NMR spectroscopy for Structural Biology NS
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Lecture: 12
Practical Aspects of FT-NMR-4

So, we were discussing about the various aspects of FT-NMR. We discussed about the water suppression and we have discussed about the spin echo and I want to show you here how a spin echo based water suppression scheme which we discussed last time as a water gate how it will achieve the water suppression.

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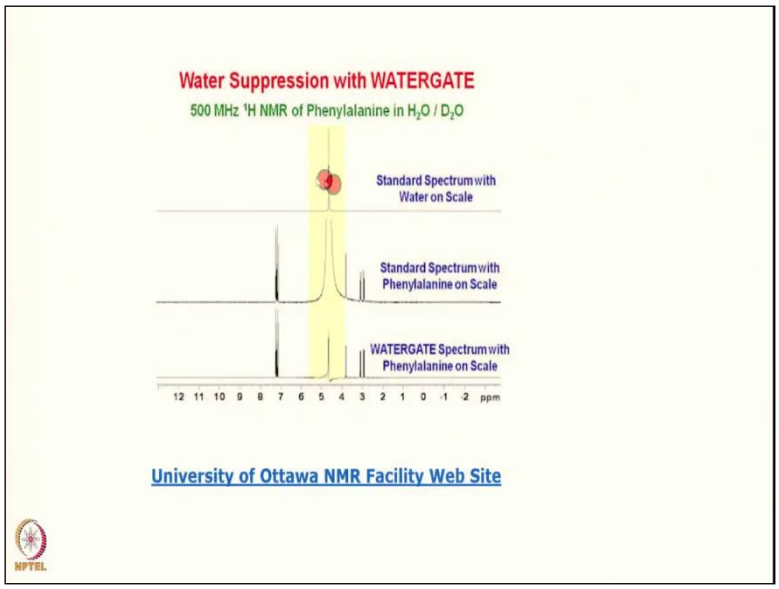

So, here is an experimental spectrum you can see here this is the normal experimental spectrum recorded in water the sample is phenylalanine dissolved in water phenylalanine in water with a small concentration. This is the normal factor without doing anything the top scale is the normal thing without doing anything you can only see water nothing else this is water.. Now you just blow it up just scale it up here somewhat more then you start seeing little this tiny signals which are already present here.

But then of course they are not seen clearly because of the huge water signal you adjusted the scale such a way that the water signal completely comes in the screen here then you do not see the other signals.. Now you scale this up so, you see the water gets saturated here saturated

meaning actually this is cut off here and you start seeing little tiny signals here of this phenylalanine.

So, this again the same spectrum as this except that it is just scaled up. Now you do an experiment with a water gate. Now you suppress the water and. Now you see where is the water? This is the water signal water signal is much less than your actual sample signals. So, these are our sample signals the phenylalanine signals and this is also all these are belonging to the phenylalanine. And now you see those ones are stronger than the water itself.

So, this is what is achieved by the water gate pulse sequence which I described to you last time and this is taken from this source here University of Ottawa NMR facility and you will find many of such words in the in the Google search if you do of course you will find many examples of watergate suppression and also many other pulse techniques which are there for water suppression and I have taken one simple example here to illustrate how the water gate is useful for recording good spectrum.

So, that was just a continuation of the last slide last topic. So, now we move on to another topic which is called as dynamic NMR.

