Lessons from previous lecture

1. We discussed the behavior of a population of spins in the context of a frame of reference rotating at the frequency of precession of the spins about the axis of the applied field (the z-axis). The population of spins is “frozen” at this frequency.
2. The spectrometer is set up so that pulses of RF photons can be generated in the x,y-plane, orthogonal to the applied field. These provide an effective magnetic field that can apply a rotational force, - torque, - to the spin population.
3. The detector coils measure magnetization in the x,y-plane, also orthogonal to the applied field.
4. We can design sequences of pulses which rotate the spin vector of a population through any angle, about the axis of the applied field. Most useful are $\pi/2$ and π pulses, which rotate the spin vector through 90° and 180° respectively.
5. After rotation of the spin vector by a pulse, the system relaxes back to the equilibrium state before the pulse (with the vector along the z-axis). Relaxation involves precession at a frequency determined by the difference between the spin frequency and the reference frequency of our rotating frame.
6. Because we detect in the x,y-plane, we design our pulse sequences so that the population of interests has its vector in this plane.

The NMR spectrum is obtained by Fourier transformation of the time domain signal into the frequency (energy) domain. An NMR spectrum has absorption bands due to the following:

1. The energy levels for the spin flipping transitions of the nuclear spins. These have characteristic values (frequencies), determined by the gyromagnetic ratio, γ, of each nucleus, and the field, B_0. The dominant spin population is that of protons (there are more of them). Few if any lines in the spectrum actually have the frequencies expected, because of the effects below, but all nuclei of a type will have frequencies in a range close to the "parent" frequency. ^{1}H, ^{31}P, ^{14}N are seen at natural abundance, and ^{13}C and ^{15}N, on isotopic enrichment of a sample.
2. Each particular spin in a molecule has its resonance the energy (its spin flip) shifted by a small amount due to the magnetic field of the electrons circulating in the molecule. This is the chemical shift, as measured by δ, in ppm from a reference frequency. Chemical shifts are characteristic of particular substituents, and are similar in different molecules with the same substituents.
3. Nuclear spins connected through three or fewer bonds to one or more non-equivalent nuclear spins have their energy levels split through spin-spin coupling. This increases the complexity of the spectrum but provides useful information on connectivity through bonds.

1. Spins relax through interaction with neighboring spins. Appropriate sequences can be used to measure the three main pathways for relaxation in spin systems:

    a. Spin-lattice relaxation, with characteristic time, T_1. These reflect energy exchanges with the environment associated with the random tumbling of molecules (and their associated nuclear spins) in the surroundings. Strongly dependent on local mobility (viscosity, temperature, molecular size, etc.). T_1 can be measured using the Inversion Recovery approach.

    b. Spin-spin coupling through interaction between spins connected through bonds, with characteristic time, T_2. The couplings are seen in the spectrum as fine-structure with characteristic patterns, and energy separations, *J*. The spin population in the x,y-plane dephases due to the energy (frequency) differences arising from spin-spin coupling. The T_2 rate is more or less independent of the applied field along the z-axis.

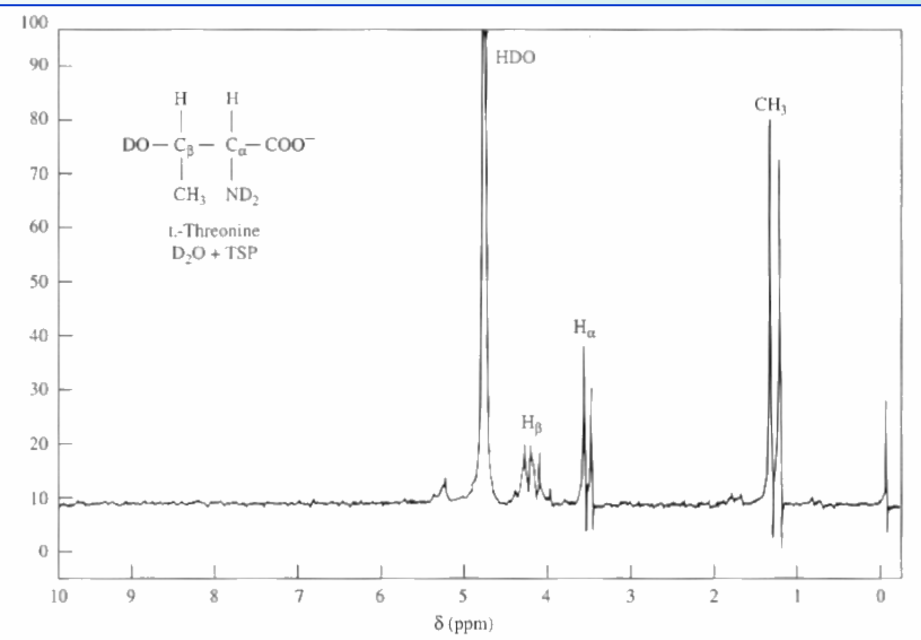
    c. Dipole-dipole interactions detected through the NOE effect. The single-quantum transitions of one component are saturated, and the effect is observed in the rates of single-quantum transitions of the other component(s). These rates are determined by "invisible" double-quantum transitions through normally forbidden dipole-dipole exchanges. These latter interactions are strongly dependent on distance, and appear only for spin pairs within ~5 Å.

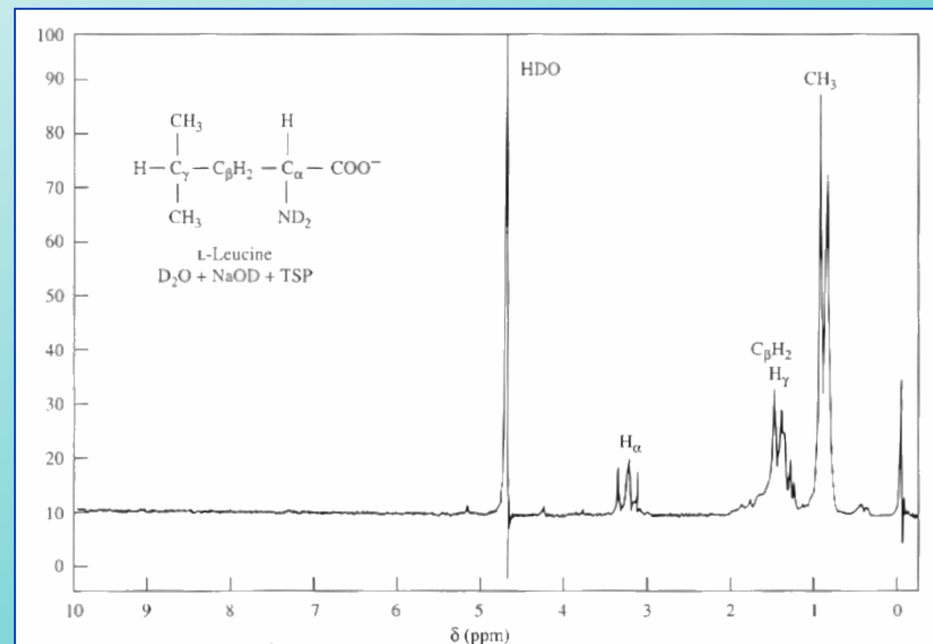
So what's all this NMR stuff good for?

We'll look at a few examples of application of simple 1-D NMR to biological systems, and then get on to more complicated applications that depend on fancy pulse sequences, timing, 2-D and 3-D analysis of data.

1D-NMR. In combination, the data about nuclear (chemical) species (from chemical shifts), connectedness and orientation (from *J* splittings, and relaxation through spin-spin coupling), and distances and angles (from dipole-dipole interactions), provide the information on distances and connections necessary for determination of chemical structure and bond orientation.

The spectra below demonstrate application of 1D-NMR to chemical structural studies. Were looking at the ^1H region of the spectrum. The samples are threonine (left) and leucine (right) dissolved in D_2O (^2H_2O). Because ^2H has a different γ, its spectra are off scale, and we see only ^1H here. Protons that can easily exchange are diluted out by ^2H, so we see only lines associated with the non-exchangeable protons (and a small amount of HDO). The fine structure and areas under the curves identify the two amino acids.





The spectrum on the right is that of an intact frog muscle, with the frequency range selected to excite ^{31}P. The major phosphorous-containing species are those involved in energy metabolism of the cell, - ATP, ADP, Pi, and creatine phosphate, which is the cell's reserve of "high-energy" phosphate. Under the metabolic conditions prevailing, the spectrum is dominated by ATP, but the peaks of ADP would show up in similar positions. In other cells, the metabolite phosphates might contribute recognizable peaks.

