**Principles and Applications of NMR Spectroscopy**
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**Module 6**
**Lecture No 26**
**Introduction**

In the last class we started with what is 2 dimensional NOESY experiments we saw the basic idea behind that, what is nuclear overhauser effect. The idea in a NOSEY spectrum is to capture cross peaks between 2 atoms which are less than 6 Armstrongs typically 5 to 6 Armstrongs and we use the method of polarizations transfer to do that and this is through space interaction that means there is no need not be any direct in through bond interaction between the 2 atoms.

So, I need 2 hydrogens which are close in distance between in less than to 5 Armstrongs to 6 Armstrongs can be studied by this spectrum experiments and it is very useful technique for determining structures of molecules Because, when we talk about structures in general structures means we want to look at distance between atoms and the distance between atoms you have to obtain by only this technique of NOSEY very difficult to obtained distance information by any other method and this is therefore a very direct to approach to calculate the distances and by using distances we can get structure.

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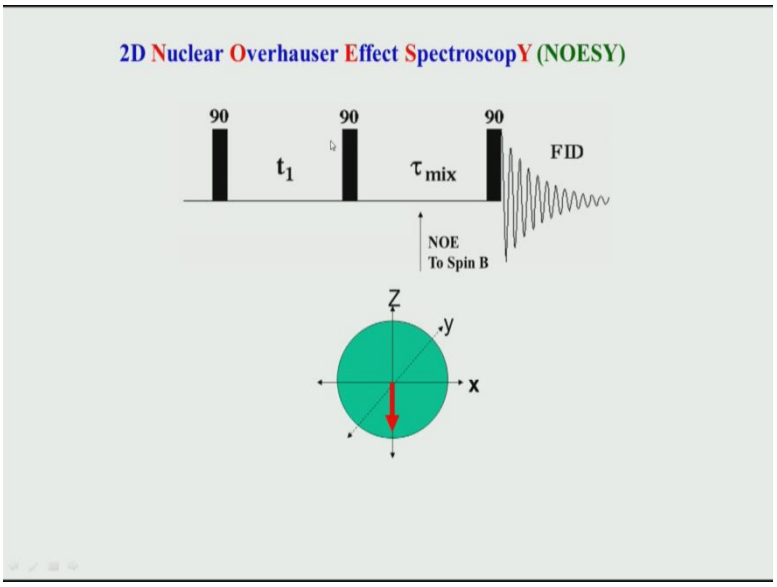


So, let us continue a little bit on how to how do this NOESY experiments work because this is one of the very important experiments in NMR. So, one should therefore grasp the basics

of this experiment. So this is the pulse sequence which is shown here, this include 3 pulses, so this is what is a schematic drawing here. So, what we do is we have a 90 degree RF pulse which is applied on the proton channel that is on the hydrogen then when it moves after excitation there is a T1 evolution.

This is similar to what we have been doing in all the other experiments. So, once you do that you allow it to evolve, so this is what we will see mathematically shortly and then you apply one more 90 degree pulse after some time and after application of this pulse, what happens is the magnetization vector comes to the - Z axis. Once this happens, it starts now relaxing back towards the positive Z by T 1 relaxation, during that period that is during this period when it is relaxing back to Z axis we start transferring the polarization to another spin B. So, suppose this is spin A it has brought to - Z axis during this period mixing time that is this is called mixing time during this period. It is transferred to spin B by the effect of NOE (Nuclear Overhauser Effect). So, this is basically the idea that you use a certain duration allow the certain duration for during this period allow the spins to mix with each other.

And when we do that after some time here the magnetization has come to be that is the Z axis, - Z of one spin A has gone to - Z little bit to of another spin B, now this transfer depends on variety of factors. Number one factor is of course the distance between the two atoms. If spin A and spin B are not very close they are far away, then spin A cannot transfer it is polarization to spin B and second factor which matters is the mixing time. So, the few take a very short mixing time that means you do not allow the 2 spins to interact for sufficiently long time, then you do not except spins to be a transferring energy to each other or polarization to each other. So therefore, you have to give some sufficient delay but too much delay is also not good idea because of several factors which we will see later.

