VII - Magnetic Resonance

Magnetic Resonance is a spectroscopic technique that exploits the fact that some atomic particles possess a magnetic moment and will therefore experience a force when immersed in a magnetic field. These particles acquire their magnetic moment by virtue of having both angular momentum (most commonly visualized as spin) and a charge. The relevant atomic particles are

**Nuclei**: Although a large number of nuclei possess a magnetic moment, in practice the method is only widely used for those nuclei that have a maximum spin of one-half. These include \(^1\text{H}, \, ^{13}\text{C}\) and \(^{15}\text{N}\). (Nuclear spin (I) depends on atomic number (A) and mass number (Z). If Z = odd, I = half-integer. If Z is even and A is even I = 0, otherwise I = an integer > 0)

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Spin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H})</td>
<td>(\frac{1}{2})</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>(\frac{3}{2})</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>(\frac{1}{2})</td>
</tr>
<tr>
<td>(^{19}\text{F})</td>
<td>(\frac{5}{2})</td>
</tr>
<tr>
<td>(^{31}\text{P})</td>
<td>(\frac{3}{2})</td>
</tr>
<tr>
<td>(^{35}\text{Cl})</td>
<td>(\frac{3}{2})</td>
</tr>
<tr>
<td>(^{39}\text{K})</td>
<td>(\frac{3}{2})</td>
</tr>
<tr>
<td>(^{63}\text{Cu})</td>
<td>(\frac{3}{2})</td>
</tr>
</tbody>
</table>

**Electrons**: All electrons have spin but most electrons are spin-"canceled"; they occur in atomic or molecular orbitals as pairs with opposed spin. There are, however, species that are not spin paired. Most commonly these are (i) certain metal ions (e.g. Fe, Cu, Mn, Co) and (ii) organic molecules that have undergone either a one-electron oxidation or one electron reduction, yielding the radical cation and radical anion respectively. These species are especially important in electron transfer reactions that occur in the mitochondrion and in photosynthesis. Laboratory synthesized spin-labels, analogous to fluorescence labels, are also used as experimental tools.

For an intense signal the species to be observed should (i) Have a large magnetic moment; (ii) Be present in high abundance; (iii) Have a spin = 1/2.

Our Approach: (a) Traditional (cw magnetic resonance: nmr & epr); (b) Contemporary (FT-nmr).

The basic theory is identical for nuclei and electrons. I will describe it for the nucleus although, historically, certain important concepts were first developed for the electron.

There are **three** basic relationships: The first is:

\[
\mu = g_n \beta_n I
\]  

(VII-1)

This equation relates \(\mu\), the magnitude of the magnetic moment of the particle, to its spin (I) *via* the quantities \(g_n\) and \(\beta_n\). The quantity \(\beta_n\) is the nuclear magneton; it is the unit of nuclear magnetism and is defined as
The first ratio in this definition arises from a derivation of the magnetic moment expected for a classical particle of atomic charge \( e \) and (nuclear) mass \( m \) circulating in a Bohr orbit at the velocity of light, \( c \). The second ratio, Planck’s constant divided by \( 2\pi (\hbar) \) is the unit of quantum mechanical angular momentum and is the only correction needed for converting to an orbiting, charged, quantum mechanical particle. \( \beta_n \) has the magnitude \( 5.05 \times 10^{-24} \) erg G\(^{-1}\) (G = gauss) or \( 5.05 \times 10^{-27} \) JT\(^{-1}\) in MKS units (\( T = \) tesla, 10\(^4\) gauss).

The second quantity, \( g \), is called the g-factor or spectroscopic splitting factor. Originally \( \beta \) was derived for the electron (and later adopted for nuclei by using the mass of the proton). Subsequent to the classical derivation of this relationship between an electron’s orbital motion about the nucleus and its magnetic moment it was discovered that the electron also had an intrinsic magnetic moment; this is usually visualized as arising from the electron spinning about its own axis. This spin magnetism was found to be twice as intense as that anticipated from early theory and this fact was accommodated by introducing \( g \) into the expression for \( \mu \). The value of \( g \) depends on the particle. For protons \( g = +5.6 \); nuclear g-factors can be either positive or negative depending on the nucleus. For the electron \( g \) is very close to 2 (actually 2.0023).

The last quantity in equation VII-1 is \( I \), the symbol for the total spin associated with the nucleus. In elementary discussions \( I \) is usually visualized as a vector and we will have reason to use this representation; in more sophisticated calculations \( I \) is taken as a quantum mechanical operator. Note that both operators and vectors are written with a bold typeface.

The changes to VII-1 necessary for working with electrons are:

(i) \( I \) is replaced by \( S \), the electron spin. We will be principally concerned with \( S=1/2 \).
(ii) Because the mass of the electron is ca. 1/2000 that of the proton the electron magneton \( \beta_e \) has the value \( 9.285 \times 10^{-21} \) erg G\(^{-1}\) in cgs units and \( 9.285 \times 10^{-24} \) JT\(^{-1}\) in MKS units.
(iii) The g-factor characteristic of the electron. A precise value for the free electron is 2.0023. Organic radicals have values very close to this: however, as we shall see, electrons in metals usually exhibit g-values very different from 2.0.
(iv) In the case of the electron the expression for \( \mu \) contains the minus sign; \( \beta \) is inherently positive (because \( e \), the atomic charge, is positive) and the minus sign is needed to accommodate the negative charge of the electron:

\[
\mu = -g_e \beta_e S \tag{VII-3}
\]

Our second equation is:

\[
E = -\mu \cdot H \tag{VII-4}
\]

Equation VII-4 describes the change in energy experienced by a nucleus exposed to an external magnetic field, \( H \). Because \( H \) has both magnitude (its intensity) and direction it is a vector quantity. The use of the dot product implies that \( \mu \) is also a vector quantity, which follows from its direct proportionality to \( I \). This is the classical expression for the change in energy. (To proceed to the quantum mechanical equivalent we replace \( \mu \) by its equivalent operator and \( E \) by \( U \) or \( H \)).

Substituting (1) ⇒ (4)
\[ E = -g_n \beta_n I \cdot H \quad \text{(VII-4a)} \]

What is \( I \cdot H \)? It is component of \( I \) parallel (or anti parallel) to \( H = m_I \).

\[ E = -g_n \beta_n m_I H \quad \text{(VII-4b)} \]

\( m_I \) can have the values \( I, I-1, I-2, ..., -I \); therefore for \( I = 1/2 \), \( m_I = +1/2 \) or \(-1/2\).

From V-IIb we can now get the energies:

\[ m_I = +1/2 \quad E_+ = -1/2g_n \beta_n H \quad \text{(lower, therefore stabilized)} \]
\[ m_I = -1/2 \quad E_- = 1/2g_n \beta_n H \quad \text{(destabilized)} \]

\[ \Delta E = E_- - E_+ = g_n \beta_n H \quad \text{(VII-4c)} \]

The relative occupancies of the + and - states can be obtained using the Boltzmann distribution law:

\[ N_{\text{upper}} \quad N_{\text{lower}} \quad \text{exp}(-\Delta E/k_B T). \quad \text{(VII-5)} \]

For protons \( g = 5.6 \). Using \( H = 11.744 \) tesla (as in the department's 500 MHz nmr instrument), \( T = 300 \) K at room temperature, \( \beta_n = 5.052 \times 10^{-27} \) JT\(^{-1}\) and \( k_B \) (Boltzmann's constant) = 1.38 \times 10^{-23} \) JK\(^{-1}\) we get

\[ \Delta E = 3.31 \times 10^{-25} \text{J}. \text{With} \ k_B T = 4.1 \times 10^{-21} \text{J} \quad \Delta E/k_B T \approx 0.0001. \]

Using the approx. that for small \( x \): \( \text{exp}(\pm x) = 1 \pm x \quad \text{(VII-6)} \)

\[ \text{exp}(-\Delta E/k_B T) = 1 - 0.00001 = 0.9999. \text{Therefore} \ N_{\text{upper}}/N_{\text{lower}} = 0.9999 \ (0.01\% \text{ excess in the more stable state}). \text{As a typical nmr sample is 1.0 mL of a 10}^{-3} \text{ M solution the number of molecules present} = \ (0.001)(0.001)N = 6.03 \times 10^{17}. \text{ Thus} \ N_{\text{upper}} + N_{\text{lower}} = 6.03 \times 10^{17}, \ N_{\text{lower}} = 3.0153 \times 10^{17} \text{ and} \Delta N = N_{\text{lower}} - N_{\text{upper}} = 3 \times 10^{13}. \text{In magnetic resonance we are interested in the Net Magnetization} \ (M), \text{the excess in the stable orientation times the magnitude of an individual magnetic moment} \]

\[ M = \Delta N \mu. \quad \text{(VII-7)} \]

Finally Important Equation No. 3

\[ \Delta E = h \nu = h \omega \quad \text{(VII-8)} \]

(Note the identity \( h \nu = h \omega \), with \( \omega = 2\pi \nu \) and \( h = h/2\pi \). The machines are built by electrical engineers while physicists derived the theory)
As $\Delta E = g\beta H = \hbar$

$$v = \frac{g\beta H}{\hbar} \quad (VII-9)$$

With $\hbar$ (Planck's constant) = $6.63 \times 10^{-34}$ Js and $H = 11.744$ tesla, $v = \nu_o = 5.000 \times 10^8$ Hz = 500 MHz. Thus in an applied field of 11.744 tesla the energy separation between the up and down spin-states is 500 MHz.

The relationship $g\beta H = \hbar v$ is called the **resonance condition**. For a fixed value of $v$ the value of $H$ that satisfies this condition is symbolized as $H_0$ and called the resonant field; conversely for a fixed value of $H$ the value of $v$ ($\nu_o$) that satisfies this expression is called the resonant frequency.

**Comparison of data obtained in a field-swept (fixed frequency) instrument and a frequency-swept (fixed field (e.g. FT)) instrument.**

<table>
<thead>
<tr>
<th>Fixed Field Spectrometer</th>
<th>Fixed Frequency Spectrometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta E$ smaller $g$</td>
<td>$\Delta E$ larger $g$</td>
</tr>
<tr>
<td>needs smaller frequency</td>
<td>needs larger frequency</td>
</tr>
<tr>
<td>size of gap set by frequency</td>
<td>The larger $g$ the smaller $H$ needed to meet the gap.</td>
</tr>
</tbody>
</table>

Fig. VII-1

$\Delta E$, the separation in energy between the two spin-states is variable and depends upon the intensity of $H$ as is revealed in eqn. VII-4c. It follows that there are two alternative instrumental configurations. In the first configuration $H$ is set to a fixed value and the "light" source is varied over the appropriate frequency range. This might mean varying the frequency from 500 MHz (larger $g$) down to 499.99 MHz (smaller $g$). In the second configuration the frequency is set to a fixed value, say 500 MHz, and the intensity of the magnetic field varied over the relevant range. This might mean varying $H$ from 10 tesla (larger $g$) to 10.0001 tesla (smaller $g$). Thus a plot of nmr absorption vs. frequency puts the smaller $g$ species on the left; a plot vs. field puts the larger $g$ species on the left. The two presentations are the reverse of one-another.

