(iii) Use of a combination of the above two approaches by assigning a more restrictive upper limit to b_Q [= $\{(b_1^2+b_2^2)/2\}^{1/2}$ - c_Q]. This approach has been used in programs such as CYANA.
(iv) A distant restraint based on the sum average of the NOE derived distances [<d> = $(d_1^{-6} + d_2^{-6})^{-1/6}$] which is applied to both substituents. This approach has been used in XPLOR.

It is obvious that none of these approaches can make up for the information that is available once stereo-specific assignments can be achieved. The third approach, which uses two identical upper bounds, leads to fairly satisfactory structures.

5.9 Removing Undesirable Restraints

We note that there are several NOEs, whose information is already present in terms of bond lengths and bond angles given as input in primary structure. One example is the distance between *geminal* protons. It is desirable that such restraints are not used in structural algorithms.

6. PARAMAGNETIC MOLECULES AND REAGENTS

Like proton, electron also has spin ½. However, electron spins are paired during the formation of chemical bonds. Therefore, most molecules are diamagnetic. Paramagnetism arises from the presence of a free electron spin(s) in molecules. In several molecules, it arises from a metal ion having odd number of electrons. Paramagnetic metal ions are integral part of several biological molecules, such as heme proteins and metalloenzymes. A paramagnetic centre can also be introduced exogenously.

The magnetic moment (μ_S) due to unpaired electrons in a molecule is given by:

$$\mu_S = g\mu_B[S(S+1)]^{1/2} \qquad 4.24$$

Here, g the ratio of the angular and magnetic moment of electron, S is the electron spin quantum number (1/2 for single unpaired electron) and μ_B is electron Bohr magneton. Like the chemical shift, g is also a tensor quantity. Its value for free electron is 2.0023, but is significantly different in molecules.

In the presence of paramagnetic ions, both the chemical shifts and relaxation rates change significantly. The mechanism of interaction of electron spin with nuclear spin is similar to the nuclear spin-spin interactions. However, the electronic magnetic moment is several times larger than the nuclear moments. Therefore interactions between electron and nuclear spins, dominate over nuclear spin-spin interactions.

When a metal ion is bound to a particular site in the molecule, resonances in a large volume sphere around the metal centre are broadened beyond the level of detection, due to lower T_2 in paramagnetic molecules. At high magnetic fields, the separation between the electronic Zeeman levels is higher, and consequently the line-widths are larger. Therefore, high fields may not offer an advantage to study

such systems. Even beyond the sphere of broadened resonances, chemical shifts and relaxation rates in paramagnetic molecules are dominated by the electron spins due to paramagnetic ions. Under favourable situations, such molecules show highly dispersed NMR spectra with chemical shifts extending much beyond the normal range observed in diamagnetic molecules. A number of methods have been designed to locate such broad resonances, and to exploit the advantages that such systems offer.

6.1 Contact Shifts

The chemical shifts in paramagnetic molecules are determined by two mechanisms:
(i) a direct delocalization of the electron spin at various nuclear sites (called the contact shift δ^c), and
(ii) an effect arising from the anisotropy of the *g tensor* of the electron spin (leading to pseudo-contact shifts, δ^{pc}).

Both these shift contributions are large. The changes in the chemical shifts due to hyperfine interaction ($\Delta\delta_i^p = \delta^{pc}+\delta^c$), arise solely from the interaction between unpaired electron and the nuclear spins. These can be estimated from the difference from the corresponding shifts in a diamagnetic molecule (δ^d) with a similar chemical structure ($\Delta\delta_i^p = \delta^p - \delta^d$). A convenient way to estimate δ^d in a molecule is to replace the paramagnetic metal ion with a diamagnetic ion of similar size. However, it is generally difficult to separate contributions from δ^{pc} and δ^c, in the observed paramagnetic shift. Estimates of δ^c, can be made theoretically. In some cases, one of the two terms may be small. In such cases, the observed changes are due to only one of the two mechanisms.