So, therefore the total there is an optimal value typically we use for this delay and this is typically for small molecules is in the order of 200 to 500 mili seconds and for proteins little biomolecules larger molecules peptides it will be of the order of 100 to or 60 to 150 mili seconds. So this depends on the size, so we will see that factors what governs the mixing time later. So, the basic idea is during this period the 2 spins are allow to interact with each other through space that is through the dipolar coupling method, which is basically responsible for NOESY effect and during this period for magnetization is transferred? After the magnetization is transferred you apply one more 90 degree pulse which now brings the spin B into X-Y plane and then its starts evolving, this is called evolution

This chemical shift starts evolving and that is captured by detector and that is known as an FID. So, basically what has happened is we started one spin A, we allowed the frequency of the spin A to evolve, so the information of spin A is captured here through chemical shift evolution and that magnetization of spin A is inverted and during the inversion the information of the chemical shift is carried along with it because whatever happens during this period.

That is the modulation because of chemical shift that modulates the intensity of the peak means it affects the intensity of the peak of the spin A and that intensity is now stored information in this particular vector. And therefore when it mixes that means when it transfers from A to B and in B is now print to X-Y plane, the information which is coming all the way from A is transfer to B and then B will start evolving with its frequency chemical shift.

So basically what we have done is we have correlated, connected the chemical shift from one spin with the chemical spin of another spin by during this mixing. That means only those chemical shifts which are mixed or which are transferred from one to another during this period. Only they will give rise to peak cross peaks in the final spectrum. If two spins are not interacting with each other means they are far away more than 5 or 6 Armstrong, there will be no interaction between them through space and therefore the energy of the magnetization of A cannot be transferred to B, which means in the final spectrum you will not expect to get any cross peak between A and B. So this is a very simple idea, you first allow this is a general idea of 2D which we have seen in the case of also TOCSY and COESY is that you have a first a period where you allow once spin to evolve in chemical shift then use a prepare mixing time that is a mixing pulse which mixes the magnetizations and then in gets transfer to the second spin.

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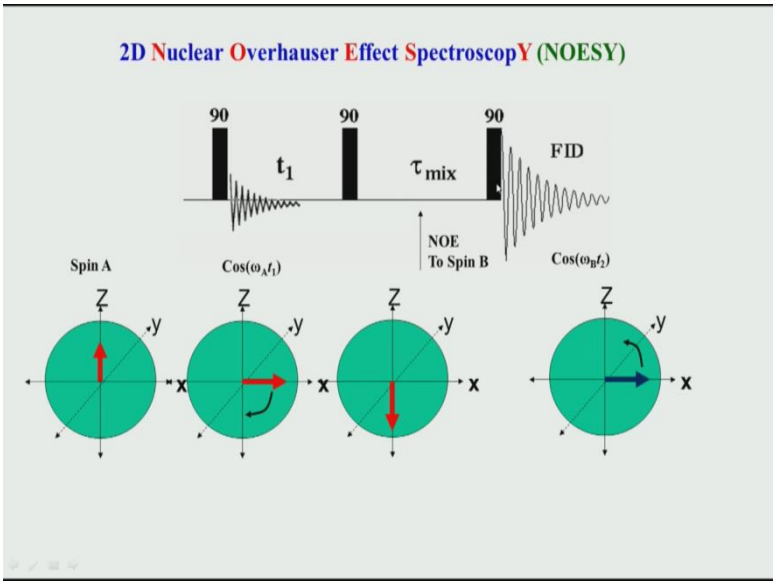


So, let us see more detail the mathematical part of this, so this is what is shown here by the vector diagram this is called a vector diagram. So, you start from spin A magnetization which is along Z axis, because before the any pulse is applied it is called relaxation delay means you allow this spins to evolve and it relax back to complete equilibrium. Then you apply this pulse this is 90 degree pulse, this brings the spin A into the X-Y plane and during this period it evolves, because of chemical shift and that evolution is represented mathematically as cosine of Omega T 1. Omega is a chemical shift, now during this mixing period it comes to -Z and then it is transfers to B because of NOE and then as we said as to the last pulse is applied. This now the spin A energy as polarization has gone to B and now B is bent plot to the X Y pane which is shown by this blue and the B will start it rotating in the X-Y plane by chemical shift.

