equilibrium concentrations of species H and G (actual concentration in solution) are not the same as the initial or 'made up' concentrations. Identification of the unknown parameters K_a, and δ_{HG} is achieved by measurements with a series of different concentrations of $[G]_0$ and $[H]_0$ and subsequent data treatment following some kind of linearisation method, or a nonlinear curve fitting procedure.

The basic methodologies were first worked out in the early 1960s during studies of hydrogen bonded and charge transfer complexes. At this time the binding equations for binary 1:1 complexes in fast exchange were solved. Later work generalised the equations to allow for ternary systems and introduced computer based fitting methods. The more recent examples described here come from the literature of molecular recognition[2,3] and host–guest chemistry;[4] and in particular, the neutral complexes[5,6] formed between small molecules and cyclodextrins,[7,8] crown ethers, calixarenes[9] and cryptophanes.[10]

[†] Throughout the following discussion it is assumed that the guest molecule is the observed species in the NMR experiment. It does not matter which molecule is observed and the most readily observed and responsive molecule would normally be chosen. The data treatment for observed host is identical, with host and guest symbols switched.

NMR has become a routine tool for the study of host–guest supramolecular chemistry and there are now hundreds of reports of studies where an NMR titration was used to measure intermolecular association. Foster and Fyfe[11] comprehensively reviewed the literature up to 1964 (the linear methods). Other reviews appearing since then that have included descriptions of the NMR methodologies have been those by Connors,[1] Bradshaw et al.[12] and Tsukube et al.[13] Chapter 5 of Connors' book and the Tsukube et al. review are particularly recommended for reading. The purpose of the present work is to provide an accessible guide to the experimental procedures and the various data treatments that are possible. It is hoped that it will be useful for newcomers to the field.

1.1. Scope of the review

The present review is focused towards host–guest chemistry where association constants are of the order of $10–10^6\ M^{-1}$. It does not consider dimerisation, or aggregation phenomena. Neither will it cover the large body of publications relating to weak complexation ($K_a<2\ M^{-1}$), except for some interesting cases that are instructive to host–guest chemistry. Some references to literature dealing with NMR studies of shift reagents, and binding of small molecules to proteins are included when appropriate.

The most common NMR experiment observable—a chemical shift change—is dealt with first. The two main types of data treatment (graphical methods and computer fitting) are described in Sections 2 and 3, and throughout these sections the emphasis is firmly on chemical shift data. Some non-chemical shift type experiments are discussed in Sections 4 and 5. Sections 6 and 7 discuss the reliability and limitations of NMR experiments, and how experiments can be devised to extend the range of the NMR method. The commonly recurring terms are defined as follows:

Chemical shift terms

δ_{obs}	an experimentally measured chemical shift
δ_H	chemical shift of a nucleus in the host molecule
δ_G	chemical shift of a nucleus in the guest molecule
δ_{HG}	chemical shift of a nucleus in the host–guest complex
$\Delta\delta$	measured change in chemical shift (upon addition of host species) referenced to that of the uncomplexed guest
$\Delta\delta_{max}$	the difference in chemical shifts between that observed in the guest molecule and that observed in the host–guest complex

Concentration terms

X_G	mole fraction of guest in equilibrium mixture
X_{HG}	mole fraction of host–guest complex in equilibrium mixture
[H]	concentration of host at equilibrium
[G]	concentration of guest at equilibrium
[HG]	concentration of host–guest complex at equilibrium
$[H]_0$	known total concentration of host
$[G]_0$	known total concentration of guest.

Table 1. Constructed data for a typical NMR titration. The virtual conditions are as follows—$[G]_0$=2 mM; $[H]_0$=1–500 mM; K_a=10, 10^2, 10^3, 10^4 and 10^5 M^{-1}; δ_G=0.0 ppm; and δ_{HG}=0.5 ppm. See Section 1.2 for details. The bold type identifies the solutions that meet the Weber criteria $0.2 \leq p \leq 0.8$ (see Section 6.2)

K_a (M^{-1})	10		10^2		10^3		10^4		10^5	
$[H]_0$ (mM)	[HG] (mM)	δ (ppm)	[HG] (mM)	δ (ppm)	[HG] (mM)	δ (ppm)	[HG] (mM)	δ (ppm)	[HG] (mM)	δ (ppm)
1	0.0194	0.005	0.1557	0.039	**0.5858**	0.146	0.9156	0.229	0.9902	0.247
2	0.0385	0.010	0.2918	0.073	**1.0000**	0.250	**1.6000**	0.400	1.8635	0.466
5	0.0935	0.023	**0.6101**	0.152	**1.5505**	0.388	1.9368	0.484	1.9934	0.498
10	0.1789	0.045	**0.9501**	0.238	1.7830	0.446	1.9754	0.494	1.9975	0.499
20	0.3288	0.082	**1.3031**	0.326	1.8953	0.474	1.9890	0.497	1.9989	0.500
50	**0.6608**	0.165	1.6572	0.414	1.9592	0.490	1.9958	0.499	1.9996	0.500
100	**0.9950**	0.249	1.8151	0.454	1.9798	0.495	1.9980	0.499	1.9998	0.500
500	1.6657	0.416	1.9606	0.490	1.9960	0.499	1.9996	0.500	2.0000	0.500

1.2. A constructed data set

It is useful to have some data to illustrate part of the following discussion. Accordingly, Table 1 is made up to be representative of typical data that might be obtained from a study of host–guest chemistry. In the virtual experiment, a series of solutions were made to be 2 mM in the NMR active species (the observed species—G), and covering a range from 1 to 500 mM in H. The non-complexed molecule G has a peak at 0.0 ppm in its NMR spectrum and this peak appears at +0.50 ppm in the 1:1 HG complex. Table 1 shows how G is distributed between the free and complexed HG species over a range of association constants from 10 to 10^5 M^{-1}, and how the observed chemical shift changes as a function of both $[H]_0$ and K_a. This data is also presented graphically in Fig. 1.

1.3. Determination of stoichiometry

Before any determination of K_a is performed it is essential always to determine the stoichiometry of the host–guest complex.[1,13] This is most readily achieved from NMR data by means of the method of continuous variations (Job's method).[14–16]

The method of continuous variations involves preparing a series of solutions containing both the host and the guest in varying proportions so that a complete range of mole ratios is sampled ($0>[H]_0/([H]_0+[G]_0)<1$), and where the total concentration $[H]_0+[G]_0$ is constant for each solution. The experimentally observed parameter is a host or guest chemical shift that is sensitive to complex formation. The data are plotted in the form $X_G\Delta\delta$ versus X_H (Fig. 2). Another technique known as the mole ratio method works well if K_a is large ($>10^5$). In this method a plot of $\Delta\delta$ versus $[H]_0$ from a series of solutions containing constant $[G]_0$ and a suitable range of $[H]_0$ produces two straight lines that intersect at the [H]/[G] ratio corresponding to the stoichiometry of the complex.

Note that the data obtained to determine stoichiometry are not the best data for abstracting the association constant and so separate experiments should be planned and executed (see Section 6).

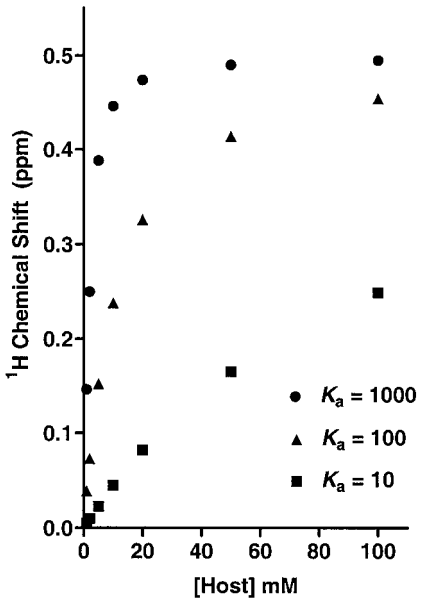


Figure 1. Simple plot of virtual NMR titration data. The data from Table 1 (for K_a = 10, 10^2 and 10^3 M^{-1}) are plotted in a simple fashion to show the relationship between the induced chemical shift change and amount of host added. The observed NMR line is a fictitous proton on the guest molecule ($[G]_0$ = 2 mM). This curve illustrates the non-linear relationship between $\Delta\delta$ and $[H]_0$. The small K_a data do not reach the limiting chemical shift $\Delta\delta_{max}$. The larger K_a data rise almost linearly to $\Delta\delta_{max}$ and then level out.

