Biological NMR Spectroscopy

- Introduction to the principles of NMR (basic phenomenon, spectral parameters, Fourier transform)
- Understand simple 1D experiments on small molecules
- Explanation of $^{1}H/^{13}C$ 2D NMR techniques on proteins
- Interpretation of 1D and 2D $^{1}H/^{13}C$ NMR spectra (problem class)

Applications of NMR:

- Analytical – in synthesis for quickly checking for correct product
- Chemical structure – Chemical shift and couplings for identifying molecular structure
- 3D structure determination –nuclear Overhauser effects in proteins, RNA/DNAs, oligosaccharides etc…
- Molecular interactions – chemical shift mapping and NOEs in enzyme/inhibitor, protein/DNA etc…
- Molecular dynamics – relaxation and the NMR time scale for monitor the kinetics of processes
- NMR imaging – encoding spatial dimensions
- Metabolomics – metabolic profiling of organisms

What is Spin?

- Fundamental particles possess an intrinsic angular momentum, **I**, known as spin (similarity to spinning top)
- Intrinsic angular momentum is quantised (has discrete values and direction) and comes in multiples of ½ such that there are (2I + 1) values between –I and +I (spin quantum number)
- Protons, unpaired electrons (EPR/ESR) and neutrons possess spin and I = ½ (note: neutrons have no net charge but possess magnetic moment)
- Nuclei with even protons and neutrons have a 0 spin angular momentum. Nuclei with odd proton and neutrons have integral spin quantum numbers. The rest have 1/2-integral spin (Nuclear Magnetic Resonance)

A charged nucleus (e.g., ^{1}H) rotating with angular frequency creates a magnetic field B and is equivalent to a small bar magnet whose axis is coincident with the spin rotation axis.

Nuclear Magnetic Resonance

A nucleus with non-zero spin has a magnetic moment and the orientation of this magnet is determined by the value of M_I → it can have 2I + 1 orientation (in a magnetic field this 2I+1 orientations have different energies).
Nuclear spin quantum number= I
Value of energies:

$$E_{Mi} = -\gamma_N \hbar B_0 M_I$$

$\gamma_N = nuclear\ magnetogyric\ ratio$

For spin-1/2 nuclei with positive γ_N, the energy is sometime written in terms of the nuclear magneton μ_N $\mu_N = \frac{e\hbar}{2Mp}$

Alternative expression of energy in terms of empirical constant nuclear g factor g_I

$$E_{Mi} = g_I \, \mu_N B_0 M_I$$

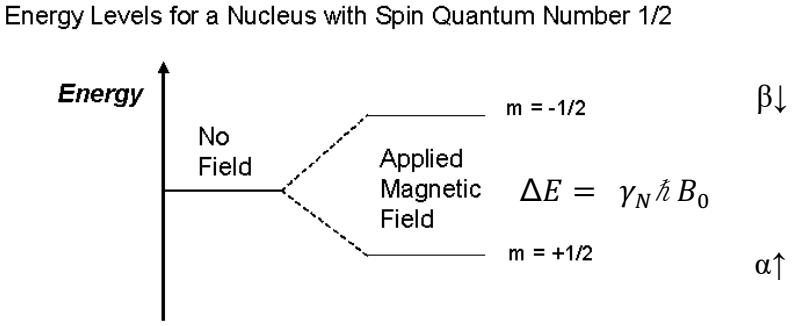
g-factors are experimentally determined dimensionless quantities that vary between -6 and +6

- Positive γ_N (and g_I) = nuclear magnet has the same direction as nuclear spin (^{1}H)
- Negative γ_N = points in the opposite direction

The energy separation of the two states (α and β) of spin-1/2 nucleus is:

$$\Delta E = E_\beta - E_\alpha = \frac{1}{2}\gamma_N \hbar B_0 - \left(-\frac{1}{2}\gamma_N \hbar B_0\right) = \gamma_N \hbar B_0$$

Energy levels of a spin-1/2 nucleus (^{1}H or ^{13}C) in a magnetic field:



Resonance occurs when the energy separation of the levels matches the energy of the photons in the electromagnetic field ($E_{EM} = E_{nucleus} \rightarrow h\nu = \gamma_N \hbar B_0$)

Because for nuclei with positive γ_N, the α state lies below the β state:

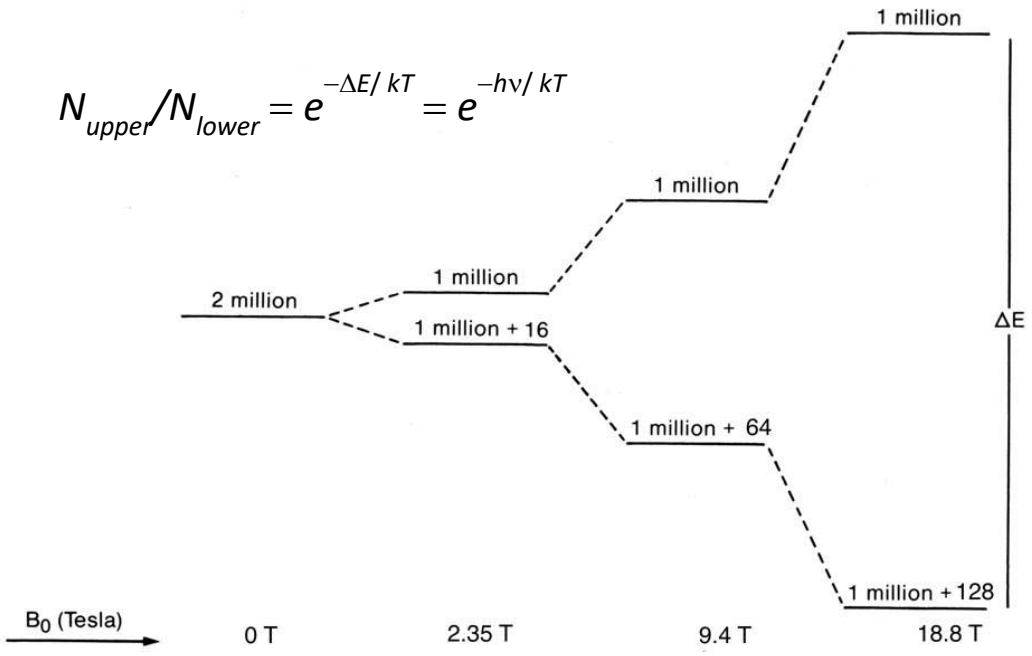
$$E_\beta - E_\alpha > 0$$

From Boltzmann distribution, the populations of the α and β states ($N_\alpha \, and \, N_\beta$) are proportional to $e^{\frac{-E_\alpha}{Kb\,T}}$ and $e^{\frac{-E_\beta}{Kb\,T}}$

The ratio of populations at equilibrium: $\frac{N_\alpha}{N_\beta} = e^{\frac{-(E_\alpha - E_\beta)}{Kb\,T}}$

So in nuclei ($E_\beta - E_\alpha > 0$), $\frac{N_\alpha}{N_\beta} < 1$

→ there are slightly more α spins than β spins (opposite of electrons)



If the sample is exposed to radiation of frequency ν, the energy separations come into resonance with the radiation when the frequency satisfies the resonance condition:

$E_{EM} = E_{nucleus} \rightarrow h\nu = \gamma_N \hbar B_0$ or $\nu = \frac{\gamma_N B_0}{2\pi}$

At resonance there is strong coupling between the nuclear spins and the radiation, and strong absorption occurs as the spins flip from α (low energy) to β (high energy)
These transitions are referred to as Nuclear Magnetic Resonance
Intensity of NMR transitions is proportional to $(N_\alpha - N_\beta) B_0$

Where $N_\alpha - N_\beta = \frac{N \gamma_N \hbar B_0}{2 Kb T}$ where N = total number of spins $(N = N_\alpha + N_\beta)$

- Decreasing the temperature increases the intensity by increasing the population difference
- Also NMR transitions can be enhanced significantly by increasing the strength of the applied magnetic field
- Also Absorption of nuclei with large magnetogyric ratios (^{1}H for example) are more intense than those with small γ_N

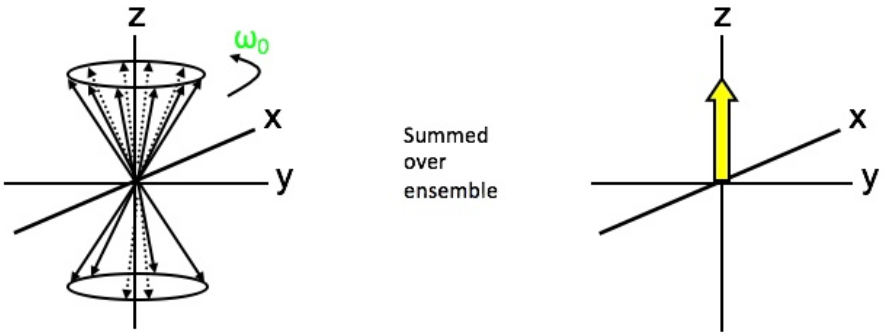
In its simplest form, NMR is the observation of the frequency at which magnetic nuclei come into resonance with an electromagnetic field when the molecule is exposed to a strong magnetic field

How to record a NMR spectrum?