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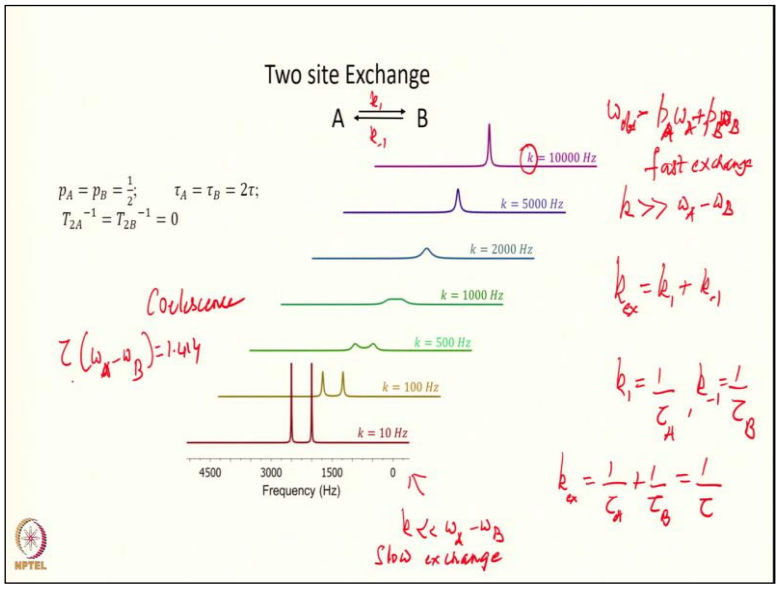

In the sense that the NMR spectra are very sensitive to the dynamic effects in your system. If the molecule is undergoing conformational exchange or chemical exchange between 2 different conformations or multiple confirmations whatever then it will show up in the NMR spectra and this is illustrated here as a by simulation I will also show you the experimental spectra.

So, here you can consider for instance a simple 2-sited exchange a chemical exchange a proton exchanges between 2 sites A and B and these ones have different chemical shifts. So, these chemical shifts are here you can see this one is these are the 2 chemicals should sign the in terms of the frequencies here. And if the exchange is happening and now it depends upon what is the exchange rate and what is the population of these individual states?

So, what does the population depend upon it depends on the energy difference between these 2. Suppose they are of similar energy they may have similar populations but there can be barrier in between and the barrier will result in different exchange rates different forward rates and for backward rates and the exchange rate is defined as the sum of these 2 rates.

So, suppose I want to write suppose I write here the to exchange rates here as the forward exchange I write it as k 1 and the backward exchange I write it as k - 1 then I have the exchange rate is defined as k exchange is equal to k 1 + k - 1. So, one single time constant describes the 2 the process of exchange and that is defined as a k exchange or this is the same as the k here which is written here this is the same as this k exchange here.

This is the simulation which has been done assuming the following parameters assumed here that the populations of the 2 states are identical p_A = ½ that means that is these are the probabilities the probabilities of meaning one the populations are the same in both cases. And the tau A and tau B these are the lifetimes these are the lifetimes of the individual states. So, lifetimes if both are equal. So, if I say $\tau_A = \tau_B = 2\tau$.

So, that is the lifetime and the lifetime is related to the exchange rate. So, the $k_1 = 1/\tau_A$ and $k - 1 = 1/\tau_B$ and. Now if you put $\tau_A = \tau_B = \tau$ then we define one rate which is the k, k exchange or k, k exchange is the same as the $k = \frac{1}{\tau_A} + \frac{1}{\tau_B} = \frac{1}{\tau}$. Now we can substitute here 1 by yeah so, this k – 1 + k.

So, if you substitute $\tau_A = \tau_B = 2\tau$ then what you will get you will get $\frac{1}{\tau}$. So, τ is the single time constant which describes the exchange phenomena how the spectra will change as a result of this exchange happening. Of course how does one change the exchange rate you can change

the exchange rate by changing the conditions like you can change the temperature you can change the viscosity by adding different things and so on so forth.

And then it also depends upon the relaxation times of the individual states how much is the relaxation time of the spin in the 2 sides. So, here are the 2 relaxation times here but those become individually assumed here that they are infinitely long that in the sense that $\frac{1}{T_2A} = \frac{1}{T_2B} = 0 = 1$ that means they are extremely slowly relaxing therefore we do not include here the effect of this relaxation.

We will include here the changes that are happening as a result of the exchange process only. So, that is why to show the effect of the exchange we are doing the simulations here. So, you see when the exchange is very slow k = 10 hertz you see 2 clear lines see you see 2 clear lines here and used and of course the intensities are also high and you increase it to 100 hertz then the lines have broadened.