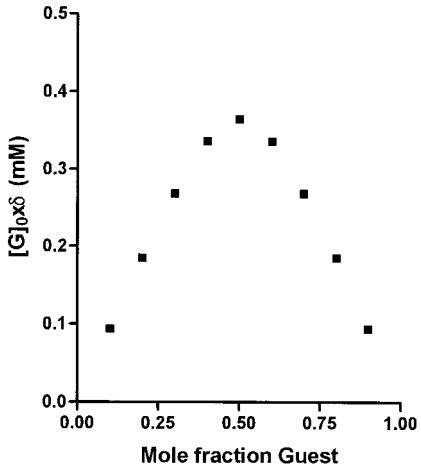


Figure 2. Illustration of the Job Plot for Determination of Stoichiometry. This figure shows calculated data for a system in which δ_G=0.0 ppm, and $\Delta\delta_{max}$=0.50 ppm, and K_a=10,000 M^{-1} for a 1:1 complex. In the virtual experiment solutions were made over a range of host/guest ratios and under the condition that $[G]_0+[H]_0$=2 mM, and $[G]_0$ (the observed species in this experiment) varies from 0.2 mM to 1.8 mM in 0.2 mM steps. The position of the maximum indicates the stoichiometry of the complex.

2. Graphical Methods

Graphical (or linearisation) methods are designed to produce a linear relationship between δ_{obs} and K_a, so that NMR data can be treated graphically. The equations that describe the 1:1 binding isotherm are those of the rectangular hyperbola, and there are three graphical methods for their solution.[1]

2.1. Benesi–Hildebrand (Hanna–Ashbaugh) treatment

The most common approach is frequently (and somewhat loosely) called a Benesi–Hildebrand treatment. The original Benesi–Hildebrand experiment was an optical spectroscopy study of the association of iodine with aromatic hydrocarbons.[17] The key feature of this method is that by working with a large excess of component H, the concentration of uncomplexed H can be set equal to the initial concentration, $[H]=[H]_0$. Relationships between known quantities (initial concentrations) and experimental observations can now be derived.

Mathur et al.[18] and Hannah and Ashbaugh[19] have independently derived the NMR version of the Benesi–Hildebrand equation.

$$1/\Delta\delta = 1/(K_a\Delta\delta_{max}[H]_0) + 1/\Delta\delta_{max} \quad (5)$$

where $\Delta\delta=(\delta_G-\delta_{obs})$, and $\Delta\delta_{max}=(\delta_G-\delta_{HG})$.

A plot of $1/\Delta\delta$ against $1/[H]_0$ (often referred to as a double reciprocal plot) should be linear, with a slope $1/K_a\Delta\delta_{max}$ and intercept $1/\Delta\delta_{max}$. The procedure is illustrated in Fig. 3 with the data for $K_a=100\ M^{-1}$ taken from Table 1. Note that this expression is only valid when observing species G in the presence of a large excess (minimum 10×) of species H and when a 1:1 complex is formed. A further limitation of Eq. (5) is that an extrapolation to high concentration of H has to be made. In systems where K_a is small, this procedure may lead to large errors in $\Delta\delta_{max}$ and consequently incorrect values of K_a. Throughout the current literature the terms double reciprocal plot, Benesi–Hildebrand approach, and Hanna–Ashbaugh approach are used interchangeably to describe this method of data treatment.

2.2. Scatchard (Foster–Fyfe) method

An alternative solution has been proposed by Foster and Fyfe.[20,21]

$$\Delta\delta/[H]_0 = -K_a\Delta\delta + K_a\Delta\delta_{max} \quad (6)$$

This is a special form of the more general Scatchard plot.[22] In the Foster–Fyfe procedure a plot of $\Delta\delta/[H]_0$ against $\Delta\delta$ (referred to as an *x*-reciprocal plot) should be linear, the gradient is equal to $-K_a$ and the intercept gives $\Delta\delta_{max}$. This procedure is illustrated in Fig. 4, again with the $K_a=100\ M^{-1}$ data from Table 1. In contrast to Eq. (5), this requires an extrapolation to infinitely dilute solution and the K_a is not dependent on the extrapolation. This appears to be a better method but it has not been as generally used as the Benesi–Hildebrand method.

2.3. Scott plot

A third linearisation approach is the *y*-reciprocal, or Scott[23] plot, in which $[H]_0/\Delta\delta$ is plotted against $[H]_0$. This technique has not been widely used for the analysis of NMR data.

2.4. Rose–Drago method

There is another graphical approach to the measurement of K_a that is worthy of comment before concluding this section. The Rose–Drago method[24] is a graphical solution to the simultaneous equations relating K_a to $\Delta\delta$, and as such it does not require the condition $[H]\approx[H]_0$. Like the

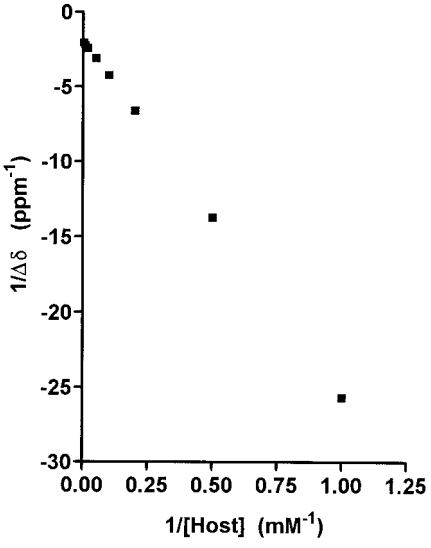


Figure 3. Illustration of the Benesi–Hildebrand Data Treatment. The data from Table 1 ($K_a=100\ M^{-1}$) are plotted as a double reciprocal plot. Non-weighted least squares fitting of this data gives $1/\Delta\delta_{max}=-1.909\ ppm^{-1}$ from the extrapolation to the abscissa and $1/\Delta\delta_{max}K_a=-23.685\ mM\ ppm^{-1}$ from the slope. Hence the Benesi–Hildebrand treatment gives $\Delta\delta_{max}=0.524$ ppm and $K_a=80.6\ M^{-1}$.

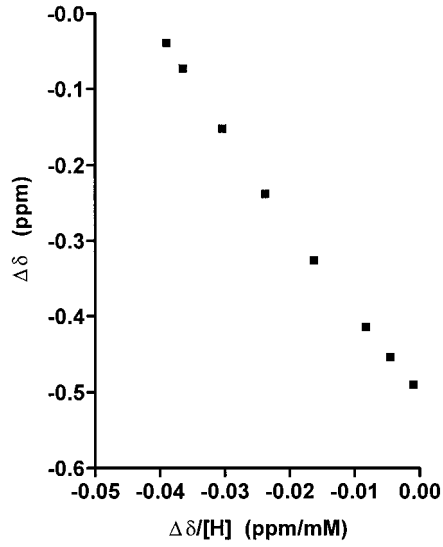


Figure 4. Illustration of the Scatchard Data Treatment. The data from Table 1 ($K_a=100\ M^{-1}$) are plotted as an *x*-reciprocal plot. Non-weighted least squares fitting of this data gives $\Delta\delta_{max}=-0.511$ ppm from the extrapolation and $-K_a=-0.08403\ mM^{-1}$ from the slope. Hence the Scatchard method gives $K_a=84\ M^{-1}$.

Table 2. Typical applications of linear methods to NMR chemical shift data (all for 1:1 complexes)

Study	System	Solvent	NMR nucleus[a]	Method[b]	$[G]_0$[c] (mM)	$[H]_0$[d] (mM)	K_a (M^{-1})	Comments
Carper[26] 1970	Trinitrobenzene π-complex	Several solvents	^1H 60	S	5–10	100–1600	0–5	
Roberts[27] 1975	Charge transfer complex	$(CHCl_2)_2$ $CHCl_3$	^{13}C 15.1	'inverse'[e] HAFF	167–333	up to 2333	0.2–1.6	Data from all carbons computer fitted
Bergeron[28] 1977	α-Cyclodextrin **1** inclusion complexes	D_2O	^1H 100	BH	5	5–50, 2–75	ca. 10^3	Computed binding curves
Bergeron[29] 1979	α-Cyclodextrin **1** inclusion complexes	D_2O	^1H 220	BH	6, 5	10–70, 1–100		
Gold[30] 1982	18-Crown-6 **3** acetonitrile complex	CCl_4	^1H 250	FF	5	800	2.1	
Haake[31] 1984	Phosphate anion-cation complex	D_2O/H_2O	^{31}P 80.9	BH (not cited)	10	20–200	<0.1–40	No citations to earlier methods
Cram[32] 1989	Cavitand complexes of CD_3CN	CCl_4	^1H 200	BH	$>10^2$	~3[f]	45–89	Iterative graphical procedure
Djedaini[33] 1990	β-Cyclodextrin **2** inclusion complex with **4**	D_2O	^1H 500, 600	FF	0.2	5–10	760	Includes Job plots
Chang[34] 1991	α-Cyclodextrin inclusion complex with **5**	D_2O	^1H 500	BH	10	4–70	53	Simultaneous fit to three ^1H shifts
Aoyama[35] 1992	Sugar/resorcinol complex	D_2O	^1H 270	BH	200–1600	0.5–2	1–100	100–800 Fold excess of guest[g]
Aoyama[36] 1994	Calixarenes of type **6**	D_2O	^1H 400	BH	1.5	10–55	33–70	Chiral host[h]
Pappalardo[37] 1998	Cyclodextrin inclusion complexes of **7**	D_2O	^1H 500	BH	<10	<10	740, 930	
Fish[38] 1998	Host–guest chemistry	D_2O	^1H 270, 500	FF		$10\times[G]_0$–$30\times[G]_0$	456–1040	
Lämsä[39] 1998	Crown ethers **8** with tropylium cations **9**	CD_3CN	^1H 200	BH	0.4–1	20–100	3–32	Similar results by UV/Vis spectroscopy

[a] Observed NMR nucleus (Larmor frequency, MHz).
[b] Methodology cited for data treatment. Most workers give brief information on data treatment and cite some previous work for details of how to calculate K_a. S, Scatchard; HAFF, Hanna–Ashbaugh–Foster–Fyfe; BH, Benesi–Hildebrand; FF, Foster–Fyfe.
[c] Concentration of the observed species (usually, but not always the guest molecule).
[d] Concentration range of the second molecule (required to be in excess to satisfy the Benesi–Hildebrand approximation).
[e] Referred to as 'inverse' because the donor was the observed species, rather than the more commonly observed acceptor for charge transfer complexes.
[f] The host protons were observed, allowing discrimination between two different binding pockets in the cavitand.
[g] Observed species was the host.
[h] The host was studied as a chiral shift reagent. K_as for pairs of guests (enantiomers) differ by approximately 10–25%.