The frequency of irradiation is swept through all resonances and the absorption of RF is measured. Continuous wave (CW) NMR is very inefficient

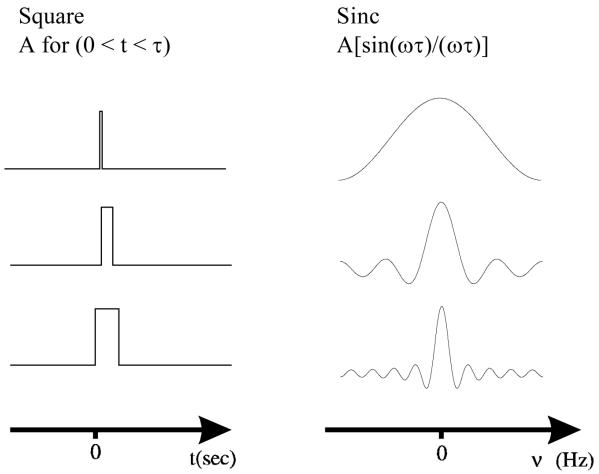
Larmor precession:
Spin can be thought as a small magnetic field which will interact with an external applied magnetic field (B_0) (note: right hand grip rule)
When placed in a magnetic field, charged particles will precess about the magnetic field. In NMR, the charged nucleus, will then exhibit precessional motion at a characteristic frequency known as the Larmor Frequency. The Larmor fequency is specific to each nucleus. The Larmor frequency is measured during the NMR experiment, as it is dependent on the magnetic field that the nucleus experiences

The vector model is to conceptualise the situation of an ensemble (collection) of spins (simply we rotate the axis with the precessional frequency)



The RF Pulse

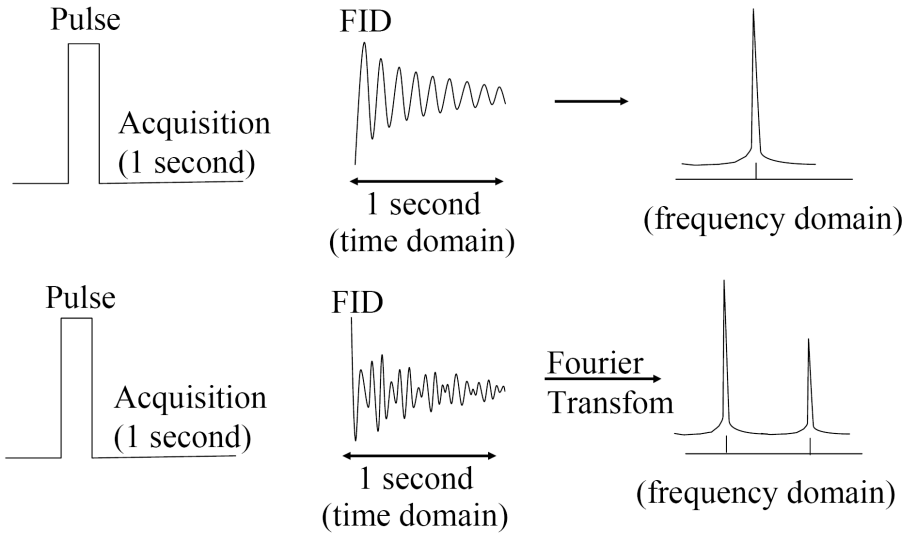
A very short radio frequency pulse contains many frequencies in a broad band and thus can excite resonances of all spins in a sample at the same time.



Fourier pairs are two functions: frequency domain form and the corresponding time domain form

RF pulse contains and linearly oscillating B and E field – can viewed as counter-rotating magnetic fields – when the frequency matches the Larmor frequency energy is absorbed

The precessing magnetization can be detected to give a signal which oscillates at the Larmor frequency – the free induction signal. This signal will eventually decay away due to the action of relaxation; the signal is therefore often called the *free induction decay* or FID. The question is how do we turn this signal, which depends on *time*, into the spectrum, in which the horizontal axis is *frequency*.



Time decay RF signal generated is called the 'free induction decay' (FID) and is related to the traditional frequency spectrum though a Fourier Transform

Parameters of NMR Spectra

1. Chemical Shift (δ)
2. Integral
3. Scalar Coupling (J)
4. Dipolar Coupling (D)
5. Relaxation times (T1 and T2)
6. Nuclear Overhauser Effect (NOE)

1. Chemical Shift

Electrons associated with atoms circulate about the direction of an applied magnetic field, which causes a small, local magnetic field at the nucleus. This can oppose or reinforce the applied field (e.g. aromatics). Electronegative groups withdraw electrons away from nucleus there reducing shield effect.
Induced local magnetic field: $\delta B = -\sigma B_0$

σ (dimensionless)= shielding constant; can be positive or negative depending whether induced field adds or subtracts to the applied field.

⇒ The ability of the applied field to induce a circulation of electrons through the nuclear framework of the molecule depends on the details of the electronic structure near the magnetic nucleus of interest → nuclei in different chemical groups have different shielding constants.

Total local field: $B_{tot} = B_0 + \delta B = (1-\sigma)B_0$

⇒ Resonance conditions: $\nu = \frac{\gamma_N B_{loc}}{2\pi} = \frac{\gamma n}{2\pi}(1-\sigma)B_0$

Because σ varies with the microenvironment, different nuclei come into resonance at different frequencies.

The chemical shift of a nucleus is the difference between ts resonance frequency and that of a reference standard
The standard for protons is the proton resonance in tetramethylsilane (TMS), highly aliphatic

Chemical shifts are reported on the δ scale: $\delta = \frac{\nu - \nu_0}{\nu^\circ} \cdot 10^6$

- If $\delta > 0$ → nucleus is deshielded
- If $\delta > 0$ → nucleus is shielded

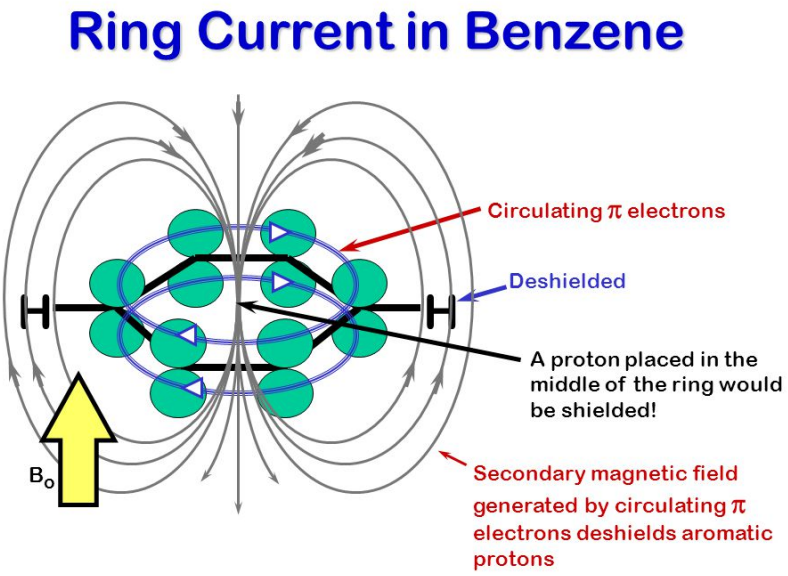
Observed shielding constant is the sum of three contributions:

$$\sigma = \sigma_{local} + \sigma_{neighbor} + \sigma_{solvent}$$

Local is form the electrons of the atom that contains our nucleus
Neighbour is from the electrons that form the rest of the molecule
Solvent is from electrons of the solvent molecules

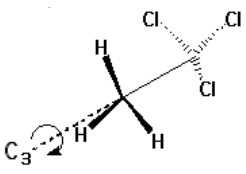
Local contribution is proportional to electron density of the atom
Shielding is decreased if electron density on the atom is reduced by the influence of electronegative atoms nearby
Reduction in shielding translates into an increase in deshielding and hence to an increase in the chemical shift as the electronegativity of a neighbouring atom increases.

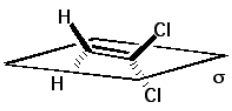
Another contribution to σ (local) arises from the ability of the applied field to force the electrons to circulate through the molecules by making use of orbitals that are unoccupied in the ground state. The neighbouring group contribution arises from the currents induced in nearby groups of atoms. The field induces a ring current, a circulation of electrons around the ring, when it's applied perpendicular to the molecular plane. Protons in the plane are deshielded, but any that happen to lie above or below the plane (substituents of the ring) are shielded.