And therefore the intensity has come down the height has come down increase it further you give it even less then you reach a state when it is like almost you are not able to distinguish between these 2 and you start getting a broad line very broad line this is known as coalescence. The 2 lines tend to merge the 2 lines tend to merge when that happens the $\tau = \tau$ or $\tau\,\omega_A - \omega_B = 1.414$ when this condition is satisfied then you will see coalescence you start getting a single line.

Now what happens after that the exchange rate increases further then the lines start narrowing, narrowing further. Now you start becoming sharper, sharper and sharper and where it will appear this line will appear at the average of the weighted average of the 2 individual chemical shifts. The final position the final position what you will observe in this situation will be ω observed will be p_A or $p_A\,\omega_A + p_B\,\omega_B$ where p_A and p_B are the populations.

So, ω_A and ω_B are the individual frequencies and this average will appear at the weighted average position when the exchange rate is far, far larger than the separation between those 2 and what is the meaning of first this is called as fast exchange. Fast exchange meaning k is much much larger than $\omega_A - \omega_B$ and this one is a slow exchange here this is slow exchange here k is much less than $\omega_A - \omega_B$ this is called as slow exchange.

So, therefore you can see by monitoring this changes in the spectra as a function of some temperature or something like that or whatever you can actually try and measure the extra you can study the dynamics of molecules inside your in the system. And by simple examination at this point you can actually find out what should be the exchange rate. Of course you should know the ω_A and ω_B.

These will be present only when there is the slow exchange when you do it as a function of temperature at very low temperature you will see these 2 separate lines. So, you take them as ω_A and ω_B and then you substitute that here then at the coalescence temperature or coalescence condition you can find out what is τ and once you know what is τ you can calculate what is the exchange rate.

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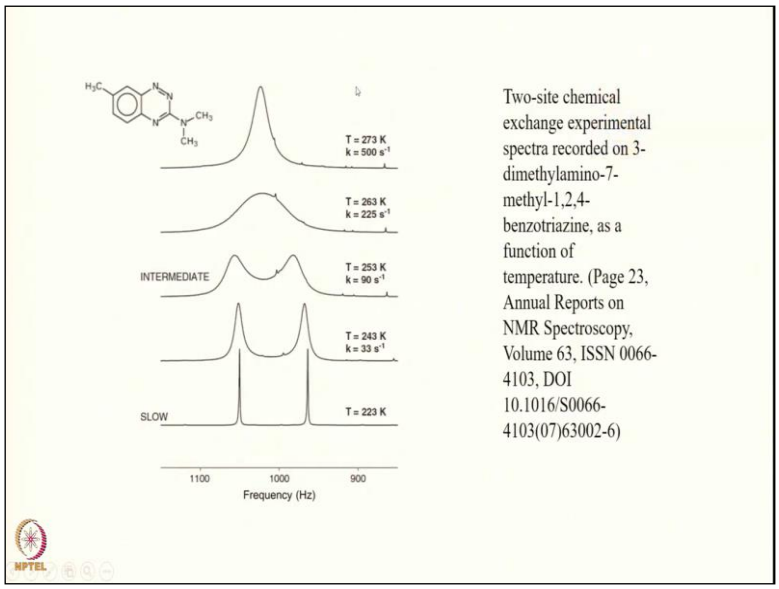

So, let me show you an experimental spectrum of this here is a molecule. So, this is what we are seeing here are the 2 lines of these 2 methyls NCH_3 CH_3 these are the 2 methyl groups which have separate positions these are 2 separate lines. And this is the spectrum recorded at 223° Kelvin and of this molecule in some solvent this is taken from this resource here. So, that is annual reports and NMR spectroscopy. So, these 2 pro methyl groups are non equivalent because the double bond character in this NC bond here. Therefore there is no free rotation around this bond therefore these 2 methyl groups are non equivalent and then you will see 2 separate lines. Now as you start increasing the temperature there will be free rotation around this bond the rotation around this bond happens.

