CHAPTER-II....

General Discussion
NMR spectroscopy has become the most important tool for the structure elucidation of organic compounds, particularly in the solution state. The method is of increasing significance for most CD application studies. There are few alternatives to NMR spectroscopy in the CD related studies.\textsuperscript{206-211}

As with many carbohydrates, it is often difficult to obtain single crystal of CD derivatives and then to analyze them by X-ray crystallography, or by neutron diffraction. Other techniques such as fluorescence, UV/visible spectroscopy, calorimetry etc. play a major role in measuring complexation energetics with CDs but usually provide very indirect and qualitative information about inclusion modes and geometries.

Structure determination is of particular importance for supramolecular host-guest complexes, which are the basis of most CD applications in medicine, catalysis, separation and sensor technology and also food chemistry. Pharmaceutical uses of CDs for drug protection or targeting now legally require structure determination of the administered compounds. NMR spectroscopy is also becoming an important tool for \textit{in vitro}, in future perhaps even for \textit{in vivo}, studies of CD interactions with biological macromolecules such as nucleic acids, proteins, or cell membranes. The most obvious incentive to use NMR techniques for the investigation of CD complexes is the interest to understand the driving forces and binding modes in these non-covalent associations, and then to make optimal use of these factors for new applications. It should be remembered that the driving force for CD inclusion often is of solvophobic nature and that most CD applications involve action in a liquid matrix, which emphasizes again the role of NMR spectroscopy as the most important method applicable in solution.

After the first publication on the use of NMR spectroscopy to study inclusion phenomenon by Demarco and Thakkar,\textsuperscript{212} there has been a virtual explosion of such studies. The older work was restricted to the observation of few CD protons, mostly at the anomeric centers which were sufficiently separated from other strongly coupled signals. The advent of high-field instruments and in particular 2D methods has completely changed the situation, and the possibilities of now available NMR techniques are far from being exploited. Nuclear Overhauser Effects (NOEs) have already becomes a major tool in structural studies of complex biomolecules.
The spectacular advances of NMR techniques, have led to much more detailed structural information of CDs and their complexes. These tasks represent a fascinating challenge for the NMR spectroscopist in view of the high complexity of the underlying cycloamylose \( ^1H \) NMR spin systems.\textsuperscript{213-215} These are characterized by signals which, apart from the anomeric proton, absorb in a range of only 0.5 ppm and are strongly coupled. In addition, the shielding effects of the CD cavity on the included guest molecules are limited to a few tenths of a ppm at most, as a consequence of a host framework being built up entirely of single, less polar and polarizable bonds and thus weak shift tensors.

The aim of the present discussion is not to discuss or even to mention all the work on the study of the CD complexes using NMR spectroscopy. Instead we will illustrate the use of NMR techniques in the structure elucidation of CD inclusion complexes with some representative examples, yet without a special focus on this method.

Demarco and Thakkar,\textsuperscript{212} first noticed the highfield chemical shift changes in the \( \beta \)-CD cavity protons, namely H-3' and H-5', in the presence of a variety of aromatic substrates in aqueous solution and envisaged from these observations that the aromatic ring is positioned in the \( \beta \)-CD cavity. This observation later became the basis for NMR spectroscopic study of the CD inclusion complexes. He used 100 MHz instrument for these studies which is actually not suited for this type of work because all the CD protons, except H-1', resonate in the 0.5 ppm range and are not easily distinguished. He established the assignment for each proton on the basis of analysis of individual splitting patterns and coupling constants at 220 MHz, decoupling experiments and expected chemical behaviour i.e. H-3' and H-5' being 1, 3-diaxial to the \( C_1 \) axial oxygen should resonate at lower fields than the H-2' and H-4' protons.