The former configuration is to be preferred because the abscissa of the spectrum is frequency and hence linearly proportional to energy. This is the common configuration in nmr.

In epr technical considerations make this preferred configuration very difficult to implement, especially when large variations in $g$ are to be studied. As a consequence the standard epr
instrumentation utilizes a fixed frequency and a variable magnetic field; the abscissa (H) is then proportional to reciprocal energy.

Summarizing: In the "fixed-frequency variable-field" instrument the smaller the shielding the "sooner" the applied magnetic field can achieve the resonance condition and weakly shielded nuclei will be found to lower field than are strongly shielded nuclei. In a "fixed-field variable frequency" instrument, weakly shielded nuclei will be exposed to a more intense $H_{\text{eff}}$ and thus a higher value of the applied frequency will be needed to achieve resonance than will be necessary for strongly shielded nuclei.

**Shielding and the Chemical Shift.**

Why is NMR so useful? The principal reason is that, when immersed in the same magnetic field, different protons exhibit different $\nu_0$. This implies that $g$ is different for protons in different environments.

Rather than describe NMR spectral shifts in terms of variations in $g$ it is customary to assert that $H_{\text{eff}}$ (the effective field or field experienced by the nucleus) $\neq$ $H$ (the applied field provided by the instrument). Why? Because the surrounding electrons screen (or shield) the nucleus from the applied field! So:

$$H_{\text{eff}} = H(1 - \sigma) \quad \text{(VII-10)}$$

$\sigma$ is called the shielding constant. It is positive and of order of $0.000001$. Thus $H_{\text{eff}}$ is slightly smaller than $H$ and $\nu$ for resonance will be slightly smaller than that one would find with a "naked" proton.

$\sigma$ has several contributions: In organic molecules the most important is DIAMAGNETISM.

When an atom is immersed in a magnetic field the electrons in that atom are driven into motion. This motion (Fig. VII-2) consists of a circulation of the electrons in a plane perpendicular to the applied magnetic field. This circulation leads to an induced magnetic field with a direction opposed to the applied field. The magnitude of the induced field is directly proportional to (i) the magnitude of the applied field; and (ii) the number and bonding arrangement of the electrons that are involved. (Not simply electron density otherwise chemical shift of protons would depend only on the electronegativity of attached group! The details of bonding are important.)

How do we measure line positions? In NMR we don't measure the absolute position because the differences are so small that it would be difficult to do so reliably on a routine basis. The strategy is to measure the position of the line from the unknown (u) relative to the position of a convenient standard (s). This difference is called the chemical shift ($\delta$).

$$\delta = \frac{10^6 (\nu_u - \nu_s)}{\nu_s} \quad \text{at constant H} \quad \text{(VII-11a)}$$

It is routinely approximated by

$$\delta = \frac{10^6 (\nu_u - \nu_s)}{\nu_0} \quad \text{(VII-11b)}$$
The $10^6$ in numerator is used to cancel MHz in denominator and give nice numbers (in ppm). Conveniently by quoting $\nu_0$ in MHz the formula becomes $\delta = (\nu_u - \nu_s)/\nu_0$

A common standard is $(\text{CH}_3)_3\text{SiCD}_2\text{CD}_2\text{COO}^-$ (TSP) which is dissolved in our sample (provided it is unreactive). It is a water soluble derivative of TMS (tetramethylsilane, which is insoluble in water).

Example: In a 500 MHz spectrometer the measured difference in frequency ($\Delta \nu$) between a chosen proton and the line due to TSP = 1000 Hz. So $\delta = 1000/500 = 2$ ppm.

A positive value for $\delta$ means that the nmr resonance of the unknown is to be found at a higher frequency than that of the standard. An important feature of $\delta$ is that it is independent of spectrometer frequency (the frequency appears in the denominator of eq. VII-11b).

The relationship between chemical shift and shielding.

As $h\nu = g\beta H_{\text{eff}}$,

$\nu = \{(g\beta)/h\}H_{\text{eff}}$

$= \{(g\beta)/h\}(1 - \sigma)H$

(VII-12)

so $\nu_i = \nu_0(1 - \sigma_i)$

(VII-13)

Making the substitutions $i \Rightarrow u$ and $i \Rightarrow s$ for $\nu_u$ and $\nu_s$ in eqn. VII-11b:

$\delta = 10^6(\sigma_s - \sigma_u)$

(VII-14)

The observed difference in frequency between standard and sample is $10^6$ times the difference in shielding. TSP contains very highly shielded protons and is (almost always) found at the lowest frequency. Almost all protons of interest are shielded less well (deshielded) than those in TMS and are thus found at higher frequencies. The approximate range of proton shifts is 0-12 ppm with respect to TSP.
<table>
<thead>
<tr>
<th>Chemical Type</th>
<th>Chemical Shift in proteins (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic NH</td>
<td>10 ⇒ 15</td>
</tr>
<tr>
<td>Peptide NH</td>
<td>7 ⇒ 11</td>
</tr>
<tr>
<td>Aromatic -CH</td>
<td>6 ⇒ 9</td>
</tr>
<tr>
<td>aliphatic CH2/CH</td>
<td>-1 ⇒ 6</td>
</tr>
<tr>
<td>-CH₃</td>
<td>-1 ⇒ 3</td>
</tr>
</tbody>
</table>

The range of values arises from secondary shifts imposed by the environment; thus denatured (unfolded) proteins and native proteins are quite different—fortunately!

By convention nmr spectra are presented with frequency (and hence chemical shifts) increasing from right-to-left. This convention arises because when nmr spectra were first recorded only field-swept instruments were available (with H increasing from left to right).

Fig. VII-3

Example: ethanol. | Proton | δ (ppm) | Intensity |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-OH</td>
<td>3.7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>-CH₂</td>
<td>2.6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>-CH₃</td>
<td>1.2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Note that the intensity of each line is proportional to the number of protons contributing to that line.
Other Origins for Chemical Shifts

1) RING CURRENTS. This is a particularly large form of magnetic field induced electron circulation that is found with planar aromatic groups. In biochemistry it is the aromatic amino acids and heme that yield the best known effects. In this situation the electron circulation is macroscopic, following the carbon skeleton of the aromatic group. The resulting induced field is shown in Fig. VII-4. Protons located above or below the plane of the aromatic ring experience an induced field that opposes the applied field; those located along the periphery of the aromatic group experiences an induced field that aids the applied field. (The intensity of the induced field is proportional to \((1-3\cos^2\theta)\), where \(\theta\) is the angle subtended by the location of the proton and a line drawn perpendicular to the plane of the aromatic species.) The protons at 6 and 12 o’clock see a ring current of relative size -2, those at 3 and 9 o’clock see a filed of +1 and those rare protons located where \(\theta = 54^\circ\) escape the effect of the induced field.

2) PARAMAGNETIC EFFECTS. These are only observed in molecules that contain an unpaired electron; usually this means a metal such as Fe (III, II) or Cu(II). The unpaired electron has an associated magnetic field and those atoms that are connected to the metal via some kind of bonding pattern will experience some of this magnetic field; the extent of the effect depends upon (i) the number of bonds from the atom to the metal and (ii) the type of bond (saturated vs. unsaturated, the former are much less effective than the latter). Were a whole electron located at the proton a contribution to the applied field of about 10 tesla would result. Thus at 500 MHz the presence of only \(1:10^6\) of a metal electron at a proton will contribute 1 ppm to the chemical shift. Shifts that arise by this process are called Contact Shifts. They are easy to diagnose because the shift will be temperature dependent (via eqn. VII-5).

SPIN-SPIN COUPLING

Chemical shifts is the first of the two pieces of useful information in the nmr spectrum. The second is spin-coupling. Spin-coupling is the process whereby the line due to one class of protons is split into two-or-more components due to interactions with protons on adjacent atoms. How does this arise? The basic picture: the magnetic field at the first class of protons is altered by the second class of protons in the same molecule ( \(< 4\) bonds in saturated hydrocarbons). This phenomenon is called spin-spin splitting.
For example: consider acetaldehyde (CH$_3$CH=O). This molecule has two kinds of protons:
1) the single carbonyl proton.
2) the three methyl protons.
We define $H_{\text{res}}$ as the field necessary for resonance and $H_{\text{loc}}$ as a local field at the first proton(s) due to adjacent protons:

$$H_{\text{res}} = H_{\text{eff}} \pm H_{\text{loc}}$$

(a) The methyl protons see 1 aldehydic proton ($\uparrow \Rightarrow H_{\text{loc}}$); if the aldehydic proton is "up" ($\uparrow$) $H_{\text{loc}}$ is added to $H_{\text{eff}}$. If the aldehydic proton is "down" ($\downarrow$) $H_{\text{loc}}$ is subtracted from $H_{\text{eff}}$. The numbers of "up" and "down" are the same (to $1:10^4$). There will be two resonant frequencies located at $H_{\text{eff}} \pm H_{\text{loc}}$. The separation ($2H_{\text{loc}}$) expressed in Hz is called the coupling constant ($J$); in this example it is 2.9 Hz ($\approx 1$ milligauss).

(b) The aldehydic proton "sees" three methyl protons.

<table>
<thead>
<tr>
<th>Possible combinations of $H_{\text{loc}}$</th>
<th>No.</th>
<th>% molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) $\uparrow \uparrow \uparrow$</td>
<td>1</td>
<td>$1/8 = 12.5$</td>
</tr>
<tr>
<td>(b) $\uparrow \uparrow \downarrow \uparrow \uparrow$</td>
<td>3</td>
<td>$3/8 = 37.5$</td>
</tr>
<tr>
<td>(c) $\uparrow \downarrow \downarrow \uparrow \uparrow$</td>
<td>3</td>
<td>$3/8 = 37.5$</td>
</tr>
<tr>
<td>(d) $\downarrow \downarrow \downarrow \uparrow \uparrow$</td>
<td>1</td>
<td>$1/8 = 12.5$</td>
</tr>
</tbody>
</table>

This can be represented as a "stick spectrum":

![Stick spectrum](image)

Again $J = 2.9$ Hz. Reciprocal couplings have the same coupling constant. The relative intensities of the 4 lines will be determined by the statistical probabilities of the four alternatives as shown in the last column of the table above ($\Rightarrow$ binomial theorem (Pascal's triangle)).