So, when there is a rotation around this bond. So, therefore the exchange is happening the lines will start getting seeing the exchange between these 2 lines the lines start getting broadening broadened here and then at some intermediate value you see the lines have become so, broad that almost they are overlapping they are there is a small dip here that the depth of this one is decreasing and you get a coalescence condition here.

At 263° Kelvin see there is only one line the broad line is here you see this is the coalescence condition and that this happens at 2 and so now from here you can estimate what is the exchange rate. And because you know this ω_A and ω_B here of these 2 positions and then you know you can therefore you can calculate here what is the exchange rate you can calculate tau.

And you can calculate the exchange rate and this happens at 263° Kelvin and if you increase the temperature further this will start narrowing down again and you will see this peak at an average position. Notice here that this is not quite in the middle of this line this is not here this is not quite in the middle. So, it is slightly shifted onto this side this is because the 2 populations of this are not necessarily the same.

They can be slightly different as a consequence of that you will see a line which is slightly at one side or the other this is an experimental spectrum.

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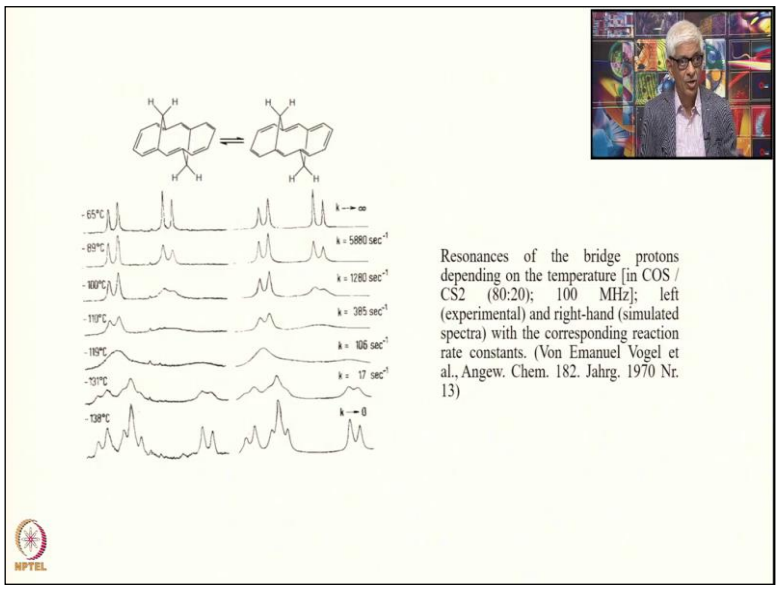

And I will show you another experimental spectrum and this is of a molecule which is undergoing equilibrium like this so here you have a molecule. So, and we are talking about these 2 protons here. So, these 2 protons this is the bridge here and the bridge there are these 2

protons which are there and then you see this undergoes conformational equilibrium of the tautomerism here between these 2 positions these 2 conformations.

So, and therefore you see the signals which are presented this is the signal at slow time scale and you see the separately these signals here all of them are separately seen and you start increasing the temperature to -138° Centigrade here you see this is extremely at a very very low temperature and I just start increasing the temperature exchange rate starts increasing and you see here at this point.

At this point this has become raw and this is sort of vanished here and then actually it started appearing again the broadening has happened already here this is already broadened here. The coalescence has already happened here and this happens differently for different groups you see it is not the same for this group and for this group it is different for the different groups. Therefore the exchange positions in the groups itself there can be different rates in the given molecule as because the barriers can be different for the different rotations.

And therefore for this group you have a coalescence temperature at one point for this group there is a coalescence temperature at another point. So, therefore you can measure these exchange rates for the 2 things at different. So, at this point here this is the fine final thing at $k = \infty$ these extremely fast motions then you start seeing only these 2 lines here right this is this happens at -65° Centigrade and you measure the exchange rate as 5880 seconds inverse.

