Principles and Applications of NMR spectroscopy
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Module 5
Lecture No 25

In the last class we looked at an experiment which is called as 2D TOCSY which stands for total correlation spectroscopy and we saw that it is it contains all the peaks on a from a COSY but it has much more information that is all the spins, all the nuclei in a spin system are coupled to each other and that is reveled in a TOCSY so you can actually unravel at spin system based on a TOCSY experiment. So now we come to another very important experiment known as 2D NOESY .

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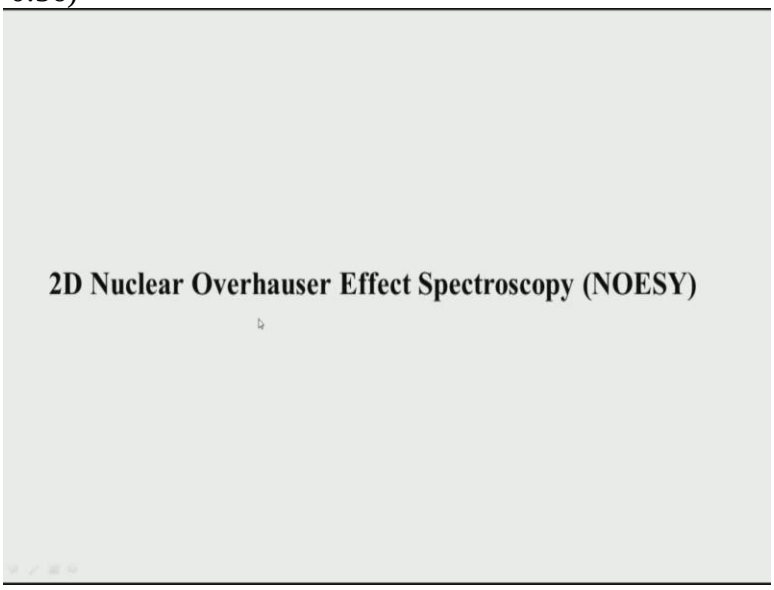

As you can see here the NOESY word stands for this acronym, NOESY acronym stands for this word that is Nuclear Overhauser Effect Spectroscopy. So Overhauser is the name of the person who discovered this effect but it was not in organic com., it was in a metal but the idea was we observed that the polarization of one nucleus or one magnetic early active nuclei or electron can be transferred to another nucleus.

So there is what we exploit in the case of chemistry or in biology that two hydrogen atoms if they are close by means located in space closer to each other then there is a possibility of

transferring polarization from one to another. So remember what is polarization? Polarization in NMR means the population difference. So the population difference depends on the factors such as the gamma value it depends on the magnetic field and the temperature.

So assuming that we are looking at protons only now, so gamma is same for (()) (02:00) protons, temperature is same and magnetic field is same. So what we are trying to do now is that one particular proton will have a given polarization is now transferred to second spin through this effect known as Overhauser effect and that helps us to find out which two protons are close to each other and they interact through space, ok. So let us start from this.

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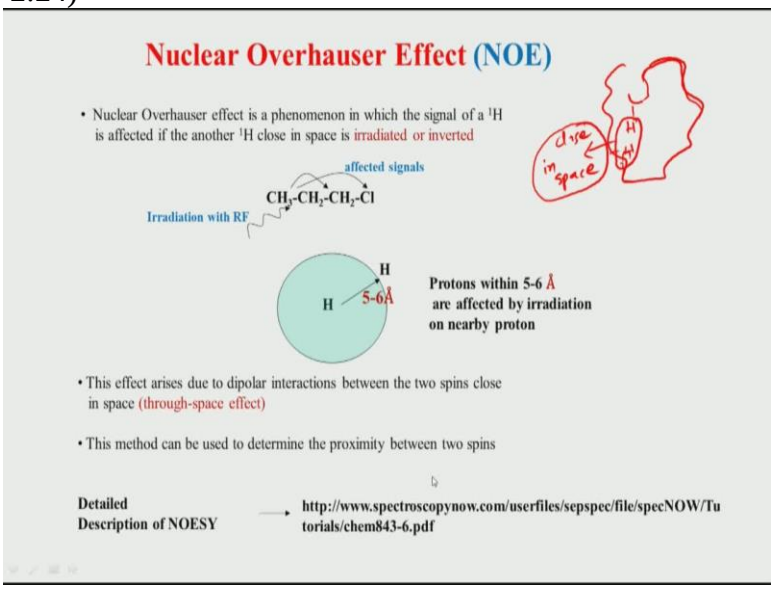