The complete spectrum is a 1:1 doublet centered on the methyl proton chemical shift and a 1:3:3:1 quartet centered on the chemical shift of the single aldehydic proton.
Comments: (i) The value of J is independent of the spectrometer frequency; its value is always quoted in Hz. (ii) Why don't the methyl protons "see" each other and produce additional splittings? Because they are all "flipping" at the same, characteristic frequency. A comment as to why methylene protons can be distinguished is given in the additional material at the end of the chapter.

Spin-spin couplings are divided into 2 general classes: AX and AB.

Class 1; AX: $\Delta\delta$ (expressed in Hz) between the interacting protons $>> J$. Example: Acetaldehyde; CHO ($\delta = 1$ ppm), CH$_3$ ($\delta = 10$ ppm), $\Delta\delta = 9$ ppm = 4500 Hz in a 500 MHz machine; $\Delta\delta >>> J$ (2.9 Hz). Acetaldehyde is called an A$_3$X system! Our intuitive model works!

Class 2; AB: J is comparable to $\Delta\delta$. Cannot be solved intuitively but needs an explicit quantum mechanical calculation (as summarized in Fig. VII-6).

AB behavior illustrated for a two proton system.

The limiting conditions are:

1) $J = 0$ (top left). The A and B lines are seen unmodified.
2) $J \ll \Delta\delta$ (top right) This is analogous to the AX case discussed earlier.

Fig. VII-6
3) $J < \Delta \delta$ (middle left). The lines are still doublets as in (2) but the relative intensities are unequal as shown.

(4) As $J$ begins to get larger relative to $\Delta \delta$ the inner lines move closer and intensify while the outer lines move further apart and lose intensity.

(5) $\Delta \delta = 0$; only one line is seen at the common value for $\delta$.

$$C = 0.5(j^2 + \delta^2)^{1/2}$$

Another Example: 3 classes of proton: $\Rightarrow$ AMX system - illustrated below for component A only.

![Diagram of AMX system](image)

Procedure: Start with the largest $J$, then proceed onto the next largest etc., in order. Identical patterns will be obtained for components M and X. The final spectrum would consist of three groups of four lines each centered on the chemical shift for the respective nucleus. This is the spectrum of vinyl acetate; (the methyl protons are sufficiently distant that they do not couple with the vinyl protons).

The Bottom Line! It's simpler to evaluate an AX system. The method of successive splittings is valid when the $J$'s are significantly smaller than the $\Delta \delta$'s. Experimentally this can often be accomplished by working at the highest possible spectrometer frequency! High Fields: AB $\Rightarrow$ AX. This is one of the principal motivations for the development of instruments operating at the highest possible frequency.

Typical spin-spin couplings between protons attached to tetrahedral carbons are:

<table>
<thead>
<tr>
<th>No. of bonds between protons</th>
<th>$J$ (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10-50</td>
</tr>
<tr>
<td>3</td>
<td>1-10</td>
</tr>
<tr>
<td>4</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

The magnitude of these couplings, especially for the 3-bond case, are sensitive to geometry, and thus contain structural information; this is quantified in the Karplus equation.

![Diagram of Karplus equation](image)
For NH-C$_\alpha$H the equation is:

$$J(\phi) = 6.44 \cos^2(\phi - \pi/3) - 1.4\cos(\phi - \pi/3) + 1.9 \text{ (in Hz)}$$

where $\phi$ is the angle (radians) subtended by the two dihedral planes. Fig. VII-9 is a graph of this function.

Fig. VII-9
Precession-A necessary first step to FT-NMR

The previous material was presented using the language and pictures of classical magnetic resonance. But to get some understanding of modern, pulsed nmr a different approach is required.

The description of magnetic resonance using energy levels is most useful when one is trying to understand the position of the epr/nmr spectrum and any structure that might be present in the spectrum. However to understand Fourier Transform nmr (FT-NMR) and related pulsed resonance methods, and to get an insight into relaxation phenomena the most intuitive approach is via a classical model called precession.

In this model one views the individual magnetic moments (as well as the net magnetization) as precessing around the direction of the applied magnetic field, in precisely the same way that the axis of a spinning top slowly changes direction in response to the earth's gravitational field. Thus the motion of the magnetic moment defines the surface of a cone as indicated in figure VII-10. The rate of spinning ($\omega$, radians per sec) is specified by the gyromagnetic ratio ($\gamma$) and is directly proportional to the intensity of $H$.

$$\omega = \gamma H$$ (VII-15)

$\omega$ is called the Larmor frequency. The direction of spinning is opposite for electrons and nuclei; the direction for an electron is shown in Fig VII-10. As $H$ is increased the rate of the precessional motion increases. What is $\gamma$? Recall that $h\nu = h\omega = g\beta H$

$$\omega = \frac{g\beta H}{h}$$

So:

$$\gamma = (1/h)g\beta$$ (VII-17)

$$\beta = \frac{eh}{2mc}$$

$$\gamma = (\pm) \frac{ge}{2mc}$$ (VII-18)

(±) only implies that $\gamma$ is a signed quantity with the sign specifying the sense of rotation.

The precessional motion of the magnetic moment can be visualized as having two components Fig. VII-10):
1) The first of these is parallel to $H$ and is called $\mu_z$ (also called $\mu$-parallel). The value of $\mu_z$ is independent of where $\mu$ (called $u_{tot}$ in the figure) lies on the surface of the cone. Consequently it is called a "constant of the motion". It is the only part of $\mu$ that can be observed directly.

2) The second component, $\mu_{x,y}$, (also called $\mu$-perpendicular), is the component of $\mu$ perpendicular to $H$; its absolute magnitude is also independent of direction but its value changes from positive to negative as $\mu$ rotates in the xy plane. Consequently the value of $\mu_{x,y}$ averages to zero over one revolution of $\mu$ about $H$. This component was not normally considered to be an experimental observable though it can be studied indirectly, principally through a study of relaxation phenomena (as we will see below).

In the magnetic field $H$, the spins precess around $z$ at a frequency $\gamma H$ (Fig VII-10). For a proton $\gamma = 2\pi \times 4278 \text{ Hz/gauss} = 26753 \text{ rad G}^{-1} \text{ s}^{-1}$; for an electron with $g=2$, $\gamma = -2.8 \text{ MHz/gauss}$; remember that the sign of $\gamma$ defines the sense of the rotation.

Two cones exist, one pointing up, the other down (Fig VII-11) reflecting the two orientations of the spins (magnet). The cone pointing up is a little "denser" because it contains slightly more spins. This excess density is the net magnetization ($M$) which we defined earlier as $\Delta N\mu$. 

Fig VII-10

Fig VII-11
In magnetic resonance it is this net magnetization that is really being studied; thus once can think of the cone shown in Fig.VII-10 as being the time dependence of a single magnetic moment or as representing the spatial distribution of the large number of individual \( \mu \) (ca \( 10^{13} \)) that contribute to the net magnetization; in the absence of other forces, \( M \) is aligned along the stable orientation.

To flip an individual spin we must move it from one cone to the other—from the up-cone to the down-cone (Fig VII-11; \( H_0 = B_0 \)). This requires re-orienting a spin in a direction perpendicular to \( z \). This re-orientation can be achieved, for example, by a magnetic field (\( H_1 \)) directed along \( x \) for this will cause the spins to precess about \( x \), in the \( yz \) plane, and the spins will pass from \(+z\) to \(-z\) to \(+z\) and so on.

There is however a glitch. For precession to occur in the \( x \)-direction the spins must see a constant field along \( x \). But the spins are precessing about \( z \). So while they might see a field (\( H_1 \)) when the spin points along \(+x\) they see the opposite field when they point along \(-x\). Thus a static field along \( x \) has no effect.

However by making \( H_1 \) a rotating field in the \( xy \) plane and making sure that the direction and rate of this rotation is the same as that of the ensemble of spins then we have two synchronous rotations and the spins see a constant field (Fig VII-12). This is achieved by making \( H_1 \) a linearly polarized field along \( x \); then \( H_1 \) can be viewed as two counter-rotating fields, one rotating with the same sense as the spins, the other with the opposite sense. The latter is irrelevant.

The easiest way to understand such systems is to use rotating coordinates. These are probably unfamiliar to you but are another example of a smart coordinate system. An analogy is to place yourself at the center of a carousel. As the carousel turns you turn with it and you will always be facing the same riders; you could, for example, easily pass cookies (energy) to them because the rotation has no influence on your relative position. In fact in this rotating coordinate system you and the riders are not moving. Contrast this with an observer who is standing outside the carousel and who sees a continually changing group of riders that is repeated every rotation. Any attempt to transfer material between observer and rider that requires contact for a time that is long compared to the rate at which the carousel rotates will obviously be very difficult.

The strength of \( H_1 \) is very much smaller than that of \( H_0 \). For nmr \( H_0 \) is around 11 tesla while in steady-state nmr \( H_1 \approx 0.1 \) milligauss. (For epr \( H_0 \) is around 3000 gauss while \( H_1 \) is about 0.1 gauss.) Thus the protons rotate rapidly about \( z \) (ca 500 million/sec) and slowly about \( x \) (about 1/sec.). The motion that results is complicated and can be pictured as a slow "widening" of the upper cone such that the motion of its tip describes an ever-opening spiral until it lies in the \( xy \) plane whereupon the tip begins to move down and the spiral begins to close until it lies along the lower cone.

However, after reaching the lower orientation, \( H_1 \) is still present and so the reverse journey begins; the tip of the cone opens up once more until it lies along the equator whereupon it begins to close and ends up in its original position along the upper cone.

This motion continues as long as \( H_1 \) is present. In the "downward journey" energy is transferred from \( H_1 \) (the light beam) to the spin system. In the "upward journey" energy is restored.
from the spin system to the light beam. Consequently over one up-and-down journey there is no net loss of energy from the light beam. This back-and-fro motion is the resonance phenomenon.