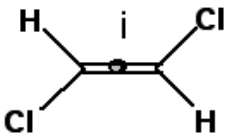


Chemical shift equivalence

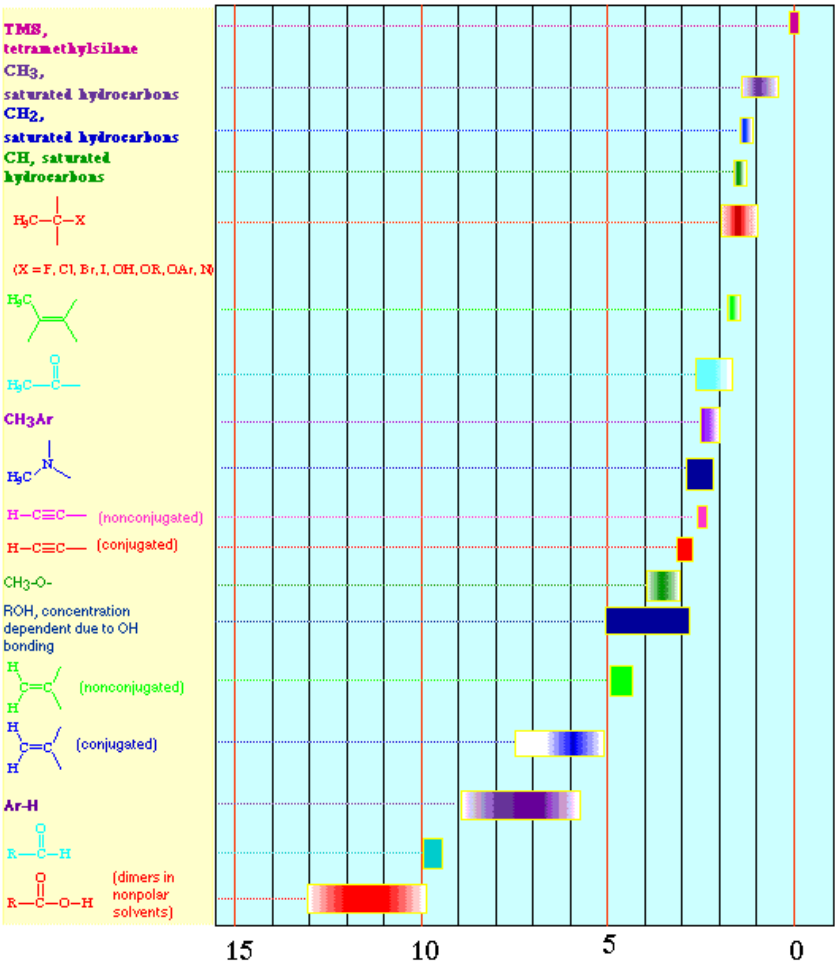
Nuclei that are interchangeable by a symmetry operation





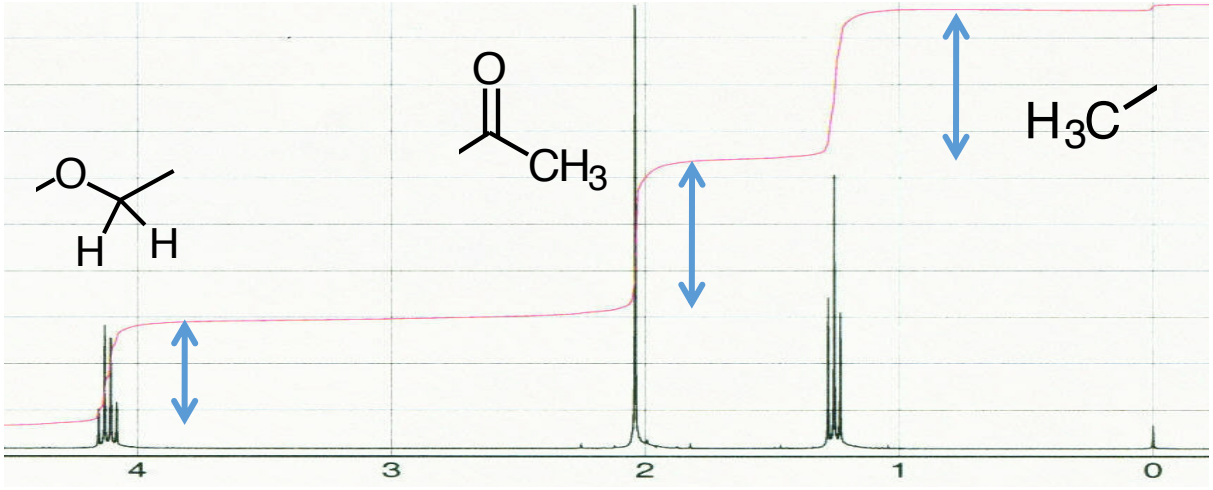


... or rapid exchange on the NMR timescale have the same chemical shift



2. Integral

The area under the peak gives us the relative numbers of nuclei
(*note: not always true – the NOE in ^{13}C)



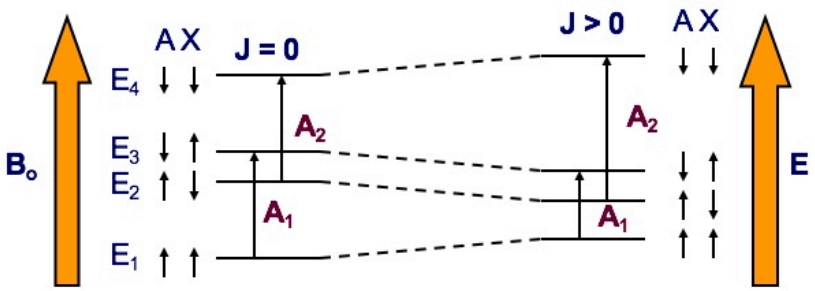
3. Scalar Coupling

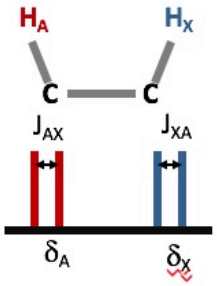
Two adjacent spin ½ nuclei will experience an electron coupled spin-spin interaction. So the frequency of A is difference dependent on the alignment of X

Observed as the splitting of the resonance signal, the linewidth in the spectrum

The splitting of the resonance signals into multiplets is not caused by direct dipolar interactions between magnetic dipole moments (dipolar coupling)
But the interaction is mediated by the electrons of the bonds between the two nuclei. This interaction is known as spin-spin coupling. The strength of this interaction is measured by the scalar coupling constant ${}^{N}J_{AX}$
n= number of covalent bonds between the nuclei (usually between 1 to a max of 4)

Scalar coupling modifies the energy levels of the system, and the NMR spectrum is modified correspondingly.
A weakly coupled two-spin system is referred to as an AX spin system
A strongly coupled two spin system is referred to as an AB

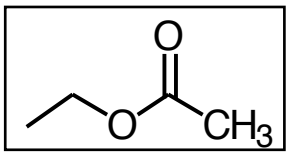




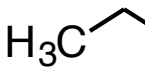
Structure	Pattern
A	1
AX	1:1
AX_2	1:2:1
AX_3	1:3:3:1

If two spin-1/2 nuclei (A and X) are scalar coupled with a coupling constant J_{AX}, evolution of transverse magnetization of the A spin leads to splitting of the resonance signal into multiplet components at frequencies

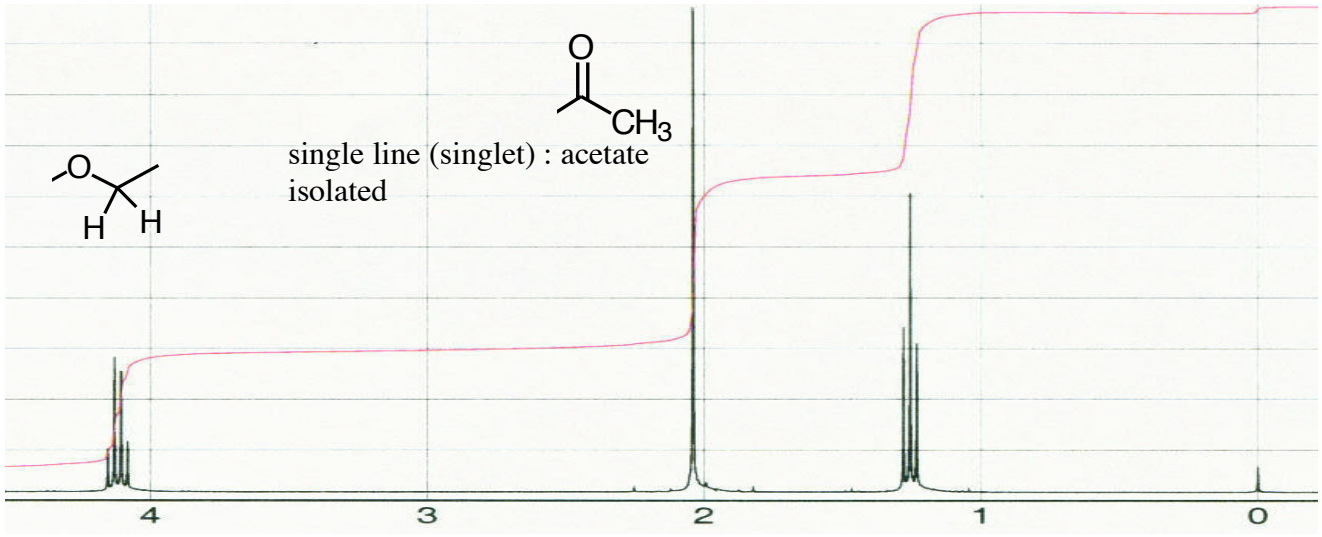
$$\nu_A + \pi J_{AX} \text{ and } \nu_A - \pi J_{AX}$$



