Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 09
2D Nuclear Overhauser Effect Spectroscopy

Welcome back to the course. In the last class, we began looking at 2 D NOESY which is which stands for 2 D Nuclear Overhauser Spectroscopy. This along with another exponent called Roesy is a very set of a very important experiments in NMR, especially biomolecule NMR of a protein structure determination. So, will well some more time on these two experiments and understand them thoroughly before we actually start using them when we lead on the line when we solve the structure.

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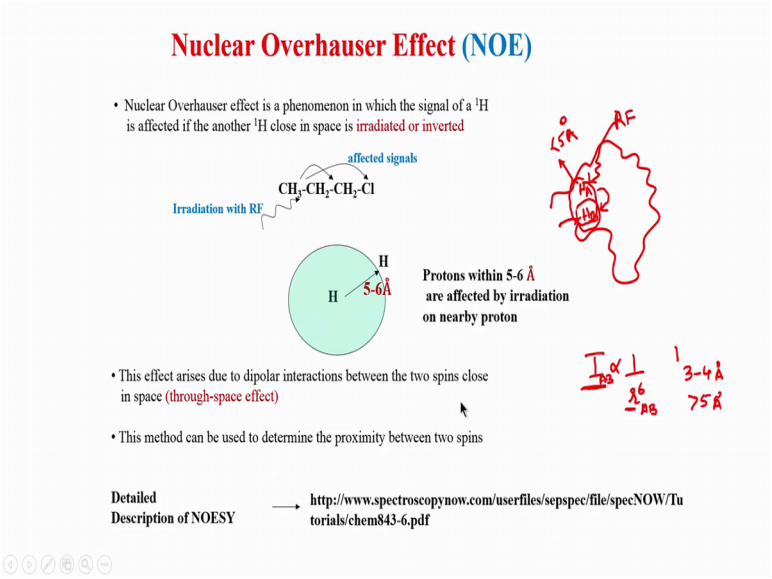

So, let us look at just the NOE effect first, what is Nuclear Overhauser effect. This effect was this historically discovered actually in metals not in nuclear, not in solution or not in molecules, but the idea was that there is a dipolar interaction between electrons and metals and that was resulting in some kind of magnetization transfer and the same thing happen is what is exploited now in molecules. So, the basically the idea is the following, any spin any in NMR, any NMR active spin such as the hydrogen or carbon 13, they are actually small tiny magnets this is something which we saw in the very beginning of the course. This magnetic they have what is called as magnetic dipole.

So, when you have two dipoles close to each other, just two hydrogens or carbons what happens is this two dipoles start interacting with each other and that is called dipole dipole interaction and this an interaction between which is something which happens between all any two magnetic dipoles.

It can happened between proton and carbon, it can also happens between carbon and carbon but by for proton to proton is a strongest dipolar coupling because this dipolar coupling depends on the gyro magnetic ratio of the nucleus involved. So, gyro magnetic ratio proton being the highest in the NMR, the two protons which interact will have the highest dipolar coupling.

So, what do you we a do in Nuclear Overhauser effect in spectroscopy what is NOE? So, suppose let say I take this molecule which is a very simple linear chain molecule and I irradiate means I apply an RF energy, this RF energy, it can be a pulse, it can be a continuous RF increasing energy so on, but if it applied on a particular hydrogen, let us say I chose this methyl proton. This methyl proton of this is perturb meaning it is excited or it is perturb from it is equilibrium state and that information now is actually starts getting transmitted to this proton. In other words, when this proton starts relaxing back to it is original equilibrium situation position this starts effecting the relaxation of this nucleus as well.

So, these two hydrogens, this hydrogen now which is close to this hydrogen gets effected because this hydrogen is irradiated and if I irradiated this hydrogen, it can effect this hydrogen or it can effect this hydrogen as well. So, the important point here in NE is that there is a distance or approximately dependencies or vicinity hydrogens which are closed to each other in space are the ones which are effected by each other. So, here we do not consider j coupling, this phenomenon is purely through space effect meaning it is in through in the in the in the 3 dimensional space.

In, in a just small molecules of course, they are all close to each other, if you consider biomolecule such as protein, it has much longer change it has a consist of amino acids as we will see later in the course and there many amino acids joint together linearly and the proteins starts folding meaning it calse up and it forms as a specific 3 dimensional structure..