Remember fluorescence: If this were all that happened then we would not have any spectroscopy, for spectroscopy usually operates by measuring the net decrease in the intensity of the light beam as it passes through the sample. Again it follows that there must be some process that can take spins from the high-energy (down) orientation to the low-energy (up) orientation without returning the excess energy to the light. Such processes do exist; they have the generic title of SPIN-LATTICE RELAXATION and have a characteristic time (T1) that is a measure of their efficiency (a small T1 being more efficient; 1/T1 is a rate constant). The overall process thus consists of energy entering the system from the light to raise the spins to the unstable state. Once in this state two processes are in competition, both of which return the spin to the stable orientation. (i) The energy can be returned to the light and (ii) the energy can be lost to the lattice i.e. the solvent -which gets warmer (by millidegrees) as a consequence. This and other relaxation processes will be covered shortly.


In pulsed magnetic resonance H1 is only turned on for a short, well-controlled period. For example a H1 field of ca. 6 gauss turned on for 10 usec is just sufficient to take the spins originally in the upper cone down into the xy plane. After 10 usec. H1 is turned off. This is called a 90° pulse-by convention it is applied along +x (in the rotating frame); it is seen as a rotation of the magnetization about x, initially to +y. The sense of the rotation is given by a left-hand rule (place the thumb along +x, the stretched index finger along ±z (as is relevant) then the remaining curled fingers give the direction that the spins rotate-for positive H1).

{How did I arrive at the above numbers? \( \gamma = 4258 \text{ Hz/gauss} \) is the number of precessions per sec so \( 360 \gamma = 1,533,000 = \text{number of degrees per sec per gauss} \). Thus \( 1/(360 \gamma) \) is the time needed to tip through 1 degree for a H1 of 1 gauss = 0.6524 usec. For 90° we will need \((0.6524)90 = 58.5 \text{ usec} \). However as \( \Delta t \) was chosen to be 10 usec H1 will have to be \( 58.5/10 = 5.85 \text{ gauss} \)}

This event is detected by a coil of wire placed in the xy plane, say along +x, which will detect a current as the rotating magnetization "cuts" the coil; the size of this current will be proportional to the number of spins that have been tipped into the xy plane. (A common design is to make the coil that generates the pulse and the coil that detects the magnetization after the pulse has been turned off, one-and-the-same piece of wire.)

We start by first considering a single class of protons (i.e. protons with the same satellite δ) that have no J-coupling.

In the standard FT-NMR experiment we place the sample into H0 whereupon a net magnetization is created. We then apply a 90° pulse along x to tip the net magnetization into the xy plane where it initially lies along y (the left hand rule above). We then observe the behavior of this magnetization with time.

(If flipping the spins into the xy plane bothers you an alternative picture might help. Before H1 is turned on the spins that comprise the net magnetization of the cone are randomly distributed over the surface of the upper cone. \( M_z \) is finite but both \( M_x \) and \( M_y \) are zero. After the 90° pulse half of the
excess spins have been flipped so $\Delta N$ and hence $M_z$ is zero, and the spins "bunch-up" on one side of the cone (Fig VII-18). By bunching the spins in this way we say that we have induced a *phase-coherence*; this phase coherence results in a net $M_{xy}$, a net magnetization in the xy plane which is the fundamental requirement for the detection of NMR and it is the time dependence of the behavior of $M_{xy}$ that contains the information about the chemical shifts and the linewidths.)

Two examples follow. The first example (Fig VII-13, lower) shows the signal detected in the xy plane for the case when $\delta$ is such that the difference between the precession frequency of the proton and the spectrometer frequency ($\Delta \nu$) is 20 Hz e.g. the spectrometer frequency is 500,000,000 Hz and the proton frequency is 499,999,980 Hz. We obtain a sine wave of frequency 20 Hz, with an envelope that decays exponentially with time-the decay of this envelope has a time constant $= 1/T'_2$ (this will be explained later). In the upper example (Fig. VII-13) the difference in frequency is 10 Hz and so the frequency of the sine wave is now 10 Hz. These oscillating signals are called the FIDs (the free induction decay). The Fourier transform of these FIDs gives peaks (shown on the right located at the appropriate frequency with a width determined by the rate of decay.

Note that we are now dealing with FT's which interconvert data which varies with time (Fig 13, left) into the representation of the same data as a function of frequency (Fig. 13, right; (in x-ray crystallography the FT operation converts electron density as a function of space into its representation as a function of reciprocal space.)
Fig VII-14 provides a simple explanation of how the signal is actually detected; remember the spins are precessing at 500 MHz! Where did the 500 MHz go?

![Diagram](image)

The data input to the detection system are two signals, $\nu_o$ the operating frequency of the spectrometer and $\nu$, the frequency of the precessing proton. The "black box" mixes these two signals and the result are two frequencies $\nu_o \pm \nu$. We ignore the plus combination and the minus combination is simply the difference between the chemical shift (in Hz) of our proton, as shown on the right. This is the frequency of interest.

We now complicate the picture by adding J coupling using as an example a compound in which the single class of protons are split by a $I = 1/2$ nucleus with $\delta = 50$ Hz and $J = 10$ Hz; one-half of the protons precess at $\delta + J/2$, the remainder precess at $\delta - J/2$. The FID is represented by the picture (Fig. VII-15) in which the free induction decay is no longer a simple oscillating exponential but is modulated (has rapid variations in amplitude). The pattern consists of the sum and difference frequencies. The rapid frequency component of small amplitude yields $\delta$; the frequency of the modulation (the slower oscillations of larger amplitude)
yields J and the overall decay of the envelope yields the linewidth. The FT of this FID yields the spectrum (Fig. VII-15, right) with two lines split by 10 Hz (J) and centered at 50 Hz (δ).

This is the essence of the 1D nmr experiment.

However in a typical molecule we have

1) a large number of protons
2) a number of δ's possibly as great as the number of protons.
3) Many J's reflecting the possible interaction of each class of protons with one-or-more other classes of protons.

The FID of such a system is a mess-see the example provided in Fig. VII-16, top! It seems like a miracle but the FT of this mess will still lead to a sensible nmr spectrum with all the δ's, J's and linewidths correctly displayed (Fig 16, bottom.).

Pulsed Magnetic Resonance and Spin-Echoes

FT nmr as just described is the simplest of the pulsed nmr experiments. We are now going to consider some of the other cases; this lead eventually to nD nmr which is where the action is! (we will only consider n = 2; n = 3 and 4 are now being used).

We turn on a field of ca. 6 gauss for 20 usec; this is just sufficient to take the net magnetization originally in the upper cone and invert it into the lower cone. After 20 usec. H1 is turned off. This is called a 180° pulse.

In this circumstance we have disturbed the equilibrium composition of the spin-system that existed before the pulse was turned on. We have increased the number of spins in the down-cone at the expense of spins originally present in the up-cone so that the equilibrium difference in population has been decreased; indeed it has been inverted. If left alone the inverted magnetization would last forever. However the original equilibrium population is slowly restored as individual spins return to the upper-cone by the process called spin-lattice relaxation, which is the magnetic resonance equivalent of the "internal conversion" we encountered in fluorescence. Spin-lattice relaxation is a first-order process because the number of spins that return to the upper level at any time t is directly proportional to the deviation from the final, equilibrium population that exists at that time t.

\[
\frac{d M_t}{dt} = \frac{-(M_t - M_e)}{T_1} \\
(M_t) = -(M_e) \exp\left(-\frac{t}{T_1}\right) \\
(M_0) = -(M_e)
\]

where \(M_t\) is the z-magnetization at time \(t\) and \(M_e\) is the equilibrium value, which existed immediately before the pulse. The solution looks like

\[
M_t - M_e = (M_0 - M_e) \exp\left(-\frac{t}{T_1}\right)
\]

\(M_0\) is the z-magnetization at time = 0, i.e. immediately after the 180° pulse. The exponential term varies from 1 (at \(t = 0\)) to zero (at \(t = \infty\)) and so \(M_t\) varies from \(-M_e\) to \(M_e\) as time evolves (assuming the 180° pulse was powerful enough to completely invert the population, \(M_0 = -M_e\)). The magnetization returns to its initial value exponentially (its a first-order process).
Fig. VII-16. The FID and its FT of a 21 base oligonucleotide (provided by Ed. Nikonowicz).
This phenomenon can be used to measure $T_1$. This measurement cannot be made directly because nmr spectrometers function by detecting changes in magnetization that occur in the xy plane as was explained above; the phenomenon just described only results in magnetization changes along the z axis. However if we follow the 180° pulse with a 90° pulse i.e. a pulse half-as-long as the first (10 usec. in this example) (Fig VII-17) any (excess) magnetization along -z gets tipped back into the xy plane (along -y) where it can be detected

\[ e^{-t/T_1} \]

![180 pulse applied here.](image)

90 pulse applied at intervals

Fig VII-17

for as $H_0$ is still present the spins still rotate about z and so they will rotate in the xy plane (at ca 500 MHz). Thus in our experiment the detector will first report a negative voltage because the excess magnetization before the 90° degree pulse is along -z. By varying the time (t) between the inversion pulse (180°) and the "reading" pulse (90°) we will sense the change in z-magnetization, starting at its most negative value (Mo), passing through zero ($t_{null}$) and finishing at its positive equilibrium value.

\[ T_1 = \frac{t_{null}}{\ln 2} \quad (VII-21) \]

($t_{null}$ is the time at which $(M_t - M_e)/(M_o - M_e) = 0.5$).

**The Spin-Echo Experiment:** A second important exploitation of pulses reverses the order of the 90° and 180° pulses. Initially after a 90° pulse (i.e. a 10 usec, 6 gauss pulse) the spins comprising the magnetization will look like a very "tight" cone (Fig VII-18) but over a period of time (which requires very many rotations) the cone fans out (Fig VII-19, A then B); the maximum voltage generated in the detector coil mounted at +x is seen to shrink as the cone spreads out.
Why does the cone fan out? Because the individual spins are inequivalent! Perhaps they are located in slightly different regions of $H$ and because $H$ is spatially slightly inhomogeneous the individual spins have slightly different precession frequencies; other reasons will be considered (much) later. Thus the cone fans out, the xy component shrinks in size and the amplitude of the signal in the detector decreases. This decay is also a first-order process and so can be described by an exponential. The characteristic parameter of this exponential is denoted $T_2$ and called the spin-spin or transverse relaxation time.

(Remember: The rate at which the amplitude decays is related to the linewidth of this set of protons in the conventional nmr spectrum; it is the Fourier Transform of the linewidth. Fat lines decay rapidly, narrow lines decay slowly.)