At magnetic fields above 9.4 T, corresponding to 400 MHz for the \( ^1H \) NMR spectra, the dispersion is high enough to locate, in conventional one dimensional spectrum, most of the CD protons, eased by high symmetry of the macrocycles. The \( ^1H \) NMR spectrum of \( \beta \)-CD in aqueous solution has already been assigned in detail. It has been established that, on the NMR time scale, all the seven glucose units have identical conformations and the molecule is highly symmetrical. Furthermore, the magnitude of the vicinal coupling constants \( J_{1,2} \) through to \( J_{4,5} \) are consistent with the \( C_1 \) chair form for the glucose units of CDs.\textsuperscript{216}
It is now well accepted that the signals of the cavity protons move highfield when the guest molecule or a part of it enters the CD cavity. The chemical shift change data obtained from $^1$H NMR titration experiments can be used to determine stoichiometry, binding constant and is thus helpful in the determination of structure of the complex.

The magnitude of the chemical shift changes for the protons positioned in the CD-cavity, $\Delta\delta_{H-3}^{\beta}$ and $\Delta\delta_{H-5}^{\beta}$, increases with an increase in the concentration of the guest$^{217,218}$ while that for guest protons increases with an increase in the concentration of the CD.$^{219}$ The magnitude of the $\Delta\delta_{H-3}^{\beta}$ and $\Delta\delta_{H-5}^{\beta}$ also depends on the nature of the guest. The chemical shift changes are high in the case of aromatic guests while these are relatively small if the aliphatic guest enters the CD cavity. Salbutamol (25) forms a 1:1 inclusion complex with $\beta$-CD whose structure has been confirmed by detailed NMR spectroscopic and molecular modeling studies (Fig. 5).$^{220}$ It has been established that aliphatic part of the guest enters the CD cavity. Fig. 6 shows very small shift changes in the $\beta$-CD cavity protons in the presence of salbutamol. On the other hand these shift changes are quite large when aromatic ring of (1S, 2R)-(+)-ephedrine (26) enters the $\beta$-CD cavity.$^{221}$

![Salbutamol-$\beta$-CD complex](image1)

![Salbutamol-$\beta$-CD complex](image2)

**Fig. 5** Structures of inclusion complexes of salbutamol and (1S, 2R)-(+)-ephedrine.
Fig. 6 Comparative chemical shift changes in the β-CD cavity protons upon inclusion of an aromatic guest [A] and an aliphatic guest [B]. The [H]/[G] is same in both cases.

The CDs generally act as one site ligand and the guest enters the cavity from wider rim side. This is always true for α-CD but in the case of β- and γ-CDs, complexes formed by the penetration of the guest from narrower rim side have also been reported. The relative chemical shift change data for the cavity protons (Δδ₃' and Δδ₅') is sometimes taken as an evidence for the mode of penetration of the guest. It has been suggested that in the cases where Δδ₃' > Δδ₅' the guest entry is from wider side and vice versa. This statement may be true only when the guest is bulky but can not be generalized.

Kano studied the complexation of pure enantiomers of binaphthyl derivatives with various CDs. The chemical shift changes observed for H-3' of heptakis(2, 3, 6-tri-O-methyl)-β-CD (TMe-β-CD) were quite high compared to those for H-5' (Δδ₃' > Δδ₅') in the presence of 1, 1'-binaphthyl-2,2'-diyl hydrogen phosphate (27). It was established that
27 is shallowly bound to the wider side of the TMe-β-CD cavity. The guest being bulky cannot penetrate deep into the cavity. Moreover, the mode of penetration of the two enantiomers was found different (Fig. 7).

Fig. 7 Modes of penetration of two enantiomers of 27 into the β-CD cavity.