And that now, depends now what happens is two hydrogens. So, let me show you this semantically now. See if I have protein which is a chain which goes to like something like this and it will come close back and it can form kind of a close or loop structure or this is a 3 dimensional structure approximately of a protein. See if I have two hydrogen suppose I have an hydrogen here, proton A and I have another hydrogen B, let me call it proton B.

Now, these two are now coming very close in space, this could be less than angstrom we will look at this distance concept little bit later, but let us say these two hydrogens are very close in space. So, what we can see here the bond, if bond wise if you go through this entire chain they are really far away it could be 200 bonds, 300 bonds and so on, but when it come to being in space, there were very close to each other.

So, now if I irradiate this hydrogen by RF frequency similar to what I am showed here this irradiation is transmitted to this irad, to this proton. So, the magnetization or a the energy of this hydrogen or a population of this hydrogen is affected because of perturbation of the population of the other hydrogen H A. So, now, this information, this perturbation of B because of A is happening only because of this dipole dipole interaction which happens through space.

So, you see it is very useful to do a NOE because it immediately gives us an idea about what is the effect of distance on the magnetization.

So, this is basically very useful experiment to be carried out in general if a structure is determination of biomolecules. So, now, we can see here this is semantic again for the same this. So, you can see here the aero is slightly displace, but this irradiation of this methyl proton affects the methylene here and not only methylene proton, it also will affect this methylene proton here. Why this is because these two also are not very far away these two hydrogens are also pretty close by they are within 5 angstrom.

So, anything which is within this 5 to 6 angstrom is basically affected even the protons are irradiated. So, this brings as essentially to this concept of distance which I was talking about. So, now, you can see here if two hydrogens, if two hydrogens are within the radius of 5 to 6 angstroms, they are affected by irradiation of nearby proton. So, essentially if I irradiate this hydrogen, this hydrogen will be affected if irradiate this hydrogen, this hydrogen will be affected.

Now, why is this concept of 5 to 6 angstrom from where is it come from this comings. This limit that we cannot observe irradiation or affect irradiation beyond this comes from the distance dependence of the irradiation intensity. So, let me show you this again. So, this intensity which will see again later as we go in the class is proportional to R to the power 6; that means, the intensity at by which is affected the 2 hydrogens are related to the distance between those 2 hydrogen, A and B.

So, they can see there is a 6 power distance. So, if I go from if one angstrom is going to be strong of course, no two hydrogens can come within one angstrom, they very close. But if I have 3 to 5, 4 angstrom, they are pretty strong, but the moment I go more than 5 angstrom, this R to the power 6 reduces this intensity what is this intensity? This is the intensity of the interaction you can say between the two protons.

So, the intensity of the interaction or the intensity of how one is perturb by the other is proportional to this inversely proportional to the R to power 6. So, this is the very strong dependency. If you very short range, interaction being the moment you go little bit far the interaction energy or interactions energy or interactions strength decreases rapidly and therefore, this limit of 5 to 6 angstrom comes into picture. If you go little bit away from this, if you go little bit away from this if you go to 7 or 8, there is no effect of hydrogen which is 7 angstrom away from this hydrogen if I irradiate that.

So, basically what essentially happens is by irradiating one proton I am able to get in the information of which are the other proton present in that radius of 5 to 6 angstrom. So, that is why you see this NOESY experiment is a very useful experiment for getting distance information. This information will not be available through any of the other experiments we have seen earlier such as 2 D Cosy, 2 D Tocsy which are pure j dependence and j dependent coupling is waste 3 to 4 bonds away it is over.

So, we cannot go beyond 3 to 4 bonds. So, therefore, distance if you have two proton very faraway in space, but very close in space, sorry very far away in the sequence or very faraway j bond, they want be affected in the 2 D Cosy and Tocsy experiment, they want be energy transfer between these two in a Cosy or a Tocsy, but in NOESY, this will happen because of the close approximate.

So, essentially this say experiment therefore, we can be used to determined an distance between two protons. How do we use is to determined distance will see subsequently as

we go along, but this is a basic idea. So, now, let us see what are the other detail experimental schemes, which are used in NOESY. So, I would recommend to for you to go to this web page, this pdf which very nicely explains in a very lucid manner how this NOE effect works.