Ethyl Acetate Coupling Systems



3 lines (triplet) : ethyl adjacent to CH_2

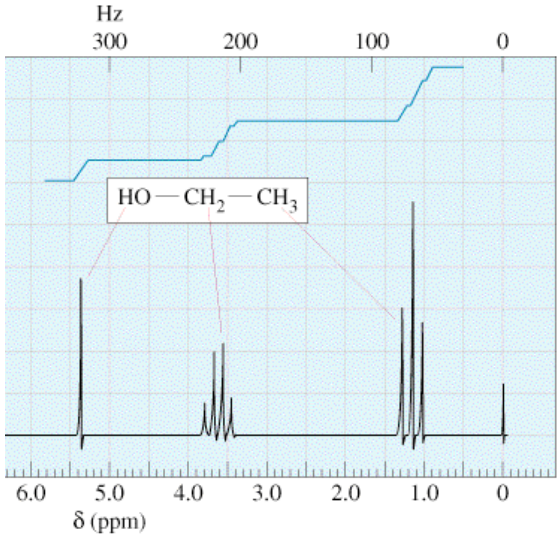


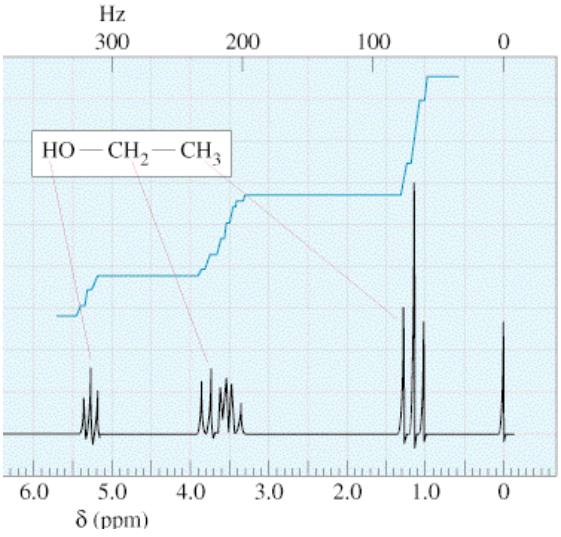
single line (singlet) : acetate isolated

Four lines (quartet) : ester Adjacent to CH3

3 lines (triplet) : methyl adjacent to CH_2

Scalar Coupling to OH/NHs





Pure samples of ethanol show splitting to OH but a small amount of acidic/basic impurities will remove splitting (D_20 shake)

Scalar Coupling Constants

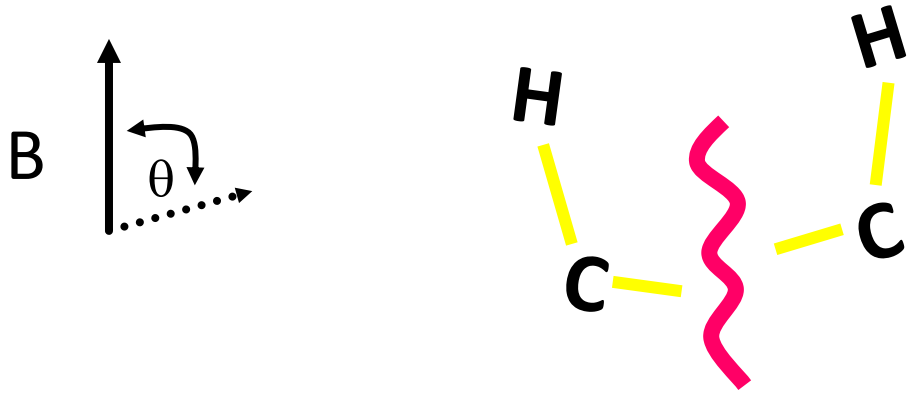
Pair	J/Hz
^{1}H-C-^{1}H	-12 to 15
^{1}H-CC-^{1}H	2-14
^{1}H-NC-^{1}H	1-10
^{1}H-^{13}C	~140
^{1}H-C-^{13}C	5
^{1}H-^{15}N	89-95

4. Dipolar Coupling

The dipolar coupling is defined by a direct magnetic coupling and is dependent on $1/r^3$

It is a through-space interaction (gives rise to Nuclear Overhauser Effect)

Dipolar coupling results from each spin generating a magnetic field that is oriented parallel to the nuclear spin vector. Two spins that are close to each other in space experience each other's magnetic field, which leads to a slightly different effective magnetic field B_{eff} at one spin that depends on the orientation of both magnetic dipoles.



Magnitude of coupling also has an orientation dependence as the second order Legendre polynomial of the cosine of the angle between the vector connecting the interacting particles and direction of the magnetic field.

Dipolar interaction causes spin relaxation, but it's not the only way of relaxation.

5. Relaxation Times

As resonant absorption continues, the population of the upper state rises to match that of the lower state. We can expect the intensity of the absorption signal to decrease with time as the populations of the spin equalize. This decrease due to the progressive equalization of populations is called **saturation**.

Relaxation: the non-radiative return to an equilibrium distribution of populations in a system

⇒ T1: spin-lattice relaxation time (time in which the system returns to equilibrium distribution, excess of α over β spins) This is relaxation in the *z*-direction and leads to restoration of Boltzmann equilibrium

- ENTHALPIC

⇒ T2: spin-spin relaxation time (exponential return of the system into random arrangement of spins) is used to quantify the rate of the decay of the magnetization within the *xy* plane. After a 90° pulse the nuclear spins are aligned in one direction (are said to be phase coherent), but this arrangement is gradually lost

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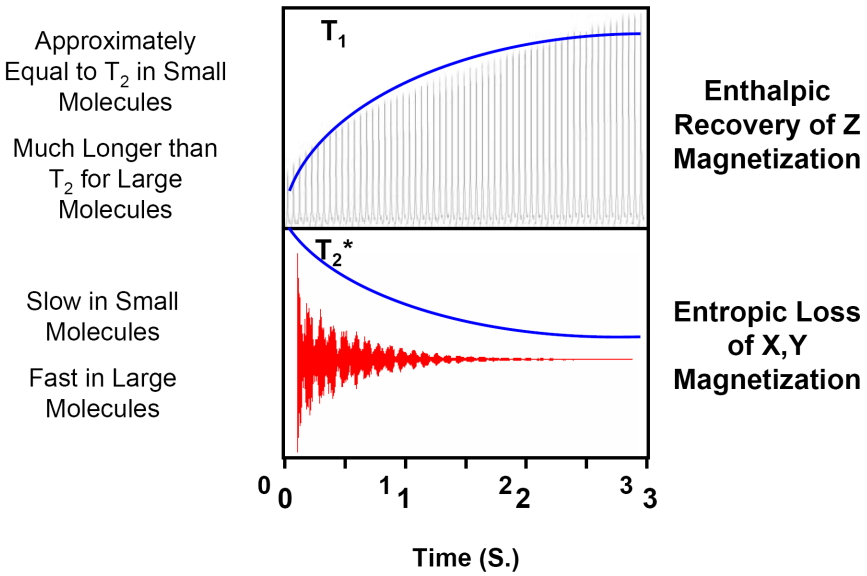
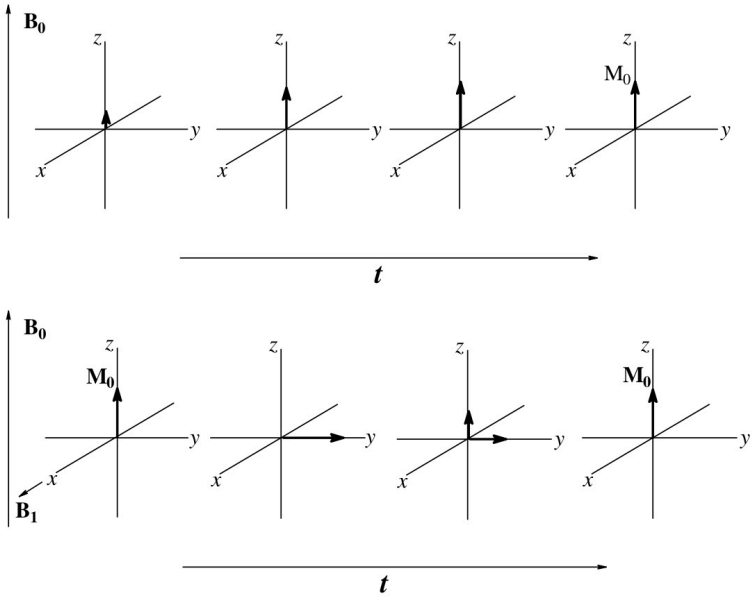
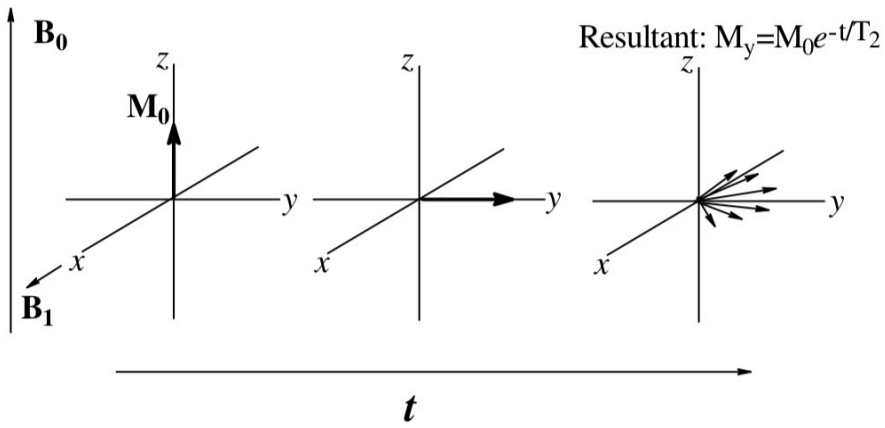