So, what basically we have done is we have got captured the FID contains now this term and this term together, so if you recollect what we saw in the case of theory of 2D in the last few classes, when you multiply these two that is the FID and it has now two chemical shifts connected to each other, Omega A in one dimension that is T 1 and omega B in the second dimension which is T 2. So if I plot a 2D spectrum, I will get a cross peak between A and B. But remember as we discuss in the last classes that it is not that the entire polarization of A can be completely given to B, it is only a stage of A polarization goes to B, the remaining 90% remains on A itself. So that means during this mixing period A just transfers only 5 to 10% of the polarization to B that is a kind of efficiency you would expect at best.

So therefore, when you apply the last pulse you do not only have the B magnetization but you also have the magnetization from A which was not transferred at all, so that will also evolve as cosine omega A T 2, which is not written here but you can think of it that when the second time again A will evolve because A is polarization is has not been completely transferred to B, so that we will cause a diagonal peak because during both T 1 and T 2, it has omega A and Omega A because that will be the non-transferred part of the magnetization. So that is a diagonal peak and then you get cross peak which is B.

So, as for as the analysis or interpretation of NOESY is concerned we normally we never care for the diagonal peak you only worry about or we are only look at the cross peak, why is that so, because diagonal peak any way does not have any information. It is only telling me that A remains on A which has no nothing interesting try to say but A going to B is lot of information because that tells me that A and B are now close in space by less than 5 to 6 Armstrong. So, just an observe an observing a peak observing a peak in NOESY spectrum itself tells us that there is a distance information because only if they are less than 5 to 6 Armstrong you would except them to show any interaction.

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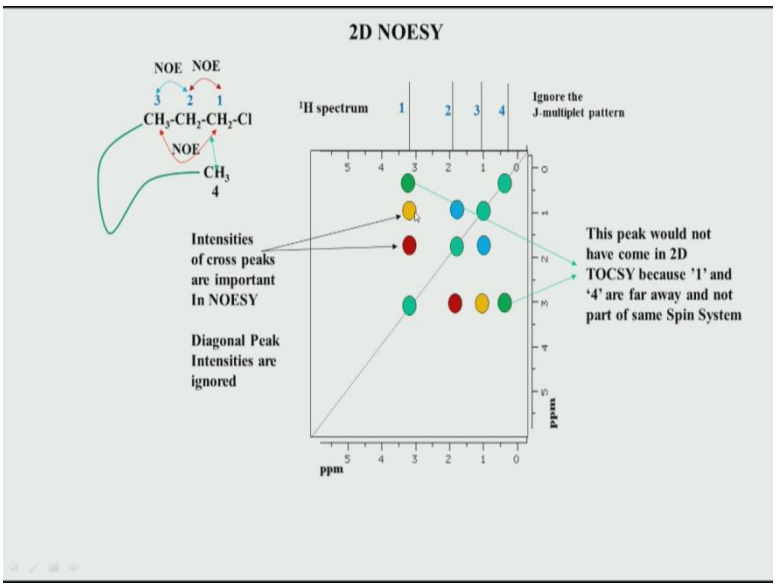


So, we will go further you can see an example here, so this is a propyl chloride, this is same molecule which we saw in the case of TOCSY. So, we will take the same example here, so here let us say again ignore the J multiplied pattern you basically get a 3 peaks because there are 3 types of hydrogens, we are looking at the proton spectrum and then let us say based on the electro negative effect that is shielding a electro shielding inductive effect A will be the

most down field shifted B will be the second, the second and the third will be the last, so this is what is shown here.

So, now I draw a 2D spectrum here, so now we will look at how the NOESY cross peaks or NOESY spectrum will look like. So, first remember always we get a diagonal peak in NMR this is in homonuclear proton and proton experiment. So, if you see in this case the 3 to 3 is here, 1 to 1, 2 to 2 and 3 to 3, so this is always the case because we can never transfer complete polarization or a magnetization form one nucleus to another, okay.

So, now let us see the cross peak, so in this case if hydrogen one and 2 are close in space. So, how much will be this, this is only about the 3 bonds away and remember one bond is typically around 1 Armstrong, so if you say the 3 bonds are together with in 3 Armstrong, 3 to 3.5. So obviously they are less than 5, so I would accept a cross peak between 1 and 2 in a NOESY spectrum, so this is what is schematically drawn here. Now, similarly between 2 and 3 I except a cross peak because 2 and 3 are also not far away they will be again within 3 to 3.5, so 2 and 3 also will show a cross peak in a NOESY spectrum.