He also studied the complexation of several helical metal complexes with modified CDs. Λ-Ru(phen)$_3^{2+}$ ion (28) forms a complex with heptakis(6-carboxymethylthio-6-deoxy)-β-CD (per-CO$_2$-β-CD) (Fig. 8). It has been shown that the guest approaches the cavity from narrower rim side and for this complex the chemical shift for H-5' was quite high compared to that for H-3' ($\Delta\delta_{H-5'} > \Delta\delta_{H-3'}$).$^{223}$ These examples show that the relative values for $\Delta\delta_{H-3'}$ and $\Delta\delta_{H-5'}$ can be used in support of the mode of penetration of guest into the CD cavity.$^{224}$

Fig. 8 Mode of inclusion of 28 into the (per-CO$_2$)-β-CD cavity.
Rekharsky, however, made a detailed $^1$H NMR study of the CD inclusion complexes of a variety of guests and showed that while the magnitude of the chemical shift changes for H-3' and H-5' protons is a quantitative measure of the stability of the complexes, their ratios, $\Delta \delta_{H-5}/\Delta \delta_{H-3}$, are related to the depth of penetration of the guest into the CD cavity. In all the studied cases, the guest approached the β-CD cavity from wider rim side though $\Delta \delta_{H-5} > \Delta \delta_{H-3}$. He showed that higher magnitude of $\Delta \delta_{H-3}$ and $\Delta \delta_{H-5}$ values is due to higher stability of the complex. On the other hand, their ratios, $\Delta \delta_{H-5}/\Delta \delta_{H-3}$, which were found as high as 1.2-3.0 for β-CD, indicate a deep penetration of the guest into the host cavity. It becomes obvious from these examples that the use of relative magnitude of chemical shift changes for cavity protons as an evidence in support of the mode of penetration of guest into the CD cavity cannot be generalized (Table 7). In fact the mode of penetration can better be determined by NOE experiments.

**Table 7** Chemical shift changes ($\Delta \delta$) of H-3' and H-5' of β-CD protons upon complexation with selected ligands in buffered aqueous solutions at $pD = 7.0$ and $T = 298.15$ K. The mode of penetration of the guest into the β-CD cavity is from wider rim side in all the cases.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>[G]/[H]</th>
<th>H-3'</th>
<th>H-5'</th>
<th>H-5'/H-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenethylamine</td>
<td>0.94</td>
<td>0.03</td>
<td>0.06</td>
<td>2.0</td>
</tr>
<tr>
<td>L-α-O-Benzylglycerol</td>
<td>0.59</td>
<td>0.03</td>
<td>0.09</td>
<td>3.0</td>
</tr>
<tr>
<td>l-Benzylimidazole</td>
<td>0.77</td>
<td>0.09</td>
<td>0.18</td>
<td>2.0</td>
</tr>
<tr>
<td>4-Benzylpiperidine</td>
<td>0.94</td>
<td>0.14</td>
<td>0.22</td>
<td>1.6</td>
</tr>
<tr>
<td>l-Butylimidazole</td>
<td>0.95</td>
<td>0.05</td>
<td>0.09</td>
<td>1.8</td>
</tr>
<tr>
<td>(1S, 2S)-(+)-Pseudoephedrine</td>
<td>0.98</td>
<td>0.07</td>
<td>0.12</td>
<td>1.7</td>
</tr>
<tr>
<td>(1S, 2R)-(+)-Ephedrine</td>
<td>1.00</td>
<td>0.07</td>
<td>0.12</td>
<td>1.7</td>
</tr>
<tr>
<td>(1R, 2S)-(-)-Ephedrine</td>
<td>0.97</td>
<td>0.06</td>
<td>0.10</td>
<td>1.7</td>
</tr>
<tr>
<td>Hydrocinnamate</td>
<td>0.92</td>
<td>0.04</td>
<td>0.10</td>
<td>2.5</td>
</tr>
<tr>
<td>Phenyl-β-D-glucopyranoside</td>
<td>0.81</td>
<td>0.02</td>
<td>0.05</td>
<td>2.5</td>
</tr>
<tr>
<td>3-Phenyl-1-propylamine</td>
<td>1.03</td>
<td>0.10</td>
<td>0.12</td>
<td>1.2</td>
</tr>
</tbody>
</table>
The formation of the CD inclusion complex also results in the concomitant chemical shift changes for the guest protons and generally all the guest protons, and not only the part included in the CD cavity, show downfield shift changes, but sometimes upfield changes are also observed. These changes have mostly been found to be of qualitative significance though these may also provide important information regarding the structure of the complex.