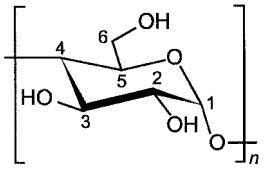
Benesi–Hildebrand experiment,[17] the original Rose–Drago method was devised to deal with UV–visible spectroscopy data.[24] Wachter and Fried published the NMR version for 1:1 complexes and derived the following relationship.[25]

$$(\Delta\delta_{max} - \Delta\delta)K_a = \Delta\delta\Delta\delta_{max}/(\Delta\delta_{max}[H]_0 - \Delta\delta[G]_0) \quad (7)$$

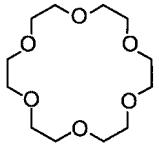
Values of K_a are calculated for a series of assumed $\Delta\delta_{max}$ values for each experimental host concentration. A graph of K_a^{-1} versus $\Delta\delta_{max}$ is constructed which contains a curve for each $[H]_0$. The intercept of these lines gives $1/K_a$ and $\Delta\delta_{max}$. The method is not currently used because it has been made obsolete by the curve fitting methods described in the following sections, but it still remains an ingenious solution.

2.5. Examples of Benesi–Hildebrand and Scatchard methods

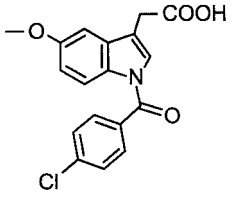
The traditional linearisation methods require measurements in the presence of a large excess of one of the reagents. These conditions are difficult (often impossible) to maintain, particularly for NMR experiments. Despite their limits, linearisation methods are often used to extract K_a from NMR titration data. Table 2 reviews some uses of these methods. The articles cited in Table 2 were chosen from a large number of published studies to give a flavour of the kind of work that has been done. One criteria for inclusion in the table was that the paper should include some detailed discussion of the data treatment used for the determination of K_a, or it should be a useful leading reference to other relevant work.



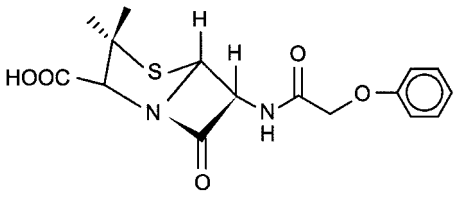
1 n=6 α-cyclodextrin
2 n=7 β-cyclodextrin



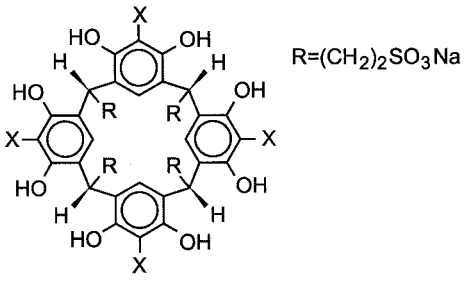
3 18-crown-6



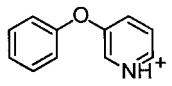
4 indomethacin



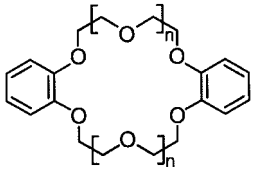
5 phenoxymethyl penicillin



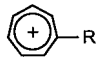
6a X=H
6b X=CH_3
6c X=OH



7 3-phenoxypyridinesulphate



8a n=1
8b n=2



9a R=H
9b R=OH
9c R=NH_2

The following conclusions may be drawn from Table 2. The Benesi–Hildebrand technique is being routinely used to study K_as in the range 10^2 to 10^3 M^{-1}, and the experiments require approximately 1–10 mM of the observed species. The requirement to work at the highest possible magnetic field (to maximise the frequency shift) is recognised. The requirement for at least a ten-fold excess of species H is often violated and hence the Benesi–Hildebrand approximation is sometimes used inappropriately. Workers do not always correctly cite the useful methodology papers, and this is probably a reflection of the fact that NMR is regarded as a routine tool in host–guest chemistry. References to the original Benesi–Hildebrand report alone are not useful.[‡]

[‡] The Benesi–Hildebrand paper is probably more often referred to than read. It is to be expected that most workers who have cited Benesi and Hildebrand, but have not provided further details of their data treatment, have probably used the Hannah–Ashbaugh or the Foster–Fyfe treatment.

Most of the experiments reviewed in Table 2 are concerned with observation of ^{1}H. This is because the success of the method requires a nucleus which is a sensitive reporter of its environment, ^{13}C chemical shifts being not very sensitive to environment changes.

It is likely that the Benesi–Hildebrand and Scatchard based methods continue to be used because they are simple and are universally accessible.

3. Curve Fitting Methods

The principle of curve fitting methods is that with knowledge of the complex stoichiometry a binding isotherm may be calculated and compared to the experimental data. $\Delta\delta_{max}$ and K_a are separate variables and the correct values of $\Delta\delta_{max}$ and K_a are those that produce the best fit of calculated to observed data.

3.1. Early iterative approaches

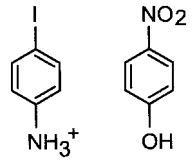
Several approaches developed in the late 1960s occupy the methodological middle ground between the previously described Rose–Drago method and fully computerised curve fitting.

Creswell and Allred studied the association of chloroform with benzene in cyclohexane.[40] In their data treatment they calculated X_{HG} for a series of assumed values of K_a. Only the series based on the correct K_a gives a linear plot of δ_{obs} against X_{HG}. Higuchi et al. described an iterative approach applicable to situations where $[G]_0 \approx [H]_0$.[41] The first step in their data treatment gives an approximate value of $1/\Delta\delta_{max}$ which is then used to obtain an approximate value of [HG]. A new value of $1/\Delta\delta_{max}$ is calculated and iteration continues until successive cycles yield convergent values.

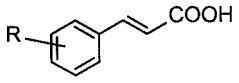
Lang's method[42] has sometimes been cited in the experimental sections of reports describing the estimation of K_a from NMR data.[43–45] The original paper by Lang described a modification of the Benesi–Hildebrand treatment for larger K_as.[42] Tentative values of ϵ (equivalent to $\Delta\delta_{max}$) and K_a were obtained and repeated cycling through a graphical method refines the first estimates. The restriction that $[H]_0 \gg [G]_0$ does not apply to this treatment.

3.2. Modern curve fitting procedures

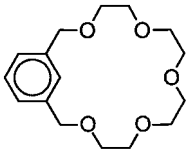
Curve fitting methods require no approximations and allow an almost unrestricted distribution of experimental points (concentrations). They are *correct* data treatments and should produce the most reliable and accurate measurements of K_a. A minor problem with curve fitting methods is the investment of effort required to establish a working procedure. An appraisal of the literature in this area shows that most workers have independently produced local solutions (i.e. written computer programs, or adapted commercial packages), and hence there is a proliferation of programs available to do the job.



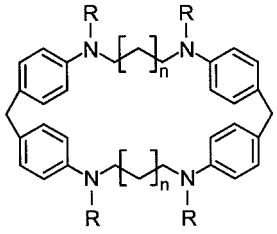
10 **11**



12a *trans-p*-methylcinnamate
12b *trans-m*-methylcinnamate



13 1',3'-xylyl-18-crown-5



14a n=4 R=CH_3
14b n=6 R=H, CH_3, or *p*-$SO_2C_6H_4CH_3$

Monographs on the determination of K_a by potentiometry and spectrophotometry have included compilations of computer programs, but these are not directly applicable to NMR data.[46,47] Previous reviews by Leggett et al.[48] and Tsukube et al.[13] noted some NMR specific programs. A survey for this report identified seven fully documented[§] computerised methods or programs for treatment of NMR data—KINFIT,[49] MICMAC,[50] Unnamed,[51] Unnamed,[52] EQNMR,[53] EMUL/MULTIFIT,[54] HYPNMR[55] and CALCK[56]—and many other programs, including the useful HOSTEST and NMRTIT that are described in varying detail in the methods sections of primary communications.