The contact shift arises from the presence of finite electron spin-density at the site of the nucleus. The interaction of the nuclear and electron spins is given by:

$$H = A\boldsymbol{I.S} \qquad 4.25$$

Where A is a constant, which is related to the spin density at the site of the nucleus and is called the Fermi contact hyperfine coupling constant. A general expression for the contact shift, taking into account of the anisotropy of the **g** and the magnetic susceptibility tensor $\boldsymbol{\chi}$ (which are related to each other), is given by:

$$\Delta\delta_i^c = \Delta\nu_i/\nu_0 = A[\chi_{xx}/g_{xx} + \chi_{yy}/g_{yy} + \chi_{zz}/g_{zz}]/B \qquad 4.26$$

where, B is a constant.

6.2 Pseudo-Contact Shifts

A more useful parameter for structural studies is the pseudo-contact shift. This interaction arises from dipole-dipole interaction between the electron and nuclear spins. It is similar in nature to the dipolar nuclear spin-spin interaction. For systems

having axial symmetry, the change in chemical shift due to the pseudo-contact term is given by:

$$\Delta\delta_i^{pc} = \Delta\nu_i / \nu_0 = D\,(\chi_{zz} - \chi_{xx}) \langle (3\cos^2\theta_i - 1)/r_i^3 \rangle \qquad 4.27$$

In this equation D is a constant which depends on the properties of the ion, r_i is the distance between the nucleus i and the paramagnetic metal centre and θ_i is the angle between the vector $\mathbf{r_i}$ and the principle symmetry axis of the molecule. Thus, the ratios of pseudo contact terms provide information on molecular shapes, much in the same way as the nuclear dipole-dipole interactions. However, the interaction between nuclear and electron spin can manifest much beyond the 5 Å distance limit of in NOE measurements and can provide long-range information.

Equation for pseudo-contact shift can be generalised for a molecule having several paramagnetic centres. It is expressed in terms of the direction cosines l_{ij}, m_{ij} and n_{ij} of the position vector of the atom i with respect to the magnetic susceptibility vector tensor for the j^{th} paramagnetic centre and the distance r_{ij}.

$$\Delta\delta_i^{pc} = \Sigma_j \; 1/(12\pi r_{ij}^3)\,[\Delta\chi^j_{ax}(3rr_{ij}^2 - 1) + 3/2[\Delta\chi^j_{rh}(l_{ij}^2 - m_{ij}^2)] \qquad 4.28$$

To use these equations in protocols for structure calculation, one needs to have a suitable diamagnetic reference compound such that the contribution to the experimental chemical shift arising from the pseudo-contact shift ($\Delta\delta_i^{pc}$) can be estimated. This problem may be critical for regions where $\Delta\delta_i^{pc}$ is small.

The $\Delta\chi$ tensor can in principle have five independent components. These have to be estimated as part of the structural protocol. Similar to the approach used for other NMR observables, a pseudo-potential term (V_{pc}) is introduced such that the difference between observed and calculated $\Delta\delta_i^{pc}$ is minimized as one approaches the final structure in the iterative process. Such constraints have been introduced both in DYANA (PSEUDODYANA) and energy minimization and molecular dynamics calculations.

6.3 Relaxation Rates

As has been discussed, nuclear spins in paramagnetic molecules relax mainly through the fluctuating fields arising from the interactions between electron and nuclear spins. The presence of electronic moment makes dramatic changes in both the T_1 and T_2 relaxation times of molecules. The contributions arise from three terms;

(i) the electron relaxation, corresponding to correlation time τ_s;
(ii) molecular tumbling (τ_r); and
(iii) chemical exchange (τ_M).

The total correlation time (τ_c) is therefore given by:

$$\tau_c^{-1} = \tau_s^{-1} + \tau_r^{-1} + \tau_M^{-1} \qquad 4.29$$

It is difficult to quantify different contributions to τ_c, though usually only one of the three terms dominates.

The relaxation contributions have important bearing on our ability to observe NMR spectrum. For example, the electron relaxation times τ_s of several metal ions are in the range 10^{-8} to 10^{-10} sec, which produce NMR line-widths of more than 100 Hz. These ions do not provide useful high-resolution NMR spectrum. From the view point of NMR, the useful ions are those which have τ_s of less than 10^{-11} sec. The list of such ions includes low spin Fe^{+3}, Co^{+2}, copper in tetrahedral sites and most of the trivalent lanthanides.