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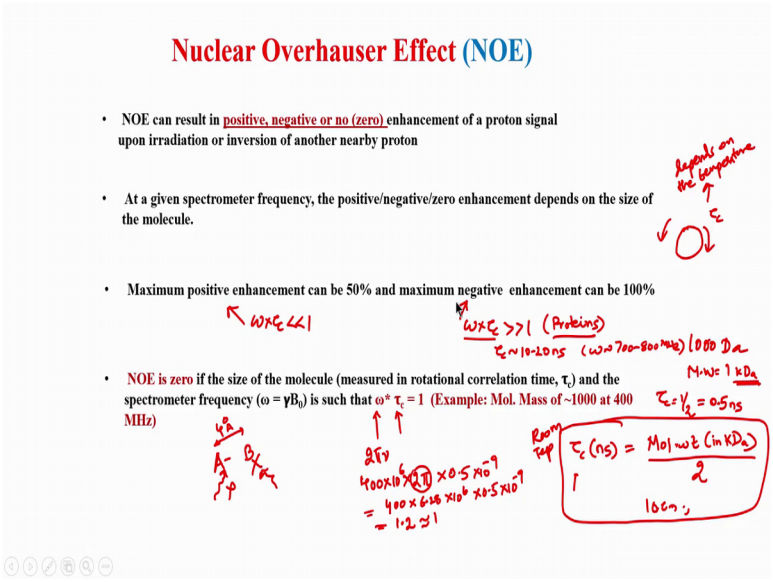

So, now let us look at the different aspects of NOESY experiment that is how actually it is carried out. NOE has written here can be positive, negative or 0 which means suppose I irradiate one hydrogen, second hydrogen may get enhanced in it is intensity or population it may get decrease in it is intensity or population or a third possibility it has no effect.

So, no effect is basically because the two hydrogens may not be nearby, but even if they are nearby, there is a possibility that there is no effect between in two hydrogens and that is something very interesting which will see down the line, but it. So, the so, the point in this case in this here is that the two hydrogens are really close, but we are not getting any NOE effect. So, what do we do in such a cases? So, will look at alternative experiment but otherwise generally, the proton if two protons are close in space the if I irradiate one hydrogen, the second hydrogen may either experience an increased in intensity or population that is positive or it may experienced decreased intensity.

On this increasing and decreasing basically comes from the population difference between the energy levels. So, that is why the intensity changes. So, now, this positive,

negative or 0, when do we get positive enhancement? When do we get negative enhancement or when do we get no enhancement of a signal of a proton when you irradiate a nearby proton depends on two parameters; it depends on the spectrometer frequency at what we are operating that is examples 600 megahertz or 400 megahertz and so on. It depends on that and it second factor which is depends on it is size of the molecule that is how big your molecule, what is the molecular weight of your sample of a molecule and so on.

So, I will this is basically the equation which is shown here that basically NOE either positive and it become 0, NOE as I mentioned here it can have a either positive or negative or it can be come 0 that that, then NOE become 0 when this equation is satisfied. So, you can see what is this equation this equation now has two parameters here you as you can see, one is the frequency that is the spectrometer frequency and one is the Tocsy which is the rotational correlation time rotational correlation time is a parameter which captures the size of the molecule.

So, for example, if I molecular wise of weight of 100 that is 1000 Daltons, if you can calculate the Tocsy as follows. So, I wish I will see that very rule of some which is used. So, let us say the molecular the Tocsy in nanosecond is given by molecular weight in kilo Dalton divided by 2. So, this is a very rough estimate of molecular weight of a correlation time. There are many assumptions in this formula in the form assumptions are that the molecules percale, it is like a football like globular spherical shape and it has no internal dynamics.

Now, you can see let us apply this formula. So, let us say I have a molecule which is 1000 Dalton if I have thousand Daltons, then my molecular weight is actually 1 kilo Dalton in kilo level in a kilo units. So, when it is 1 kilo Dalton, the Tocsy is 1 divided by 2 according to this formula here and that becomes 0.5 nanoseconds. So, when you have a molecular weight of 1 kilo Dalton, your Tocsy in nanoseconds is 0.5 that is 0.5 nanoseconds. So, this is roughly the way to calculate Tocsy. Now, Tocsy is rotational correlation time it basically gives you the, how fast the molecule is tumbling in a solution.