So how does an axis of the experiment is carried out so before that let us understand this effect little bit more. So this is what I said Nuclear Overhauser Effect is what we study in NOESY. So this effect basically is a phenomenon in which the signal of one hydrogen is affected if another hydrogen or proton is located close in space, ok. Now this is not just a presence of two hydrogen nearby results in NOE what is happen what happen is you have to first irradiate one hydrogen. Now the irradiation of that hydrogen disturbs the population level in that particular hydrogen.

So we saw that when you do not apply a pulse that is known as Boltzmann distribution of population equilibrium. But when you apply a pulse it becomes equal in population that means the two levels we will say the word saturated they are saturated means they are equalize a

population so there is no more any population difference. Now that polarization transfer that polarization which is 0 now it starts creating going back towards equilibrium when you remove the pulse.

So that the creation of this 0 polarization basically is affects the neighbouring hydrogen if it is dipolar it is coupled to this hydrogen. So the polarization of this is affected by disturbing the polarization of this nucleus. So why is this so? This is because they are close in space and there is an interaction know as dipolar coupling. So remember till now in the case of COSY and TOCSY and also in 1D NMR we only looked at J coupling, J coupling is just a interaction between two neighbouring hydrogens either through three bonds or two bonds.

So that is because of the connection the physical connection between two hydrogens. But this is not the case in this is not the only way of interaction between two hydrogens. The two hydrogens can also interact through space means not through bond because they are close in space and that is called as dipole interaction. Because this is a dipole remember NMR the spins are all magnetic so we call them a magnetic dipoles.

So there is a dipole movement associated with one hydrogen and dipole movement associated with the second hydrogen. So these two dipoles can interact with each other through the mechanism known as dipolar coupling. And this interaction affects the population of one hydrogen when it is irradiated. So if this hydrogen is irradiated it affects all hydrogens which are neighbour to it in space.

So we have to remember the point we are talking about through space interaction. Whereas those hydrogens which are very close far away they are not affected, ok. So this is the main point in NOE although we have not got gone into details mathematical analysis of this interaction but it has a qualitatively one can think of it that when the population of one hydrogen is perturbed disturbed through irradiation or inversion or any other mechanism that disturbance is transferred to the second neighbouring hydrogen, ok.

So this is very important word transfer, so whatever you do with one spin that information or that perturbation is sort of transferred to another spin to which through we by which it is interacting

via space. Remember this is not J coupling again this is only through space. That means I do not need a bond here, ok.

So for example let us say you have a very large let us look at it more in carefully. So let us say you have a molecule which has a structure like this and there is a hydrogen here let us say and there is one hydrogen here. So you see these two hydrogens are now close in space but they are not connected through bond . Why? Because this bond is very far away. So the only thing they are coming close in space.

So in such scenario now if I irradiate, or I disturb, or I perturb, or I invert whatever I do with one hydrogen atom population of the one hydrogen atom that perturbation or disturbance will be communicated to this hydrogen, ok. Or in the other words it will be interacting so this will now perturb this hydrogen also. So although we interrupt perturb only this hydrogen also gets perturbed or vice versa if I disturb this hydrogen this will be affected because now they come very close in space.

And now the question is how should how much is the closeness how much should be the closeness for them to interact. So there has to be some limit. Typically the limit as we will see here is 5 Armstrongs that 5 to 6 Armstrongs so if the two atoms comes within 5 to 6 Armstrongs you will start seeing a communication between them in terms of dipolar or interaction I would not use the word communication although we are talking about qualitatively here but the more appropriate word would be an interaction between the two hydrogens because of the dipolar coupling when they are less than 6 Armstrongs in space, ok.

So that is a major important point here for as for as your consistence so this is what is shown. So if I have an hydrogen any other hydrogen in the sphere in a radius of 5 to 6 Armstrongs will be affected by irradiation of the nearby proton. So this proton will be affected if this is perturbed or vice versa if this have a hydrogen will be affected if anything in this vicinity of 5 to 6 is perturbed, ok.