Now if you see 1 to 3 they also or not going to be far away because if we look at the 3 dimensional structure, which we have not shown here, but you can if you imagine a 3 dimensional structure this will be a also within 5 Armstrong, this is not very far away even in the case of real 3 dimensional molecule, so therefore I would except a NOE between them and that is also we show of in the NOESY spectrum. So, you see this looks like a TOCSY experiment remember, in TOCSY also we saw that 1 to 2 is J coupled one is J coupled to 2, J is J coupled to 3, so 1 2 3 will get J coupled through intermediate to and we saw a 3 by 3 cross peak pattern same thing here. So then what is the difference between NOESY and the TOCSY, the 2 things number one in NOESY is of course the transfer was not through J coupling, it was through space okay.

So, therefore the intensity of this peaks will be proportional as we will see in next slide, it will be proportional to the intensity having the distance of the peaks between the atoms inversely proportional. So we will see that relation more carefully in the next slide, so now let us see what is the difference between the NOESY and a TOCSY. Now suppose imagine that you are actually molecule had something like this structure, okay, so where this was a long chain there was something else we will ignore what was here, but there was a long chain and more molecules turned around folded and there was a methyl group here which you will label it as 4. So, this is just an example which I am trying to show you that how is NOESY further

different from TOCSY. So, now these 4 let us say has a 1D proton peak shows here is a methyl group. So, it will be expected somewhere between 0 to 1ppm, so let us say it will comes here.

So now because of this structure of the molecule there is a fold here, this is called folding. So, when the molecule has folded, it has this atom has come close this number 1 and therefore I would expect a cross peak in NOESY between these 2 atoms because there will be very close to each other and they may come bit less than 5 to 6 Armstrongs. So, if I see a cross peak like this between 1 and 4, immediately I can say that I can conclude that there is a distance of less than 5 to 6 Armstrongs between 1 and 4, but this will not be possible to see in TOCSY.

So, this peak would not have come in 2 D TOCSY why because 1 and 4 are far away and therefore are not part of the same spin system. Remember, the word spin system which we define in the case of a TOCSY spectrum, spin system is a set of spins which have some J coupling within between each other, not all of them are coupled to all, but there is no spin which is not coupled to any other spin means there is no isolated spin. So, that is the set of spins which are all having coupling within them is spin system.

So, this does not dubiously belong to this spin system which is these 3 hydrogens because by bond it is very far away, but NOESY we will give you that because it is close in space. So, this is how NOESY helps a lot to get the structure information which you would not have got from TOCSY. So that is why NOESY occupies a very important role in NMR spectroscopy because of this feature. Now how do we get the distance information that is what is shown here and again we will see in the next slide that intensity of cross peaks are very important in NOESY, intensity means the volume the area of the peaks.

If, I integrate this peak whatever area I get has some relation with respect to distance okay. But remember as I said, diagonal peak intensities are ignored because diagonal peak has no information it is only telling me this 4 is come to closer to 4 what is that going to help us, no how it is not going to help us similarly 3 is with 3, so had it has no additional. So, diagonal peaks are ignored, when intensities are concerned in fact, diagonal peaks are a problem in life because you want suppose there is a peak somewhere close it is a diagonal it will be covered by this diagonal. Remember, diagonal peaks are very strong in NOESY and we will down the lines will see some real spectrum but this is just a schematic, but typically what happens is a diagonal peak in an NOESY spectrum is very strong, typically about almost 100 times stronger 50 to 100 times compare to the cross peaks.

So, that means if I intensity of this is 100 the cross peaks will be only about 1 or 2 and therefore, if this intensity the some peaks is let us say comes very close to diagonal, it will obviously be hidden or covered by the diagonal peak. So getting a diagonal less without diagonal NOESY itself has been a focus of research in NMR spectroscopy over the years, several papers research publications have been papers have been published, where people have tried to design a diagonal less NOESY and NOESY without diagonal because peaks which are close to diagonal can now be then observed that would not observed if there was a strong diagonal present.