In Figs VII-19 (A-C) I show 5 individual spin vectors, (B) representing the state of affairs some time later than (A). Now suppose at the time appropriate to (B) I subject the sample to a 180° pulse. These spin vectors get flipped about z by 180° leading to the situation depicted in (C) which shows that 1 and 5 have interchanged their position. Originally #5, the fastest "precessor", was in front but now it is in back, while the slowly precessing #1 is now in front of the pack. However the sense of precession about z hasn't changed. So #5 and its neighbors begins to catch up on the pack while #1 and its neighbors begin to fall back towards the pack. At some time later, which is equal to the time between the original 90° and subsequent 180° pulse, the components of the cone have returned to their original positions and the cone has recovered its original "compactness". The signal in the detector is as large (not quite; see below) as it was immediately after the 90° pulse (Fig VII-19a). This phenomenon is called a spin-echo. This spin-echo phenomenon can be used to measure $T_2$. 
The above picture (Figs VII-19) was presented on the assumption that spin-lattice processes did not exist. But, in reality, during the period of time that the cone was fanning out in the xy plane (and subsequently reassembling), some spins were returning to lie along +z. In addition there are processes that cause the cone to "fan-out" that cannot be reversed by the 180° flip. Both of these phenomena lead to a "shortening" of the cone and hence to a decrease in the size of the echo signal detected in the pick-up coil. For a more anthropomorphic picture of spin-echoes see the end of the chapter.

![Fig VII-20](image)

So if we now do this 90°-180° pulse experiment a number of times increasing the time (τ) between the 90° and 180° pulse with each repetition. We then plot the size of the echo as a function of τ. The size of the echo is found to decrease exponentially as τ increases and the time constant for this exponent is $T_2$ (As τ gets longer more spins have had a chance to jump back along z or to "irreversibly dephase").

$$\frac{1}{T_2'} = \frac{1}{T_2} - \frac{1}{T_1} \quad (\text{VII}-21)$$

The difference between the two measured values ($T_1$ and $T_2$) yields $T_2'$; this time is the characteristic time for those processes responsible for the loss of the xy magnetization which do not involve the spins returning along the z axis.
Two Dimensional NMR: A Technique for Enhancing the Information in a NMR Spectrum

Even a small protein contains a large number of protons. For example BPTI (basic pancreatic trypsin inhibitor) contains 58 amino acids. There must be a proton on each of the 58 α-carbons plus the 57 amide protons plus the amino terminus plus the protons present on the 58 R-groups that are present on the α-carbons. Clearly there will be several hundred protons contributing to the nmr spectrum of this protein. If these protons can be resolved, and if the data is obtained using nmr methods to be described in this section then one stands a good chance of deducing the 3-D structure of the protein.

![Fig VII-21: Random-coil chemical shifts for the 20 common amino acids. H_N, solid circles; H_α, solid squares; H_β, solid triangles; H_γ, open square; H_δ, open triangle; H_ε, open diamond; aromatic CH, star.](image)

Fig VII-21 summarizes the approximate position of the relevant classes of proton signals that will be found in a protein. All of the signals are up-field from TMS (i.e. to the left) with the exception of the so-called high-field shifted methyl resonances (not shown); these arise from ala, val, leu and ileu that are situated sufficiently close to aromatics to experience ring-currents.
Presented in this linear way the direct (1D) proton NMR spectrum would be extremely "busy" and it would be difficult (impossible?) to identify the individual components; imagine that all the symbols in the above figure were plotted on the x-axis. The 2D approach is a technique to spread the spectral information out in such a way that the individual lines are readily identified and, importantly, the presentation of the data is such that the resonances of each residue are identified and the "connection" that each residue makes to its two neighbors are established (items 1 and 2 below).

In principle the 2D method is very simple:

1. Identify enough of the signals in the spectrum so that you can identify the $\alpha$-carbon and amide protons of each residue and the type of each residue, that is ala v.s. trp etc. This requires the use of a technique called COSY (correlated spectroscopy). The COSY spectrum of BPTI is shown in Fig. VII-25.
2. Identify which residue is attached to which using a second technique called NOESY (Nuclear Overhauser Spectroscopy). Superficially a NOESY spectrum resembles a COSY spectrum. It yields the amino acid sequence.
3. Identify secondary and tertiary structure using NOESY in conjunction with "distance geometry" computer programs that check the assignments against known conformational constraints (Van der Waals radii, Ramachandran angles).

To understand 2D nmr we begin with an informative experiment, first described by Jeener in the early '80s. His sample was chloroform (CHCl$_3$) which has a single proton. The experiment consists of two pulses (Fig. 22).

![Fig VII-22](image)

The first 90° pulse is the same as in the 1D case and tips the magnetization along the +y-axis (as before). But unlike the 1D experiment we do not begin acquiring data immediately. Rather we wait a period of time $t_1$ and then we subject the sample to a second 90° pulse after which we immediately begin acquiring data. The time during which data is acquired is called $t_2$.

In the following numeric explanation the chemical shift ($\delta$) has a value such that $\Delta \nu = 80$ Hz (i.e. the rotating vector returns along +y every 1/80'th of a second). To understand what happens during these pulses consider the following simple cases:

(a) $t_1 = 0$. In fact we have applied a 180° pulse and M now lies along -z. There is no magnetization in the xy-plane and hence the FID = 0 (Fig. 23, upper picture, bottom trace).

b) $t_1 = 1/320$ sec $\approx 3$ msec. In this time M will have rotated to from +y $\Rightarrow$ +x. As the second pulse is along x it will have no effect on M (left-hand rule) which remains in the xy plane. The maximum FID will be observed during the acquisition period and the FT of this FID yields a normal nmr spectrum located at 80 Hz on the $\nu_2$ axis (Fig. 23, upper picture, 4'th trace).

c) $t_1 = 2/320 = 1/160$ sec $\approx 6$ msec. M will have advanced to -y and the second pulse will flip it back to +z. Again the FID = 0. (Fig. 23, upper picture, about the 7'th trace).
(d) $t_1 = 3/320 \approx 9$ msec. The magnetization will have advanced to $-x$; as in (b) it will be unaffected by the second pulse and the FID will have its largest amplitude. However as data collection starts at $-x$ the FID will be upside down and FT will give a nmr spectrum that resembles that obtained in (b) but is inverted (Fig. 23, upper picture, about the 9’th trace).

(e) $t_1 = 4/320 \approx 12$ msec. Back to case (a) above (Fig. 23, upper picture, 12’th trace).

At intermediate values of $t_1$ the FID will be of intermediate amplitude—as specified by the magnitude of the projection of M along x (or -x).

Fig. VII-23 (upper) shows the set of spectra obtained by Jeener as $t_1$ was incremented in 1 msec steps (each spectrum is a separate experiment). Spectra shown in this way are called "stacked spectra".

Pick a point on the lowest spectrum shown in Fig. VII-23 (last page, upper). For example, pick the point at $v_2 = 80$ Hz; this corresponds to the position of the peak in the 4’th spectrum. Measure the intensity at that point—its essentially zero (case (a) above). Now move along the stack of spectra (in the direction of $t_1$) measuring the intensity at the same location each time. The intensity clearly increases to a maximum (the 4’th spectrum), then decreases to zero (between the 6’th and 7’th) then goes to a negative extreme (the 12’th) then returns to zero etc. We now plot this amplitude as a function of the time $t_1$; it clearly oscillates (Fig. VII-23 (lower)). In fact this variation in amplitude as a function of $t_1$ describes a 80 Hz sine wave, reflecting the fact that the response of M to the second pulse is determined by its value of $\delta$. So, if we now take the FT of this sine wave we get an absorption curve that also has a peak at 80 Hz. We plot this spectrum parallel to the $v_1$ axis (derived from $t_1$ because of the FT) and obtain a peak at the location 80 Hz on $v_1$.

We now generalize this procedure by not taking just one time slice through the stacked spectra but many time slices, say one at each increment in Hz as we move from left to right along $v_2$ and use each of these "time slices" as input to the FT. Each resulting spectrum is plotted vs. $v_1$ at the appropriate location on $v_2$. The result is a 3D plot of nmr absorption vs. $v_1$ and $v_2$ (Fig. VII-24, left). In practice we treat the original set of FIDs that led to the stacked spectra as a two dimensional data set, each point in the set, $P(t_1, t_2)$, being a function of two parameters, with $t_1$ denoting the value of the delay between the two 90° pulses experienced by this datum and $t_2$ denoting the time this datum was acquired during the recording of the FID.
In this plot $\nu_2$ (from left to right) represents the traditional axis (the 1D case) while $\nu_1$ (from top to bottom) represents the variation in amplitude created by the changes of $t_1$. $\nu_2$ represents just the chemical shift of the proton. But in this simplest of cases so does $\nu_1$ because the sine wave we generated with our first timeslice was oscillating with the frequency of 80 Hz. Cross-sections through the peak parallel to either $\nu_1$ or $\nu_2$ are NMR peaks with linewidths $1/T_2$. The peak is located at the same position in both dimensions because $\nu_2$ monitors $\delta$ directly while $\nu_1$ monitors it indirectly via the amplitude response of $\nu_2$. The same frequency component is present during both $t_1$ and $t_2$ (the acquisition time).

Now repeat the whole operation with a different proton for which $\delta = 20$ Hz. One can repeat all the above arguments exactly except that all references to 80 are replaced by 20 and the increments in $t_1$ are $1/(n*20)$ rather than $1/(n*80)$.

Now repeat the experiment with a material containing both a 80 Hz proton and a 20 Hz proton but which are not coupled. Each of these two sets of protons are oblivious to one-another and so the result is simply that obtained by combining the two scenarios from above. The recorded FIDs will yield 2 peaks after the FT, one at 20 Hz and one at 80 Hz. The peak at 80 Hz will be amplitude modulated at 80 Hz with respect to $t_1$ while that at 20 Hz will be amplitude modulated at 20 Hz.

The resulting 2D plot shows peaks exclusively on the diagonal, running from lower left to upper right, with the position of each peak reflecting its chemical shift, that can be read off either axis. All we have done is to measure the chemical shift twice—which is of no practical value.

Most commonly the 2D spectrum is not represented as shown on the left of Fig. VII-24 because when a large number of classes of protons are present the figure gets very cluttered; the practice is to use contour maps as shown in Fig VII-24, right. See Fig VII-25 for a 3D plot and the corresponding contour plot for bovine pancreatic trypsin inhibitor (For the moment ignore the fact that there are many peaks that are not on the diagonal).
From Jeener’s experiment we learnt:

1. A single species (we call A) will exhibit an FID that is a simple sine wave with respect to $t_2$ with a period of 80 Hz.
2. The amplitude of this FID oscillates sinusoidally with respect to $t_1$ and the frequency of these oscillations is also 80 Hz.
3. A 2D plot will show a peak at 80 Hz on the $v_2$ axis and a peak at 80 Hz on the $v_1$ axis–on the diagonal.