For example let us say molecule is spherical like this the molecule is actually tumbling in solution. So, how fast or how quickly there is a tumble this characterized by Tocsy. So,

this is very important number to keep it in account when we are low working with NOESY. So, now, let us calculate this 2 pi this is you can see in this formulas here it is omega is 2 pi into mu, mu is basically the frequencies spectrometer frequency. Say if I have 400 megahertz spectrometer, I can see 400 into 10 to the power 6 into 2 pi that is this omega into Tocsy. So, Tocsy for 1000, we just calculated as 0.5 nanoseconds. So, I multiply this 5, 10 to the power minus 9. So, nanoseconds.

So, now if you do this calculation, this is roughly 2 pi is 400 into 6.28 which is 2 pi into 10 to the power 6 into 2.5 into 10 is to minus 9. This for further we can calculate, this is about 1.2; you should calculate it you will get it around 1.2. So, this is coming close to 1 although it is not a exactly 1, it is coming closed to 1 and because of that this NOE effect according to this rule says that if omega into Tocsy becomes comes close to one your NOE effect is 0 meaning if there are two hydrogens which are close by the distance may be let us say 4 angstrom, but if I irradiate this it has no effect on B.

Similarly, if I irradiate B, it has no effect on a. So, this 2 although they are close in space, still do not interact through space by dipolar they interactive dipolar coupling, but the transfer of magnetization between A and B because of the NOE effect does not happened at this magic number that is omega into Tocsy is 1. So, that as I said depends on the molecular weight which in this case we saw found it has 0.5 or in this case when the frequency now I can change the molecular weight. So, for example, if I double the molecular weight and I reduce the frequency by half again, I will get the same value. So, which means if you it depends on these two together.

So, let us say you are getting value 1 here as we saw in this case can I do something. So, the question may be you can ask is can I do something to still get the NOE effect what should I do for example, So, that I can get the NOE effect even though this it is turning out to be close to 1. So, one trick which you can do is that you lower the temperature. So, when you lower the temperature, the molecules so, look here. So, this rotational tumbling correlation time depends on temperature depends on the temperature.

So, typically the calculation is showed you is for room temperature. So, this whole calculation here is for room temperature, but if I lower the temperature, I will start getting higher molecular higher Tocsy because the tumbling starts slowing down because the rotation of the molecule is slow down because of lower in temperature, it is starts

rotating in a slower space. So, when it starts rotating in a lower phase it is Tocsy basically increases because it takes longer to rotate by same angle compare to what it does at a higher temperature.

So, as you lowered the Tocsy increases, when the Tocsy increases here let us say the 0.5 becomes 0.8 or even one then this number will change completely. So, by lowering the temperature, we can actually change this value, we can as well go to higher temperature if I go to higher temperature the Tocsy will decrease because the molecule is rotating faster. So, the Tocsy is basically the time or it takes for the molecule to tumble by a certain degree and that time reduces if you increase the temperature because it is now rotating faster at a higher temperature. See if Tocsy is goes down, let us say from 0.5 comes on to 0.25, then again this number will change and it will be not equal to 1 any more.

So, by changing the temperature, either increasing or decreasing we can come out of this constrain for a given molecule for a given spectrometer. So, what we have done we can see here we have not change the molecular structure, we are not done anything to the sample we are simply change the temperature either increased it or decreased it. This is one option. The second option is suppose you cannot decrease or increase the temperature because the sample may be critically dependent on the temperature, then you can go to another spectrometer. So, instead of 400, if I got to 700 megahertz or 800 megahertz again, I am doubling the frequency here that will double this number here and again I will come out of this constrained of mega Tocsy is equal to 1.

So, now I can change the spectrometer frequency as well and choose another spectrometers. So, therefore, one has a choice what one can do to get rate of this problem in particular cases. This will not normally happened in small molecules or in big proteins. Typically, this happens in the peptize you see this molecular mass here is thousand and roughly one amino acid is about 100 or 110. So, this corresponds to a peptize which is about 9 to 10 amino acids long. So, small peptize is this 1 to 10 amino acids or 9 to 12 amino acid peptize typically have this problem that you cannot do NOE experiment at room temperature give with a specular given spectrometers like 400 or 500 megahertz because of the problem that there is no NOE effect.