T1 Relaxation:



T2 Relaxation:



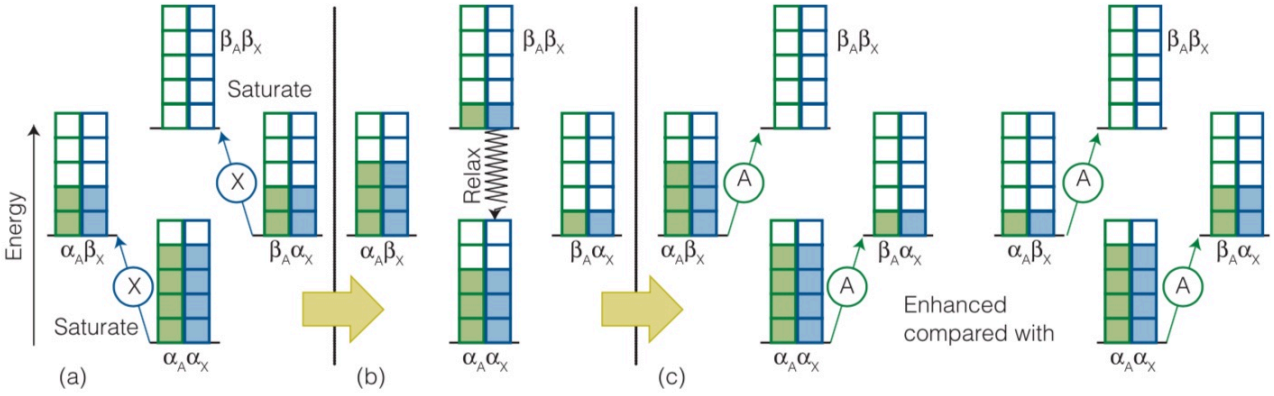
6. Nuclear Overhauser Effect

Modification of one resonance (A) by saturation of another (B)
NOE is the change in intensity of an NMR signal when the transition of another are saturated. This is a through space effect and is dependent on $1/r^6$ and τ_C

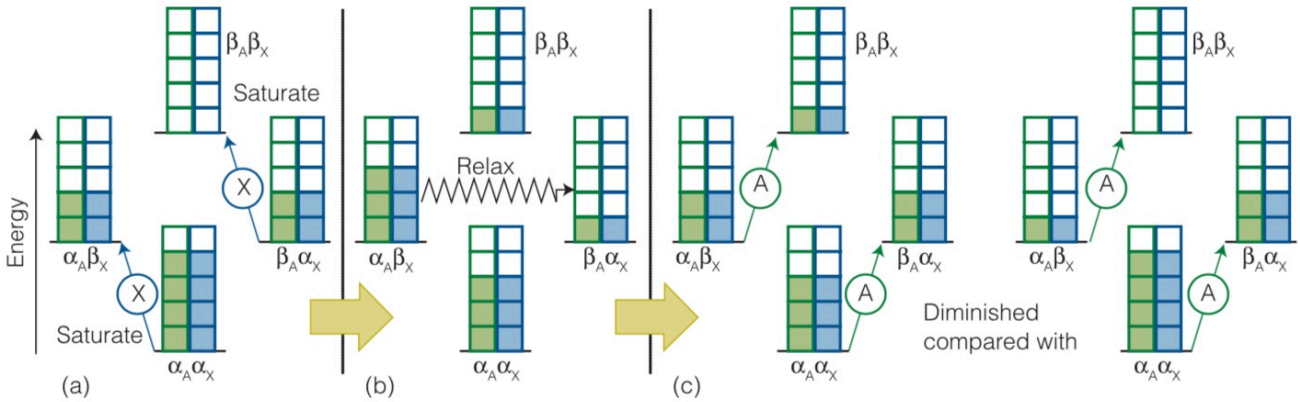
Consider AX system: two spins interact by magnetic dipole-dipole interaction. Expect two lines in spectrum (one from A and from X). However when irradiate system with RF at resonance frequency of X using high intensity to saturate transition (equalize populations of X level), A resonance is modified.

Relaxation can occur in a variety of ways if there is a dipolar interaction between A and X spin

1. Magnetic field acting between the two spins can cause them both to flip from α to β, so the $\alpha_A\alpha_X$ and $\beta_A\beta_X$ states regain their thermal equilibrium population. However the populations of the $\alpha_A\beta_X$ and $\beta_A\alpha_X$ levels remain unchanged at the values characteristic of saturation. The population difference between the states joined by transition A is ow greater than at equilibrium → so resonance absorption is enhanced



2. dipolar interaction between two spins can cause α to flip to β and β to flip to α. This transition equilibrates the populations of $\alpha_A\beta_X$ and $\beta_A\alpha_X$ but leaves the $\alpha_A\alpha_X$ and $\beta_A\beta_X$ populations unchanged → resonance absorption is diminished



Carbon-13 (^{13}C) NMR

⇒ Problems
- Low abundance (1.1%)
- Low magnetic moment – m (1/4 of 1H)
- Long relaxation times
- Integrals do not correspond to # of Cs

⇒ Advantages
- Spin =1/2
- No effective ^{13}C-^{13}C coupling
- Large chemical shift range (>200ppm)
- Easy to interpret

^{13}C is a dilute-spin species → unlikely that more than one ^{13}C nucleus will be found in any given small molecule (need to isotope label sample)

It is not necessary to take into account the ^{13}C -^{13}C spin-spin coupling

Protons however are abundant-abundant spin species (many coupling of one ^{13}C with many protons)

To avoid difficulty in ^{13}C-NMR spectra, they are observed with proton decoupling:
Irradiate protons with a second, strong resonant radiofrequency → they will undergo rapid spin reorientations and ^{13}C nucleus senses an average orientation → its resonance is a single line and not a 1:3:3:1 quartet.
Also proton decoupling enhances the sensitivity because the intensity is concentrated into a single transition frequency instead of being spread over several transition frequencies.

Basic ^{13}C- spectra

- ^{13}C nuclei are split by directly attached protons and proton attached to adjacent C atoms
- Give complex spectra – **saturate** ^{1}H signals to remove coupling

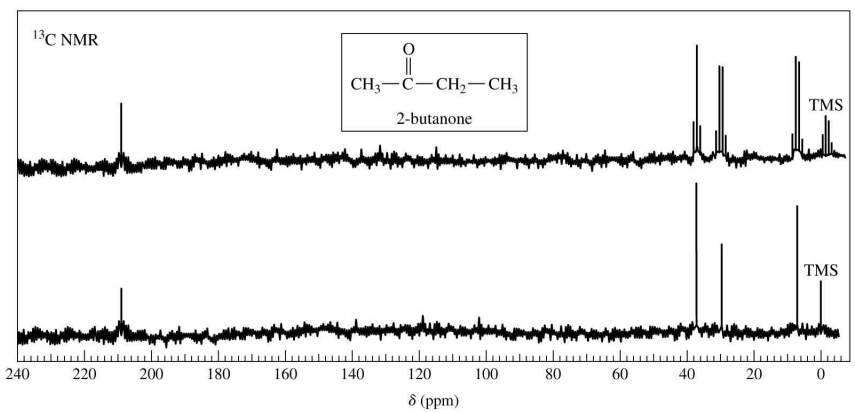
With off-resonance ^{1}H decoupling

- ^{13}C nuclei are split only by the protons attached directly to them.
- A carbon with *N* number of protons gives a signal with *N* + 1 peaks.