When the electron-nuclear spin dipolar term dominates the relaxation mechanism, both T_1 and T_2 depend inversely on the sixth power of the distance from the ion. This information can be used for obtaining relative distances between the paramagnetic centre and the nuclear spins.

6.4 Metallo Proteins

Paramagnetic ions exist naturally in a number of metalloproteins. High-resolution NMR studies on such molecules, is a flourishing area of research and has been discussed in Chapter 6.

6.5 Lanthanides Shift Reagents

Paramagnetic ions such as lanthanides (Ln^{3+}) can be used as exogenous probes to modify the properties of diamagnetic molecules. Because of similarity in the ionic radii, Ln^{3+} is an ideal probe for substitution of Ca^{2+}, in Ca^{2+}-binding proteins. The changes resulting from substitutions by Ln^{3+} in the chemical shifts and relaxation rates can be used to study conformations of large molecules.

A dynamic equilibrium may exist between the paramagnetic and the diamagnetic states of the molecules. In such situations, a small amount of bound ion may be sufficient to measure the large effects induced by paramagnetism. The use of lanthanides to replace diamagnetic metal ions in metalloproteins serves as a useful probe for obtaining additional constraints in structural studies.

The number of metal ions in the lanthanide series is large. Their magnetic properties vary significantly, such that different ions can be used for different purposes. The sign of $\Delta\chi$ changes as one goes to lanthanides with higher atomic numbers. Therefore ions with both positive and negative $\Delta\chi$ can be introduced. Certain paramagnetic lanthanides give relatively sharp lines. These can be used for measuring pseudo-contact shifts. Ions such as Gd^{+3} make large contributions to relaxation times and these ions can be used to enhance relaxation effects. Diamagnetic lanthanides such as Lu^{+3} can be used to estimate the properties of the diamagnetic metalloproteins.

6.6 Use of Spin Labels

One of the ways to obtain long-range constraints is to use a paramagnetic spin label. Nitroxide spin labels have been used in ESR spectroscopy for a number of years. The free electron in these labels is stabilized by the molecular frame in which nitroxide radical is embedded. A useful spin label reagent for NMR studies is 1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl (PROXYL). PROXYL can be covalently attached to a site in the macromolecule, where it may not influence the structure of the later. In proteins, Cys offers a convenient site for attachment.

The electron spin on PROXYL causes enhanced relaxation within a radius of about 15 Å. The changes in relaxation rates as measured from changes in the line-widths of the labelled molecule and the diamagnetic state have been used to estimate the distance of the nuclear site from PROXYL. Results can be analysed quantitatively to obtain ranges of distances. The method is particularly useful for information on intermolecular interactions and orientations of different domains in protein structures.

7. FROM NMR PARAMETERS TO STRUCTURES

A large number of NMR experiments on biological molecules are performed with the aim to determine their 3D structures. However, it is obvious that while NMR provides a wealth of information on the structure of biological molecules, the same cannot be translated directly into the 3D structure. The final step in structural studies is to unlock the indirect structural information in the form of NMR parameters, in order to obtain the 3D structures.

A number of software packages based on different approaches have been developed for this purpose. They can be divided into the following categories.

(i) Metric matrix distance geometry (programs such as DISGEO, DSPACE, EMBOSS, TINKER);

(ii) Variable target function (DIANA, DISMAN); Molecular dynamics in Cartesian space (AMBER, CHARMM, DISCOVER, GROMOS, SYBYL, XPLOR);

(iii) Torsion angle approaches (DYANA, CYANA, TANDY).

There is certain amount of overlap among these approaches. They all attempt to find a 3D structure of a given molecule which is consistent with the experimental NMR parameters, the correct stereochemical geometry, satisfactory covalent and hydrogen bonds, and is free from steric violations. Other experimental evidences are also incorporated as constraints in such programs.

7.1 Starting Structures

To reach a final conformation, an initial structure is often assumed as a starting point. This is altered by minimization of a suitable function, such that the final conformation is a *good fit* to the input data. In this endeavour, the initial structure has to be chosen carefully. An unsatisfactory starting structure may end up with erroneous results due to the minimization process leading to a false local minimum. There are several ways by which the initial structures can be generated.