Table 3 is a summary of the most prominent work in this area and shows examples of the use of curve fitting procedures. Most of the reports that are cited in Table 3 give some explanation of the data treatment. The dates highlighted with bold text indicate papers that provide a particularly thorough explanation of the method and/or complete derivations of binding equations. The most frequently cited computer programs are highlighted with bold text and contact information is given where possible.

When discussing Table 3 it is convenient to use the terms host and guest, even when the terminology is not appropriate

[§] Description of the binding equations and the fitting algorithms is the main focus of a primary paper.

Table 3. Procedures used to extract K_a by curve fitting of NMR data

Study	System	NMR nucleus	H:G ratio	Computer program	Comments
Foster[57] 1971	Small molecule charge transfer complexes	^{1}H and ^{19}F	1:1, 2:1	Not named	Non-linear Scatchard plots are due to the formation of ternary complexes at high $[H]_0$
Wilson[58] 1972	DDT with small molecules and solvents	^{1}H	1:1, 2:1	BMDX85[a]	Four parameter fit for K_1, K_2, δ_1 and δ_2
Popov[59] 1973, 1977	Alkali metal solvation and crown ethers[b]	^{7}Li, ^{133}Cs, ^{133}Cs	1:1 1:1 1:1, 2:1	**KINFIT**[c]	A general purpose program fits several types of spectroscopy data
Reuben[60] 1973, **1978**	Shift reagent with DMSO	^{1}H	1:1, 1:2	Not named[d]	Four parameter fit for K_1, K_2, δ_1 and δ_2, plus details of data analysis and reliability criteria
Shapiro[61] **1975**	Shift reagent with ketones and alcohols	^{1}H	1:1, 1:2	LISA2[d,e]	Four parameter fit for K_1, K_2, δ_1 and δ_2, plus a general discussion of reliability
Foster[62] 1976, 1985	Small molecule donor/acceptor complexes	^{1}H, ^{13}C	1:1, 2:1	MINDS	Four parameter fit for K_1, K_2, δ_1 and δ_2
Hruska[63] 1976	Complexes of **10** and **11** with α-cyclodextrin	^{1}H	1:1	Not computed[f]	Noted that $\Delta\delta_{obs}$ is not sensitive to $K_a>10^3$
Laufer[64] 1978	Cyclodextrin complex with **12a**	^{13}C	1:1, 2:1	Not named[g]	Multiparameter fit for K_1, K_2, δ, δ_1, and δ_2 for every observed carbon in the guest molecule
Fujiwara[65] **1979**	$Sn(CH_3)_2Cl_2$ and pyridine	^{1}H {^{119}Sn}	1:1, 1:2	DAVID[h]	K_1 and K_2 estimated from ^{1}H chemical shift data, and from Δ^2J_{H-Sn}
Reinhoudt[66] 1982	Complexes of crown ethers **3** and **13**	^{1}H	1:1, 1:2	Not given	Variable temperature experiments gave ΔH and ΔS, in addition to K_1 and K_2
Horman[67] 1983, 1984	Small molecule complexes and caffeine dimers	^{1}H	1:1	Not named[i]	Both δ_0 and $\Delta\delta_{max}$ are fitted for equimolar solutions $[H]_0=[G]_0$
Lincoln[68] 1984	α-Cyclodextrin inclusion complex[j]	^{19}F	1:1, 2:1	DATAFIT[k]	Four parameter fit for K_1, K_2, δ_1 and δ_2
Wilcox[69] 1986, 1988	Cyclophane inclusion complexes	^{1}H	1:1	**HOSTEST**[l]	Two parameter fit to K_a and $\Delta\delta_{max}$
Shinkai[70] 1988	Calixarene complexes	^{1}H	1:1, 1:2	Not described	No details of binding measurements or data treatment
Schneider[71] 1988, 1989	Cyclophanes **14a** and **14b**	^{1}H	1:1	Homewritten PASCAL programs	K_a determined from the average of values obtained independently from each observed proton
Dougherty[72] **1988**, 1993	Macrocycle host–guest chemistry	^{1}H	1:1	**MULTIFIT** and **EMUL**[m]	K_a determined from a simultaneous fit to δ_{obs} of each guest proton
Koga[73] 1989	Cyclophane	^{1}H	1:1	DELTA[n]	No details
Whitlock[74] 1990	Cyclophane macrocycles	^{1}H and ^{31}P	1:1, 1:2	**NLSQ**[o]	No details
Djedaïni[75] 1991	β-Cyclodextrin steroid complex	^{1}H	1:1	COMPLEX[p]	Computer fitting of data from Job's plots
Izatt[76] 1992	Crown ether and NR_4^+ cation	^{1}H	1:1	EQDD	No details on K_a ΔH from variable temperature experiments
Yannakopoulou[77] 1993, **1995**	Cyclodextrin pheromone complexes	^{1}H	1:1	COMPLEX[q]	Two parameter fit, plus an approximate solution for the 2:1 complex

Table 3 (*continued*)

Study	System	NMR nucleus	H:G ratio	Computer program	Comments
Anslyn[78] **1993**	Bisguanidinium/phosphodiester complexes	^{31}P ^{1}H	1:1, 2:1 and 1:2	Not named	Double ended experiment $\Delta\delta_{GUEST}$ with ^{31}P (1:2), $\Delta\delta_{HOST}$ with ^{1}H (2:1)
Brown[79] 1994	Cyclodextrin bile salt anion complexes	^{1}H	1:1	Not described	Derivation of a simple relationship between $\Delta\delta_{obs}$ and K_a
Diederich[80] 1990, 1995	Cyclophane steroid complex	^{1}H	1:1	**ASSOCIATE**[r]	Two parameter fit to K_a and $\Delta\delta_{max}$.
Jaime[81] 1996	β-Cyclodextrin benzoic acid complex	^{1}H	1:1	**CALCK**[s]	Using $\Delta\delta$ values from both host and guest[t]
Loukas[82] **1997**	β-Cyclodextrin haloperidol complex	^{1}H	1:1	Not detailed	Three improved non-linear mathematical binding models
Hunter[83] **1998**	Ternary complex amide oligomer and nitrophenol	^{1}H	1:1, 1:2 and 2:1	**NMRTIT**[u]	Equations are given for binary equilibria and for the formation of ternary complexes
Dodziuk[84] 1999	α-Cyclodextrin camphor complex[v]	^{1}H	2:1	NMRTIT	Includes a comparison with results obtained from a modified B–H method

[a] Health Sciences Computing Facility, UCLA.
[b] For a recent review of the use of multinuclear NMR method to study crown ether metal complexes, see G. W. Buchanan *Prog. NMR Spectroscopy* **1999**, *34*, 327–377.
[c] Ref. 49.
[d] Cubic equations solved by Newton–Raphson method.
[e] Implemented on an IBM 360/65 computer and available from the authors.
[f] Order of magnitude estimate (lower bound) of K_a from a visual fit of the calculated curve to the experimental data.
[g] Schwartz, L. M.; Gelb, R. I. *Anal. Chem.* **1978**, *50*, 1571.
[h] Fletcher, R.; Powell, M. J. D. *Computer J.* **1963**, *6*, 163.
[i] Flow chart of program implemented on a Hewlett-Packard 3000 computer system.
[j] For a more recent report of the use of ^{19}F NMR to determine K_a, see Brown, S. E.; Lincoln et al., S. F. *J. Chem. Soc. Faraday Trans.* **1991**, *87*, 2699–2703.
[k] T. Kurucsev, University of Adelaide.
[l] v5.1 available from C. S. Wilcox, University of Pittsburgh.
[m] http://www.cco.caltech.edu/~dadgrp/dadftp.html
[n] A. Itai, University of Tokyo.
[o] Available from lilprofessor@chem.wisc.edu
[p] Djedaïni, F.; Berthanlt, P.; Perly, B. In Proceedings of the 6th International Symposium on Molecular Recognition and Inclusion, Berlin, L14, 1990.
[q] Djedaïni, F. PhD Thesis, Université de Paris Sud, 1991.
[r] Available from brpeters@chem.psu.edu
[s] Ref. 56.
[t] Data from both species were fitted.
[u] Available from c.hunter@sheffield.ac.uk
[v] Chiral discrimination between the two enantiomers of camphor.

in the context of the original study (i.e. early work on small molecule charge–transfer complexes, donor–acceptor complexes, and shift reagents). In these 'inappropriate' cases the term guest is used to indicate the NMR observed molecule.