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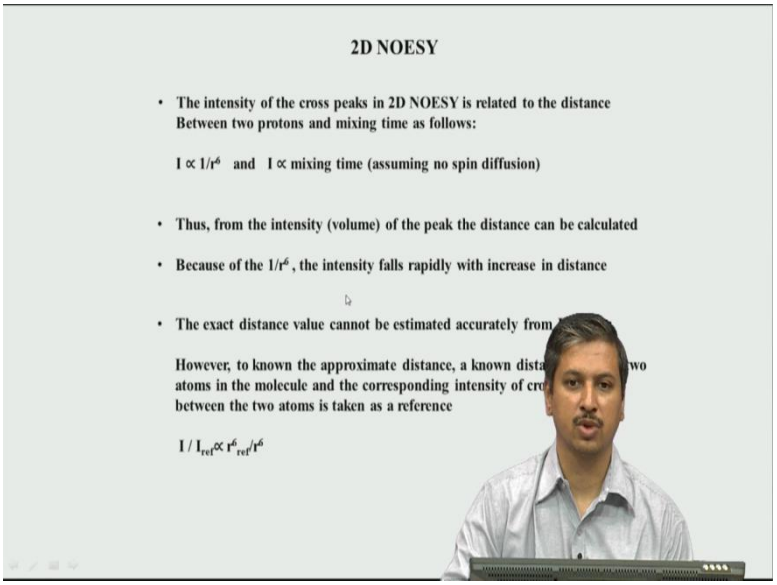


So, these are the basically the ideas is to behind NOESY, now let us see how the distance information is used come from the intensity. So as I said in the last slide, the intensity of the cross peaks in 2 D NOESY is related to the distance between 2 protons and as well as the mixing time. With this we will see the effect of mixing time a little bit later, let us see look at how the distance between proton varies with distance a with intensity, so this is the very famous formula I is intensity that is volume, so when we talk about intensity in NMR, we say typically we mean we imply area.

So keep that in mind that area in intensity or volume of a peak, peak can also be a 3 dimensional structure. So the volume of a peak is intensity they are all kind of interrelated okay. So, the intensity is proportional to 1 by R to the power of 6 and as far as mixing time is concern, intensity is directly proportional to mixing time, but there is one phenomenon which known as spin diffusion, which we will discuss little bit later which spoils this equation.

It does not you should not have spin diffusion only if you there is no spin diffusion, intensity will be directly proportional to mixing time. So we will talk about spin diffusion little bit later, so let us remain on this here that intensity is proportional to the inverse 6 power of distance. So therefore, from intensity or volume of the peak distance can be calculated. I would stress on this fact that we actually do not calculate like this, we use the word estimated, It is working I would say the peak of space of the intensity of the peak the distances can be estimated. Okay.

So, because of this 1 power R 6 you see this course as R to the power 6, the intensity falls rapidly with distance in increase in distance. Therefore, we use this cut off of 5 to 6 Armstrongs will because if you go from 6 to let us 8 or 7 Armstrong, the intensity will fall very rapidly because 1 by 6 to the power 6 versus 1 by 7 to the power of 6, 7 Armstrongs, where R = 7, whereas R = 6, if you can see compare there will be an order of magnitude difference. Therefore, these orders of magnitude difference their peak intensity falls by 10 times if I go from 6 to 7 Armstrongs.

Therefore, we say that there is a sharp kind of a decrease in the intensity of the peak as the distance is increase. So therefore, this is a very interesting thing because of this we can safely say that peaks which are seen in the NMR NOESY spectrum means they are within 5 to 6 Armstrong. Now as I said in the here point here this exact distance cannot be calculated accurately from NOESY spectrum. So, this is a big issue there are many reasons for this, so what are the ways to come out of this, there are 2 ways, number 1; like we do for chemical shift referencing you can take a fix distance, let us say you have a molecule if let us say you have a system in your molecule like let us say an aromatic system in your molecule, so remember in aromatic is very rigid NMR structure.