So this is the major point here that is through space effect and the effect as I said arises due to the dipolar interactions between two spins which are close to each other. And this method so now if you see here what has happened when if I irradiate this hydrogen I am affecting this hydrogen

the signal intensity of this. Remember in NMR signal intensity is all coming because of the population difference.

So if I change the intensity of one hydrogen if I change the population of one hydrogen, the intensity of that hydrogen is affected because its population has changed, ok. So therefore the intensity of the signal is what is monitored to get the information on the interaction so you see effect of any hydrogen in this radius, or of a sphere, or a circle anything within 5 to 6 is affected which means that if I can find out which two which hydrogen if I irradiate and which other hydrogen is affected then I get the information between those two because there should be within now 5 to 6 Armstrongs.

So therefore the distance information the proximity the information on the proximity of two hydrogens can now be obtained using this method of NOE. That is why NOE is a very powerful method in NMR because it gives you the distance information between two hydrogen atoms which you will not get by any other approach. Because if you see here these let us say look at this picture again this is a (mol) hypothetical molecule which has a very complicated shape but there are two hydrogens which are very close to each other and because of that if I perturb one hydrogen I see a intensity signal disturbance in the second.

The intensity may go down or it may go up either can happen, that we will see in the next slide. But this perturbation of each other now tells us that these two hydrogen should be now within 5 to 6 Armstrong. So you see we directly get the distance information not the exact distance, ok. We are not talking about the exact distance here, we are talking about rough estimate of proximity between two hydrogens which we get now because of the effect of each other.

That information would not have come from any other method in NMR like COSY, TOCSY and so on it only comes from this experiment knows as NOESY. So therefore NOESY occupies a very important place in NMR in fact for biological molecules this is very useful because biological molecules remember have a very complicated shape and the distance structural information can only be obtained through this kind of an experiment.

And that is one of the reasons why Kathbutrick was given noble prize because he pioneered the use of NOESY for structure determination of biomolecules, ok. So that is why it occupies infact

all structure determination in NMR has to have a NOESY component because no other experiment uses a direct structural information as you get in NOESY. So therefore NOESY is **is** worth spending time on NOESY reading about it more and we will see this more and more as we go along in different applications.

So the detail description of NOESY ofcourse is beyond the scope it a lot of mathematics involved because we talk about dipolar interaction and so on but I would suggest recommend this particular website which has this pdf which gives a very nice and detail description but it gives in a very simplified manner. So you can go through this in addition to the books which textbooks which we have recommended.

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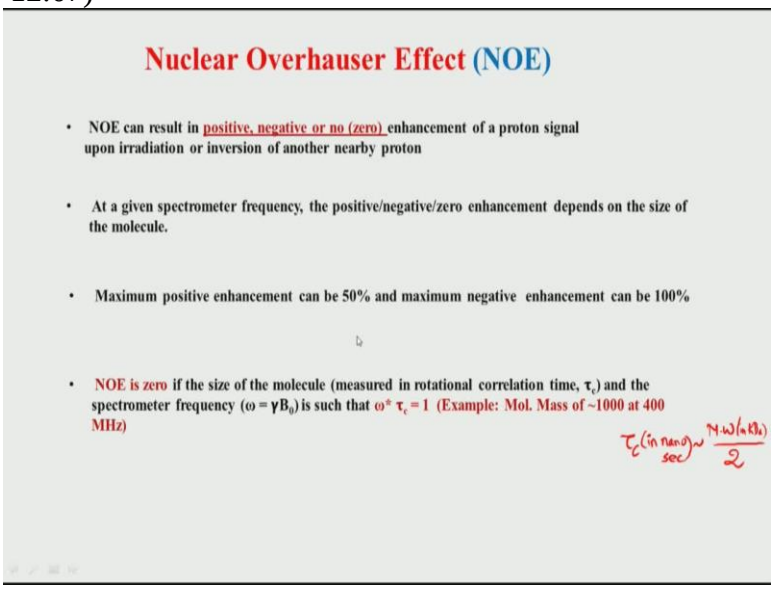

Now as I said this this perturbation that is which affects the signal intensity when we irradiate one nearby hydrogen this the resulting perturbation in the neighbouring hydrogen can either result in increase of its signal we use the word positive enhancement or it can decrease the signal or it will have no effect, no enhancement. No enhancement means there is no either there are two reasons, one is the two hydrogens are not close to each other or it can be by chance it has a particular value we will see that of what is called as a frequency which results in zero enhancement means no effect.