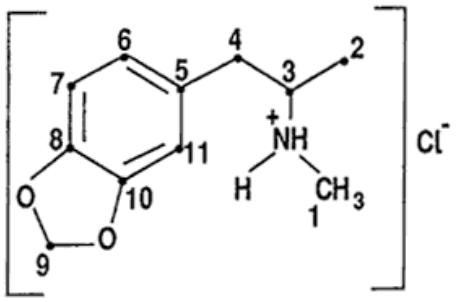
With broadband ^{1}H decoupling

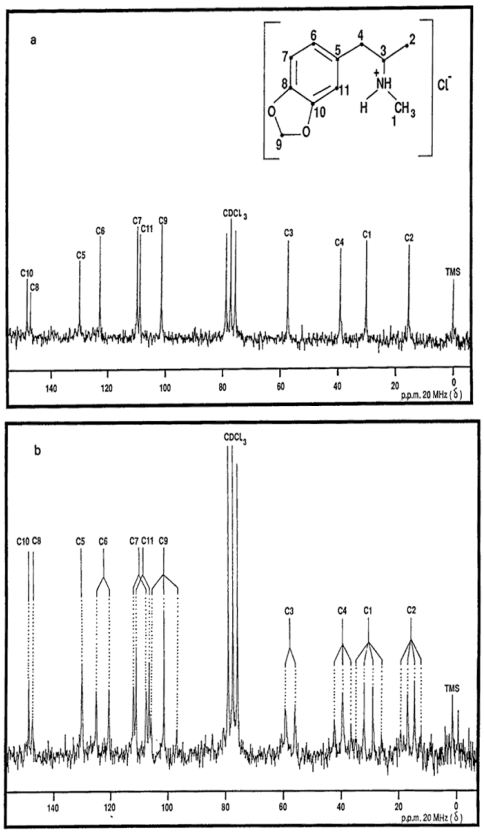
- Singlets for all carbons

2-Butanone ^{13}C- spectra:

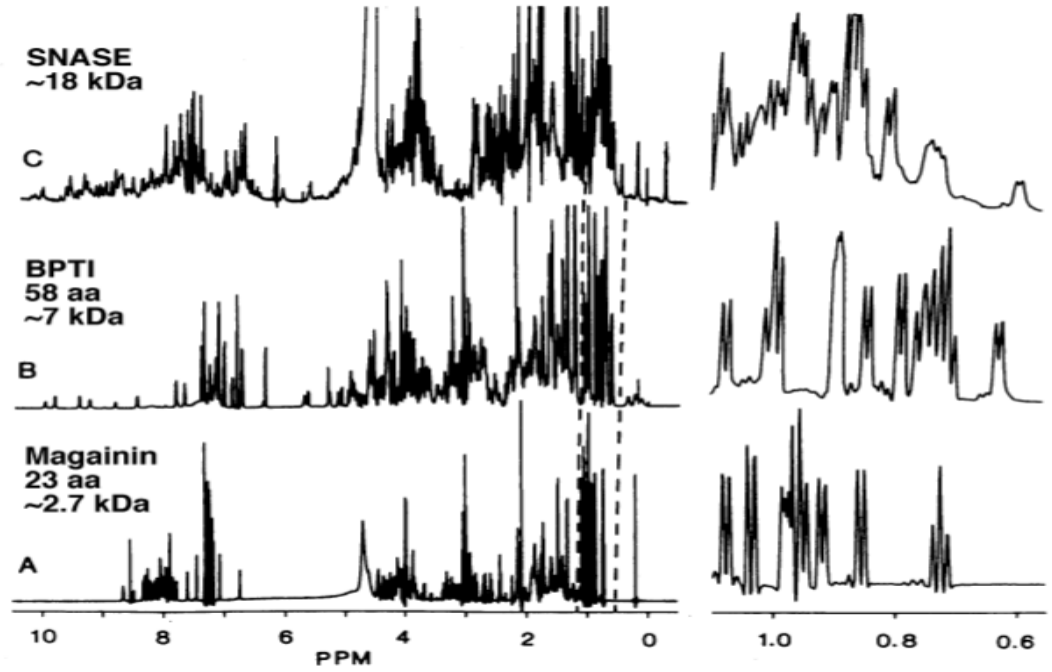


MDMA ^{13}C- spectra:





^{1}H NMR spectra of larger biomolecules (peptides and proteins)
overlap and broad lines

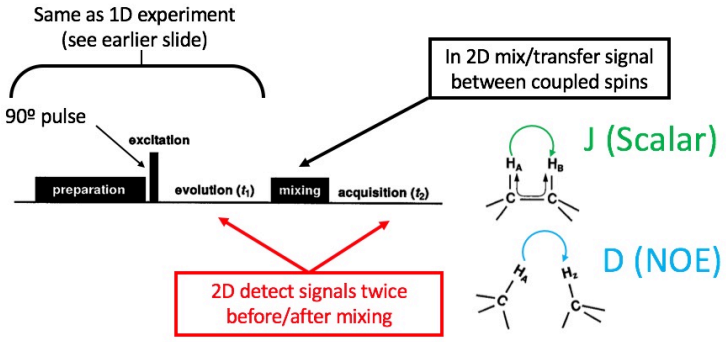


2D NMR

An NMR spectrum contains a great deal of information and, if many protons are present, is very complex. e complexity would be reduced if we could use two axes to display the data, with resonances belonging to different groups lying at different locations on the second axis. is separation is essentially what is achieved in **two-dimensional NMR**.

Much modern NMR work makes use of **correlation spectroscopy** (COSY) in which a clever choice of pulses and Fourier transformation techniques makes it possible to determine all spin–spin couplings in a molecule.

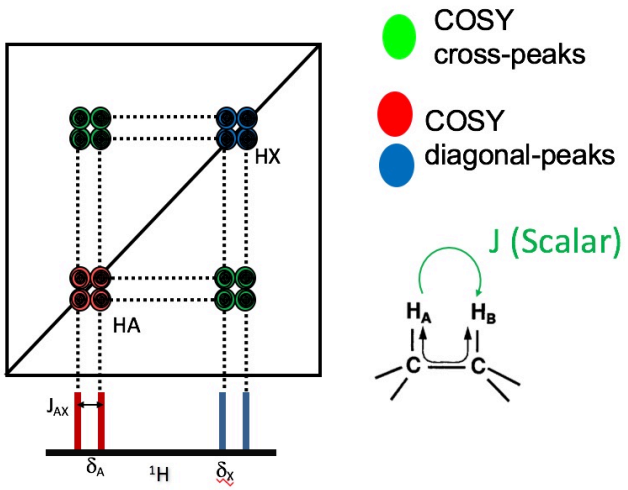
Cross-peaks reveal a correlation between frequencies on two frequency axes – either through bonds - based on J or through space - based on D (NOE)



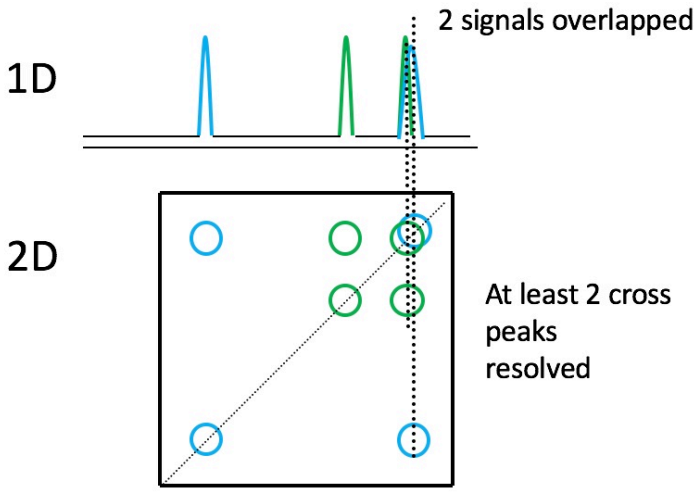
The **diagonal peaks** are signals centered on (d_A,d_A) and (d_X,d_X), and lie along the diagonal where $n_1 = n_2$. at is, the spectrum along the diagonal is equivalent to the one-dimensional spectrum obtained with the conventional NMR technique. e **cross peaks** (or *o -diagonal peaks*) are signals centered on (d_A,d_X) and (d_X,d_A), and owe their existence to the coupling between the A and X nuclei.

Although information from two-dimensional NMR spectroscopy is trivial in an AX system, it can be of enormous help in the interpretation of more complex spectra, leading to a map of the couplings between spins and to the determination of the bonding network in complex molecules. Indeed, the spectrum of a biological macromolecule that would be impossible to interpret in one-dimensional NMR can o en be interpreted reasonably rapidly by two- dimensional NMR.