A clear advantage of the curve fitting approach is that it is amenable to consideration of none 1:1 stoichiometries. When ternary complexes are formed, two equilibrium constants describe the system

$$H + G \rightleftharpoons HG \qquad K_1 = [HG]/[H][G]$$

$$HG + H \rightleftharpoons H_2G \qquad K_2 = [H_2G]/[HG][H]$$

δ_1 and δ_2 are used to distinguish the chemical shifts of the measured nuclei in the complex HG and H_2G, respectively (there are two $\Delta\delta_{max}$ values). The H_2G complex will be referred to as a 2:1 complex. A 1:2 complex implies two guest molecules associated with one host (HG_2).

Determination of K_a for ternary systems is essentially a problem of determining speciation. All of the programs are based around solutions of the general speciation Eq. (8) for 1:1 complexes (2 parameter fits), and more complicated (cubic) equations for 1:2 and 2:1 complexes (4 parameter fits).

$$[HG] = \frac{(Ka[H]_o + Ka[G]_o + 1) - \sqrt{\{(Ka[H]_o - Ka[G]_o)^2 + 2Ka[H]_o + 2Ka[G]_o + 1\}}}{2Ka} \tag{8}$$

Independently developed and adapted computer fitting programs are routinely applied. In the past year (1999), EQNMR,[85] AGRNMRL,[86] GRAFIT,[87] and GRAPHPAD PRISM[88] were also used to fit NMR data arising from complex formation between small molecules and macromolecules.

Are computer curve fitting methods superior to graphical methods? Provided that the experimental constraints are adhered to and appropriate weighting is used for the linear fitting, graphical methods do give correct results. The benefit of direct data fitting is that the experiments are not required to be performed in a large excess of one species, and ternary complex formation is tractable.

4. Diffusion Experiments

The pulsed field gradient (PFG) NMR technique has been used for some time as a direct measure of the molecular self-diffusion coefficient (D). The Stejskal–Tanner equation[89]

$$\ln A_g/A_0 = -\gamma^2 g^2 \delta^2 (\Delta - \delta/3) D \tag{9}$$

relates the signal intensity recorded from a PFG spin echo experiment (PFGSE) to the nuclear gyromagnetic ratio (γ, rad s g^{-1}), the strength (g, gauss cm^{-1}) and duration (δ, s) of the magnetic field gradient pulse, and the interval between the field gradient pulses (Δ, s) and the diffusion coefficient (D, $cm^2\ s^{-1}$). In the basic sequence, a 90° RF pulse transfers magnetisation to the xy plane where the magnetisation dephases. A 180° refocussing pulse produces a spin echo after an appropriate interval. Only spins that have undergone no net displacement during the interval Δ are refocussed, and hence the echo amplitude is related to D. The data are treated by plotting the log of the signal intensity against $\delta^2 g^2(\Delta - \delta/3)$ and the slope of this linear plot then gives the diffusion coefficient.

Pulsed field gradient NMR techniques have been widely applied to measurements of D in chemical systems. A stimulated echo experiment (STE) is additionally available,[90] and there are many variants of the basic pulse sequences.[91–94] The two modifications that have been most widely adopted and which have found routine use, are the longitudinal eddy current delay sequence (LED),[95] and the bipolar pulse pairs-LED sequence (BPPLED).[96]

The hardware necessary to perform PFG experiments (actively shielded z-gradients probe and a gradients driver) are now standard accessories from NMR spectrometer manufacturers. The hardware is the same as that required to perform gradients versions of regular 2D experiments (gradient enhanced spectroscopy),[94,97–100] and these experiments can therefore be implemented easily on modern machines. The accessible range of diffusion coefficients is from around 20 to $0.01\times10^{-6}\ cm^2\ s^{-1}$. Typically, the experiment times require about 15–20 min of spectrometer time to measure 10–20 gradient pulse increments (Δ or δ).

4.1. Direct measurements of D

The relevance of D to measurements of K_a is that D is a direct reporter of events such as molecular association and aggregation. It is well established that the molecular self-diffusion coefficient is related to molecular size—small molecules diffuse faster than larger molecules. In the field of host–guest chemistry, it is therefore reasonably expected that guest molecules (small) will have faster diffusion coefficients than host molecules (large). Additionally, in the case of fast exchange of the host–guest complex, the measured diffusion coefficient of, for instance, the guest molecule will be the mole fraction weighted average of the diffusion coefficients of bound and free molecules. This is exactly the same as that for any other NMR observable parameter, e.g. chemical shift or relaxation time as discussed in the other sections, and the treatment is the same.

$$D_{obs} = X_G D_G + X_{HG} D_{HG} \tag{10}$$

There is, however, an advantage in measuring D instead of δ, namely that the diffusion coefficient of the host–guest complex may not need to be treated as an unknown. It is assumed that, for binding of a small guest molecule to a large host molecule, the diffusion coefficient of the host is not greatly perturbed and the diffusion coefficient of the host–guest complex can be assumed to be the same as that of the non-complexed host molecule. An unknown parameter hence drops out of Eq. (10) and the system is in principle defined by a single experiment. Titrations are no longer required.

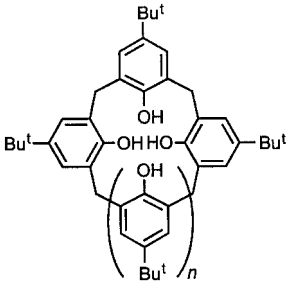
Table 4 provides an overview of the various systems that have been studied by the NMR PFG techniques and illustrates the ranges of diffusion coefficients that are typically

Table 4. Applications of PFGSE diffusion experiments to the determination of K_a

Study	System	D_{guest} ($cm^2\ s^{-1}$) ($\times 10^6$)	D_{host} ($cm^2\ s^{-1}$) ($\times 10^6$)	K_a (M^{-1})	Comments
Stilbs[101] 1983	Alcohols in α- and β-cyclodextrins	6.8[a]	2.7[b]	13–2100[c]	First application to host–guest chemistry. Methodology clearly described
Kuchel[102] 1994	2,3-Bisphosphoglycerate with hemoglobin	1.8–2.4	0.1	500–2500[d]	A study by ^{31}P NMR of diffusion in intact erythrocytes
Cohen[103] 1994	Methylammonium chloride in 18-crown-6, and [2.2.2]cryptand	13.8	4.5	34	K_a measured at 1:1 mole ratios (at 50 mM) in methanol and water solutions[e]
Larive[104] 1995	*cis* and *trans* Phenylalanylproline with β-cyclodextrin	5.7	3.2	(*cis*) 95	Data processed with DOSY methodology (see Section 4.1)
Cohen[105] 1995	Toluene, MeCN and $CHCl_3$ in *p-tert*-butylcalix[n]arenes **15**	20–23[f]	5.6–7.8[g]	Not determined	Several calixarenes studied. D_{host} correlates reasonably well with the size of the host
Cohen[106] 1997	Several macrocycles[h] with γ-cyclodextrin	ca. 5–6	3.0	ca. 10–187	Discusses the advantages and disadvantages of D, as a handle on K_a
Chang[107] 1998	16-Residue peptide binding to SDS micelles[i]	2.5	0.92	12,500	
Cohen[108] 1998	Arylammonium ions[j] with alkylated α- and β-cyclodextrins	5.9	2.8 to 3.3[g]	(+) 222 (−) 67	Demonstration of enantioselectivity of cyclodextrins
Larive[109] 1998	Two simple tripeptides[k] with SDS micelles	GHG 5.6 FHF 5.1	0.86	GHG 17 FHF 8[l]	Raw data analysed by DOSY methodology. A comparison between δ and D methods
Larive[110] 1999	Binding of TSP[m] to a 17-residue peptide	7.9	1.8[n]		TSP-peptide dimer binding equilibrium exhibits anti-cooperative bahaviour
Cohen[111] 1999	Encapsulation of C_6H_6 by a tetraurea calix4.arene dimer	c.a. 21	3.2–4.7[g]	8	Proof that $D_{guest}=D_{host}$ for fully encapsulated guest molecules

[a] Value for *n*-butanol.
[b] D of the host was unaffected by complexation of guests and D for α-cyclodextrin was not measurably different to β-cyclodextrin.
[c] Range of K_as measured for *n*-alcohols.
[d] Different K_as reported for carbomonoxygenated, oxygenated and deoxygenated hemoglobin.
[e] The example given in the table is for complexation with [2.2.2]cryptand in D_2O.
[f] The solvent molecules were all in the range $20–23\times10^{-6}\ cm^2\ s^{-1}$.
[g] Data are provided for several host compounds under a range of conditions. The values given here indicate the typical range of D_{host} values.
[h] 12-Crown-4 and its tetraaza and tetrathia analogues, cyclen and 1,4,7,10-tetrathiocyclododecane.
[i] Sodium dodecyl sulphonate.
[j] Propranolol, ephedrine and amphetamine. The example given in the table is for (+)- and (−)-propranolol.
[k] Glycyl-histidyl-glycine (GHG), and phenylaniline-histidyl-phenylaniline (FHF).
[l] Too high to measure.
[m] (Trimethylsilyl)propionic acid.
[n] For peptide dimer.