This leads to the rule: Frequencies present in both $t_1$ and $t_2$ will fall on the diagonal. Thus a system with protons A and X which are not aware of each other (i.e. uncoupled) will give 2 peaks on the diagonal; A at 80, 80 and X at 20, 20. The FID with respect to both $t_1$ and $t_2$ is described by an expression similar to

$$A \sin (\nu_A t) + B \sin (\nu_X t)$$

with $\nu_A = 80$ Hz and $\nu_X = 20$ Hz; A and B are proportional to the number of A and X protons in the sample, respectively.

The power of nD NMR is a consequence of something the nmr spectroscopist calls Transfer of Phase Coherence! Phase coherence means that the net magnetization of a species stays bunched up in the xy plane. Transfer implies that the precession frequency of that magnetization is communicated to a second spin system, provided that there is some mechanism for coupling the two spin systems. For example, the phase coherence of X is transferred to A. This transfer leads to variations in the amplitude of the FID of A as a function of $t_1$ which contain both the frequency of A and that of X. Thus a plot similar to that of Fig 23 (lower) will oscillate in a way that is more complicated with both a large amplitude 80 Hz component on which is “imprinted” a smaller amplitude 20 Hz component. (Actually the figure would be more complicated because the data contains the intrinsic frequencies of both A and X (as shown in the above equation), that of A being modulated by X and that of X being modulated by A. Thus the above equation becomes modified to something like

$$A \sin (\nu_A t) C \sin(\nu_X t) + B \sin (\nu_X t) D \sin (\nu_A t).$$

For equal numbers of A protons and X protons $A = B$ and $C = D$; but $A, B > C, D$.

How is this transfer accomplished i.e. what couples the two spins A and X? There are two basic mechanisms. The first, called COSY, is simply the through bond spin–spin coupling which we considered earlier (the phenomenon characterized by the parameter J). The second, called NOESY, is a through-space phenomenon known as the nuclear Overhauser effect—it depends on the dipole-dipole coupling formula we have seen previously in several chapters (now the two dipoles are now the magnetic moments associated with A and X).

Which of these two mechanisms is being exploited depends on the sequence of pulses that is applied to the sample. The simplest nmr protocol use a two pulse sequence to monitor COSY precisely as in Jeener’s experiment (Fig VII-22); to exploit NOESY a 3–pulse sequence similar to Fig 26 is used. In this 3-pulse sequence $t_m$ has a fixed value which is established by trial-and error in preliminary experiments.
All 2D experiments contain a minimum of two time periods. The first is called the evolution time and is given the symbol $t_1$. The value of $t_1$ is increased in small steps in consecutive experiments, from 0 to some maximum value. For each $t_1$ we eventually record an FID; this FID is acquired vs. the acquisition time (called $t_2$). Thus we have multiple $t_1$s while $t_2$ varies over a fixed, constant range. The essential component is that phase-coherence is first established in one group of spins and then this phase coherence is transferred to a second group of spins (which can be nuclei of the same or of a different element); this will only occur if the two groups are coupled to one another, either by through–bond coupling or through–space coupling. Through–bond coupling is the basis of the COSY technique and through–space coupling the basis of the NOESY technique.

For the purposes of this class it is sufficient to know that there are two mechanisms and that these can be observed using the pulse sequences just illustrated. If you wish to gain some insight into how these pulse sequences work “hand-waving” explanations are given in the section on additional material present at the end of the chapter. Our concern in this course is how the data from these two classes of experiments are used to elucidate molecular structure.

**COSY:** Because J-coupling is a through–bond process it basically involves protons on adjacent atoms (i.e. 3 bonds apart, $^3J$). This information can therefore be used to identify peaks in the nmr spectrum that arise from atoms present on the same amino acid residue. (To get correlations on protons more than 2 carbon atoms apart more elaborate COSY techniques are required; these are called relayed COSY).

Fig. VII-27 shows an idealized result for alanine. The box at the lower right contains the information that connects the $C\alpha H$ on residue i with the NH on residue i.

Note that the COSY and NOESY data are symmetrically arranged about the diagonal. Fig. 27 thus shows only one-half of the recorded data.
The triangle at the upper right contains the information that identifies the amino acid via $C_{\alpha}H \Rightarrow CH_2$, $CH_2 \Rightarrow CH_2$, $C_{\alpha}H \Rightarrow CH_3$ and $CH_2 \Rightarrow CH_3$ couplings. There is enough information in these couplings to uniquely identify almost all the common amino acids. For example, in the above figure the solid circles on the diagonal represent the NH, $C_{\alpha}H$ and CH$_3$ protons of an alanine as we read from left to right. The open square is a point associated with a $C_{\alpha}H \Rightarrow CH_3$ J coupling (and only alanine has a methyl on the $\alpha$-C) and the solid square signifies a point for the same $C_{\alpha}H$ to its associated NH; we thus have identified the chemical shift for an alanine residue and the peptide bond for which this alanine contributes the NH. Note however that we don't know which alanine this is; undoubtedly there will be several in the protein.

Normally this experiment is done in both D$_2$O and H$_2$O; the former solvent simplifies the spectral data in a predictable way by eliminating contributions from exchangeable protons such as those on the amino nitrogen.

**NOESY:** The NOESY spectrum contains the same set of diagonal and off-diagonal peaks as the COSY spectrum reflecting e.g. through space couplings between protons on adjacent carbons. In addition there are new off-diagonal peaks that reflect three inter-residue proton-proton couplings of interest. These are

a) The $C_{\alpha}H$ of residue $i \Rightarrow$ NH of $i+1$ (called $d_{\alpha}N$ or $d_1$).

b) The NH of $i \Rightarrow$ NH of $i+1$ ($d_{NN}$, $d_2$).

c) The $C_{\beta}H$ of $i \Rightarrow$ NH of $i+1$ ($d_{\beta}N$, $d_3$).

The 2D regions ($d_1$, $d_2$, $d_3$) that contain this information are shown in Fig. VII-28.

A point in region $d_1$ connects two amino acids that are adjacent. This is accomplished by identifying a $C_{\alpha}$ connected to the NH on the subsequent amino acid ($d_{\alpha}N$). If the structure of a
Protein is to be solved the number of peaks in this region must equal the number of peptide bonds (a minor glitch is that proline residues, which do not have a proton on the amino N, do not contribute). But the COSY tells us which residue type is associated with each Cα and thus from the first Cα and then connecting the NH to its Cα, we obtain the sequence of a dipeptide. We then look in region d1 for connections from this dipeptide to the following residue and thus we build up a tripeptide and so on.

Thus the sequence analysis has the following parts:

1). Record the COSY spectrum in D2O. This suppresses all information from exchangeable protons (e.g. NH) and gives the simplest spectrum with only C-C connectivities. These are sufficient to allow many of the amino acids to be identified.

2). Repeat the experiment in H2O: exchangeable protons now give rise to additional peaks due to intra-amino acid Cα-N couplings. Ideally this will allow the assignment of all peaks to amino acid types (alanine vs. valine etc.)

3). Do a NOESY expt. in H2O. Many of the peaks from the COSY expt. will be present in the 2D map. The additional peaks identify couplings between two different amino acids. These amino acids will be adjacent with a high probability.

4). Combine the data displaying the COSY data in the lower right triangle and the unique NOESY data in the upper left triangle. A simplified data set is shown in Fig. VII-29.
5). (a) Pick a spot on the diagonal in the $C_\alpha$ region that you have already assigned to amino acid A.
(b) Draw a horizontal line from right to left into the NOESY NH region until you hit a spot.
(c) Drop a vertical to the diagonal. You arrive at a spot which is the NH of the next amino acid.
(d) Draw a horizontal line from left to right into the COSY $C_\alpha$ region until you hit a peak.
(e) Draw a vertical back up into the diagonal where you should hit another peak. This is the $C_\alpha$ of
the adjacent residue (towards the C-terminus); you have AB.
(f) Repeat b-e until you run into noise. Sequences of up to 10 residues can be identified.
Frequently broken by a proline which doesn't have a NH.

Variations on the above include:

(a) Pick the NH of A and draw a vertical into the NOESY region looking for the cross-peak to the
preceding amino acid. i.e. run procedure 5a-f backwards.

(b) Use N-N couplings or $C\beta$-N couplings. These are longer range and therefore weaker, but
usually provide enough information on overlapping sequences that the sequences deduced using step
5 can be aligned.

The method works well and there are a number of cases where published sequences deduced
from traditional chemical approaches on both proteins and nucleic acids chemical had to be revised.
At this point the sequence of the protein is to hand and the remaining task is to assign secondary and tertiary structure.

The common structural motifs (alpha-helix etc.) have characteristic NOESY spectra. For example a run of $d_{NN}$ connectivities of 2.8 Å and $d_{\alpha N}$ of 3.5 Å are diagnostic of $\alpha$-helices while runs of $d_{\alpha N} = 2.20$ Å and $d_{NN}$ of 4.3 Å are characteristic of antiparallel strands.

The desired crude distances between the protons involved in the NOE's can be obtained from either:

(i) the intensity of the off-diagonal (cross) peaks at a fixed value of $t_m$ or
(ii) from the rate of increase of the intensity of the cross-peaks as $t_m$ is increased.

In the first case the most intense peaks arise from the nearest pairs, in the second case the intensity of the cross-peaks of the nearest pairs increase more rapidly. As dipole-dipole interaction falls off with $r^{-6}$ these methods measure distances in the range 2-5 Å. Both approaches are “calibrated” by using the same experimental protocol on protons from a reference compound of known geometry.

In addition wherever proton J couplings between adjacent C atoms can been measured the Karplus relationships can be used to establish the dihedral angle between e.g. $C_{\alpha}H$–$C_{\beta}H$ or between $NH-C_{\alpha}H$.

So one determines as many NN and $C_{\alpha}N$ distances as possible together with stereochemistries of individual residues (J couplings) This information is presented to a so-called distance algorithm program. This program addresses the question:

"Given many pairs of points that are separated by some distance (actually minimum and maximum values) what is the arrangement of these points in space that will account for these distances?"

The program is constrained by certain facts e.g. peptides are planar; the geometry of helices and sheets can't be violated; atoms can't approach closer than their van der Waals radii etc. A summary of the approach plus a flow-chart of the nmr method for protein structure determination is given at the end of the additional material to this chapter.