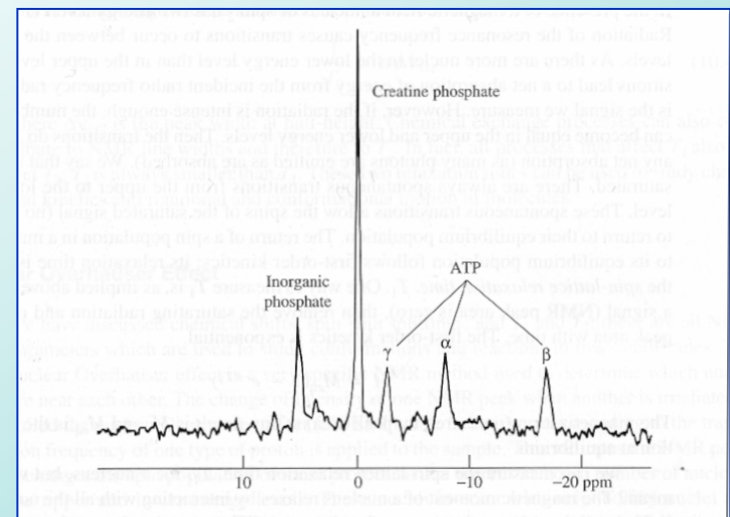
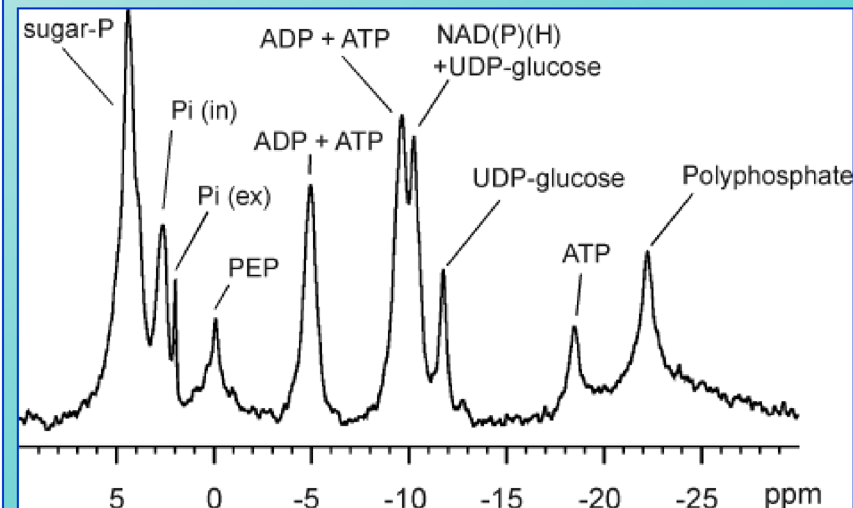


Fig. 10.33 ^{31}P NMR spectrum of intact frog muscles. The phosphorus peaks are from inorganic phosphate (a rapid equilibrium between $H_2PO_4^-$ and HPO_4^{2-}), creatine phosphate, and adenosine triphosphate (including some adenosine diphosphate). The resolution is not high enough to show that the α and γ peaks of ATP are doublets split by the two neighboring ^{31}P, and that the β peak is a triplet split by the two neighboring ^{31}P. (Figure from D. G. Gadian, *Nuclear Magnetic Resonance and Its Applications to Living Systems,* Clarendon Press, Oxford, 1982.)

In general, the ^{31}P NMR can be used to assay the energy metabolism of the cell.

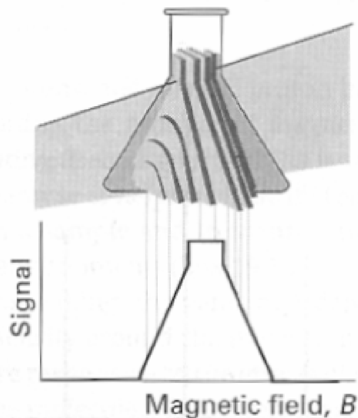
The bottom picture is from a study of metabolism in cells of the bacterium *Corynebacterium glutamicum*, showing some additional peaks.

Because inorganic phosphate (Pi) has a pK in the physiological range, its position varies with pH, and can be used to measure ΔpH.

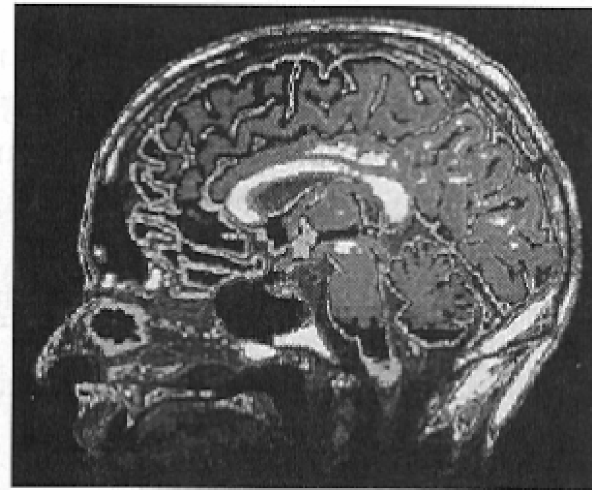


MRI – Magnetic Resonance Imaging

In MRI, the system to be imaged is placed in a field that varies linearly over the sample. Because the resonance frequency for the ^1H depends on B_0, the protons at each slice of the field will have a unique value for their resonance. The method therefore builds up an image slice by slice of the proton density. By rotating the sample, a 3-D image can be generated by computer tomography. In order to image a body, the bore of the magnet has to be wide enough. Since the sensitivity depends in field strength, state-of-the-art MRI facilities are expensive.



In a magnetic field that varies linearly over a sample, all the protons within a given slice (that is, at a given field value) come into resonance and give a signal of the corresponding intensity. The resulting intensity pattern is a map of the numbers in all the slices, and portrays the shape of the sample. Changing the orientation of the field shows the shape along the corresponding direction, and computer manipulation can be used to build up the three-dimensional shape of the sample.



The great advantage of MRI is that it can display soft tissue, such as in this cross-section through a patient's head. (Courtesy of the University of Manitoba.)

Structural studies of more complex molecules

In principle, we can apply the methods discussed in the previous slides to any molecule. In practice, the spectrum gets so crowded by all these lines that we can't get useful information from it. We therefore use 1 dimensional NMR only for relatively simple molecules.

For more complex molecules we need to find a way to eliminate parts of our spectrum, spread the interesting bits out a bit, and detect more subtle features. There are a whole range of clever tricks involving timing, decoupling, and saturation that make this possible

One way to simplify the spectrum is to decouple groups of atoms to eliminate J splittings. Another is to enhance weakly absorbing species by saturation transfer. Artifacts can be eliminated by careful use of phases. In general, the spectral information can be spread out by using of 2 or 3 (or more) dimensions (2D- or 3-D NMR). This allows us to see interactions between spins more easily.

In the next few slides we will look to see what these protocols involve.

The Nuclear Overhauser Effect, NOE:

A third type of relaxation process is important in macromolecular structural work

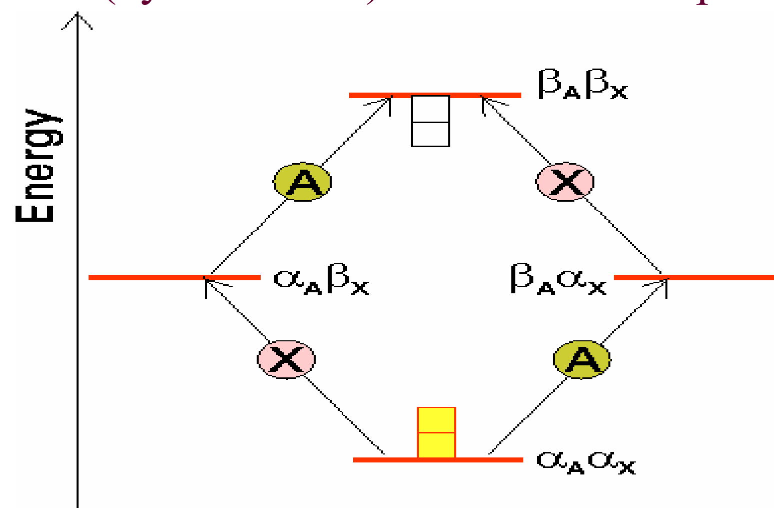
The NOE is a relaxation pathway due to dipole-dipole interactions. As such it is very distance dependent, falling off with $1/r^6$. It is this distance dependence that makes it particularly useful from the point of view of structural determination. The NOE relaxation is different from spin-spin relaxation associated with T_2. In NOE, ${}^{n}J_{AX}=0$, and the effect is not dependent on bonding. It therefore provides information about the distances of non-bonded interactions that determine secondary and tertiary structure.

NOE, adapted from Moyna, and from Atkins (Figs.)

• The ***NOE*** is a different way in which particular spin states in the system can release energy and relax. It is another relaxation pathway, and a component of the overall relaxation processes. In particular, the NOE is related to exchange of energy between two spins that are not scalar (through-bond) coupled ($J_{AX} = 0$), but have ***dipolar coupling***.

• The NOE is evidenced by enhancement of certain signals in the spectrum when the equilibrium (or populations) of other nearby spins are altered (by saturation). We use a two spin system energy diagram with four states to explain it:

The boxes represent ***equilibrium occupancies***
A filled box shows excess occupancy of the favored spin state, relative to the empty boxes. As we expect, the $\alpha_A\alpha_X$ state is favored over the $\beta_A\beta_X$ state

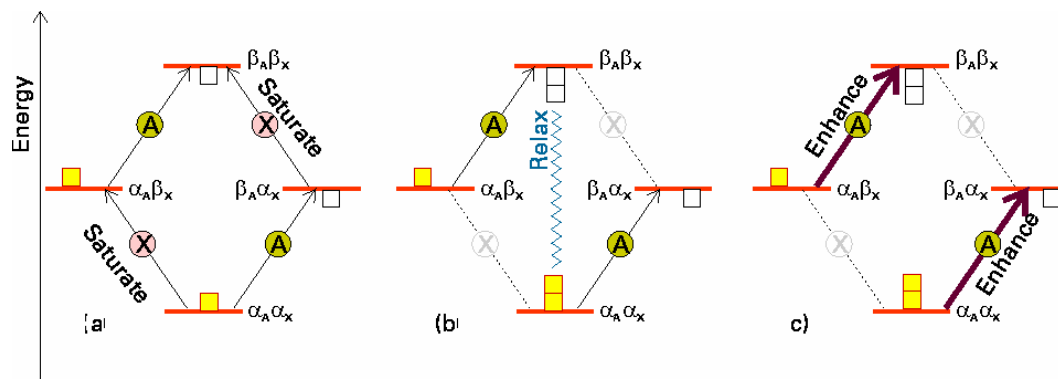


• **The arrows** represent ***transition probability***, or the rate at which certain allowed transition can take place. For the system in equilibrium we can have **A** and **X** transitions, which represents ***single quantum*** α to β transitions of spins A and X.