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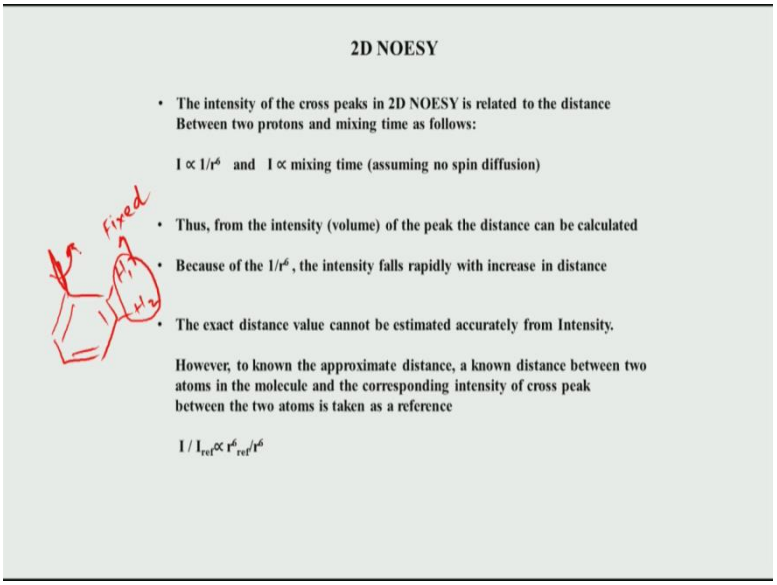


So the orthoprotons if you look at let me draw the structure here. So, if you can if you have an aromatic spin system like this a speromatic (())(21:48) in your molecule let us called this H 1, H 2 typically this distance is fixed, why is it fixed because remember according to chemistry this is a planar structure and there is a S P 2 hybridize carbon and this angle cannot be a cannot keep fluctuating. So, if the angle cannot be fluctuated that means it is fixed angle, so obviously these 2 are at a fixed distance from each other. Even if this whole aromatic molecule rotates, how does it rotate remember in molecules in typical it rotates around, so yeah so in a molecule it rotates around this axis okay, so this is axis of rotation typically in any aromatic system.

So this rotation will only change the distance between this hydrogen to some other hydrogen, but between the two hydrogens which are marked here there distances between H 1 and H 2 are fixed even if there is a rotation. So, therefore this distance if I know the distance, how will I know the distance, we know it from standard geometry or structure of any aromatic molecule. So, and if I see a cross peak between these 2 atoms in the NOESY, which I aspect to see because these two atoms will definitely B less than 5 Armstrongs okay, you can calculate yourself with taking some values and therefore if I know the distance and I know the intensity of the cross peak, then I know that this relation will hold.

So, then I take a ratio, so suppose now I have unknown atoms 2 unknown atoms whose distances I want to calculate between them, but I know only the intensity I do not know the distance. So you can see here for a fixed reference molecule like this or moiety like this I know ‘I’, I know ‘R’ okay. Now for the unknown case I know ‘I’ that is from the NOESY

spectrum but I do not know ‘R’. So, that is ‘R’ is what I want to calculate. So, I simply take a ratio then I by I reference will be = in fact, you can either say proportional or I would say =. So ‘I’ upon ‘I’ reference the reference is the intensity between these 2 hydrogens = R to the power 6 reference that is it distance between these two hydrogens divide by the unknown distance. So now in from these equations I have 3 knowns and I know this from the spectrum I know this from the spectrum I have this from the structure here and this is what I need to calculate.

So, this is one way to calculate distances between atoms based on a reference. So similar to what we do in case TMS remember in NMR everything is with respect to reference, there is nothing like absolute thing in NMR everything is relative to something which is we called as a reference, so therefore here also we use a reference. Now this is one option, the second option is that you do not calculate distance like this you will simply say that look I am seeing a distance and I am seeing a cross peak between two atoms okay, so therefore it has to be within 5 Armstrong.