Due to lower water solubility of the guest, sometimes it is not possible to use high concentration of the guest in the NMR titrations and in such cases the chemical shift changes observed for the CD cavity protons may be very insignificant while large chemical shift changes are observed for guest protons. The chemical shift change data for guest protons can be used for determining stoichiometry and binding constant of the complex but whether guest enters the CD cavity or not can only be said on the basis of chemical shift changes in the cavity protons. ROESY experiments may be required in such cases to ascertain whether guest has actually entered the CD cavity.

**Nuclear Overhauser Effect (NOE)**

Beside the chemical shift changes, observed for hosts and guests, upon complexation relative to unbound state, detection of the through-space dipole-dipole interactions (NOE and/or ROESY) between the CDs and the guests is another important probe of the structure of complexes. NOEs have been used for the study of CD complexes in solution since the pioneering work of Bergeron and Rowan. Though the advent of high magnetic fields has greatly enhanced the dispersion of signals; at the same time, however, the unfavourable correlation times of complexes with molecular weights around $10^3$ lead to a drop of the observed NOEs, e.g. from 34% at 90 MHz to 9% at 250 MHz. To obtain sizeable NOEs on a 400 or 500 MHz instrument, application of spin-lock techniques such as ROESY are required. In the ROESY spectra, artifacts which possess phase properties different than genuine NOE cross peaks are frequently observed between resonances within a common $J$-coupling network, thus being easily distinguishable. Intermolecularly, as in case of CD complexes, these COSY-type peaks are not observed; however, false cross peaks with the same phase as genuine interactions may arise from scalar transfer from H-3' and/or H-5', the cavity protons being most prone to interact dipolarly with the guest.
The use of NOEs in the structure elucidation of CD complexes is exemplified by few representative examples. A typical NOE application is illustrated with α-CD complexes with phenol derivatives. Fig. 9 describes different inclusion modes for the cyclodextrins complexes with phenol derivatives and, obviously, all three modes should lead to quite different NOEs and should be distinguishable this way.

![Mode I](image1)
![Mode II](image2)
![Mode III](image3)

**Fig. 9** Various possible modes of penetration of phenol derivatives into the CD cavity.

For Mode I with no or little immersion of the phenyl ring into the cavity there is a sizeable contact only between the guest Hₘ and H-3’ of α-CD and one can expect only small ROE at Hₘ upon irradiation of H-3’. For Mode II irradiation at H-3’ should lead to ROE of both Hₒ and Hₘ signals, at H-5’ only of the m-proton. In contrast, no effect on the Hₘ signal upon irradiation of H-5’ can be expected for Mode III. The 2D ROESY spectrum of the p-iodophenolate with α-CD (Fig. 10) shows only those cross peaks discussed for Mode II.²²⁸
Another interesting example is structure elucidation of a 1:1 complex between benzoic acid and β-CD.\textsuperscript{217} 1D ROESY experiments were performed to confirm the structure of the complex. The NOEs on the CD protons were studied on saturation of various aromatic protons. The irradiation of \( H_p \) did not show any NOE on the CD cavity protons. However, intermolecular NOEs were observed on saturation of \( H_o, H_m, H-3' \) and \( H-5' \). Almost equal NOE values were observed on both the cavity protons when \( H_o \) was irradiated, while the NOE value for \( H_m \) to \( H-3' \) was larger than for \( H_m \) and \( H-5' \). These results clearly suggest inclusion geometry with the aromatic \( H_p \) proton located at the CD equatorial plane, \( H_p \) on the chiral vertical axis, and \( H_o \) at similar distances from both the cavity protons. Both the geometries are compatible with this NOE data. However, NOEs on both the cavity protons upon saturation of \( H_m \) can only be explained when a fast equilibrium exists between the two inclusion complexes (Fig. 11).
Fig. 11 Structures of two 1:1 β-CD-benzoic acid inclusion complexes.