So, this provides a useful technique to measure the exchange rates and we will also show you how you can use this phenomena to understand dynamics in proteins because in proteins there are all kinds of dynamics present there are conformational exchanges domain motions and these are micro second time scale motions the millisecond time scale motions nanosecond time scale motions.

So, in proteins you have all kinds of motions all these various kinds of motions will show up in your NMR spectra how to extract that? That is an important point there are other method this is the very simple method of obtaining the information from looking at the one dimensional spectra. But when you go into the proteins this spectra may not be so well resolved you may have to do other techniques and then of course the principles remain the same of course you have the slow exchange intermediate exchange fast exchange.

So, the appearance in the spectra will change and you can use those data to estimate the conformational dynamics in proteins. Now there can be other effect of the exchange phenomena.

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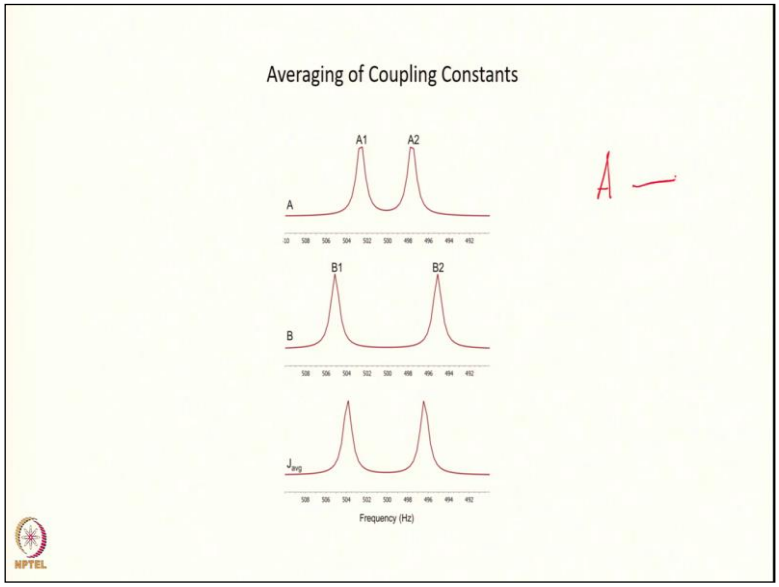

So, there is let us say there are 2 conformations of a particular molecule suppose I have a molecule which is in conformation A and these exchanges with a another confirmation B and here I have one coupling constant there is a particular proton pair let us say I have a coupling constant here and this is a another coupling constant here J_2 and I represent here the 2 spectra like this. So, I will have a line splitting here and I will have a line splitting here and this is J_1 and this is J_2 separation between these 2 and that is what is shown in this here.

So, for the A side. So, I will have 2 lines here I will add 2 lines because of the splitting the separation between them is the coupling constant. For the B side again I will have 2 lines and the separation between them is the coupling constant. So, now if there is an exchange here the coupling constants also will get averaged out the coupling constants will get averaged out and then you will get the $J_{average} = p_A J_1 + p_B J_2$.

And that shows up that is what you will see in the bottom spectrum here this is the bottom spectrum see where does it appear you see it is neither here nor it is in between if you draw a vertical line from there to here down. So, this line is lying in between this position and this position it is somewhere in the average position between these 2 lines. And similarly this is also present at the average position between these 2 lines.

So, therefore what you will measure is this coupling constant you will measure this coupling constant because of that conformational averaging. So, when this happens if you try to interpret this coupling constant in terms of the structure as one commonly does because the coupling constants are related to dihedral angles in molecules. So, you have suppose I have a situation like this a three bond coupling.

So, there is a J here then I measure here the J then I try to interpret the torsion angle this as a consequence we use the Karplus relationship and things like that. Now you should be careful that if there is a conformational averaging what you will directly interpret may be wrong because the average coupling constant will give you a certain value here 5 but that may not be the actual structural value it be the it is the result of an averaging between these 2 positions.