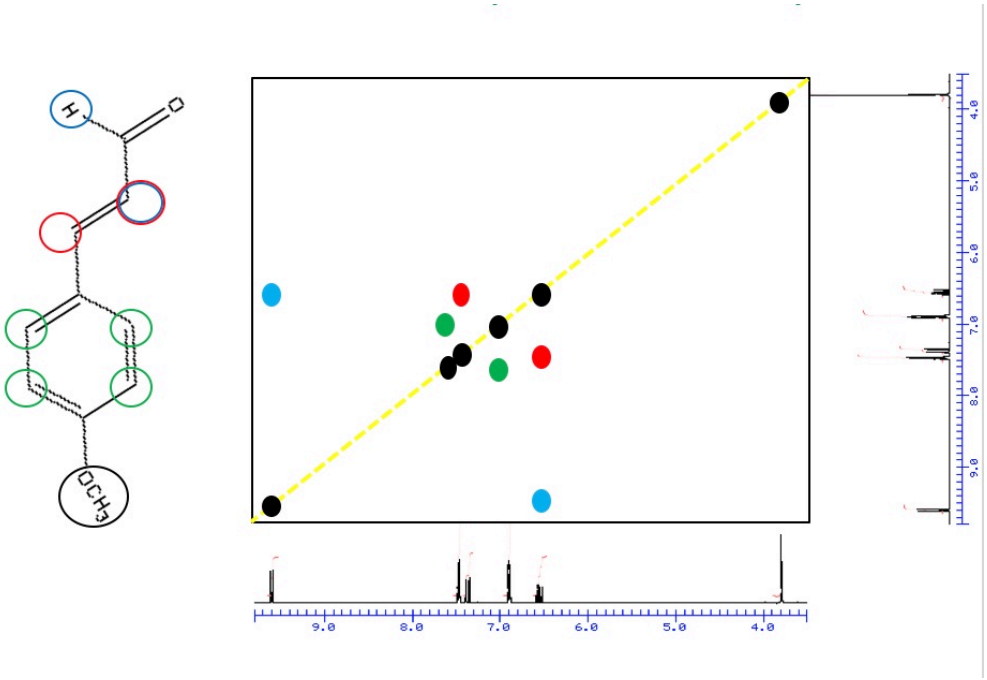
2D ^{1}H-^{1}H COSY:



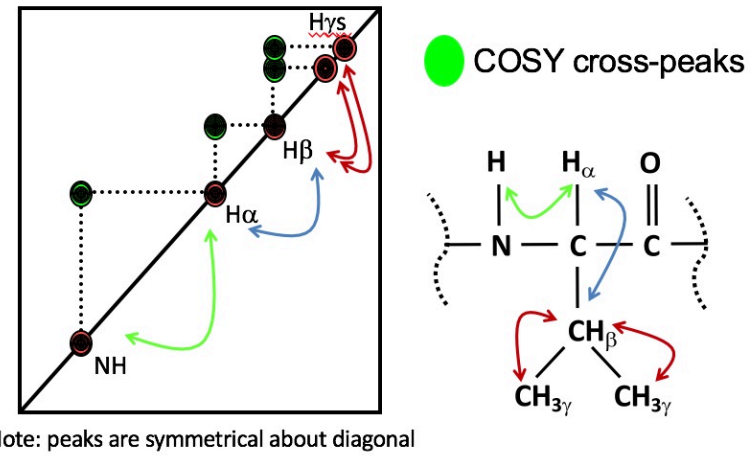
2D NMR also resolves overlaps:



2D COSY: 4-methoxycinnamaldehyde

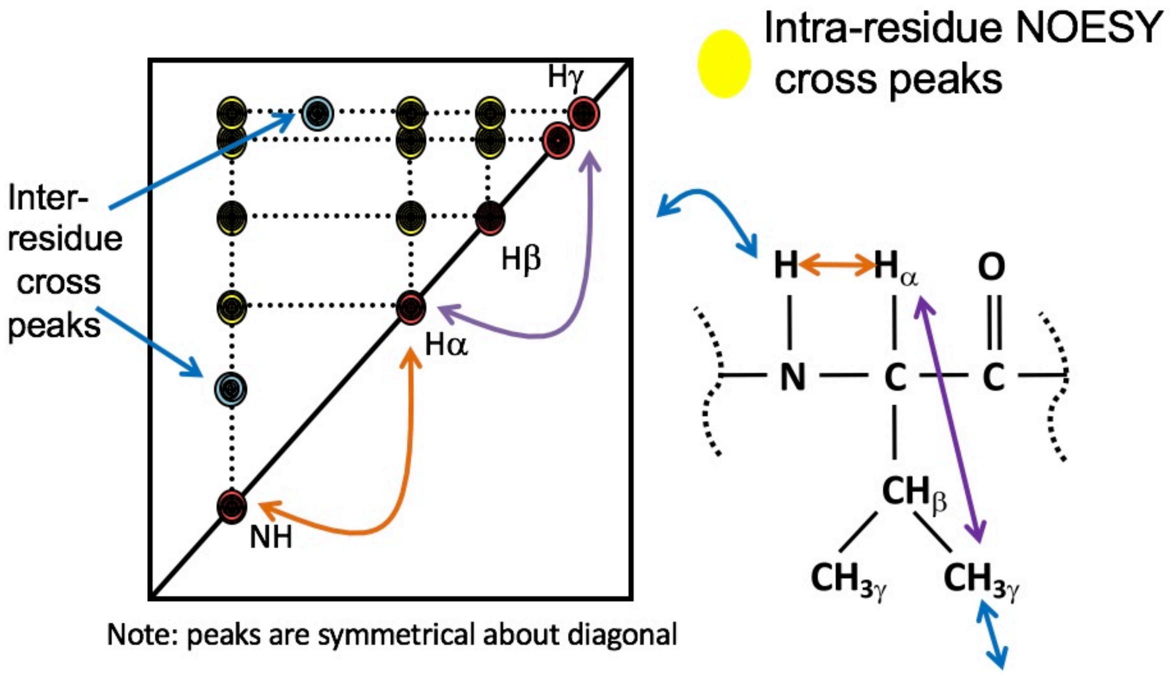


2D COSY spectrum of valine in a protein

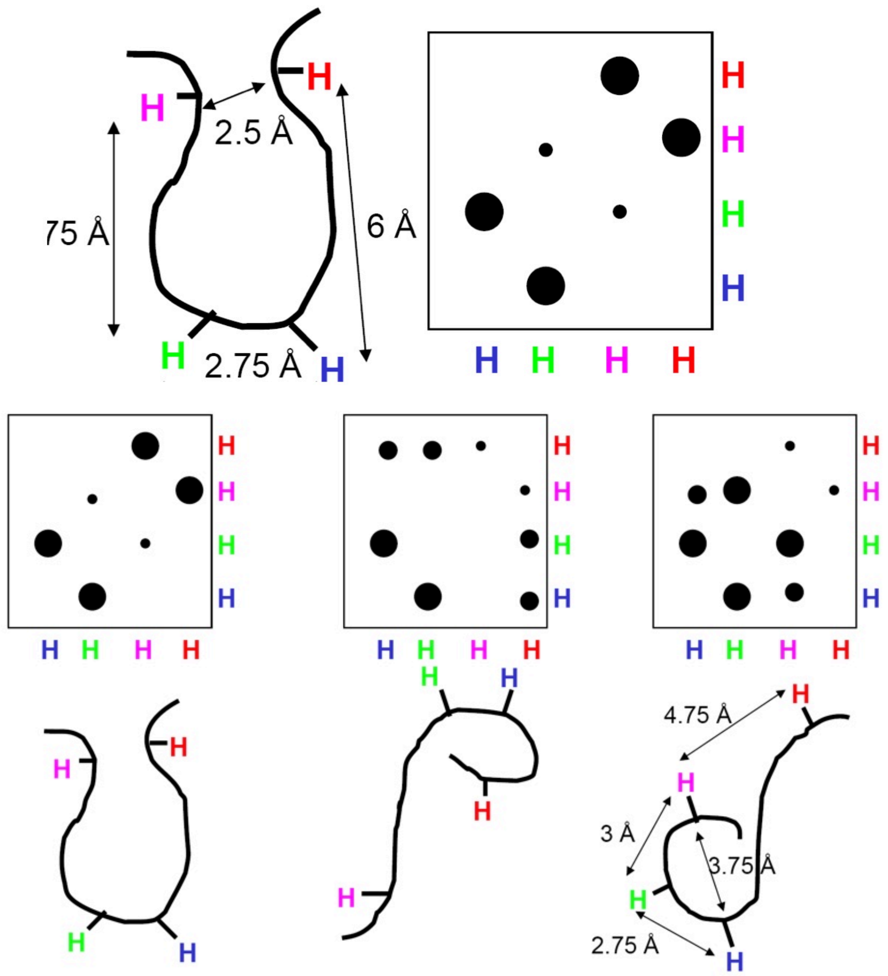


Note: peaks are symmetrical about diagonal

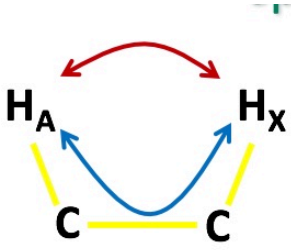
2D NOESY spectrum of valine in a protein