Figs 30 & 31 have been deleted.
Medical Magnetic Resonance

The field of whole body magnetic resonance can be divided into two areas (Fig. VII-32, below):

(i) Magnetic Resonance Imaging (MRI), which has the goal of mapping cross-sections of the body providing the spatial distribution of a particular nmr parameter. One-or-more of the pulsed methodologies that we have encountered are employed. This is clinical magnetic resonance. It is the method of choice for imaging the head because ultrasound is reflected by the skull while X-rays can't distinguish white and gray matter very well. We will restrict our attention to MRI.

(ii) Magnetic Resonance Spectroscopy, which studies a well-defined volume element and records detailed spectra from this element thus allowing the study of metabolic processes. The $^{31}$P resonance of organic phosphates and the phosphate anion are most commonly observed.

MRI is the pre-eminent imaging techniques for probing the human body because it is non-invasive, provides excellent soft-tissue contrast and does not use ionizing radiation. Clinical MRI utilizes NMR signals from protons. The high natural abundance of protons (as water and fat) and high intrinsic sensitivity make it a very important tool for diagnostic imaging. Water accounts for about 55% of human body weight and 60-90% of soft tissue weight—the remainder arises from proteins and cell membranes. However the large molecular weight of proteins suppresses their proton nmr signals while phospholipids are not an important source of signal; it is the lipids found in fatty "droplets" within the cells of adipose tissue that are significant. As they have a chemical shift slightly different from water they can “blur” the picture taken using water protons.

The nmr part of this technique is simply the application of what we have already learnt. Thus simple FT-nmr is used to monitor proton concentration, magnetization inversion (180°-90°) to study $T_1$ (of utility in discriminating between fat (a shorter $T_1$) and water), spin-echo to study $T_2$ (has been used to demarcate tumors in head and neck) and a COSY look-alike is used to facilitate the selection of volume elements (voxels).

The novel feature of MRI is the ability to observe spatial distributions. We first consider a 1-dimensional example.
A 1-dimensional MRI spectrometer subjects the sample to two static magnetic fields. The first (\(H_0\)) provides a large constant field in the z-direction. This requires a superconducting magnet; the value of \(H_0\) is typically 1.5 tesla. The second field, denoted \(G_x\), provides a field that increases along x but is directed along z (See Fig. VII-33); this is provided by special coils called gradient coils. The gradient is typically 1 gauss/cm.

\[ H_{\text{tot}} = H_0 + x \frac{dG_x}{dx} \]  

(VII-22)

The location where \(H_{\text{tot}} = H_0\) is called the iso-center and is the origin of the coordinate system. Fields to the left of the iso-center are smaller than \(H_0\) etc.

At any point along the x-axis all of the nuclei in the yz plane experience the same magnetic field (\(H_{\text{tot}}\)).

Now locate a sample of protons (a drop of water) at \(x = -1\) cm. The field at that sample will be 1.5 - 0.0001 tesla. The same sample located at \(x = +1\) cm. will experience a field 1.5 + 0.0001 tesla. But \(\omega = \gamma H\) and if \(H\) is different at the two locations then the \(\omega\) of the sample will differ at the two locations.

A standard 1D FT-NMR experiment (90° pulse and observe the FID) on the first sample will yield a peak at one position on the frequency scale; the same nmr measurement on the second sample will yield a peak at a second position. And if we were to place drops of water at both positions a nmr spectrum would show two peaks. The NMR spectrum reflects the distribution of the spins in the direction established by the gradient. We can calibrate the nmr frequency in terms of distance by repeating the experiment with a sample holder shaped like a miniature dumb-bell in which the two spheres are separated by a known distance and the spheres are filled with water.
This is the key concept in MRI imaging; The technique is called frequency-encoding; the
distance information has been translated into frequency shifts of the nmr spectrum. Note that the size
of the gradient is much larger than any variations in chemical shifts of the protons, so all water
protons contribute equally.

Suppose the sample did not consist of two discrete blobs of water. For example suppose the
sample is a 1 mm x 20 mm slice of pastrami laid along the x-axis centered at the iso-point. The
proton content in the pastrami will vary considerably; in some places there's protein, in other places
there's fat and the water content will also depend on the variations in dryness.

The 1-dimensional MRI absorption of this sample will be
a spectrum that will originate at the frequency corresponding to \( x = -1 \) cm. and will terminate at \( x = +1 \) cm. In between it will show
variations in amplitude that directly reflect the variations in proton content along the strip; more accurately from each yz cross-
section as one moves along \( x \) (e.g. Fig II-34, bottom). The image obtained can be thought of as a "shadow". We now repeat the
process using additional coils that provide field gradients in other
directions (as before these gradient fields are z-directed but their
intensity varies with the chosen direction, e.g. y). Each time one
obtains a shadow-as viewed from the chosen direction-and a
computer can reconstruct these shadows to yield a 3D image of
the object (this is in fact the technique used in the X-ray CAT scan).

While the above procedure works today a different approach is employed. There is a
selective, single 90° pulse. During this pulse \( G_x \) is applied. The magnetization from a unique slice in
the yz plane (determined by \( H_0 \) + the z-contribution of \( G_x \)) is tipped into the xy plane. This
magnetization is left alone for a time \( t_1 \), but during this time \( G_x \) is turned off and a linear gradient \( G_y \)
is present. Different spins from the slice precess at rates dictated by their location in the y-gradient.
At the end of \( t_1 \) \( G_y \) is turned off, \( G_z \) is turned on and the FID is acquired. The experiment is
repeated many times changing the magnitude of the field gradient provided by \( G_y \). Each FID
contains the normal information about the z-axis as was described above for the 1-dimensional case.
But each FID contains information about how the spins evolved (variations in precession frequency)
with increases in \( G_y \). This information is present in the phase of the FID (The phase is determined
by the place on a sine wave where the FID began. For example a crossing point that is rising is a
phase of zero, a crossing point that is falling is a phase of 180° (see the first discussion of 2D nmr)).
Consequently a second FT with respect to \( G_y \) (converted into time units) yields the intensity
information with respect to the y-axis. This is called phase-encoding (note the difference with 2D-
nmr in which the second FT is done with respect to \( t_1 \)).

The third-dimension is studied by incrementally "moving" the slice forward in the x-
direction i.e. repeating the phase encoding method with a large number of x-gradients; hence
obtaining a stack of yz slices. Combining the data from all the slices yields the required 3D image.

The above sequence is the essence of the spin-warp method (I don't know why it got this
name). An extension to this method has the sequence 90°-\( t_1 \)-90°-\( t_2 \)-90°-observe(\( t_3 \)); \( G_y \) is present
during \( t_1 \), \( G_z \) during \( t_2 \) and \( G_x \) during the observe period. There is a 3-dimensional data set and the
data is FT'd 3-times, with respect to \( t_3 \), \( G_z \) and \( G_y \).
The actual techniques are somewhat more complicated than that just described primarily because of refinements (e.g. negative gradients) that serve to enhance the signal-to-noise ratio.

Depending upon the order in which the pulses and gradients are employed the reconstructed image will show (i) proton density; (ii) T1; and (iii) T2. An example of such data is shown in Fig. VII-32 (not available in electronic form) which contains images of the same head obtained by measuring (a) proton concentration; (b)T1 and (c) T2.

An extension to the technique are the use of “contrast enhancers”. These are chelates of high Z transition metals (frequently Gadolinium which has 7 unpaired electrons) that contain many unpaired electrons and are potent T1 enhancers. They are used to distinguish normal vs. abnormal tissues for the latter tend to absorb these chelates more rapidly than the former.

Some jargon: The time during which the FID is recorded is called the Image Formation period; The early phases of sample preparation is called the Preconditioning period.

Comments on Miscellaneous Points

1. "Why do the 2 hydrogens of a methylene group experience a J coupling but the 3 hydrogens of a methyl do not?"

The short answer is that the H of a methylene are prochiral and thus are in a different stereochemical environment; consequently they have slightly different δ. A longer, and possibly more satisfying, answer follows.

Consider two protons Hₐ and Hₐ attached to the same carbon which is attached to a chiral center with substituents X, Y and Z. The Newman projection of this molecule is shown above. There are three stable conformations: I, II and III, though the relative abundance of each (P_I, P_{II} and P_{III}) is not necessarily the same (also P_I + P_{II} + P_{III} = 1). Notice that Hₐ and Hₐ never swap positions; when Hₐ ⇒ Hₐ then Hₐ ⇒ R etc.

If the chemical shift of a proton located "between" X and Y is δ_XY etc. then

\[ \delta (H_a) = P_I \delta_{xz} + P_{II} \delta_{xy} + P_{III} \delta_{yz} \]
\[ \delta (H_b) = P_I \delta_{xy} + P_{II} \delta_{yz} + P_{III} \delta_{xz} \]

Since P_I ≠ P_{II} ≠ P_{III} then \( \delta (H_a) \neq \delta (H_b) \).

Because the chemical shifts are different the two protons are not equivalent and thus the logic used to explain why the 3 methyl protons do not interact with each other is not applicable.
2. An Elaboration on Spin-echoes; the "runner" analogy.

After the first 90° pulse all the magnetization (M) lies along +y-the starting line.

The runners (individual spins present in M) take off for a period of time $t_1$ during which time they spread out, the faster runners being ahead and the slow-pokes behind.

We then have the 180° pulse, the command to reverse direction. The runners commence their return to the starting line. There are several possible scenarios:

1]. IF no runners drop out (no spins return to +z via $T_1$ processes) AND all runners maintain their original speed everyone reaches the starting line at the same time, $t_1$, after the 180° pulse. The size of the echo is the same size as the original magnetization present in the xy plane following the 90° pulse.

2]. IF the only effect is some runners dropping out (sitting down) the remaining runners all reach the starting line at the same moment: now the size of the echo is reduced-there are less runners finishing. (This is the $T_1$ process.)

3]. IF the runners have a different speed on the return journey than they did on the forward journey they will not all reach the starting line at the same moment. Rather there will be a small spread-a bell shaped curve with its peak at $t_1$; the number of runners passing the line precisely at $t_1$ is only a fraction of the total number returning. This will also reduce the height of the echo. This broadening is called dephasing.

Why should the speed be different during the two $t_1$ periods? The precession frequency of each molecule-while nominally identical-exhibit small variations because of each molecule's interaction with its environment. The environment typically means the arrangement of all the other molecules around the molecule of interest.

This arrangement will change as the individual molecules move around. Thus each molecule's environment is changing and this leads to variations in the "environmental" perturbation of a molecule's intrinsic precession frequency. The longer the value of $t_1$ the greater the variation that is experienced and the fatter and shorter the bell-shaped curve.