•The vertical and horizontal transitions are omitted because they are ***double quantum*** transitions, are forbidden, and normally have a much lower probability.

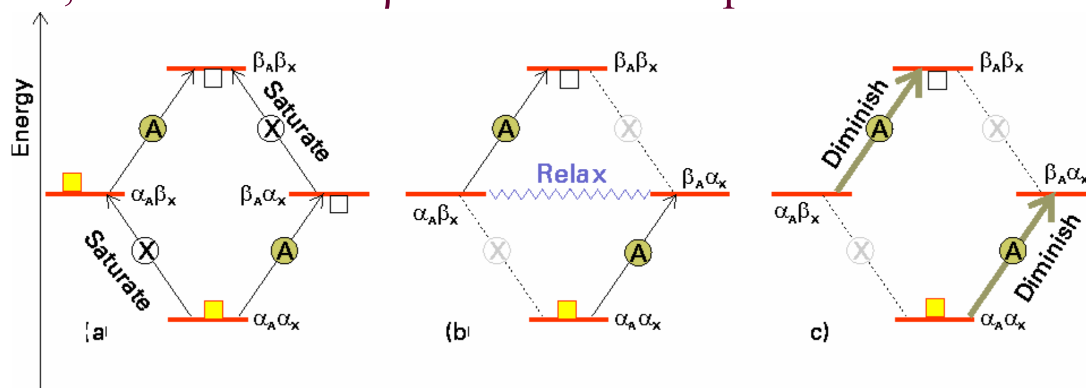
We now ***saturate*** the **X** transition with a pulse, which means that we make the populations on either side of each transition equal. The forward and back **X** transition rates are now the same, so they are effectively frozen:

- In this diagram, the changes in levels affecting the **A** transitions favor an increase in rate.



The **A** transitions no not occur during the pulse because we do not excite them, so the populations in the levels that affect their rates change only due to **X**. The **horizontal** and **vertical transitions,** though normally disallowed, become the only way **the system** can relax back to equilibrium. They can occur through dipole-dipole interaction. This will flip **both** spins, causing a **double quantum** transition, between α and β states for both spins.

- In this diagram, the changes in levels affecting the **A** transitions favor a decrease in rate.



These relaxation pathways deplete or enhance populations involved in A transitions, and thus the enhance their signals. **Vertical transitions** will give positive enhancement of **A**, and **horizontal transitions** will diminish **A** (negative enhancement).

We cannot detect these **double quantum transitions (dq)**, but they affect the way the spin system relaxes, so we can see their effects on the signals due to the allowed transitions. The two dq transitions have different rates, - one has a rate close to twice ω, while the other one is almost zero. So one will be related to very slow motions, and the other one to fast tumbling...

- If we now put all this in a big equation (the Solomon equation) we get something that will help us see several things. Using $\mathbf{V_{dq}}$ and $\mathbf{H_{dq}}$ for the rates of the vertical and horizontal dqs we have the enhancement given by η:

$$\eta = \gamma_A / \gamma_X * \frac{V_{dq} - H_{dq}}{2 * X + V_{dq} + H_{dq}}$$

- First, if the molecule tumbles rapidly (all small organic gunk) we find that under saturation of the **X** transitions **vertical dq** will dominate, so the maximum enhancement for **A** is γ_A / γ_X. If we are looking at the ^{13}C signal while decoupling (saturating) ^{1}H, we can get an enhancement of ~ 4.

- If the molecule tumbles slowly, as for a protein, **the horizontal dq** dominates, and we have a maximum NOE of - γ_A / γ_X. Since here we are interested in ^{1}H - ^{1}H NOE, the theoretical enhancement will be ~ -1.

Nuclear Overhauser Effect (summarized)

•It is useful to compare the frequency of the spin system to the molecular tumbling rate or ***correlation time***, τ_c.

• $\omega * \tau_c << 1$ -This means that the molecule tumbles fast, and we have positive enhancements. It is called the ***extreme narrowing condition*** (small molecules, non-viscous solvents).

• $\omega * \tau_c >> 1$ -This means that the molecule tumbles slowly, and we have negative enhancements. It is called the ***diffusion limit*** (proteins, viscous solvents).

• $\omega * \tau_c \approx 1$ - These are the "in the middle's", and we can have situations in which the NOE goes to zero. It will happen for certain medium sized molecules and it depends on the base frequency of the NMR

•There is one important point that we left out from our treatment, which is the dependence of the NOE on the distance between our A and X spins. Because of the $\mathbf{1/r^6}$ dependence, we will only see an NOE of our spins are **5 Å or less apart**. This therefore becomes a powerful tool for assaying distance, and therefore the "connectedness" of macromolecular structure in secondary and tertiary folding.

NMR acronyms

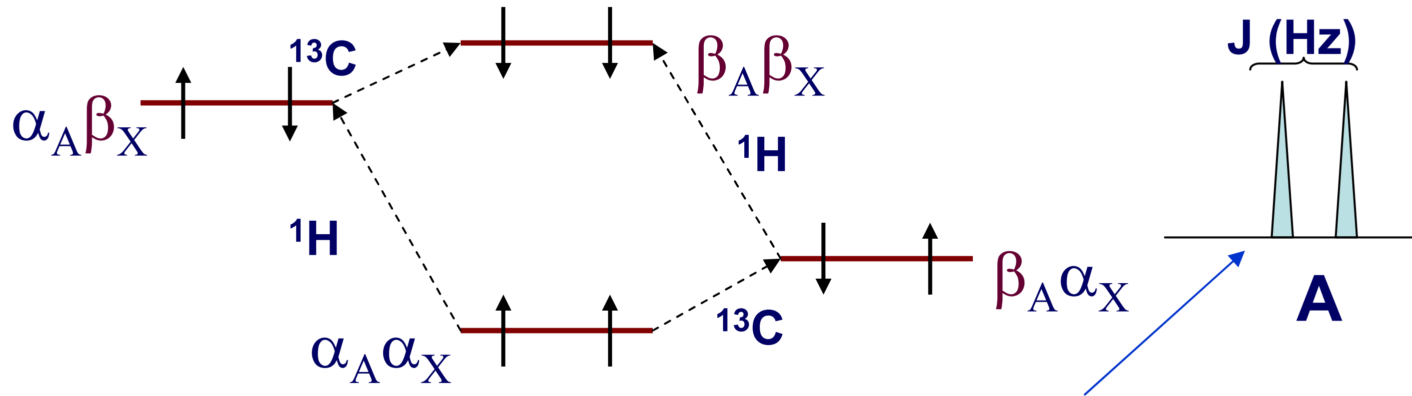
COSY – a 2D homonuclear **co**rrelated **s**pectroscop**y** and its variants. This explores the *J* couplings between nuclei in a complex mixture such as a protein. Since *J* couplings depend on bonded connections (out to 3 bonds), this allows us to measure the backbone connections of a macromolecule. If we know the sequence, we can identify splittings, and assign them to specific residues.

NOESY – a 2D **n**uclear **O**verhauser **e**ffect **s**pectroscop**y**. This explores the dipolar interactions in a complex mixture. These allow us to explore tertiary structure.

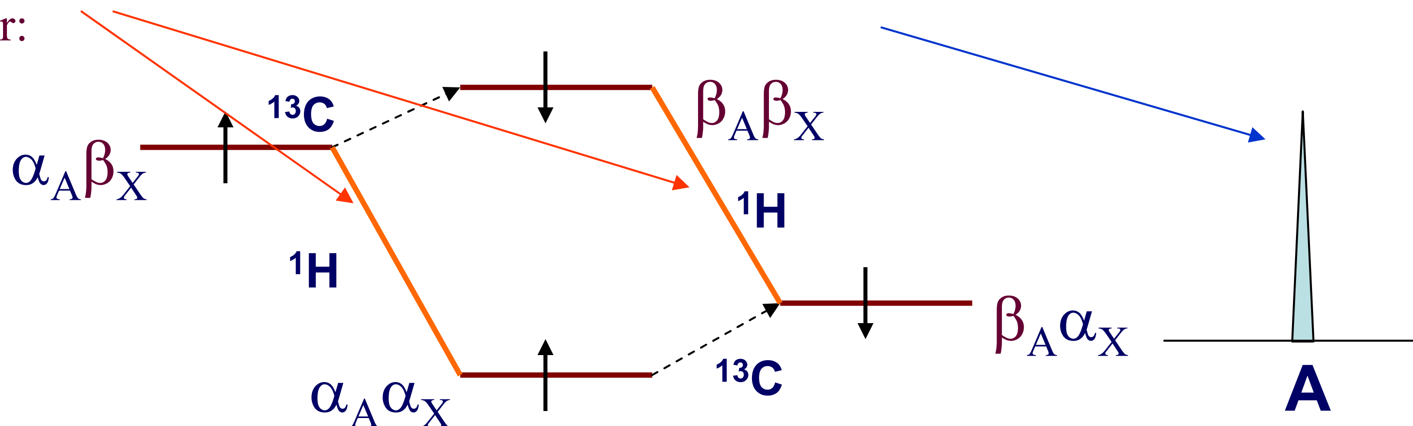
These are the two most important types of 2D-NMR. However, there are many variations on each, and many other pulse sequences that have their own acronyms. In addition, there are many protocols for refinement of the FID, - they get rid of useless noise, and enhance the resolution of the FT algorithm, also with their own acronyms.