measured. Some of these examples are not host–guest complexes as the term is conventionally used. In these cases the terms guest and host are used to describe the smaller and the larger species, respectively. The use of the PFG-NMR method to characterise macromolecular interactions in biological systems has been reviewed.[112]



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The apparent plethora of different pulse programs for the same task can be disconcerting to the non-specialist. It is worthwhile reiterating that only two basic pulse sequences are used to obtain an echo (the observed signal) in NMR gradient diffusion experiments. These are the $90°–\tau–180°$ spin echo sequence,[89] and the $90°–\tau–90°–T–90°$ stimulated spin echo sequence.[90] Both of these pulse sequences exist as a family of experiments, each incorporating various extra features designed to achieve some particular improvement. To further summarise the studies reported in Table 4, the report from Stilbs[101] and the five reports from Cohen[103,105,106,108,111] are all based on the simple PFGSE[89] experiment, Larive[104] and Chang[107] report data from the PFGLED[90,94] sequence, and Larive[109,110] used the BPPLED[96] sequence. The data reported by Kuchel[102] are from both PFGSE[89] experiments and modified PFGLED[90,94] experiments. These pulse programs have additionally been popular for the study of protein oligomerisation.[113]

Shapiro et al. have examined the impact of chemical exchange[114] and nuclear Overhauser effects[115] on PFGSE

diffusion measurements. They show that both phenomena are capable of interfering with D measurements and need to be considered during studies of host–guest systems. They recommend the BPPLED experiment because it is immune to chemical exchange modulation,[114] and they advise observing protons that are not involved in intermolecular NOEs.[115]

4.2. DOSY, Affinity NMR and DECODES experiments

Diffusion and pulsed field gradients have been hot topics in the 1990s and several other interesting concepts have emerged which relate to the measurement of aggregation and binding.

DOSY (diffusion ordered spectroscopy) is an attempt to display the results of NMR diffusion experiments on a chart with a conventional chemical shift spectrum in one dimension and a 'spectrum' of diffusion coefficients in the other.[116,117] As such, it should be viewed more as a novel and sophisticated way to manipulate and display data, rather than anything different about the way the NMR diffusion experiments are performed. The principles and applications of DOSY spectroscopy have recently been fully reviewed.[118]

The principal advantage of DOSY over the PFGSE and PFGSTE experiments is its ability to fully resolve multi-component mixtures.[119] DOSY could therefore become a powerful tool for the study of binding equilibria in complex systems. This power seems rather unnecessary however for studies of simple two-component systems, and so DOSY is unlikely to be widely used in studies of host–guest systems. The only reported examples to date are the two entries listed in Table 4.

Finally, it is appropriate to draw attention to the most recent applications of pulsed field gradient diffusion spectroscopy as an aid to rapidly screening and identifying new drug compounds. 'Affinity NMR' identifies ligands from multi-component mixtures, resulting perhaps from combinatorial synthesis. The diffusion coefficient of a small molecule is altered by complexation with a receptor and becomes significantly different from the small molecules that are not complexed. Diffusion encoded spectroscopy (DECODES) uses the different diffusion coefficients as a spectral editing filter so that only the spectrum of the compound that binds can be seen and can be identified. These techniques have only been developed in the last few years, and so far, they have been used in an entirely qualitative way. The question has been simply 'does a molecule bind or does it not?' A recent review of this area is the best source of leading references.[120]

5. Relaxation Time (T_1) Measurements

The longitudinal or spin–lattice relaxation rate ($1/T_1$) and the transverse or spin–spin relaxation rate ($1/T_2$) are NMR parameters that may additionally be used to measure binding. In practice, most published studies relate to T_1 measurements. There has been little use of the relaxation time method in the field of host–guest chemistry. This is probably because measuring T_1 is a more tedious and more time-consuming process than measuring δ. T_1 measurements are most likely to be useful when complexation-induced chemical shifts are too small to be significant.

The relaxation time of a nucleus is the time taken for the nucleus to dissipate the energy absorbed by the RF pulse.[121] Without going into the details of relaxation theory, it is sufficient to note that the nuclear excited state is somewhat stable and requires an external stimulus in order to relax. For $I=1/2$ nuclei (i.e. ^{1}H and ^{13}C) the dominant source of this stimulus is the oscillating magnetic dipole field produced by other nearby nuclei and that this interaction is modulated by molecular motions. Relaxation is allowed when the fluctuating magnetic field matches the precession frequency of the observed nucleus (the Larmor frequency).

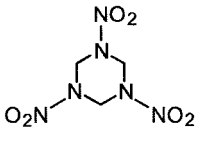
The molecular determinants of relaxation phenomena are well understood (the principal types of magnetic interaction are identified) and despite the complexity of the process, it is thus possible to make general statements about what might happen to relaxation times when a host–guest complex is formed. The details depend upon which nucleus is being observed, the dominant relaxation mechanism, and the relationship between the Larmor frequency and the motions of the molecule. Usually, for small organic molecules in non-viscous solvents, the motion that best matches the Larmor frequency is molecular rotation. Generally, the rotational correlation time (τ_c) is faster than the Larmor frequency and therefore slowing of the correlation time leads to a shorter T_1 (it is possible that large molecules may be tumbling at rates slower that the Larmor frequency and then slowing the correlation time further decouples the relaxation process and increases T_1). In simple terms therefore, binding will increase the rotational correlation time of the smaller molecule and hence, in general, the relaxation time will decrease. The smaller guest molecules would always be the observed species in relaxation studies.

There is a similarity between T_1 measurements and the diffusion based measurements described in the previous section. Both techniques are reporting on K_a via a parameter that is related to the size of the molecule (D or τ_c). Does the analogy with D go any further? When a small molecule binds to a large molecule, the small molecule takes on the diffusion characteristics of the larger species—translational motion is measured.[111] This statement is not true of τ_c modulated data where rotational motion is measured. A guest may be completely encapsulated by a host molecule, but it is not necessarily constrained to the same τ_c as the host molecule.

Behr and Lehn[122] studied the effect of encapsulation of *m*- and *p*-methylcinnamates **12a 12b** with α-cyclodextrin **1** on ^{2}H and ^{13}C relaxation times and found that the molecular motions of the encapsulated guest molecules were only weakly dynamically coupled to those of the host, i.e. in the host–guest complex, the host and guest have different molecular motions. Other studies of dynamic coupling in host–guest complexes have reached the same conclusion.[123–125]

The practicalities of determining K_a from relaxation data are no different than for any other NMR data. If the guest and

host–guest complex are in fast exchange the usual two-parameter fit is applied. Fast exchange during relaxation measurements of K_a is operationally defined[126] as when (a) the line shape of the resonance is Lorentzian, (b) the T_1 relaxation is a single exponential, and (c) the measured T_1 varies continuously as the ligand is added. The spin–lattice relaxation time (T_1) is usually measured by a $180°-\tau-90°$ pulse sequence. It should be noted however, that although spectroscopists generally talk in terms of relaxation *times*, it is the relaxation *rate* ($1/T_1$) that is directly proportional to chemical changes. When graphical methods are used, T_1 (reciprocal of rate) therefore appears on the *x*-axis and the graphs are not immediately obvious as reciprocal plots. If the system is in slow exchange it should be possible to fit bi-exponential functions to the data. Normally this would require at least a factor of two difference between the bound and free relaxation times to obtain a unique fit.



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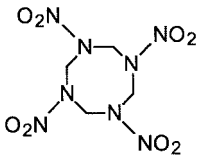

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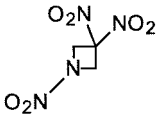

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A good example of the application of relaxation measurements to the determination of K_a for a host–guest complex has been reported by Cahill and Bulusu,[127] who studied the binding of the explosive nitramines RDX **16**, HMX **17** and TNAZ **18** with cyclodextrins. The complexation-induced shifts were negligible, but the T_1s of the nitramine methylene protons decreased on forming a complex. A simple two site exchange model with 1:1 binding was assumed,

$$R_{obs} = X_H R_H + X_{HG} R_{HG} \quad (11)$$

where R is the relaxation rate and the subscripts have their usual meaning. Data were obtained in the presence of a large excess of host and the data were analysed by the double reciprocal plot (Benesi–Hildebrand) method. A similar method and data treatment were used to determine K_a for the outer-sphere complex between pyridine and $[Co(CD_3OD)_6]^{2+}$.[128]

The ^1H relaxation time (T_1) of the solvent water was used as an indirect handle on the association between paramagnetic Gd(III) macrocycle complexes with β-cyclodextrin. Inclusion by cyclodextrin caused a decrease in the correlation time of the Gd complex and this in turn led to a decrease in the T_1 relaxation of the solvent protons. The data were analysed by the Scatchard method.[129] Again with cyclodextrins, ^{81}Br NMR linewidths (a T_2 observation) have been used to study competitive complexation of various anions against Br^-.[130]

The reports of James and Noggle on ^{23}Na NMR binding studies,[126,131] are of additional interest. Whilst not fitting in with the theme of host–guest chemistry, these are very lucid accounts of the use of relaxation time data, two-parameter computer fits[126] and graphical methods[131] to determine K_a. A review by Laszlo provides more information on the use of ^{23}Na NMR to measure binding constants.[132]

6. Errors, Reliability and Limitations

'NMR based determinations of K_a are usually only reliable for association constants in the range $10–10^4\ M^{-1}$.' This statement is of course a broad generalisation and requires some elaboration. The experimental data from a K_a measurement are concentrations and chemical shifts (or another NMR observable), and these need to be measured precisely. But what issues determine the accuracy of the resulting data? The key factor is that of separating the combined contributions of K_a and $\Delta\delta_{max}$ to $\Delta\delta$ in the binding isotherm.