The use of ROESY in the study of chiral recognition mechanism is exemplified by the following example. Chiral recognition of an anionic tetrahelicene (29) has been studied by native CDs. Fig. 12 shows the ROESY spectra of β-CD-(M)-29 and β-CD-(P)-29 systems in D$_2$O which show different connectivities of the cavity protons with the protons of two enantiomers. It was interpreted from these results that both CO$_2^-$ groups of 29 are placed near the rim of the secondary OH group side of β-CD and ring A of the 29 penetrate the β-CD cavity. It was shown that there was somewhat deeper penetration of the ring A of (P)-29 compared to that of (M)-29 into the CD cavity.229
Stoichiometry and Binding Constant of the Complex

The most commonly claimed stoichiometric ratio for CD complexes is 1:1 which is usually justified. Nevertheless, other ratios are known, most common of these probably being 2:1 (H/G) while the ratios 1:2 and 2:2 have also been reported. Before any structural or association constant determination is performed, it is always essential to determine the stoichiometry of the host-guest complex which is readily achieved from NMR titration data. There are several methods used to determine the stoichiometry, namely continuous variation method (Job’s plot), molar ratio method and several modified Benesi-Hildebrand methods.

Job’s Plot

The method of continuous variations involves preparing a series of solutions containing both the host and guest in varying proportions so that a complete range of mole ratios is sampled (0 > [H]/[H]+[G] < 1) and where the total concentration [H]+[G] is kept 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 [H]/[H]+[G]×Δδobs.
versus \([H]/[H]+[G]\). The position of the maximum indicates the stoichiometry of the complex (Fig. 13).

\[\text{Fig. 13 A typical Job's plot for 1:1 inclusion complex.}\]
The Mole-Ratio Method

In this method a series of solutions is prepared in which the formal concentration of one of the components is held constant while that of the other is varied. A plot of the chemical shift change ($\Delta \delta$) versus mole ratio of the components is then prepared. If the formation constant is reasonably favourable, two straight lines of different slopes are obtained; the intersection occurs at a mole ratio corresponding to the combining ratio in the complex (Fig. 14).

Fig. 14 A typical molar ratio plot showing 1:1 stoichiometry of the complex.
**Modification of Benesi-Hildebrand Method**

The stoichiometry and association constant of the inclusion complex can also be determined by any of the following methods. Assuming that the composition of the complex is 1:1, the following expression can be written:

\[ G + H \rightarrow GH \]

The association constant of the complex \((K_a)\) is given by

\[ K_a = \frac{[GH]}{[H][G]} \]

Where \([H]\), \([G]\) and \([GH]\) are equilibrium concentration of host, guest and complex, respectively. Benesi-Hildebrand studied the complexation of iodine with aromatic hydrocarbons by UV-visible spectroscopy\(^{232}\) and derived the following linear equation (equation 1) for the calculation of \(K_a\):

\[ \frac{1}{\varepsilon_{\text{obs}}} = \frac{1}{(K_a \varepsilon_{\text{max}} [H])} + \frac{1}{\varepsilon_{\text{max}}} \]  

where \(\varepsilon_{\text{obs}}\) is the extinction of a layer of solution 1 cm deep containing \(m\) moles of \(I_2\) and \(\varepsilon_{\text{max}}\) is the molar extinction coefficient of the complex at the wavelength of maximum absorption. NMR version of the Benesi-Hildebrand equation was independently derived by Mathur et. al.\(^{233}\) and Hanna and Ashbaugh\(^{234}\) (equation 2).

\[ \frac{1}{\Delta \delta_{\text{obs}}} = \frac{1}{(K_a \Delta \delta_{\text{max}} [H])} + \frac{1}{\Delta \delta_{\text{max}}} \]

where \(\Delta \delta_{\text{obs}} = (\Delta \delta_G - \Delta \delta_{\text{obs}})\) and \(\Delta \delta_{\text{max}} = (\delta_G - \delta_{GH})\).