Decoupling, saturation transfer and heteronuclear coupling

• In order to simplify the spectrum, we can get rid of some lines. Consider a ^{13}C nuclei (A) coupled to a ^{1}H (X):

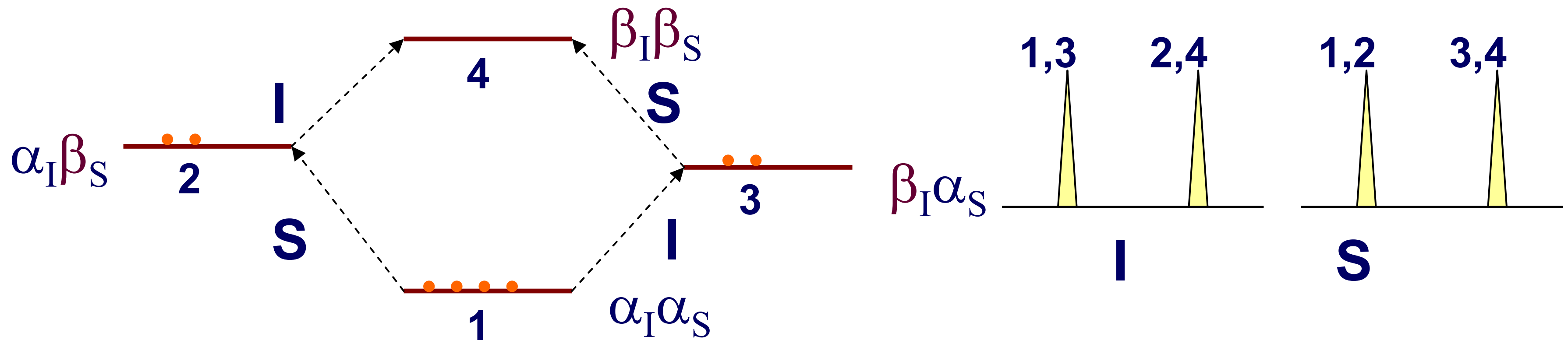


If we took the ^{13}C spectrum we would see the lines split due to coupling to ^{1}H. These couplings are from 50 to 150+ Hz, and make the spectrum really complicated and overlapped. We can decouple the ^{1}H spin interactions by using a pulse or continuous irradiation to saturate ^{1}H transitions. The ^{13}C multiplets are now single lines because they are now the only ones that can occur:

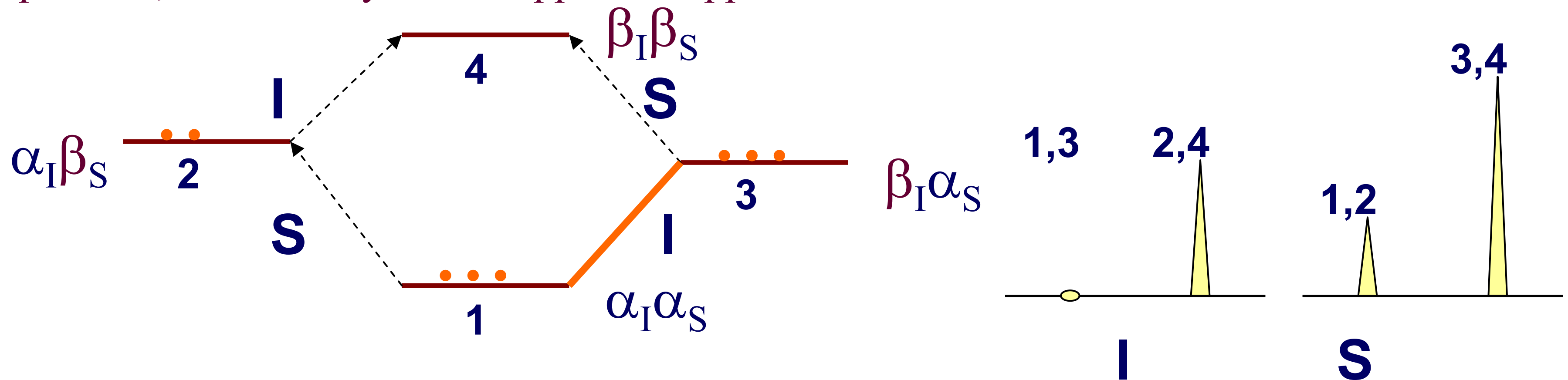


Polarization transfer

• To explain this effect, we'll use a diagram for two protons that are *J*-coupled weakly and have a large δ difference. We name them **I** and **S**, and we indicate with • the excess population from one state to the other:



• Now we irradiate and saturate only one of the lines (the 1→3 transition of **I**) of one of the nuclei ***selectively*** (called soft pulses). After a certain time, the population differences for that transition become equalized, and the spectral line disappears. Because of the changes in population, the intensity that disappears reappears in the line for the 3→4 transition.



2D-NMR. Adapted from Moyna – with explanatory notes!

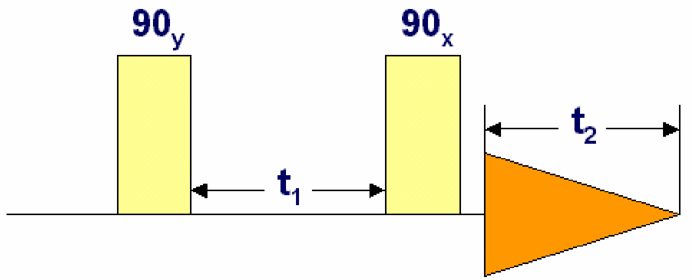
Top: notice that we have two different $\pi/2$ pulses, one along the y-axis, one along the x-axis. Remember that **the torque is applied at 90º** so the y-pulse flips the vector from z to x, the x-pulse from y to ±z.

Bottom: after the first pulse, our spin population has been torqued out of the z-axis into the x,y-plane (3-D view). On the left, we're looking down at that plane. At $t_1 = 0$, we have no spins with a vector along the y-axis, so the x-pulse does nothing, and we see all our spins.

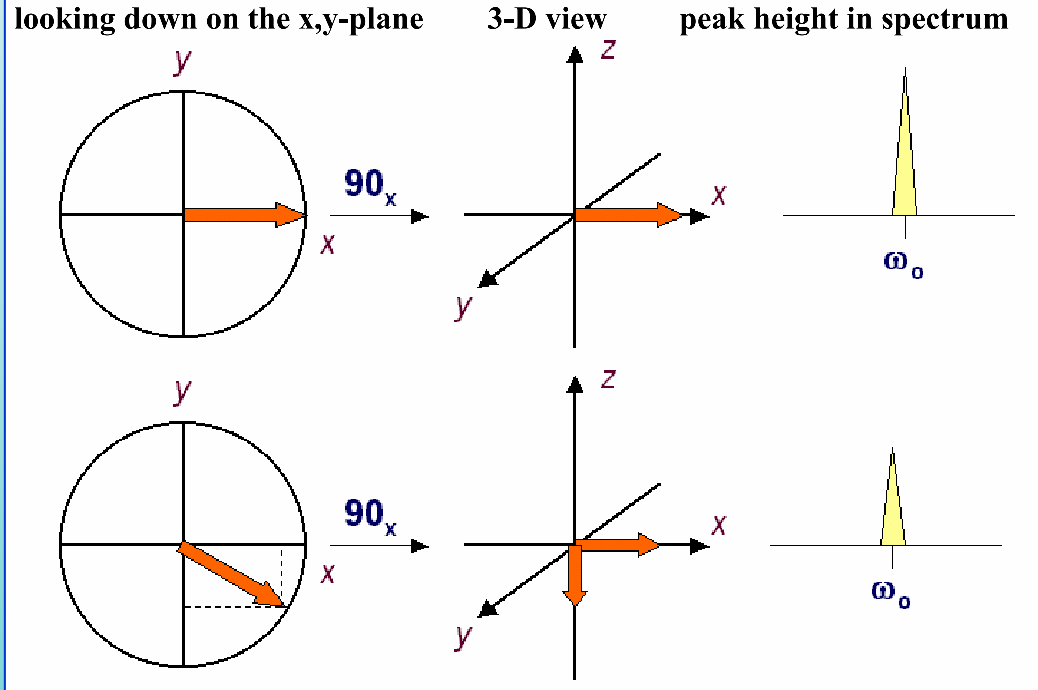
As we extend t_1, the y-axis vector becomes populated, and our x-axis pulse depletes the detectable spins in the x,y-plane (right).