Therefore you must look at the stable conditions change the temperature change the conditions and find out the individual coupling constants there and then you will be able to figure out from this what are the populations of the individual conformations. This is an important application also in structural biology and this typically happens in proteins there are rapid rotations in the physician angles in proteins.

This we will see as we go along that is the stuck I am preparing the ground for understanding the phenomena in proteins and nucleic acids which will be your focus in the area of structural biology. So, so far as the dynamism in the in the spectra.

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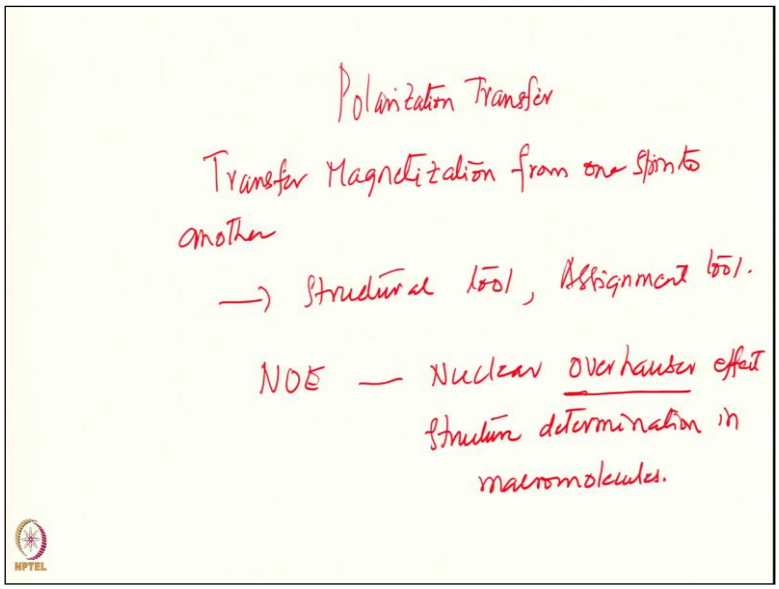

And now we move on to another important concept in NMR and that is the so called polarization transfer or NOE or polarization transfer is a very general term polarization transfer meaning that we transfer magnetization from one spin to another spin that means we transfer magnetization from one spin to another. And this is an extremely useful structural tool or it can also be an assignment tool.

Among these there is one important thing which is called as NOE this is nuclear Overhauser effect. Now what is NOE? Nuclear Overhauser is the name of the person who invented it and of course the there was initially it was electron nuclear overhauser effect and subsequently this is the overhauser this is the nuclear-nuclear overhauser effect that has become extremely useful for structure determination in proteins.

I will tell you what this means this is application I am just writing straight away structure determination in macromolecules.

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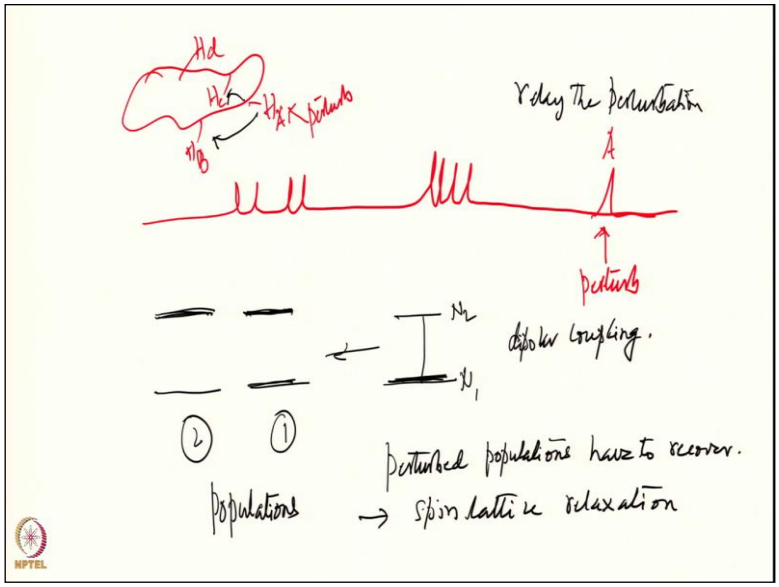

So, what is NOE? Let us say I have a spectrum. So, there are so, many lines there when there are so, many lines they of course have different chemical environments and therefore you have different lines. Now what I do is I do a perturbation here perturb this line what is the meaning of perturbation either I can do a saturation there apply RF another RF which is exactly online or that one and what is the consequence of this perturbation.