(An analogy in terms of the track is that after the 180° pulse the fastest runner is at the rear and now has to negotiate all the other runners on the return journey; this leads to an effective slowing down that was not experienced on the outward journey).

The spin echo experiment measures both contributions to the echo size as the parameter $T_2$. It should now be clear that $T_2$ contains both the $T_1$ processes and the "dephasing" processes; these latter are quantified by the parameter $T'_2$ with

$$\frac{1}{T_2} = \frac{1}{T_1} + \frac{1}{T'_2}$$
3. COSY (Chemical Shift Correlation)

The important thing to remember about 2D nmr is that the x-axis (labeled $\nu_2$) specifies the characteristic frequencies of the classes of nuclei studied while the y-axis contains information about interactions between each class and the other classes that occurred during the time period $t_1$ (which yields the $\nu_1$ label—see Fig VII-30).

As initially introduced Jeener’s experiment wasn’t too useful—but we now come to the important bit. Consider an AX system with two protons coupled by spin-spin (J) interactions.

We can represent the relative energies of the spin system as shown in Fig VII-35 (upper) in which the first $\alpha/\beta$ represents the spin alignment of the X proton and the second $\alpha/\beta$ that of the A proton. $\alpha\alpha$ is the most stable alignment and $\beta\beta$ the least stable. Because A and X have different chemical shifts the energies of the levels denoted $\alpha\beta$ and $\beta\alpha$ are slightly different (exaggerated in the figure). The nmr spectrum is idealized in Fig VII-35, lower (recall higher energies are to the left).
The COSY pulse (a la Jeener) sequence is shown in Fig. VII-36. To get some understanding of what is going on we do the thought experiment diagrammed in Fig 37.

Fig. VII-36

We subject the sample to a 90° pulse designed only to excite the transition $A_1$; the A protons in one-half of the molecules will be flipped into the xy plane. I will call these the probe protons. Some time later we apply a second pulse designed to affect all the transitions. The remaining protons will now be flipped into the xy plane but the fate of the probe protons depends on the delay between the two pulses.
1) The delay is zero (Fig. VII-37, top row). The probe protons are tipped down to -z and the population of this set of protons is inverted. (We have depopulated $\beta\alpha$ and overpopulated $\beta\beta$). Any other transitions that involve the levels $\beta\alpha$ and $\beta\beta$ will now be affected because their population differences will have changed.

2) The delay is sufficient that the probe protons lies along +/- x. The second pulse has no effect and all protons now lie in the xy plane.

3) The delay is long enough that the probe protons lies along -y (Fig. VII-37, middle row); the second pulse returns them to +z and the normal population differences are restored.

4) The delay is long enough that the probe protons lies along +y (Fig VII-37, bottom); this repeats case 1.

The result is that the intensities of the coupled transitions will be sensitive to the precession frequency of the probe protons as revealed by the response of the amplitudes of the xy magnetization to the variations in the delay time.

The FT of the FID will show all 4 peaks on the $\nu_2$ axis while the $\nu_1$ axis will only show the frequency of the probe protons i.e. the frequency of the transition $A_1$.

In reality the first pulse is not selective but tips all protons into the xy plane. The scenario just summarized is now happening to all protons simultaneously; this is too complicated to envision—which is why I indulged in the simplifying thought experiment. Now the FT of the FID shows all frequencies in both dimensions and the resultant contour map shows 16 “peaks” as shown in Fig 39.
In practice the FT is deliberately broadened (the FID is multiplied by a rapidly decaying exponential) to obscure peaks that are only separated by a few Hz with the result that rather than getting clusters of 4 each cluster is reduced to a single feature at the average position. It is often useful to restore the resolution because the separation of the spots within a cluster (a) yield the value of J; and (b) can aid in assignment. For example, glycine with 2 C$_\alpha$H gives an “off-diagonal spot” with 8 components. Thus the diagonal shows features at the chemical shift of A and X (cf p. VII-39) and the features which lie off-diagonal shows that A is connected to X. (If you are familiar with the technique of decoupling in a conventional nmr spectrometer you can think of this as the FT way of accomplishing the same goal).
Because J-coupling is a through-bond process it basically involves protons on adjacent atoms (i.e. 3 bonds apart). This information can therefore be used to identify peaks in the nmr spectrum that arise from atoms present on the same amino acid residue. To get correlations on protons more than 2C apart enhanced COSY techniques are required; these are called relayed COSY (one such has the acronym DQF-COSY).

Fig. VII-27 and associated text describe an idealized result for alanine.

4. NOESY

NOESY is Nuclear Overhauser Enhancement Spectroscopy. It is an extension to the COSY experiment which relies upon through-space effects and can be utilized to identify nearest neighbors (as in adjacent amino acids). In essence population changes are induced as before; however in this case one is exploiting the through-space dipole-dipole interaction between spins which causes populations to equilibrate via relaxation processes. This equilibration takes time which is provided by the period $t_m$ (called the mixing time). The experiment is carried out using a pulse sequence (Fig. 42) somewhat similar to that of COSY. Indeed, the NOESY experiment characterizes the fate of the z-magnetization that was created by the second pulse of the COSY experiment.

In NOESY experiments preliminary experiments are conducted to establish the “best” value for $t_m$; this value is then fixed. $t_1$ is varied as before.

With this pulse sequence there is a net 180° pulse before the acquisition pulse. In the period $t_1$ the spins are all precessing in the xy plane. The second pulse tips some of this magnetization back along z (that which was aligned along y goes to -z, that aligned along -y goes to +z. Any
magnetization along ±z will be exposed to magnetic dipole-dipole coupling with adjacent magnetic (nuclear) moments for the period \( t_m \); in our case they can be between \( H \) in the same amino acid or between \( H \) in adjacent amino acids (recall only z-magnetization is a constant of the motion). It is the component along -y that is relevant because it represents a non-equilibrium contribution to \( M_z \) and these spins will return to +z by spin-lattice relaxation. This process is accelerated by the dipole-dipole interactions. Protons close in space interact strongly and hence return to +z more rapidly; protons distant in space interact weakly and hence return to +z more slowly. (These population changes "migrate" to the other spins as a function of distance, the closer spins adjusting first, the more distant adjusting later; thus at a fixed value of \( t_m \) different protons respond differently, depending on their distance from the spin providing the dipole field.) Any dipolar interactions between A and X will be modulated by these couplings. As these dipole-dipole interactions provide pathways for relaxation the distribution of the population of the spin system is modified. The third pulse tips spins along +z back down into xy where they can be characterized; the composition of the magnetization along +z can then be established.

A simple numeric example that might help clarify the phenomenon!

Imagine we have 2 spin systems A and B, with frequencies of 11 Hz and 10 Hz. At \( t=0, 1, 2... \) sec. both will lie along +y and both will be flipped down to -z. These two spin-systems can then interact via dipole-dipole processes (With only two spins there will be no difference in dipole-dipole coupling; this contribution to \( T_1 \) will be the same for A and B; we need A, B and C for this to be relevant). This part is obvious. The next part is not so obvious because I have been "pretending" that only magnetization that lies along +y can be flipped. The reality is that it’s the component of the magnetization along y which is flipped; this varies as \( \cos^2 \theta \) where \( \theta \) is the angle between the magnetization and the y-axis. For example

<table>
<thead>
<tr>
<th>Time (sec.)</th>
<th>Spin A degrees</th>
<th>Spin B degrees</th>
<th>( \theta_A )</th>
<th>( \theta_B )</th>
<th>( M_y A )</th>
<th>( M_y B )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1/11</td>
<td>360</td>
<td>327</td>
<td>0</td>
<td>32.7</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>2/11</td>
<td>360</td>
<td>297</td>
<td>0</td>
<td>65.4</td>
<td>1</td>
<td>0.17</td>
</tr>
<tr>
<td>etc.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A comparable table interchanging the roles of A and B could be generated by incrementing time in 1/10'th sec. intervals. This table shows that the extent of the dipole coupling occurring during \( t_m \) at A from B (and vice-versa) will depend on the relative frequencies of A and B which dictates the proportions of A and B flipped from +y to -z. The changes in population (intensity) that occur during \( t_m \) are now observed by the third pulse which tips any ±z magnetization back into the xy plane and initiates a normal acquisition period. So again we get a set of FID's each one recorded at a unique \( t_1 \) and the FT of this data set yields another 2D spectrum with the same characteristic appearance as before except that the off-diagonal peaks identify protons that are linked to one another via through-space dipolar relaxation.
5. Overview of Protein Structure determination by NMR.

1. Conduct a COSY experiment. Tell the FT software to sacrifice resolution for improved signal-to-noise thus hiding the structure present in the COSY peaks. Use the off-diagonal peaks to connect peaks on the diagonal and in this way assign the NH, CαH and R chain of each amino acid. (e.g. one alanine will contribute a particular set of 3 peaks, NH CαH and CH₃; a second alanine will have its own particular set of 3.) Every diagonal peak is to be associated with an amino acid type (ala vs val vs tyr etc.).

2. Conduct a NOESY expt.

3. Prepare a graph in which only the CαH and NH data (already assigned to specific amino acid types in part 1) is present and put the COSY data in the lower triangle and the NOESY data in the upper triangle. Start with a specific α-C and carry out a snail-trail to establish the sequence in the direction of the carboxy-terminus. If there are spots remaining go to the NH of the residue with which you started and do a reverse snail-trail to carry the sequencing towards the amino-terminus. With luck you might be able to proceed 10 residues in each direction. In a protein of any size this will have to be repeated several times to generate non-overlapping sequences of up to 20 residues long.

4. Repeat 3 using longer range NOESY couplings. This will typically yield overlapping sequences; sequences that have parts common to two sequences determined in 3 and hence determine the order of those two sequences.

5. Re-analyze your COSY data telling the FT software to use a higher resolution (at the expense of noisier data) thus allowing the component structure in the COSY peaks due to J coupling to be observed. The separation of the components gives J. Off-diagonal J's in the NH-Cα region are used to determine the dihedral angle between NH and CαH of this particular amino acid; J's in the δ = 1-5 ppm region are used to determine dihedral angles between e.g. CαH-CβH. The angles are obtained by solving the Karplus equation for that value of J. There may be ambiguities-see the figure in the notes.

6. Use the NOESY intensities of assigned cross-peaks to measure distances between the two protons responsible for the cross-peak. The distance is obtained by comparing the intensity with that observed with a standard of known distance.

7. Next page.
Distance Geometry Program

Sequence Angles  Distances  Peptide bond parameters  Helix, sheet parameters
van der Waals radii

First Guess at Global Conformation

Molecular Dynamics

Energy Minimization

Final Structure