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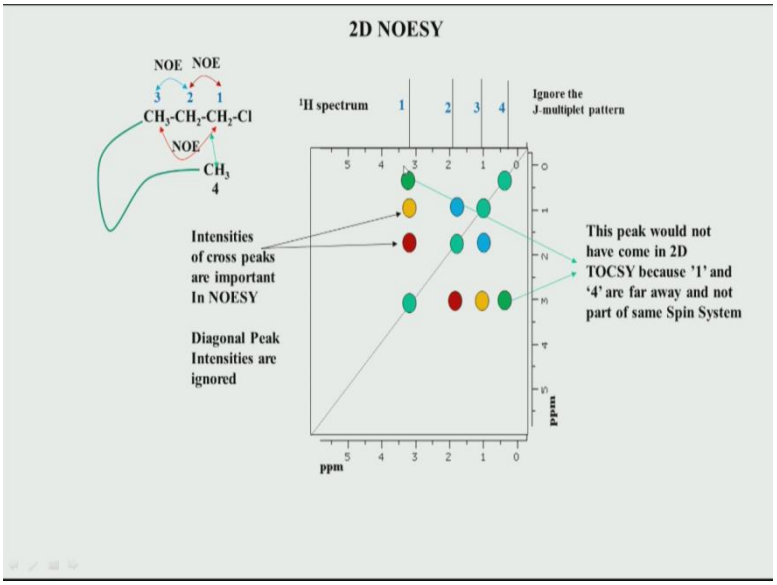


So an example let us go back to the previous slide which is here we saw that these 2 atoms or there 2 cross peaks are coming from interaction from these two. So the fact there is a cross peaks between these two atoms immediately implies that it has to be within 5 to 6 Armstrong. So you see, I already have some rough distance information from this kind of an analysis. So therefore, exact distances may not be required in fact, we will see that down the line when we look at application of NMR for structure determination, you really do not need exact distances between 2 atoms from NOESY. All you need it just an information whether they

are close or far in the sense both are going to be of course, every atom the moment you see a cross peak in NOESY, remember they are less than 5 to 6 Armastrongs, but within less than 6 Armstrongs they can be variation it can be 3 it can be it can be 2.5 it can be 4 or so on.

So, what typically a large number of people do is that just qualitatively looking at the intensity if it looks very strong peak we will say it is very short distance, if it is very weak peak you say it is long distance, but long distance means still within the limit of 6 Armstrongs. So this is there qualitative way to estimates the distance and that is also find because this procedure of using a reference and so on may not work always for a given system you may not have aromatic at all. So there you cannot because that is a only system where distances are fixed, no other moiety molecule is difficult to have a known reference in the same molecule.

So, these are the different ways a how intensity and distances can be related. Now let us see how effect of mixing time is coming into picture it happens like this. So, again we are looking at the intensity of the cross peak, so for any given 2 atoms that is let suppose I keep the distance and I vary the intensity now sorry their mixing time. So remember, if you go back to this slide here intensity is proportional to distance. So for a given to 2 atom any two atoms the distance is how does that mixing intensity now vary with mixing time.

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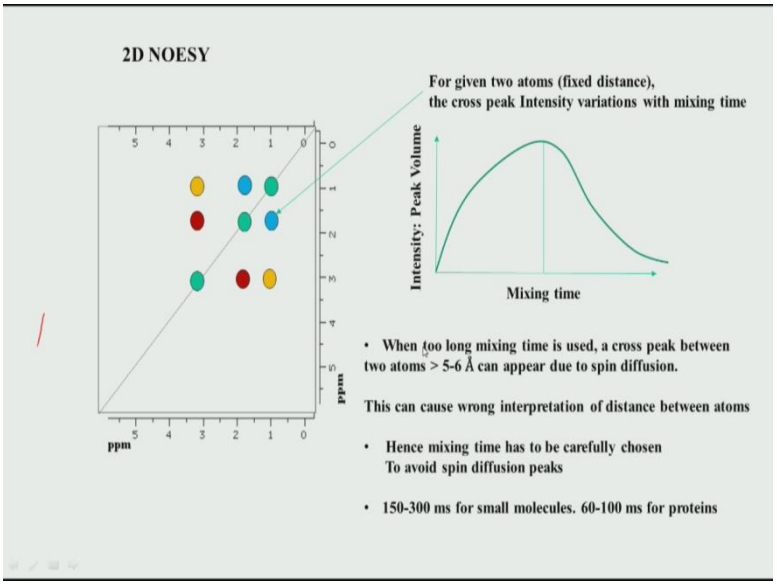


So, we are not varying that distance we are only varying the mixing time. So if the changes like this in the horizontal axis the intensity or in other words the volume of the peak shows this kind of a trend. This is a very rough sketch here, but it increases typically linearly and

then starts following down exponentially again, okay. So, this is the intensity of a cross peak that means I cannot arbitrarily choose my mixing time because mixing time has to be chosen where up to the maximum value means I should choose either here or here or here or here but not after this curve because, of T 1 relaxation and other effects that intensity now is drops going down. So when intensity is going down, it will give you a misleading information, why, again remember I my only measurement is intensity in a NOESY spectrum and from there I have to calculate the distance by using that formula we saw in the last slide.