2D-NMR – the basics

- the *COSY* pulse sequence (rudimentary version). Two $\pi/2$ pulses, separated by a variable time, t_1, the preparation time:



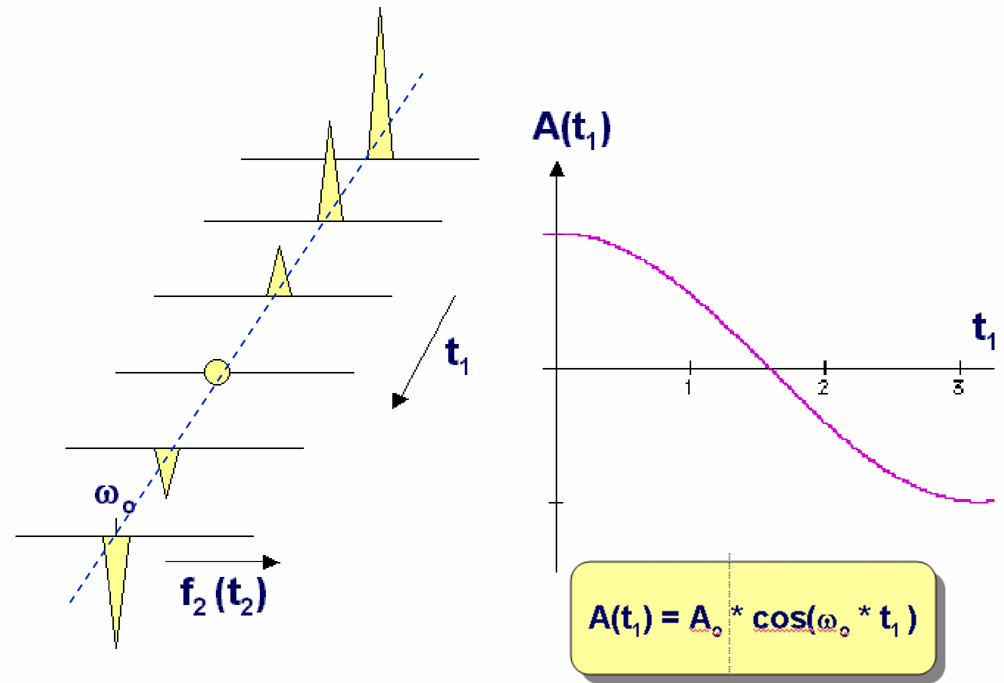
- We'll analyze it for an off-resonance ($\omega \neq \omega_o$) transition for a bunch of different t_1 values. Starting after the first $\pi / 2$ pulse:



Top left: the spectra obtained at different t_1 times are displayed with offsets to show a 3-D view of all our data (the ***stacked plot***). Remember that the spectra come from FT of our FID from the time to the frequency (energy) domain. Note that this **FID was occurring during t_2**.

If we have enough different spectra at t_1 times, we can construct a plot of the time dependence of amplitude of the spectral line (right). This represents the evolution in the time domain of this spin in the x,y-plane, - a pseudo-FID. We can find the underlying interaction energies by performing a Fourier transform into the frequency domain.

- If we plot all the spectra in a ***stacked plot***, we get:



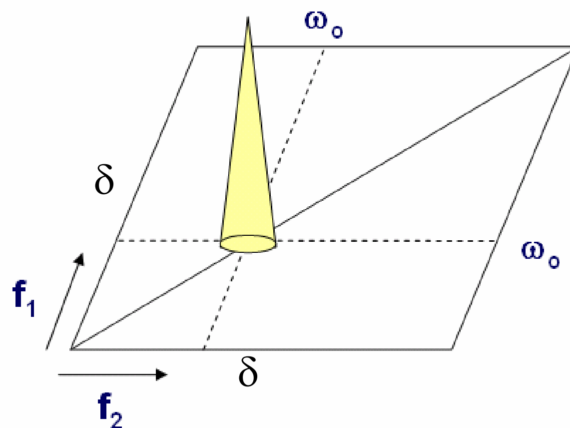
- Now, we have frequency data in one axis (**f_2**, which came from **t_2**), and time domain data in the other (**t_1**).
- Since the variation of the amplitude in the **t_1** domain is also periodic, we can build a pseudo FID if we look at the points for each of the frequencies or lines in **f_2**.
- One thing that we are overlooking here is that during all the pulsing and waiting and pulsing, the signal will also be affected by **T_1** and **T_2** relaxation.

So now we have two sets of spectra, one derived from the FID occurring during t_2, and the second from the evolution in the x,y-plane represented by the t_1 data. In both sets of data, the effects dominating the precession times will be T_2 relaxations, so if we plot one set of data against the other, most points will fall on the diagonal (top right). To make it easier to see the points, they are represented as contour plots (bottom right).

Now that we have seen this for an idealized case (a one-liner), lets see what a real 2D-NMR spectrum, for a relatively simple molecule, looks like.

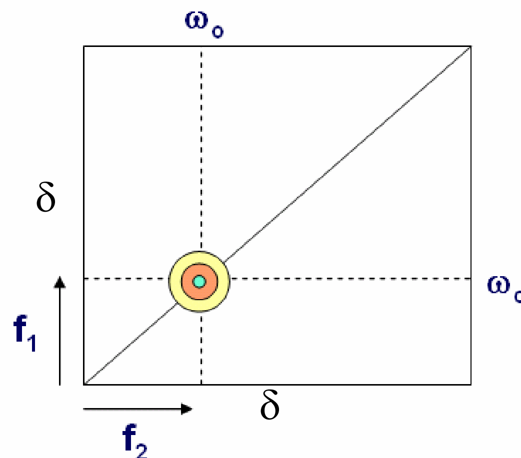
The rudimentary 2D (…)

- Now we have FIDs in t_1, so we can do a ***second Fourier transformation*** in the t_1 domain (the first one was in the t_2 domain), and obtain a ***two-dimensional spectrum***:



- We have a ***cross-peak*** where the two lines intercept in the 2D map, in this case on the ***diagonal***.

- If we had a real spectrum with a lot of signals it would be a royal mess. We look it from above, and draw it as a ***contour plot*** - we chop all the peaks with planes at different heights.



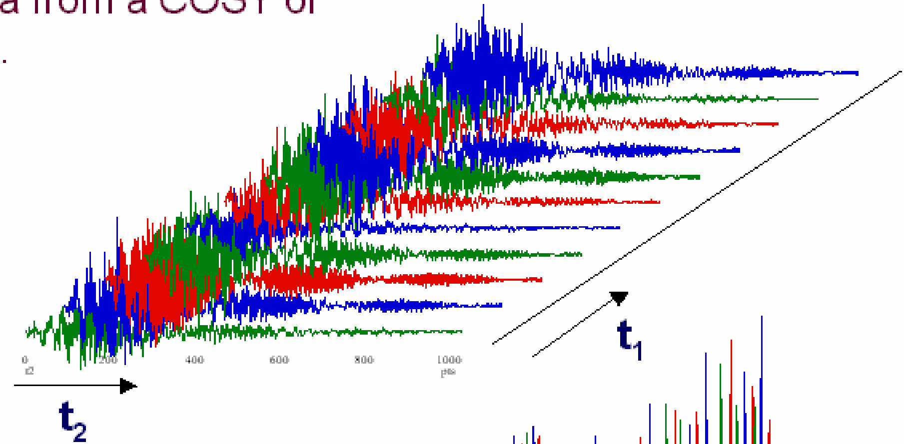
- Each slice is color-coded depending on the height of the peak.

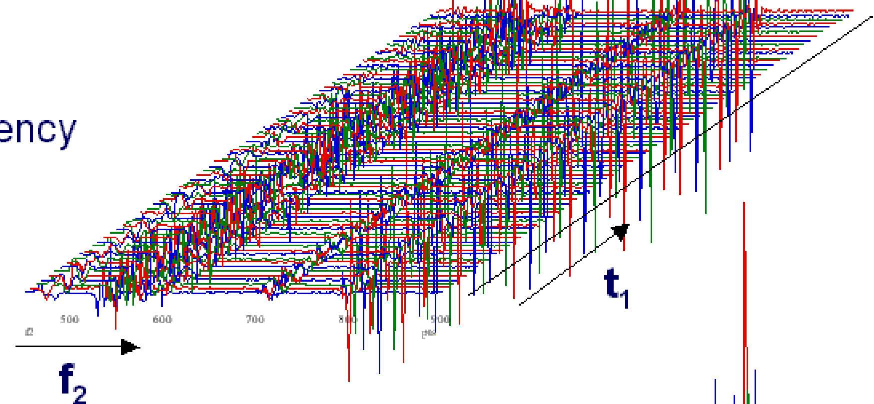
Top: the set of FIDs from the t_2 section of the pulse sequence, taken at different values for t_1.

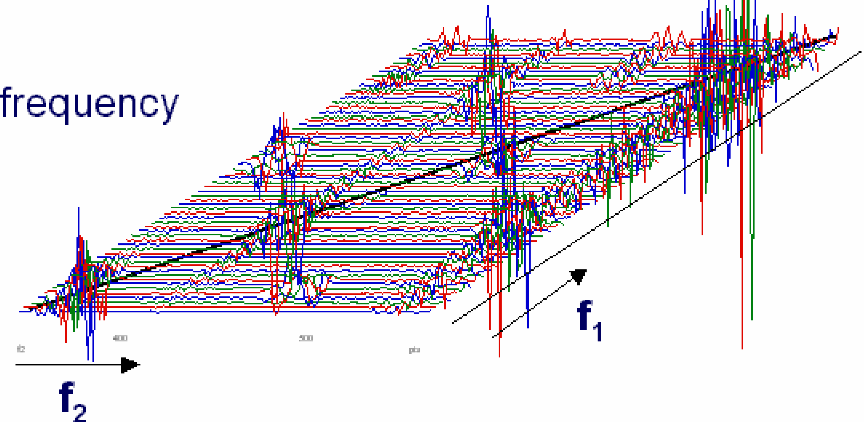
Middle: the Fourier transforms of the FIDs to give a set of spectra for different times, t_1.