A plot of \(1/\Delta \delta_{\text{obs}}\) against \(1/[H]\) (often referred to as a double reciprocal plot) should be linear for a 1:1 complex, with a slope \(1/K_a \Delta \delta_{\text{max}}\) and intercept \(1/\Delta \delta_{\text{max}}\) allowing the determination of association constant \((K_a)\). This expression is valid when observing one species in presence of a large excess of the other species.
An alternative solution of Benesi-Hildebrand equation has been proposed by Foster and Fyfe (equation 3).

\[ \Delta \delta_{\text{obs}}/\delta = -K_a \Delta \delta_{\text{obs}} + K_a \Delta \delta_{\max} \]  

This is a special form of the more general Scatchard plot. In the Foster-Fyfe procedure, a plot of \( \Delta \delta_{\text{obs}}/\delta \) against \( \Delta \delta_{\text{obs}} \) (referred as an x-reciprocal plot) should be linear for a 1:1 complex. The gradient is equal to \(-K_a\) and the intercept gives \( \Delta \delta_{\max} \). This modification requires an extrapolation to infinitely dilute solution and the \( K_a \) is not dependent on the extrapolation.

Another modification of Benesi-Hildebrand equation is Scott equation (equation 4).

\[ [\delta]/\Delta \delta_{\text{obs}} = [\delta]/\Delta \delta_{\max} + \Delta \delta_{\max}/K_a \]  

In the Scott procedure, a plot of \([\delta]/\Delta \delta_{\text{obs}}\) is plotted against \([\delta]\) (referred to as \(y\)-reciprocal plot) which should be linear for a 1:1 complex with a slope \(1/\Delta \delta_{\max}\) and intercept \(\Delta \delta_{\max}/K_a\), allowing the estimation of association constant \(K_a\).

The stoichiometry and association constant of the 1:1 tetramethrin and \(\beta\)-CD inclusion complex were determined by spectrofluorometry through double reciprocal plot (equation 5).

\[ 1/\Delta F_{\text{obs}} = 1/(K_a \Delta F_{\text{max}} [\delta]) + 1/\Delta F_{\text{max}} \]

where \(\Delta F_{\text{obs}}\) denotes the change in fluorescence intensity of tetramethrin in the presence of \(\beta\)-CD compared to pure tetramethrin and \(\Delta F_{\text{max}}\) change in fluorescence intensity of tetramethrin, when all of its molecules are complexed with \(\beta\)-CD, compared to pure tetramethrin while other symbols have their usual meaning. The equation is identical to the Hanna and Ashbaugh modification (equation 2) except that a different property of the complex is studied.

Now, assuming the complex to be 1:2, the following expression can be written:

\[ G + 2\ H \rightleftharpoons \ GH_2 \]
\[ K_a = \frac{[\text{GH}_2]}{[\text{H}]^2 [\text{G}]} \]

if \([\text{H}] \gg [\text{GH}_2] \gg [\text{GH}]\) then the following linear expression (equation 6) is obtained for calculating binding constant by fluorospectrometry.

\[
1/\Delta F_{\text{obs}} = 1/(K_a\Delta F_{\text{max}} [\text{H}]^2) + 1/\Delta F_{\text{max}} \tag{6}
\]

where symbols have their usual meaning. A plot of \(1/\Delta F_{\text{obs}}\) against \(1/[\text{H}]^2\) gives a straight line for a 1:2 complex. Thus, the NMR version of this equation can be written as:

\[
1/\Delta \delta_{\text{obs}} = 1/(K_a\Delta \delta_{\text{max}} [\text{H}]^2) + 1/\Delta \delta_{\text{max}} \tag{7}
\]

A plot of \(1/\Delta \delta_{\text{obs}}\ versus \(1/[\text{H}]^2\) should be a straight line for 1:2 complex with a slope \(1/K_a\Delta \delta_{\text{max}}\) and intercept \(1/\Delta \delta_{\text{max}}\ allowing the determination of association constant.

Throughtout the above discussion it is assumed that the guest molecule is the observed species. It does not matter which species 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.

Throughtout discussion the CD protons are numbered as H-1' to H-6'.