6.1. The NMR observation

The chemical shift difference between free and bound states of the guest obviously needs to be as large as possible. This is always a case of the bigger the better. For ^1H observations of host–guest complexation, $\Delta\delta_{max}$ can be as much as 0.5 ppm or even greater. The ideal situation is when the observed proton is proximal to a highly anisotropic moiety in the complex (carbonyl or aromatic ring). The observed maximum shift is more likely to be only one half of this value and some reports are based on a $\Delta\delta_{max}$ of 0.1 ppm. For a typical spectrometer (400 MHz ^1H frequency) observing a sharp singlet (linewidth 0.2 Hz), the chemical shift can be measured with an accuracy of ± 0.005 ppm. The NMR line frequency is therefore often the most accurate measurement of the experiment.

6.2. Solution concentrations

The species concentration is critical and not as simple as it first might appear. The issue is not one of care in the preparation and dispensing of solutions (this being taken for granted), but about creating a series of solutions that can properly represent the binding curve, i.e. what concentrations of host and guest are required in order to produce curves similar to those shown in Fig. 1? Much has been written on this issue. In the 1960s Weber,[133,134] Person[135] and Deranleau[136,137] identified the main concerns in a series of papers describing the theory of binding measurements. These early papers discuss the graphical treatment of spectroscopic data, but the conclusions are general. Wilcox has discussed these issues from the perspective of a more up-to-date NMR curve fitting context.[138]

The principal findings are as follows:

1. A 'probability of binding' (p) is defined as the ratio of concentration of complex to maximum possible concentration of complex. This definition is good for strong as well as weak complexes because titration curves often pass through the point where $[G]_0=[H]_0$. This formulation recognises that the maximum possible concentration of complex is always the initial concentration of the minor component. A 'saturation fraction' has also been defined as the ratio between the actual complex concentration and the initial concentration of the reagent, the

chemical shift of which is being measured. This term is less useful for describing the strong binding situation because it does not reflect the fact that, at the start of the binding curve (the steeply rising line of Fig. 1), the concentration of complex is limited by the concentration of added host.

2. The minimum error in the measurement of K_a occurs at $p=0.5$, and the 'best' data are obtained from the range $0.2 \leq p \leq 0.8$. In other words, the most accurate values for K_a are obtained when the equilibrium concentration of the complex is approximately the same as the free concentration of the most dilute component.
3. The maximum information on the system comes from studying the widest possible range of p. At least 75% of the saturation curve is required in order to show correspondence between the equation of the model and the equation fitting the data (i.e. to verify that the binding model is based on the correct stoichiometry). In other words any binding data will fit a straight line over a suitably short range of p. If the experimental data are limited, higher order complexes should be verified to be absent.
4. Determination of the stoichiometry of a complex requires measurements at $p=1$ (i.e. at undetectable host or guest concentrations). Since these conditions are the opposite of those required for an accurate measure of K_a, the two experiments should be separated.
5. If graphical data treatments are used, the Scatchard method is preferable to the Benesi–Hildebrand or Scott methods.
6. Weber further suggested that the optimum method of performing a binding experiment is to start with an approximately equimolar (depending on the stoichiometry of the complex) mixture of host and guest and to successively dilute this solution until the limit of detection of the experiment is reached. This method seems eminently suited to computer analysis of the data, but it has not been widely used.

The above comments on saturation fraction are illustrated by reference to Fig. 1 and the constructed data set in Table 1. It can be seen that only the data for $K_a=10^2$ and $K_a=10^3$ adequately fit the $0.2 \leq p \leq 0.8$ criteria (three points in the correct range). For $K_a=10^4$ only one data point is at a concentration appropriate to the equilibrium constant being measured, and for $K_a=10^5$ none of the data points is adequate to define K_a.

The above topics cover the most important considerations regarding experimental set-up. Further developments of Weber, Person and Deranleau's ideas (mostly for weak 1:1 complexes and in the context of graphical data treatments) have resulted in more recommendations for the optimisation of experimental conditions for the determination of K_a.[139–142]

Quantitative comparisons between the different graphical data treatments have been made. On all occasions it was concluded that as long as due consideration was given to the limitations of the method (i.e. the proper range of saturation fraction), the results are not significantly different.[143–145] Christian et al. have argued that the graphical method should provide association constants virtually identical to the curve fitting methods providing that the data are correctly weighted in the least squares fitting.[146] These conclusions are borne out experimentally.[84]

Errors in stability constants due to deviation from the condition of fast exchange have been discussed by Feeney et al.[147] They point out that the rate of chemical exchange between bound and free guest is approximately related to the binding constant and, for $K_a>10^7$, most systems would be expected to be in slow exchange. It seems intuitively correct that large binding constants might correlate with slow ligand exchange and weakly associated complexes might be in fast exchange. This generalisation is not always true however, and there are examples of host–guest complexes with K_as in the 10 to 10^3 M^{-1} range, but where the chemical exchange is slow on the NMR timescale (see Section 9).

Some thought should be given to the choice of chemical shift reference material.[148] Normally, workers use a trimethylsilyl derivative or reference to a solvent peak. It should be verified that the reference material is not itself complexed by the host molecule. For studies with cyclodextrins, tetramethylammonium ion and methanol have been shown to be satisfactory internal references.[149]

Other features of the experimental design that should be considered are control of pH and ionic strength during titrations (possibly of confusing acid–base chemistry with binding phenomena). Results from data fitted to multi-component equilibria (four-parameter fits) need to be viewed with some caution.

6.3. Summary

K_a is best defined by titration data that curve measurably and approach a limiting shift. The problem with measuring small K_as (<10 M^{-1}) is that there is a large error associated with the extrapolation to $\Delta\delta_{max}$. The problem with measuring large K_as ($>10^5$ M^{-1}) is that there is no curvature in the $\Delta\delta$ versus $[H]_0/[G]_0$ plot at realistic reagent concentrations. The guest is effectively completely complexed by any available host and the graph therefore rises linearly with increasing $[H]_0$ until $\Delta\delta_{max}$ is reached at the 1:1 stoichiometry. The computed stability constant deviates from infinity only by virtue of experimental scatter in the data. This latter limitation is fundamental to the NMR method. In order to observe curvature in the $\Delta\delta$ versus $[H]_0/[G]_0$ plot the solutions would need to be diluted by several orders of magnitude (μmol range). NMR is however, an inherently insensitive technique, and experiments are routinely performed in the mmol range.

7. The Measurement of Very Small and Large K_as

7.1. K_a for very weak complexes ($K_a<10$ M^{-1})

Because weak complexation ($K_a<10$ M^{-1}) is not usually an issue in modern host–guest chemistry, this section will be limited to a brief summary. The subject matter is closely

associated with that already discussed in Section 6. Much of the published work discussing errors and reliability of K_a measurements appeared in the 1970s when the association of small molecules were the focus of attention. K_a values were often around 1–2 M^{-1} or even less. In these circumstances, factors such as solvation,[150,151] chemical shift referencing,[152,153] nonideality,[154,155] and unspecific shielding[156–158] either negate the assumptions of the simple data treatments or have important perturbing effects and cannot be ignored. Some modified graphical[159] and curve fitting[160] methods have also been proposed.

7.2. K_a for strong complexes ($K_a > 10^5\ M^{-1}$)

Competition methods have been used several times as a means of extending the range of NMR techniques beyond $K_a = 10^5\ M^{-1}$. The experiment is set up so that two host molecules compete for binding to a guest, or two guest molecules compete for a host. One of the binding constants is known and the experiment gives the ratio of the known and unknown binding constants.