So NOESY can fall into any of these three categories in terms of the intensity effect how it is affected by a neighbouring hydrogen. So what where is the this is where the matters what parameter matters whether you will get positive or negative it depends on two factors number one it depends on the spectrometer frequency. Spectrometer frequency means that what spectrometer are we working at a 300 or 400 Megahertz or 500 Megahertz, etc.

So that frequency value is one component and second component we depends as on the size of the molecule, ok. So size here we do not mean the molecular weight because molecular weight is not really a good indication of a size of a molecule, when we talk about size we are talking in terms of parameter known as rotational correlation time. So rotational correlation time is the time taken by molecule or is indicator of a time the rotational tumbling of a molecule. So remember all molecules in solution are not static they are dynamic in nature.

So they are actually undergoing rotation, vibration and translation but we use it also when it tumbles we use the word tumbling that is rotation rotational diffusion is a correct or technical correctly correct word. So this rotational diffusion means rotation or tumbling of the molecule is characterized by a parameter known as rotational correlation time, that is in short TOUSY.

So this TOUSY is what is is typically calculated like this. So suppose you have a molecular mass a molecule of 1000 let us say so then at the rotational correlation time will be measured as follows let me give how it is calculated roughly this is not going to be an accurate calculation. So TOUSY in nano seconds is given as the molecular mass or let me say weight in kilodaltons divided by 2, ok.

So let us look at this formula more carefully this is a rough estimation of TOUSY. So molecular formula molecular weight in kilodaltons. So for example if you take a molecule which is 1000 that is equal to 1 kilodalton 1 KD, so 1 will come here so 1 divided by 2 is 0.5, so the TOUSY of that molecule will be 0.5 nano seconds, ok. So typically a molecular sizes except of course very small molecules such as water molecule or solvent molecules when you talk about molecules such as organic molecules com.s which are in the range of 500 onwards they will have TOUSY in nano second time scale, ok.

But if you go to solvent molecules like methanol water it comes in picosecond time scale. So our all our examples which we will see or generally we come across are all in the range of nano seconds. So TOUSY is generally given value is in terms of nano second, ok so what happens is whenever the spectrometer frequency that is 2 into pi into the frequency is multiplied by the TOUSY value that it becomes equal to 1 or nearly becomes equal to 1than at that kind of a for that particular com. and that particular frequency we will have no NOESY effect no NOE effect even if the two hydrogens are very close by.

So this is what comes from theory of NOESY which we cannot go into detail but the point here is one has to be one has to look carefully that what kind of molecule you are using and what is the spectrometer frequency. So for example let us say you are working on a molecule which has a mass of 1000 now if you go to 400 Megahertz spectrometer and if you do this math it is basically to remember omega is 2 pi into frequency, ok.

So 2 into pi that is 3.14 into 400 into the TOUSY if you do this calculation we will see it will be nearly equal to 1, ok. So because of this effect it if what will happen is that particular molecule you cannot use NOESY for distance information. Remember why are we using NOESY? We are not using NOESY for getting chemical shift correlation information because that information comes from TOCSY and COSY.

We are using NOESY for distance or proximity information between two hydrogens. So therefore if two hydrogens are proximal they have to show a NOESY effect but the NOESY effect becomes 0 if it happens that your molecule accidently happens to be in this particular range, ok. So it is very important to keep in mind that what kind of spectrometer you are working at and what kind of molecules you are looking at they cause by accidental by (()) (18:09) may happen that this comes close to 1.

Remember it does not have to be exactly 1, it can be close to 1 nearly. Let us say + - 10 or 20 percent also will result in a no NOE effect and you will not be able to get any information on the distance or information is molecule. So now how do we come out of this? For example let us say I have a molecule which I have to study at which is I cannot change this is what I am studying and I have only one spectrometer at in my laboratory.

So that means can I never do NOESY for this molecule? No, you can still do NOESY provided what you can do is you reduce the temperature or increase the temperature. For example remember TOUSY is what I said is a tumbling rate means it gives you the rate of tumbling of the molecule. Tumbling means rotation and rotational diffusion. Now that aspect is critically it very much depends on temperature.