We now perform a FT on this set of spectra in the t_1 direction (not shown), and plot the two frequency domain data sets against each other (bottom right).

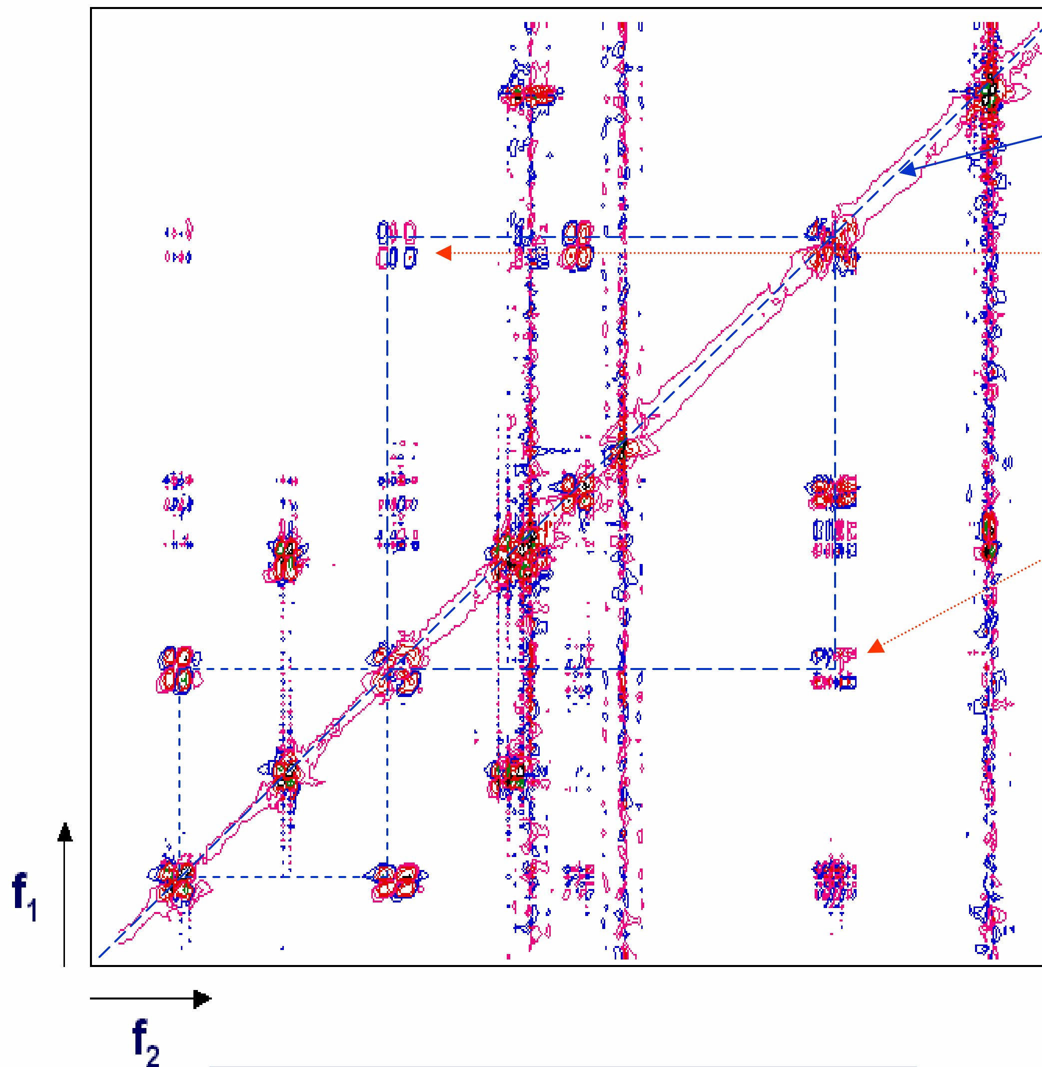
- This is data from a COSY of pulegone...







- Now the ***contour-plot*** showing all the ***cross-peaks***:



$$A_S(t_1,t_2) = A_o * \sin(\omega_I * t_1) * \sin(J_{IS} * t_1) * \sin(\omega_S * t_2) * \sin(J_{IS} * t_2)$$

The explanation so far accounts for the peaks along the diagonal. What about all those other off-diagonal peaks? First, notice that the off-diagonal peaks are related (see the dotted square).

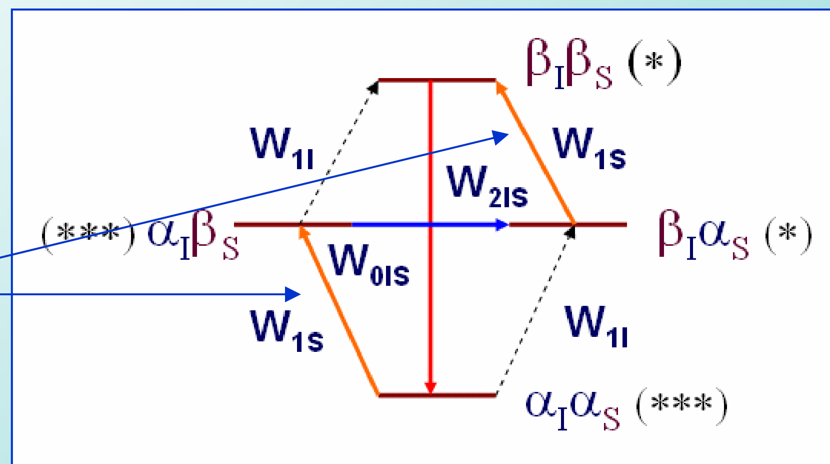
What COSY is good for is to tell which spin is connected to which other spin. The off-diagonal peaks are the useful bits of information; the two peaks found by dropping perpendiculars onto the diagonal are spin-spin coupled. The equation (bottom left) shows the relation between amplitude and *J* for the transition. After FT, these give the off-diagonal peaks; from their positions, we can get values for the *J* couplings, because the scales are in frequency units.

NOESY – 2D NOE spectroscopy

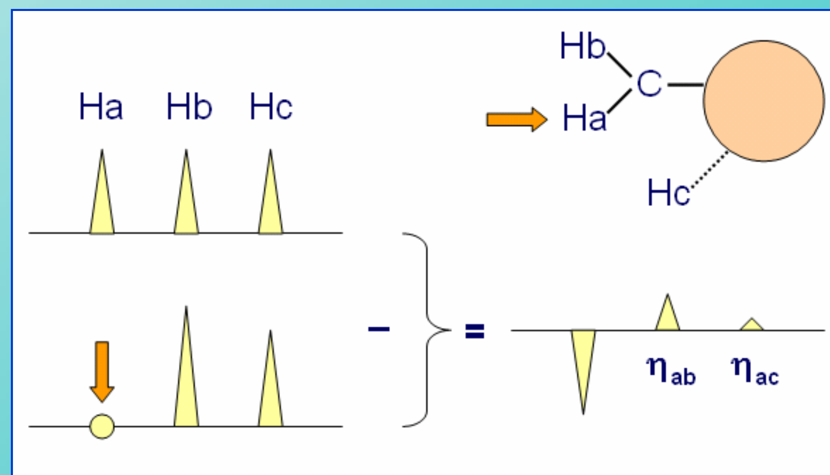
Top: This is the energy diagram for a two-spin system with similar nuclei (same energy levels for splittings) that we considered before to see how NOE works, but with Moyna's notation. The single-quantum transitions for the **S** spin are saturated, and the single quantum transitions of **I** are enhanced (if the W_{2IS} pathway is favored) or diminished (if W_{0IS} is favored).

Bottom: Steady state saturation of a system with three spins. We could use steady state irradiation to saturate Ha, and look at the dipolar coupling to Hb and Hc by taking spectra without (top) or with (bottom) saturation. The difference spectrum (right) shows the enhancement. We get an NOE from both the bonded pair (–CH_2) and the non-bonded pair. If we know the distance for the bonded pair, we can calculate the distance for the non-bonded pair.