So, you have a molecule something like that let us say we have a proton here and a proton there a proton here a proton there so on and so forth. And this line let us say belongs to let us say this

call this line is A that is this line B and there are various other ones B C D and so on. So, so many protons are there they are all giving you signals at various places if you perturb this proton a will the perturbation remain there will it always remain there or does it go to somewhere else can it transfer this perturbation to some other proton.

So, if it has to transfer the perturbation there has to be an interaction between them if there is an interaction between them this can put it is like you know pack of cards. So, you have cards put one behind the other you perturb one fellow in the front and then all the other fellows will fall down. So, it is a relay. So, the relay can happen see if I perturb this proton per turn it can relay its perturbation to another one it can relate here it can relate here and so on so forth.

Relay the perturbation and for this what is required there has to be coupling. For this there is in coupling and that is the dipolar coupling and these are magnetic dipoles all the protons are magnetic dipoles therefore there is a magnetic dipole or coupling. So, if I prefer one of those. So, when I what is what it means when a perturbed as I said suppose I have the energy levels for the spin A and these are the populations here.

So, let us say I have this N_1 here and N_2 there when a perturbed meaning I change this populations. So, I have it here like this there are something like this. So, when I perturb it here then I may make it both of them equal I can make them equal or this is one perturbation or I can also do I can make it this one here and this one down there this is the number 1 number 2. So, both kinds of perturbations are possible.

So, essentially what we are doing is both are contributing to changes in the populations of the states. So, that is like a relaxation phenomena after you change the populations the system will have to recover and the system will have to recover how does it recover it has to give away its perturbation to somebody else. So, the perturbed populations have to recover and this happens by spin lattice relaxation.

So, because of the spin lattice relaxation there is a relay of the perturbation from one spin to another spin and then it will show up in the intensity of this of this proton. So, let us say as a result of this I get a new spectrum whenever. So, what I do is I do 2 spectra so I do 2 spectra.

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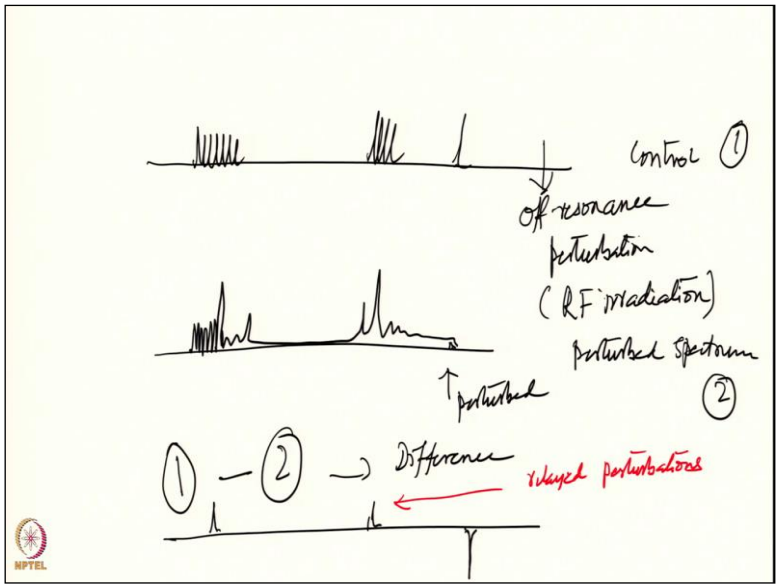

I have the same lines here this is the unperturbed spectrum well and perturb this. So, for this I do a perturbation somewhere far away this is called off resonance perturbation there is no line there I put a perturbation somewhere. So, this is just as a control of resonance perturbation. So, that I get a spectrum like this so this happens as a control just to see that the RF irradiation which I am doing and this is by RF irradiation.