So if I reduce the temperature of my sample obviously my tumbling will slow down because the viscosity of the sample will go up and so on. And therefore the tumbling will reduce and TOUSY will now decrease, actually TOUSY sorry will increase because the molecule will become a heavier molecule it is not tumbling means it is heavier and if it is heavier it has a more molecular weights.

So we have not changed the molecular mass. What we have change is the apparent molecular mass because the molecule is not tumbling slowly, it is behaving as if it is a bigger molecule, ok. So this is a very important point in NMR by changing with playing around with temperature you can play around or you can modify the TOUSY of a molecule and this is very routinely used in biomolecules because we want to go to higher temperature or slower lower temperature for exploiting the favorable properties.

So if you go to lower molecular weight in this case let us say you go to a 5 degrees this is remember this formula which I have shown here is typically for 20 to 25 degrees celcius. But let us say I go to a very low temperature 5 degrees the molecule is still not static, the sample is not frozen and let us say we are looking at water sample, so the molecule is not frozen. So when the molecule is not frozen it is having a slow tumbling because its temperature is low. So it may happen that the TOUSY which was 0.5 nano seconds in this case may become 1 nano second.

So if it becomes 1 nano second then obviously it is not equal to 1 this multiplication then we have come out of this problem and NOESY will now work for your molecule or you can go to the higher temperature. If you go to higher temperature let us say at 40 degrees or 50 degrees it may happen your TOUSY becomes less now because it is tumbling faster and that will make it small behave like a smaller molecule and the TOUSY will come down from 0.5 let us say to 0.25 and then again in 0.25 nano seconds this will work because this product will not be equal to 1.

Remember omega is fixed we are not changing the spectrometer we are only keeping the omega fixed but we are playing around with TOUSY. Alternatively in your laboratory if let us if you say have 800 Megahertz another NMR machine then you can keep the temperature same but change omega now. So omega becomes twice if you go to 800 Megahertz then there you will have that this multiplication now will be not 1 and you will be able to do NOE NOESY.

So NOESY therefore critically depends on what spectrometer frequency you are working at and it depends on the size of the molecule which is indirectly reflected in the TOUSY value. So the maximum what is the maximum possible enhancement that you can achieve with all this for given that you are not in this regime that means you are not equal to 1 you are either below 1 or you are above 1. So if you are above 1 we say that the NOE effect is negative. That means it is a negative enhancement that the peak will intensity will decrease if I irradiate a neighbouring hydrogen.

So the maximum negative enhancement can be upto 100 percent. So this is theoretical limit which you can go upto and theoretical limit on the positive enhancement side is 50 percent. That means if your omega TOUSY is basically less than 1, ok. If it is tumbling fast if it is less than 1 then on the left side that is on the positive side we say that is call positive enhancement and that you can go maximum increase of the signal upto 50 percent.

So this is theoretical limit in which we work usually in the case of larger molecules such as peptide so large peptides and proteins and we are always in the regime of negative enhancement that is omega TOUSY is much greater than 1. Because remember TOUSY is depend on the size and if we go to 10000, ok let us say 10000 which is a size of a small protein. If you go to 10 kilodaltons then your TOUSY will become 5 nano seconds that obviously if you multiply with the 400 Megahertz you will get a large number which is much greater than 1.

And therefore we say that a negative NOE is active there and there you will get a negative enhancement whereas if you go to the smaller and smaller molecules you get a positive enhancement but within these limits.

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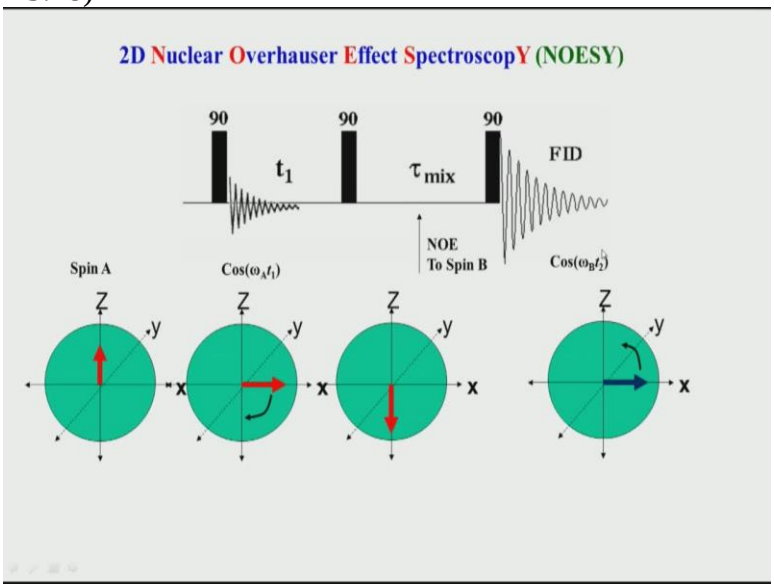