NOE energy diagram



Steady-state saturation and difference spectroscopy

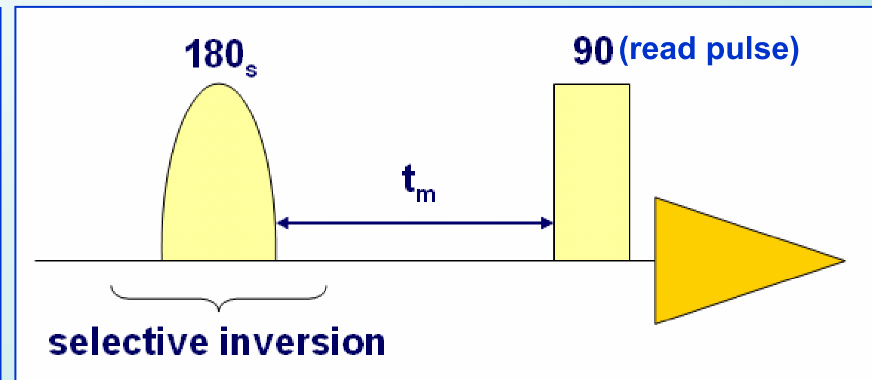


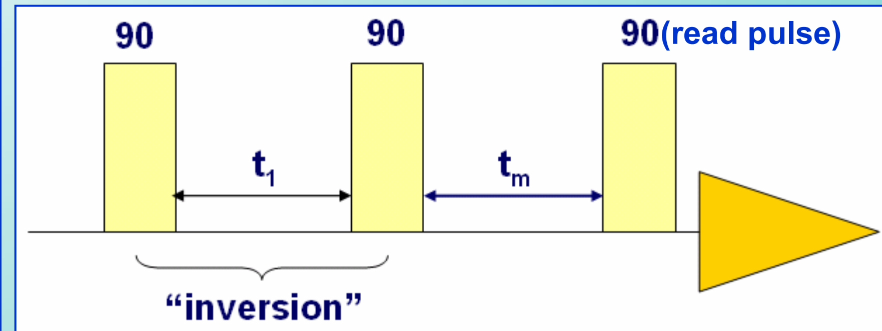
Pulsed NMR

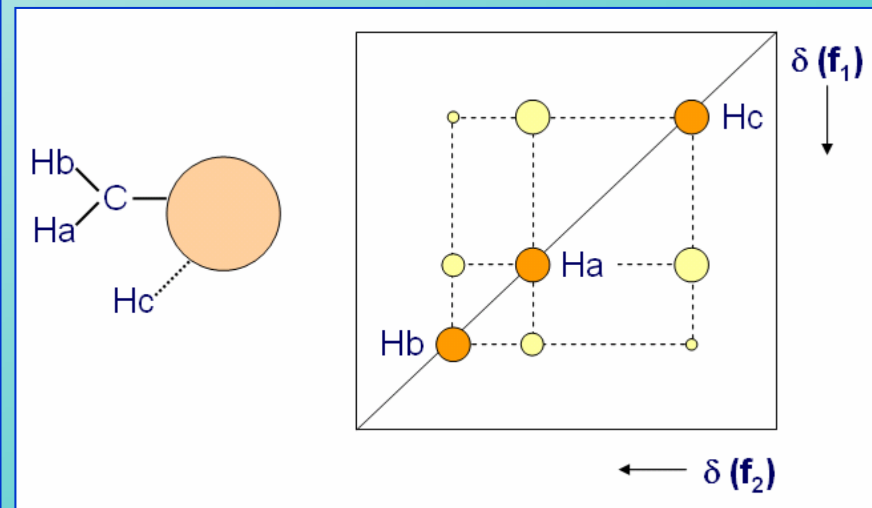
Top: Since what we need is to see how a system returns to equilibrium through cross-relaxation, we can use a pulse to selectively invert one transition and then see how the NOE grows with time The last **π/2** pulse is usually called a ***read*** pulse, it converts whatever magnetization is in **<z>** after $\mathbf{t_m}$ into **<xy>** magnetization (which we can detect). All the equations are the same, but the NOE will also depend on the mixing time, $\mathbf{t_m}$, - the time to reach the equilibrium population. The problem is that "selection" means we have to do our analysis one line at a time, - tedious.

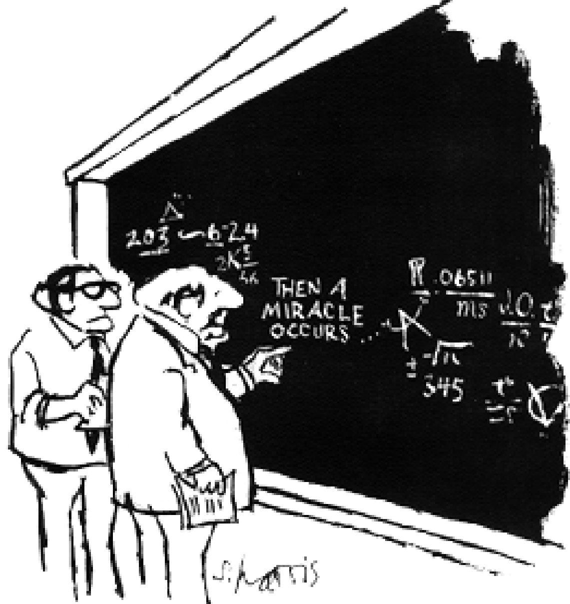
Middle: We use a 2D experiment in which we study all protons at the "same" time. This is a ***NOESY*** pulse sequence. The first two *π*/2 effectively invert all spins, but with a variable delay, t_1, and the third (the **read pulse**) detects spins still in the -z-axis after the **mixing time,** $\mathbf{t_m}$

Bottom: The off-diagonal peaks show the dipole-dipole couplings between spins.

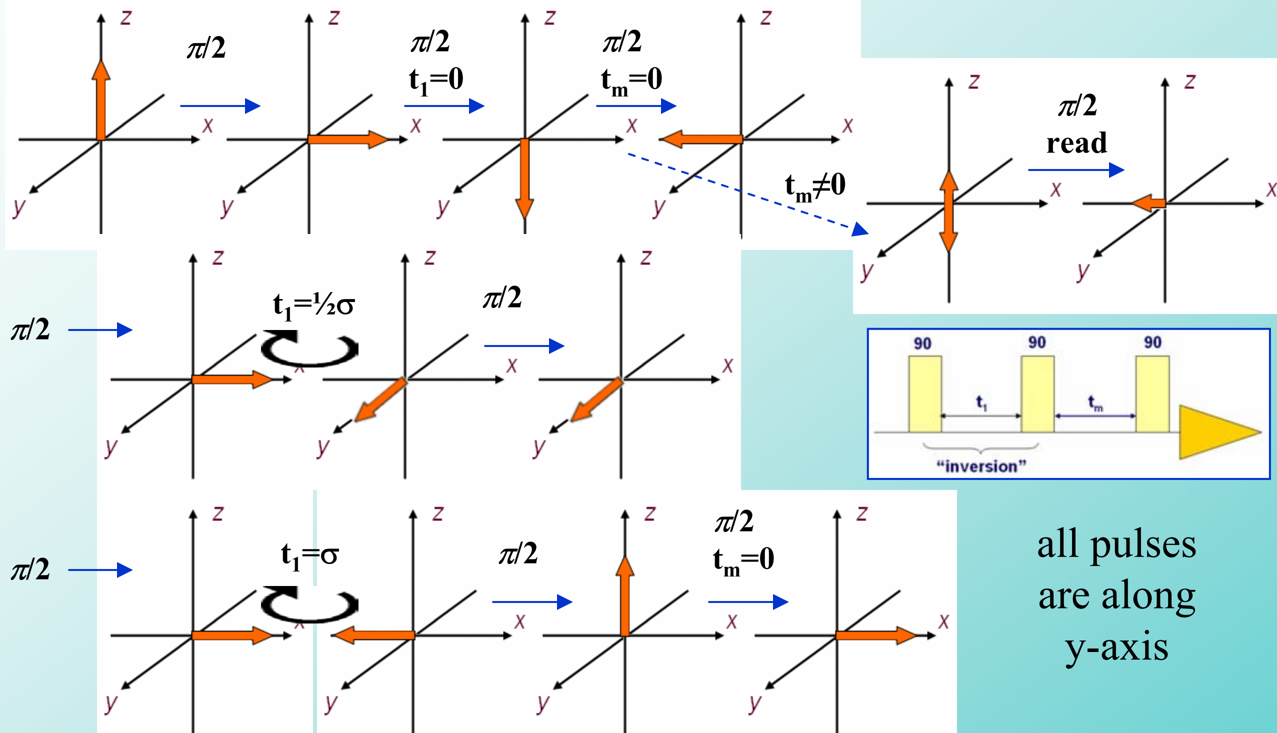








"I think you should be more explicit here in step two."