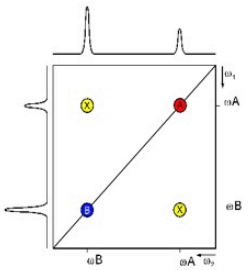
2D NOESY spectra and protein structure



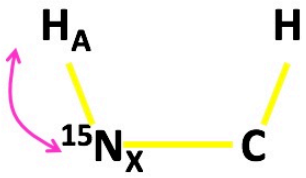
2D NMR: Other types of correlation spectroscopy



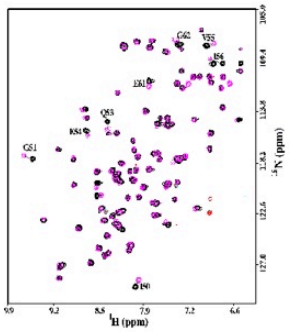
COSY
&
NOESY



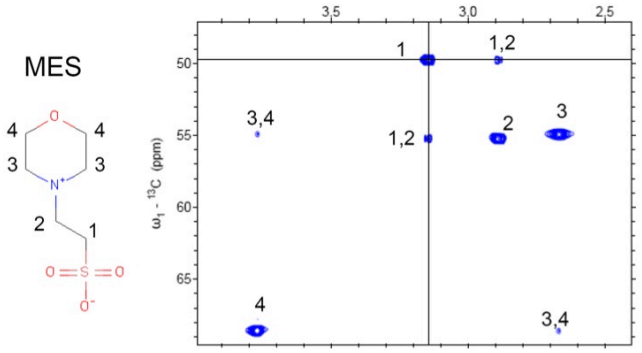
or



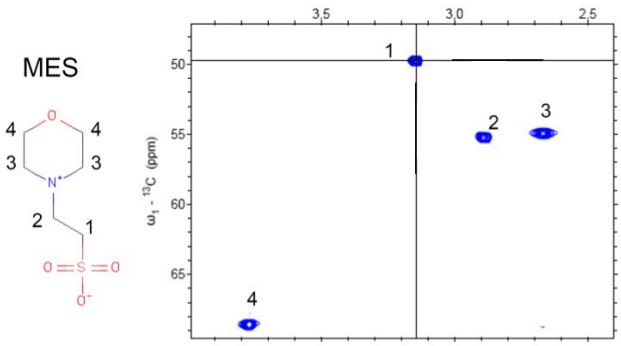
HMQC
&
HSQC



2D NMR: ^{13}C -^{1}H HMQC (Heteronuclear Multiple Quantum Coherence)



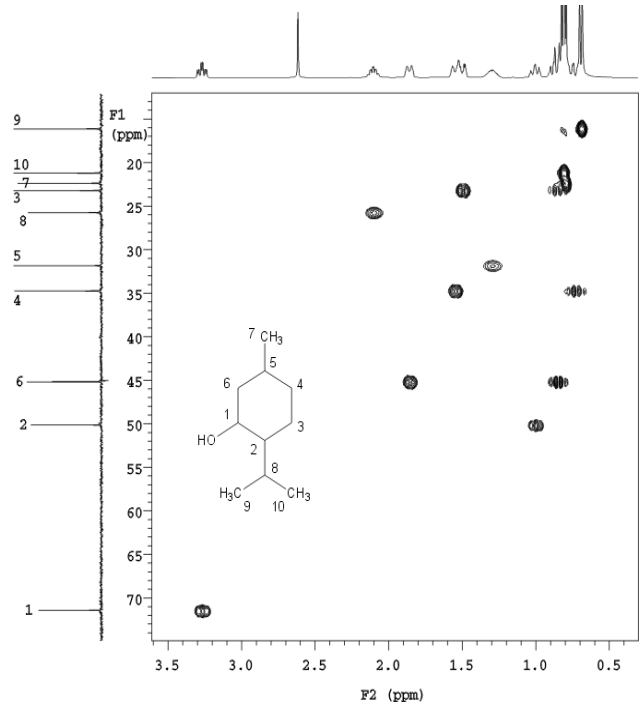
Correlates the decoupled carbon spectrum with the proton spectrum. Only directly bonded hydrogen and carbons will give cross peaks (quaternary carbons are not seen)



MES - 2-(*N*-morpholino)ethanesulfonic acid : buffering agent

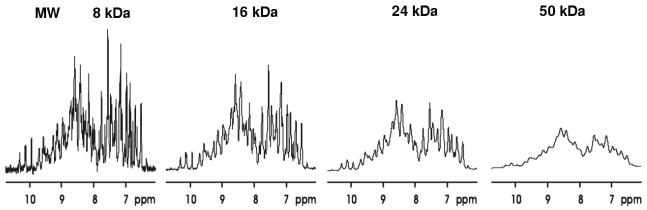
Shows long range proton-carbon connectivities

2D NMR: ^{13}C-^{1}H HMQC of sucrose

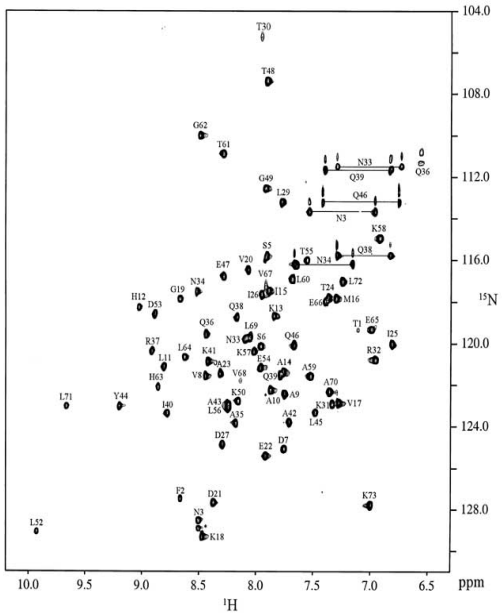


Molecular size matters for NMR

Increasing molecular weight leads to slower tumbling and faster relaxation!

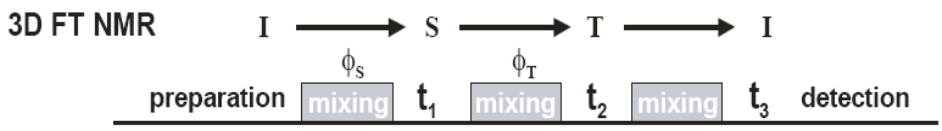


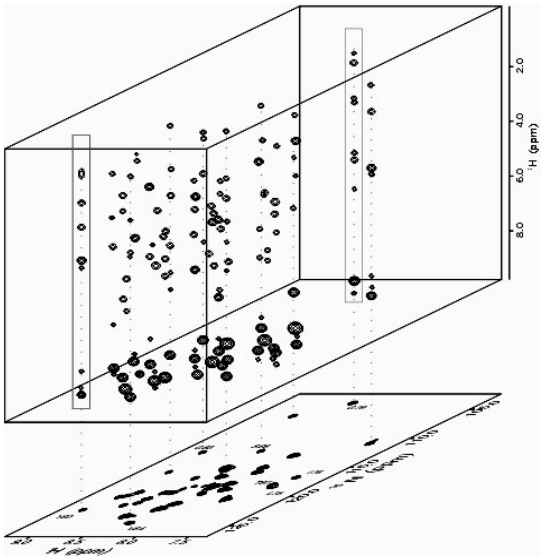
2D ^{1}H-^{15}N HSQC of a Protein

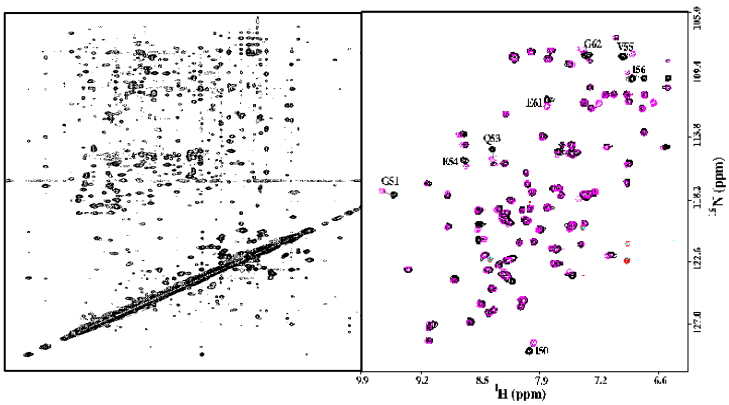


Each amide resonance has a characteristic ^{15}N chemical shift recorded in the 2nd dimension

Combine 2D expts – 3D !







NOESY

NMR Structure Calculation

Simulated Annealing (SA) is a molecular dynamics method. The starting structure is heated in a simulation (i.e. the atoms of the starting structure get a high thermal mobility). During cooling steps the starting structure can evolve towards the energetically favourable final structure under the influence of the force field

$$E_{total} = E_{bond} + E_{angle} + E_{vdw} + E_{dihedral} + E_{NOE}$$