So NOESY experiment how does it work? So this is the pulse sequence this is how we got so remember what I spoke in the last two slides we talked about irradiation so that is called as a steady state NOE. Means I am irradiating a signal waiting for it to reach a steady state saturation and then I am recording and then seeing in the effect on the neighbouring hydrogen.

But that is not the only way to do NOESY, the typical and the 2D way if you want to a 2 dimensional NOESY experiment we use the word transient NOE. That means you are actually inverting the signal of a we will see that now we actually invert the signal and that inversion of the signal is what is carried out and that polarization inversion remember this is inversion means we are inverted the population kind of so we have inverted the polarization and that is transferred to a neighbouring hydrogen if it is proximally located.

So that is how this is done and that is call transient NOE. This is not same as saturation or irradiation. We are not irradiating a nucleus here, it is only RF pulses are applied, ok. So let us see how this works. So you start from first 90 degree pulse as always, ok. And before this pulse the magnetization is along Z axis, ok.

So now we apply this pulse when you apply the pulse you have the situation which is we saw in COSY and TOCSY that the chemical shift evolution takes place of let us say some spin A, ok. So spin A hs a chemical shift evolution omega A which is shown here so I have brought the

magnetization on the Y axis, X axis and that is evolving means moving with chemical shift again it is decaying because of t2 and t1.

After sometime I will now apply one more 90 degree pulse. So because it is in this axis it will now go inverted to Z axis because it is equal to saying I applied two 90 degree pulse right. So if I apply one 90 followed by another 90, I have taken this magnetization to - Z. Now during here this period there is no t2 relaxation. Why? Because there is no signal in the XY plane. Remember t2 is for the XY plane and t1 is for Z axis so there is no relaxation in the XY plane, only thing which happens is in the Z axis.

So during that period during the t1 relaxation we give a delay that is known as mixing time, ok. So this is a typically a long delay depends again on the size of molecule and so on. so let us say we give a delay is of the order of hundreds of milliseconds, ok tens of milliseconds to hundreds of milliseconds. So typically for proteins value of 50 to 100 millisecond is used where peptides small molecules you can go upto 300, 500 milliseconds.

So during this delay this period what happens is that the polarization of one which is inverted is been A gets the affect affects this polarization of (()) (27:02) because of this effect know as dipolar coupling and we use the word NOE. So because of the NOESY interaction between two spins A and B if they are close in space so please keep in mind we are talking about proximity here.

So these two spins are going let us say are close in space less than 6 Armstrong, 5 Armstrongs then any disturbance or anything I do with spin A population will be communicated or interaction interacted with spin B. So the population of spin B is also affected. So population of spin B is affected and that is now when I exite this that means the whatever is the population of A has been transferred to B. So whatever is the chemical shift of A also gets transferred to B, ok.

So what we are basically doing is we are communicating the population difference whatever happens for A to spin B. And when does it happen? That happens during this period. So during this period the population of A is changing because of its relaxation and that whatever is the difference the decrease in population is transferred is affecting the population of B and that is communicated to B.

And that population difference also has this term because remember whatever is the intensity of a FID if you remember it is related to Cosine into e to the power –t. So Cosine omega t is also affecting the intensity of your peak of the FID that that is along with the population is transferred to spin B. So when spin B is now excited with another pulse the last pulse then spin B starts evolving according to its own FID, ok.

So now what has happen? This FID as we saw is now affected by two parameters, this has information of this as well as how much has been transferred, ok. So therefore these two effects are carried forward to B and B starts now evolving according to its natural frequency that is chemical shift. So we have got now a 2D correlation between this peak and this particular spin, ok.

So we will continue in the next class and continue with this will start up again looking at the mathematical little bit in more detail and will see how this information from one spin is communicated to other and what is the type of spectrum which you will get now and how does it compare with standard COSY and the TOCSY spectrum which we have seen in the last class. So we will continue in the next class with 2D NOESY.