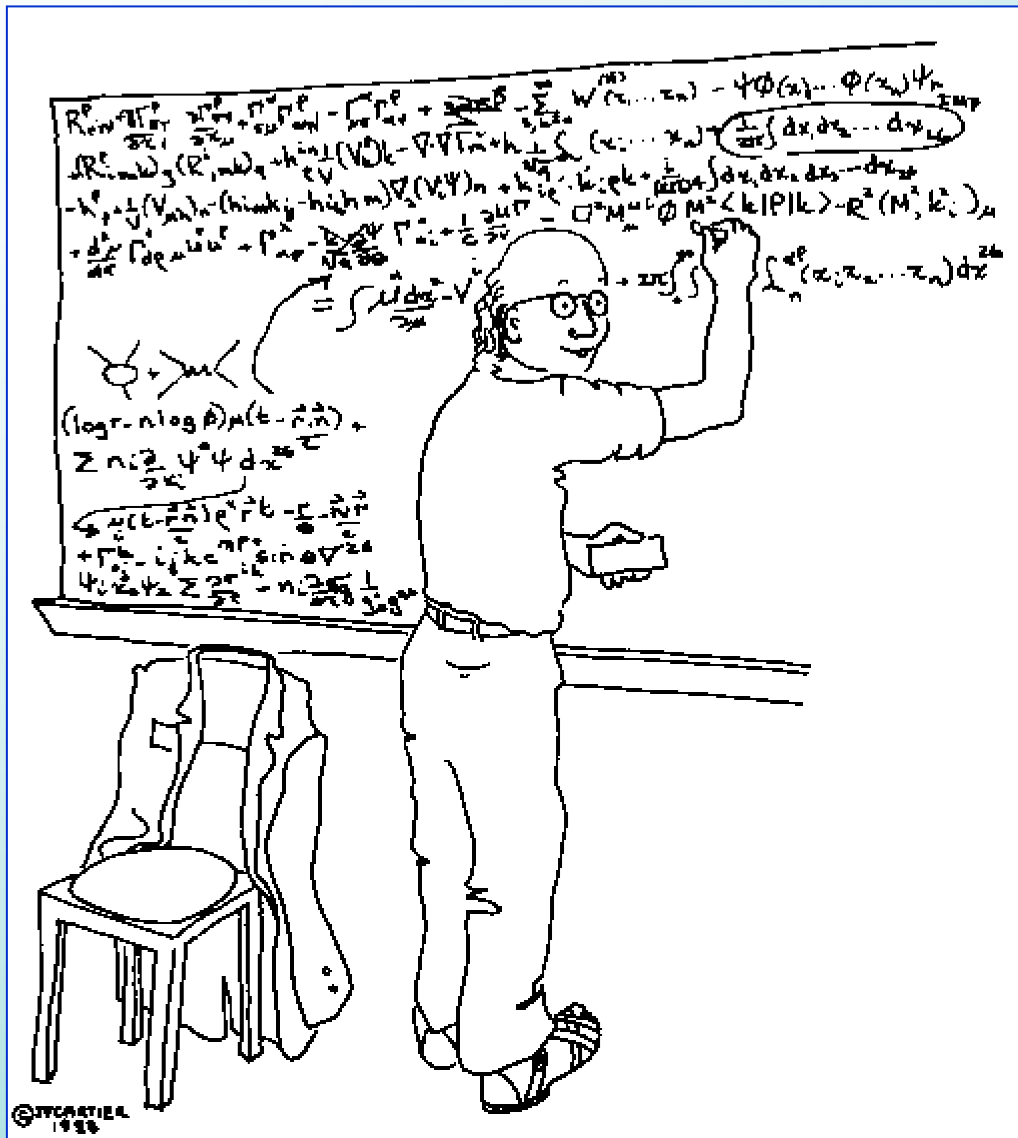


The "miracle" in NOESY is that by varying t_1, we selectively saturate different spins in the order of their relaxation in the x,y-plane. This spreads them out in the f_1 spectrum according to their J-couplings. In the FID generated after the read pulse, we sample the population that got flipped to the -z-axis by the 2nd $\pi/2$ pulse. We do this by flipping it back to the x,y-plane with the read pulse. This sample varies with the mixing time t_m. The relaxation of spins in the z-axis depends on the dipole-dipole couplings, on T_1, and on diffusion of spins by dipolar coupling to the "surroundings". Since the decay from the -z-axis depends on the dipole-dipole couplings, we can get off-diagonal peaks that show us the partners involved in NOEs.

The Nerds meet the Seven Dwarves in the shrinks office.

- ***COSY*** - 2D homonuclear **co**rrelated **s**pectroscop**y**
- ***TCOSY*** - 2D homonuclear **t**otal **co**rrelated **s**pectroscop**y** (Mad Hatter, too)
- ***NOESY***
- ***DQF-COSY*** - **d**ouble **q**uantum **f**iltered COSY
- ***MQF-COSY*** - **m**ultiple **q**uantum **f**iltered COSY
- ***SPI*** - **s**elective **p**opulation **i**nversion, - heteronuclear polarization transfer
- ***INEPT*** - **i**nsensitive **n**uclei **e**nhanced by **p**olarization **t**ransfer. It is used to increase the sensitivity (polarization) of nuclei such as ^{13}C and ^{15}N.
- ***APT -*** **a**ttached **p**roton **t**est. Simplification of ^{13}C by decoupling ^{1}H using saturation.
- ***DEPT*** - **d**istortionless **e**nhancement by **p**olarization **t**ransfer
- ***HETCOR*** - **het**eronuclear **cor**relation spectroscopy).
- ***HOMO2DJ*** – **homo**nuclear **2D J**-correlation spectroscopy
- ***INADEQUATE*** - **i**ncredible **n**atural **a**bundance **d**oubl**e** **qua**ntum **t**ransfer **e**xperiment
- ***CYCLOPS***
- ***EXORCYCLE***

No need to remember these, - just examples from the Moyna lectures.



"At this point we notice that this equation is beautifully simplified if we assume that space–time has 92 dimensions."

X-ray and NMR structures compared

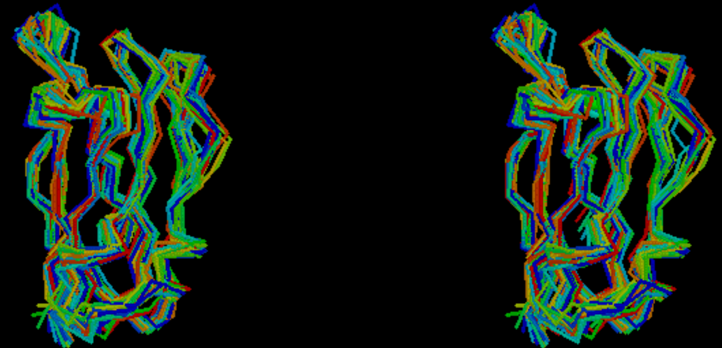
- The structures obtained by NMR are not better or worst than X-ray. NMR gives a different picture, which can be considered complementary.
- For relatively small molecules, NMR is considerably faster than X-ray. One of the reasons it is faster is because we don't need the crystals. This has a two-fold advantage. First, we don't need to spend the time growing them, and second, we can do it even if the stuff does not crystallize (small flexible peptides, polysaccharides, nucleic acids, etc.). Until recently, NMR structures were restricted to small proteins. This is because of the dependence of various relaxation processes on the tumbling time. Magic Angle Spinning (MAS) NMR of solid state (frozen) samples can get over this problem.
- NMR gives the 3D structure in water, which is the solvent in which many biological reactions take place (enzymes and drugs interact in water).
- NMR gives information on the dynamics of the molecule. It is not a static picture. What we get from NMR is a set of constraints on bonds, distances and angles connecting different parts of the molecule. We then have to use computer modeling to find structures compatible with the constraints. In general, a range of different structures will prove to fit equally well. These represent a range of possible dynamic structures. This dynamic aspect is something that crystallography does not provide, except through the B-factors of electron density (the mobile parts are more fuzzy).

The three structures shown are:

Top: a high resolution (1.9 Å) crystallographic structure of plastocyanin

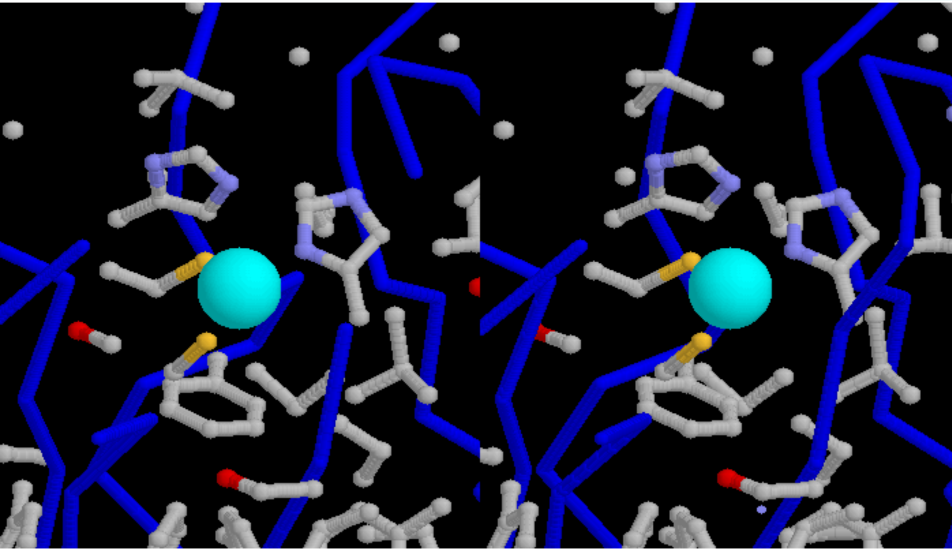
Left: The average NMR structure (from 20 solutions) for plastocyanin

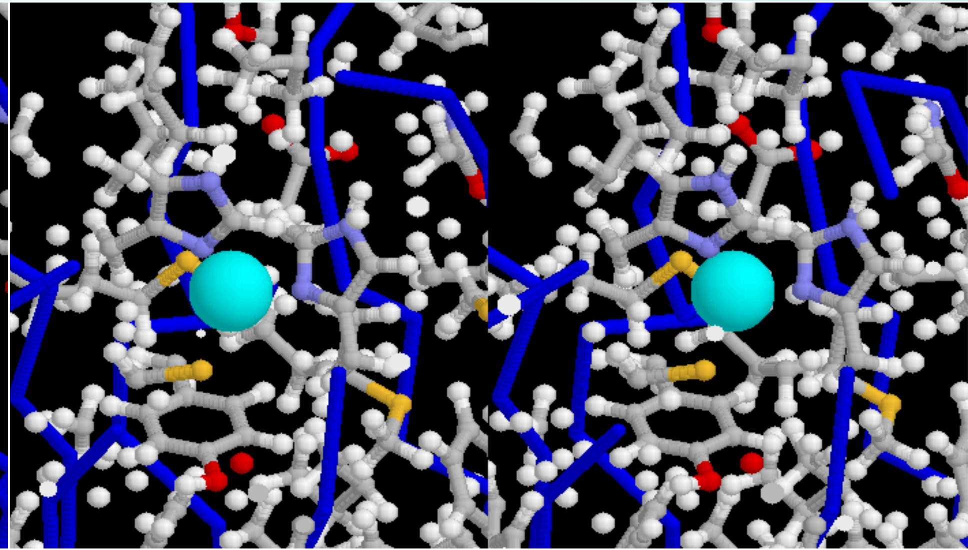
Right: The twenty structures overlaid.



The Cu atom in the X-ray structure

The Cu atom in the NMR structure





The twenty positions for the Cu-atom and the histidine ligands in the context of the backbone structure (left), and isolated for clarity (right).