And then I do another spectrum where I do the same thing and I have perturbed this, this line this is perturbed. So, what do we do now this is the perturbed spectrum. So, this is X ray let us say I call this as experiment number 1 this is experiment number 2 then what I do is I take 1 - 2 different spectrum I take a difference. So, what do I get when I perturb this I will transfer the perturbation to those protons which are dipolarly coupled.

Those which are not coupled they will not be perturbed at all there will be no difference in that so then what I will when I do this I will get a spectrum which may be like this there is a line here there is a line there and all others have gone to zero and here I will get something which is negative. Because you have perturbed this is made it 0 here or saturated made it 0 then I take this minus this or this minus this.

So, I get a negative 1 here I have written 1 - 2 but it could be 2 - 1 also it does not matter. So, you have here some change happening in this particular line some change happening in this particular line because these are the ones which are dipolarly coupled to the perturbed line. So, these are the enhancements or there can be decrease also and depending upon that we will have

some perturbations in these areas. So, these are the; so, these are the relayed perturbations and this effect is known as NOE.

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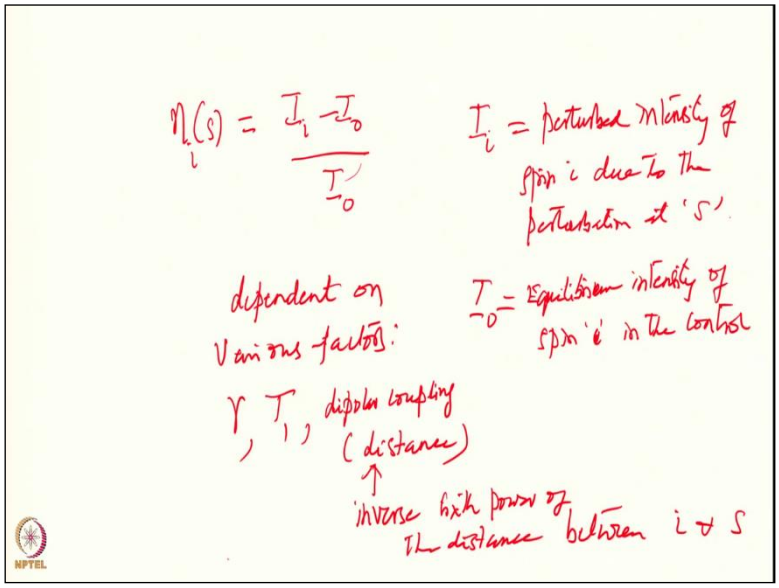

And we can quantitatively define this as NOE is defined as $n_i(s) = \frac{I_i - I_0}{I_0}$ and what is I_i is the perturbed intensity of spin I due to the perturbation at spin S at spin S and I_0 is the equilibrium intensity of spin I in the control that means in the absence of perturbation at spin S. So, when you do this you will measure this n is and you see this will be this will be dependent on various factors the important thing being the gammas.

The γ of the individual residues and the relaxation times the T_1 relaxation times and the dipolar coupling. Dipolar coupling means distance so the dipolar coupling is proportional to inverse cube of the distance for the NOE is proportional to inverse the sixth power of the distance. So, this here it is inverse sixth power of the distance. Distance between one and one between I and S these are the 2 spins which are considering we are saturating or perturbing this spin S and monitoring spin I.

And what I have here this is $I_i - I_0$ and these both refer to the spin I because the spin S we are not looking at we are looking at this pin line we have perturbed the spin s we are looking at this spin I. So, $\frac{I_i - I_0}{I_0}$. So, this is the with respect to the unperturbed intensity what is the change in the intensity that we are measuring. So, the effect of that is what is useful in quantitative estimations of distances which in turn is a structural parameter. So, we can we can stop here.