So, therefore, we have to overcome that so, let us say that I cannot change the temperature beyond certain range and let us say that I cannot even change the spectrometer have only one spectrometer in my lab, then what do I do? Then for that you will have go to another experiment and alternative to NOESY called Roesy which will see later after his NOE experiment, but that is another alternative which we can use if you cannot do NOE for because of this constrain.

Now coming to this point here that the maximum enhancement meaning how much can signal being increase by irradiating one nucleus is about 50 percent, you cannot go beyond this. This is a theoretical limit and similarly how much can it go down if I irradiated one nuclear the second reduces intensity and that can be 100 percent; that means, by you can completely go around by 100 percent if there is a negative NOE.

So, you see negative and positive NOE are possible only when this constraint is not there. So, typically the positive NOE is when omega into Tocsy is much less than one we have positive enhancement omega into Tocsy is much greater than 1, we have negative enhancement. So, this positive and negative depends on this two parameters as we already discussed. In this is typically happens in the case of proteins because in proteins we have very high Tocsy, Tocsy values typically of the order of 10 to 20 nanoseconds and omega and omega we typically work omega is 700 to 800 megahertz, this is for protein.

So, therefore, if you combine the two parameters, you will see that this number is much higher than 1 and therefore, we are in the negative enhancement region when it comes to biomolecules, but very small molecules the omega into Tocsy is much less than 1. So, there it will be positive enhancement but this is essentially that traditional 1 D NOE experiment which is to be done earlier which is no longer done today. What is done is 2 D NOESY experiment not a 1 D experiment.

So, in a 2 D NOESY experiment as we see now, we do not irradiated a particular proton.

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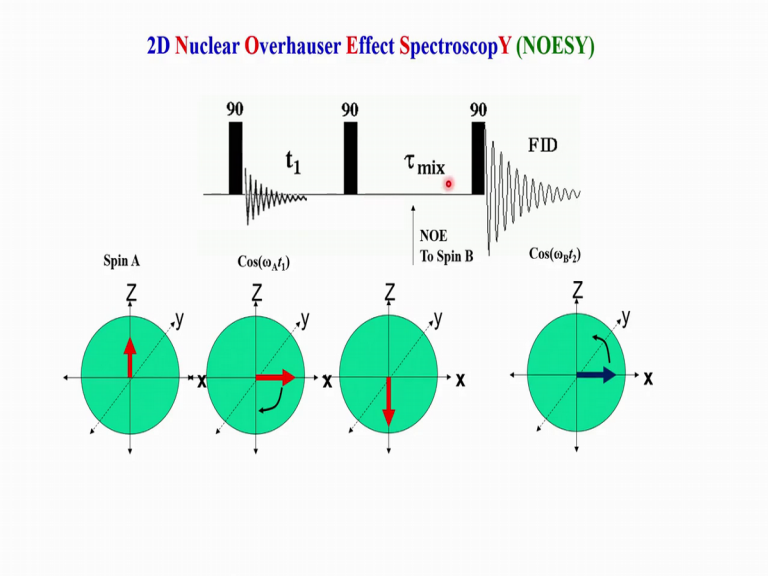

We do not irradiate us using a continuous RF as I was explaining in the previous slide. We use simple 2 D pulse sequence which is shown here for recording the data. So, this the standard three pulse experiment for 2 D NOESY and it is now a days it is use for very molecule including biomolecules. So, it is very important to understand this experiment now, how it works and then what is the information, how is information, that we get is analyze. So, let us look at start from this experiment first. So, if the first pulse is always as any 2 D experiment and excitation pulse.

So, we excite the spin particular spin A, we bring it to the transverse spin. So, this is what it shown here, we are brought it to the transverse spin by applying a 90 degree pulse. Now, during this period which is labeled as t 1, the spin starts pressing around the magnetic field. So, look at this picture here, it starts pressing in the X, Y plain, X, Y plain around the Z axis because Z axis is our main magnetic field. It starts pressing around that. So, this is similar to that we have been seeing in the case of 2 D Cocsy and 2 D Toesy. During that period, now because it is involving with a frequency omega A it is F I D is written like this because this is like F I D now in the t 1.

Although we are not detecting it way, this an indirect dimension of a 2 D experiment. So, we are not physically detecting in the physical actual detection takes place here this the second FID, but in the first FID, we do not do that. So, in the first let us say this basically cosine of mega t 1. Now, after some time when it has not completely decade although we

are showing is signal d k into 0 please keep in mind that we do not wait this, we only wait for a few points here and that is how much is the number of experiments done.