Reinhoudt et al. studied the binding of alkylammonium salts to crown ethers.[161,162] The association constant for 1:1 binding of *t*-butylammonium perchlorate with 1,3-xylyl-18-crown-5 **13** was readily determined from observations of the upfield shift of the *t*-Bu signal as a function of [**13**] and curve fitting.[162] In similar experiments using 18-crown-6 **3** however the *t*-Bu signal moved downfield by only a small amount and saturation binding was reached at low mole ratios of **3** to *t*-$BuNH_3ClO_4$ (indicating strong binding). K_a could not be reliably determined from the data. In the competition experiment **13** (H1) competed with **3** (H2) for complexation to the *t*-$BuNH_3ClO_4$. The total crown ether concentration was always kept larger than the salt concentration in order to make the free *t*-$BuNH_3ClO_4$ concentration negligible. The following relationships ensue

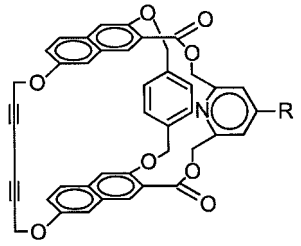
$$\delta_{obs} = X_{H1\cdot G}\delta_{H1\cdot G} + X_{H2\cdot G}\delta_{H2\cdot G} \tag{12}$$

$$X_{H2\cdot G} = (\delta_{obs} - \delta_{H1\cdot G})/(\delta_{H2\cdot G} - \delta_{H1\cdot G}) \tag{13}$$

and

$$K_{rel} = K_2/K_1 = [H2\cdot G][H1]/[H2][H1\cdot G] \tag{14}$$

The limiting chemical shift of the weaker crown ether complex ($\delta_{H1\cdot G}$) was known from the titration experiment. The limiting chemical shift of the stronger crown ether complex ($\delta_{H2\cdot G}$) was directly measurable. Hence from the observed chemical shift it was possible to calculate the concentrations of the *t*-Bu **13** and *t*-Bu **3** complexes. Using these concentrations, the free crown ether concentrations and the relative association constant as defined in Eq. (14) could be calculated. From the relative association constant and the known association constant of $tBuNH_3^+$ with **13**, the association constant of the **3** $tBuNH_3^+$ complex could be found. A prerequisite of this experiment is that K_a for the reference complex and $\Delta\delta_{max}$ for both complexes must be known, and $\Delta\delta_{max}$ values must be significantly different.



19a R=$N(CH_3)_2$
19b R=H

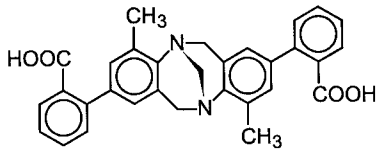
Whitlock and Whitlock described an experiment where two different cyclophane hosts **19a** and **19b** are presented with a limited amount of one guest.[163] Varying amounts of guest (*p*-nitrophenol) were added to a mixture of host H1 and host H2, and proton signals of the host spectra were followed. The following relationships were found to apply

$$X_{H1\cdot G} = (\delta_{H1} - \delta_{obs})/(\delta_{H1} - \delta_{H1\cdot G}) \text{ and } X_{H2\cdot G} = (\delta_{H2} - \delta_{obs})/(\delta_{H2} - \delta_{H2\cdot G}) \tag{15}$$

$$K_{rel} = (1/X_{H1\cdot G} - 1)/(1/X_{H2\cdot G} - 1) \tag{16}$$

$\delta_{H1\cdot G}$ and $\delta_{H2\cdot G}$ were assumed to be those which were observed when the guest/host ratio was large, and again [G] was assumed to be zero. The NMR experiment thus gives the mole fractions of each host that is bound and, from this, K_{rel}. In the above example, K_a for binding of *p*-nitrophenol to the cyclophane host was estimated by curve fitting of titration data to be 24,000 M^{-1}. The more accurate value determined from the competition experiment (with a $K_a = 6000\ M^{-1}$ for cyclophane as a reference) was 96,000 M^{-1}. An additional advantage of this method is that exact measurements of $[H1]_0$, $[H2]_0$ and $[G]_0$ are not required.

Boss and Popov[164] studied the competitive binding of two metal cations for 18-crown-6 using one of the metal ions as the NMR probe nucleus (^{133}Cs or ^{23}Na). The experiment requires titration of varying amounts of the host into a solution of the salts of two cation guests (10 mM each). The data treatment requires least squares fitting of a calculated curve to the experimental data in a manner exactly analogous to the two-parameter fit described in Section 3. In this case, the polynomial expression for the speciation is derived from the mass balance and equilibrium constant expressions for a three-component system with two association constants. The data are fitted by adjustment of the two K_as and the limiting chemical shift of the bound metal. Both 1:1 and 2:1 complexes are considered. As a test of the method, the binding constant of the K^+ 18C6 complex was determined to be $1.51\times10^6\ M^{-1}$ in a competition experiment against Cs^+ 18C6.



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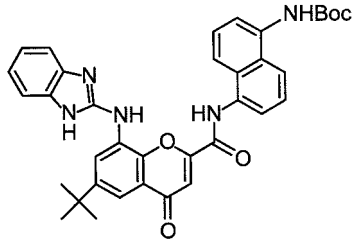

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Wilcox et al.[165] have also described a method for the quantitative analysis of continuous titration competition data from three-component mixtures (one macrocyclic host **20** and two small molecule guests). The method is based upon a careful and complete consideration of all possible intermolecular equilibria (including dimer formation) and inclusion of exact terms for these equilibria. It was possible to measure a K_a of 505,000 M^{-1}. Other treatments of NMR data from multiple equilibria systems can be found,[166,167] and there are several other reports of the use of competitive scales[168–170] including a measurement of K_a=5.5×10^7 M^{-1} for the complex of $Cl_2CHCOOH$ with host **21**.[169]

8. Miscellaneous

Homomolecular interactions (oligomerisation) have been specifically excluded from discussion in this review, however, data treatments specific for dimerisation are available.[171–174] The large body of information that has been published on lanthanide shift reagents has not been considered as a source for this article. Nonetheless, methods for obtaining association constants from NMR data are the same as those reported here and the determination of speciation is essential to the data analysis.[175,176] A mathematical model has been proposed that allows determination of K_a and $\Delta\delta_{max}$ for heteroligand complexes of 1:1:1 and 1:2:1 stoichiometries.[177] Anslyn et al. discussed the complications that arise in NMR data when host–host and guest–guest interactions occur as well as the host–guest interaction.[178] 'Single point' binding experiments can be performed if $\Delta\delta_{max}$ is known, or can be assumed to be unchanging.[179–181] Binding experiments are generally performed to obtain information on K_a and the $\Delta\delta_{max}$ data are not discussed. The complexation-induced change in chemical shift[8] contains useful structural information which is waiting to be interpreted.[182–185]

9. Slow Exchange Systems

The emphasis of this report has been on the treatment of systems that are in rapid exchange on the NMR time scale because the majority of host–guest systems are of this type. However, slow exchange host–guest systems do occur.[186–195] Treatment of such systems is simple. The bound and free molecules give rise to discrete NMR signals that can be integrated to determine [G] and [HG] directly, and hence K_a. The occasional observation of slow chemical exchange in only moderately tightly bound host–guest systems (K_a ca. 10^3)[186,187,191,193,195] underlines the statement that the rate of exchange of ligand does not necessarily correlate with the binding constant.

10. Conclusion

A major advantage of the NMR method over other techniques is that the results are not greatly affected by the presence of minor impurities and valuable structural information can be obtained.

NMR titration methods are most useful to study association constants in the range 10–10^4 M^{-1}. To maximise the reliability of NMR titration data, the experiment needs to be designed so that the binding curve covers a large range of % bound (ideally from 20 to 80%). For K_a below about 1–5 M^{-1}, $\Delta\delta_{max}$ cannot be accurately measured. Above $K_a \sim 10^5$ M^{-1}, graphs of $\Delta\delta$ vs $[H]_0/[G]_0$ become too steep to determine within convenient measurement times. More sensitive NMR probes will extend slightly the range of measurable association constants.

Graphical (linearisation) methods were developed before computers became cheap and powerful. They continue to be used, probably because they are simple and can be implemented without any resources. Curve fitting approaches are more widely used. Clear advantages of curve fitting treatments are that the experimental conditions are less constrained and more complex binding models (non 1:1 stoichiometries) can be accommodated.

Diffusion experiments are a very attractive way of measuring K_a between molecules of different sizes. This technique can be quite routine, and it is therefore likely to be increasingly used in the future.

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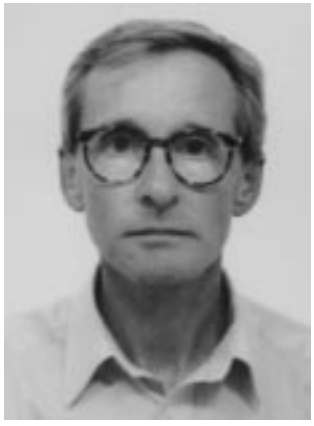
Biographical sketch



Lee Fielding was born in Widnes, England in 1953. He received his undergraduate education at Preston Polytechnic. He worked in industry, studied inorganic sulphur-nitrogen chemistry with Tristram Chivers (University of Calgary, Canada, MSc, 1978), worked again in industry, and then returned to academia to study spin-labelled iron porphyrins with Gareth and Sandra Eaton (University of Denver, Colorado, PhD, 1985). Since 1989 he has been at Organon Laboratories where he is currently head of the Spectroscopy Section, Department of Analytical Chemistry. He is interested in the structure elucidation of small molecules. He is married (1 child—4 years old) and enjoys hiking in the mountains of Scotland.