So if my intensity is not correct, my distance information will also not be correct and why will the intensity we not correct, it will not correct because as it is negative correlation if I am increasing the mixing time beyond some full duration, the intensity starts dropping. So, drop in intensity is reverse of here, so therefore typically we have to restrict our mixing time within this limit. And what is this limit this is what we will see next the limit is typically few 100s of mili seconds. So, what happens is suppose you have a too long a mixing time, suppose I choose a very ling mixing time, two things will happen one is of course this relaxations which is going on, the second is there can be a spurious or artifact or a long cross peaks between two atoms which are greater than a 6 Armstrongs. Remember, in NOESY we should not except a cross peaks between two atoms greater than this value, but I may starts seeing that and that is phenomenon known as spin diffusion.

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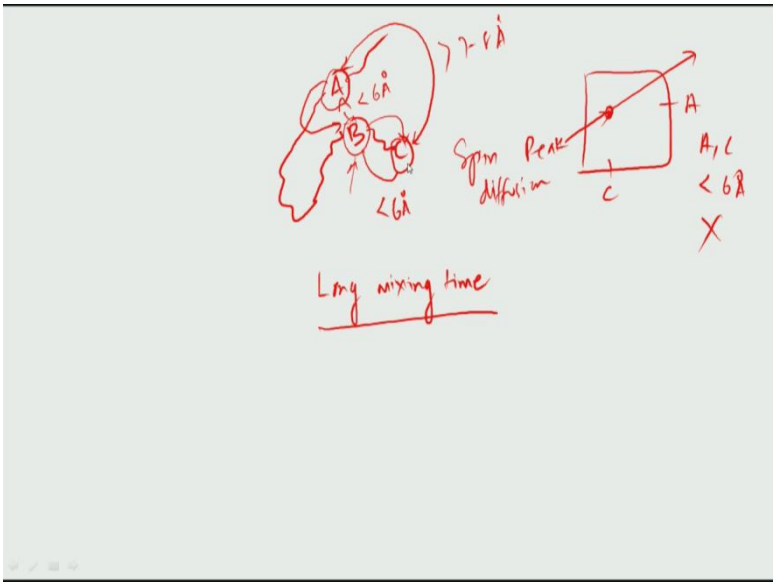


So, let us understand spin diffusion here, so what basically happens is let us say I have two atoms A and there is a change of the molecule, then there is B, then there is another change C. So let us say this is some hypothetical molecule, this may be between let us say less than 6

Armstrong and this may be also less than 6 Armstrong, but if I use a long mixing time, if I use a long mixing time what happens is this, the polarization from this first gets transfer here because of the slandered NOESY effect, form here it gets transferred to this side here in the same during the mixing time. So, therefore I may starts seeing a cross peaks between these two fellows these two atoms because of this intermediate B this is similar to what we saw in TOCSY intermediate J coupling.

But in NOESY, I do not expect direct cross peak between A and C, so if I see a cross peak between A and C in a spectrum, it will mean that A and C are less than 6 Armstrong which is wrong because this is not it may be more than 7 to 8 Armstrong, so you see this is called spin diffusion spin diffusion peak okay. So this is the problem in NOESY if you use too much or too long a mixing time the too long a mixing time will cause a wrong cross peak between A and C, which you do not except to C because A to C will be long distance, but it is coming because I used such a long mixing time that the polarization jump from here to here, then jump from here to here, so it look as if it is coming from here, but that is not real the case.

So this is basically the reason why we should avoid a very long mixing time and as written here, it is a about 150 to 300 mili second for small molecules and we choose 60 to 100 mili second for large proteins. Or in this range if you use, then the mixing time is directly relate it comes in this side and you can use safely the intensity as for cross peaks. So this brings end to the 2D NOESY experiment we will continue in the next class with a next experiment in 2 dimensional NMR that is 2D ROSEY.