It is not that like shown here that you have to wait till the FID is 0, then you have apply the second pulse it is just semantically shown here that you apply the pulse you wait for a duration and then apply the second pulse. So, when you apply the second pulse this magnetization goes to minus Z or plus Z depending on the phase of this pulse.

Now, during this portion of the time, from here to here you see this part of the sequence, there is just simple mixing time meaning there is no nothing happening here is just an is not even evaluation this is not like t 1 because t 1 is something is varies with time as we saw in the previous class how it 2 D conducted experiment.

Here, it is a fix time a fix duration. So that means, this particular time the spin now starts going back towards a axis when it goes from minus z to plus z, it is pure t 1 relaxation, there is no t 2 here by because we are not in the X Y plain. So, if you recollect our slides on t 1 and t 2, t 2 is basically something which happens in the X, Y plain that is the re defacing of the magnetization whereas, t 1 is something which happens along the Z axis and it is the magnetization recovery along the Z plain or Z axis.

So, that what happens in this particular case? This magnetization which is minus Z starts coming slowly towards this plus Z. During that period of time, it starts now effecting the other spin because two spins A and B, if they are couple to each other by dipolar coupling and if we are not in that condition that omega into Tocsy is equal to 1 where it is 0 NOE effect, let us say we are not in that condition. So, if we are away from that condition the two spins A and B can start. Now transferring magnetization to each other and that polarization transfer is what is called NOE and polarization meaning population transfer.

That means population of spin B between alpha state and the beta state is affected because of the relaxation of A.

So, this affect now this changes the intensity of B and now the B population when you apply second pulse, the B is how exited and it starts evolving. So, what happens is that the intensity of B which we excite here by this pulse is dependent on how much of the intensity A and B magnetization had been exchanged. So, that these during this mixing

period. So, during this mixing period, when A and B exchange the magnetization, the B is magnetization which exited now is not the original B magnetization. It is something depending upon how much a magnetization has come to B and that carries the information of the frequency of A as well because before applying this mixing pulse, we saw that we had an a evolution period.

So, during this period frequency of A was labeled or captured now that frequency is affects the intensity of A and the intensity of A affects intensity of B and therefore, the intensity of B carries the effect of the frequency of A information. Thus how this two gets coupled to each other meaning, they are joined to each other the in frequency because the intensity the modulation of intensity of A is transfer to the intensity of B.

Now, when the B is excited it, it gets excited and it is stats evolving like any other spin and that is captured by physically and that is called that FID, Free Induction Decay during this period this is called t 2 period, the second time evolution period which is shown here. So, that is cosine omega B into t 2. So, therefore, omega A into t 1 is now multiplied with omega B into t 2 that is the combined FID that we will get in a 2 D NOE experiment.

So, the important point here is this mixing time. If the mixing time is 0, let us say we do not have any mixing time, then there is no time for the A spin magnetization to go to B. So, therefore, it would not have any effect of B and the B magnetization will be is original magnetization, but because of this effect that a spin is now transferring his polarization to B, the B is effect is not dependent on this time. So, the longer the time you give, the higher is the transfer. But there is again limit to how long we can give. You cannot give for a very very long duration this as this depends on the t 1 of the sample. So, if the t 1 of the proton of B and A are very short the t 1 value, relaxation t 1 longitudinal relaxation, then you cannot go beyond that time period for the mixing time.

So, what is the typical value that we use for proteins or biomolecule is we use about 60 to 80 milliseconds, we do not use more than that. There are some other side effects which happens if you go beyond 80 or 100 or about 120 proteins which we will see when we analyze this NOESY for starchy determination there will see that this effect call spin diffusion. Spin diffusion is basically transfer of magnetization from A to B to B to C, C and A may not be close to each other, but because B was as an intermediate spin it affects

the trans it, it things the magnetization gone from A to C and actually which is not the real scenario. Actually it should I have stopped B, but this further transfer to C to happens because we give a very long C time.

So, the mixing time has to be appropriately chosen. So, that we only capture the magnetization transfer or polarization transfer between two very close or closely space nucleus. So, will now see in the next class, how this pulse c frequency translate into the spectrum what kind of spectral pattern we will except to see having now transfer the polarization from A to B. We will analyze that in the next class.