Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
Prof. Hanudatta S. Atreya
Department of Chemistry
Indian Institute of Science, Bangalore

Lecture - 10
2D NOESY and 2D ROESY

Welcome back to the course. Now let us look at how 2D NOE experiment the peak pattern comes about. So, we looked at the briefly at the theory of NOE in the last class we saw that to transfer magnetization from one spin to another, we use the three pulse experiment 390 degrees and between the second and the third we use a word delay called as mixing time.

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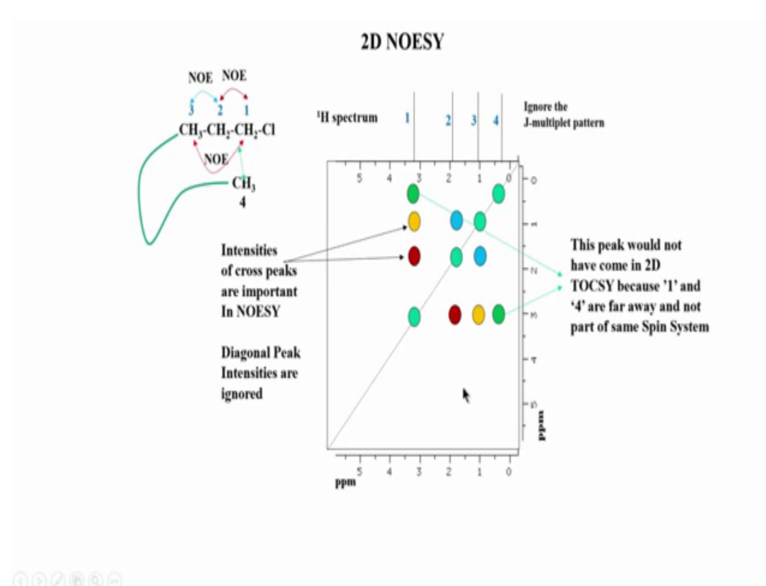

This mixing time is very useful important parameter which we will see again when we look at 3D experiments or when we look at the structures of biomolecules. So, for now let us see how does this peak pattern look in a NOESY experiment.

So, let us take again the simple molecule here propyl chloride; here you can see that these three protons are again close to each other which is this to this will be about 2 to 3 angstroms and this to this will be about 4 angstroms 4 to 5. It cannot be more than that. So, these all of these three are close to each other within that limit of 5 to 6 angstrom which we saw in the previous class. So, let us say now I record a 1D spectrum and again

let us ignore this J coupling pattern in this particular spectrum we are not interested in that that will be present, but we are not going to look at it we will not analyse that.

So, 3 2 1 is this is the 3 peaks which are seen again this is based on the inductive effect three is again very far from chlorine. So, three will come close to the 1 PPM; 2 is in the middle and 1 is close to chlorine. So, it is d shield. Now suppose I record a 2D NOESY spectrum of this molecule. So, this is how it looks. So, first is that both is axis here are protons because we are like transferring polarization in a NOESY experiment from proton to proton. So, now, let us the first thing you will see is the diagonal peak. Now diagonal peak is this is similar to what we saw in the 2D NOESY TOCSY experiments

Where what is this coming from? This come from the fact that this spin let us say 3 does not transfer any magnetization to anybody part of is. So, part of it is kept with itself and only part of it is transferred to 2 or 1. So, whatever is not transferred to anybody and is retained on itself, that part of the magnetization ends up as a diagonal peak.

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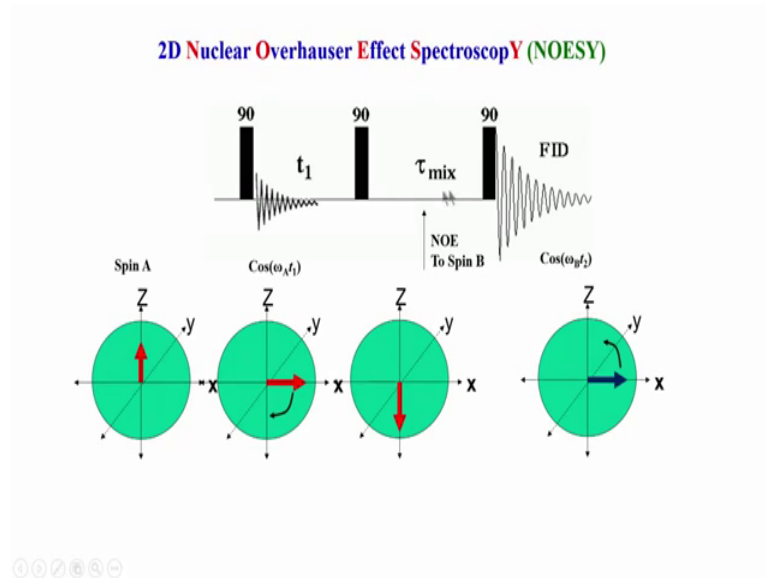

So, this is let me go back a slide and show you this experiment with reference to that. So, here we saw that when you apply this 90 degree pulse during this mixing time, the magnetization is actually polarization is transferred from a to b, but that is not 100 percent it is only about 1 to 2 percent remaining 90, 95 percent actually remains on a.

So, the bulk of it the majority of it still is on a, and only fraction of it is transferred to b and what is transferred is what we actually detect and what is not transferred is also detected. So, both of them are detected, but the one which is not transferred is essentially nothing, but a diagonal peak. It has no information in does not have any useful information for us because it is just telling us three is connected to 3, which is what anyway is known information is nothing to new about this. Similarly 2 and 1 all the protons will have the corresponding diagonal peaks and this is something you should very well keep in mind in fact.

There is a area of research in NMR and I has also eluded in the previous classes, is to how to get rid off this diagonal peak. There are several papers published known as diagonal less NOESY spectra and there because this as a said is almost 90, 95 percent of the signal is coming to the diagonal peak only a very fraction of it is in the cross peak. Now what is a cross peak? Cross peak is the peak where there is a transfer of polarization between 2 spins and this is something which we saw even in COSY and TOCSY. So, let us look at the spin number 1 it is in close proximity to 2 which is expected because there are only 3 bonds away.

So, in a NOESY spectrum you will see a cross peak between 1 and 2 ok. So, this carries actual information of the distance because only when they are close or less than 5 to 6 angstrom you will get a distance not only that the intensity of this peak. The area of this peak is also related to the distance between these 2 spins by that formula which I showed in the previous class, that is I is proportional to 1 to the power 1 by r raised is to 6. So, by looking at the intensity or integral of this peak, I can also estimate the distance with actual distance between the 2 hydrogens.

We will see that part later when we come to the structure calculation of proteins biomolecule where we actually do that, we need distance information from NOESY. So, that is based on the in area integral of the peak, but one thing is if you notice just having a peak itself is enough to tell us that these 2 protons are within 5 angstrom. If there was no peak between these 2 hydrogens and that means, they were they are away from - or they further from 5 angstrom. So, you see that distance information is already coming in without actually calculating the distance. We already know just by the presence of the peak that these 2 hydrogens are close in space.

So, this is actually the strength of this experiment that by just looking at the spectrum we are able to get the information of which hydrogens are close to which hydrogen. The actual distance at will not matter so, much as we will see later, what matters is just the information when the 2 protons are close or not. So, now, let us go further if you look between 2 and 3, again this 2 and 3 are definitely close to each other. So, you will get a cross peak between 2 and 3 this is similar to 1 and 2. Now between 1 and 3 as I said they are also very close because this is not very far away it is less than 6 5 to 6 angstrom.

So, therefore, magnetization of 1 is also transferred to 3 by NOE effect and therefore, between 1 and 3 again you will see a cross peak. So, this looks like a TOCSY pattern. So, TOCSY and NOESY actually look very similar if the within 1 molecule. So, if you record a NOESY or a TOCSY of 1 molecule let us say in your sample and this is a small molecule where all the protons are within 5 to 6 angstrom, you will not see a big difference between TOCSY and NOESY. But if you go to a larger molecule such as a protein or biomolecules, there what happens as a showed in the previous class that the 2 protons may be very close in space, but they may be very far in the sequence.

So, in such cases the TOCSY peak will not come between those 2 protons, because they are very far in terms of bonds, but they will appear in the NOESY spectrum because of very close in space. So, the proximity matters here and not how many bonds or how many how much is the J coupling, this is a very important point to keep in mind. Now this is what I showed. Let us say if we have a long chain this is something like a protein and you have a very chain which goes very long and it, but it comes close in space to one of this protons.

So, example is shown here let us say I have hydrogen which is methyl and show something is this is just an arbitrary signal it is an example. So, do not think of it as a literary this structure. This is shows that there is a long chain and therefore, it curls or comes back because of the geometry of the molecule and because of now it is close proximity to 1 and 2. So, if there is close to 1 you can see there will be a new peak now appearing between 4 and 1, but 4 and 1 are no longer J coupled there is no J coupling between them, it is only the through space interaction which is basically dominating and that gives rise to a peak.

So, this information that 4 and 1 are giving a cross peak is already a very good information because it tells us these 2 hydrogen's are close in space. So, this is the kind of information that NOESY experiment can provide us. So, you can see that this peak would not have come in 2D TOCSY, because 1 and 4 are far away in terms of bonds and they are not part of the same spin system. So, if you recollect the concept of spin system in TOCSY, we said that those network of protons, a family of protons which are coupled to each other by J coupling we call them as a spin system.

But here this hydrogen and this hydrogen are no way connected by J coupling because this is very far. So, this they do not belong to a single family and therefore, they are 2 different spin system they belong to different spin systems, but still they will give a peak because their closing proximity or space. So, this is basically the central idea and the next point is what I said in the just a few minutes ago that we need you can use the intensities or area in the integral or volume of the peaks and to get the distance information. And we can ignore this diagonal peak this diagonal peak has no information non and none for us and therefore, it is not required to analyse the diagonal peak.

But sometimes the diagonal peak and the cross peak they come very close to each other and when that happens there can be a severe problem? What happens is the high intensity of the diagonal peak starts dominating and it starts hiding the cross peaks? When that happens, you can no longer use the, I mean in the cross peaks intensity because they come very close to the diagonal peak. We will see some of the real spectra later on to get a feel of how strong this and diagonal peaks are. So, in such cases as I said there has been lot of research conducted to remove this diagonal peak by some method. So, that we can get a clean cross peak intensity.

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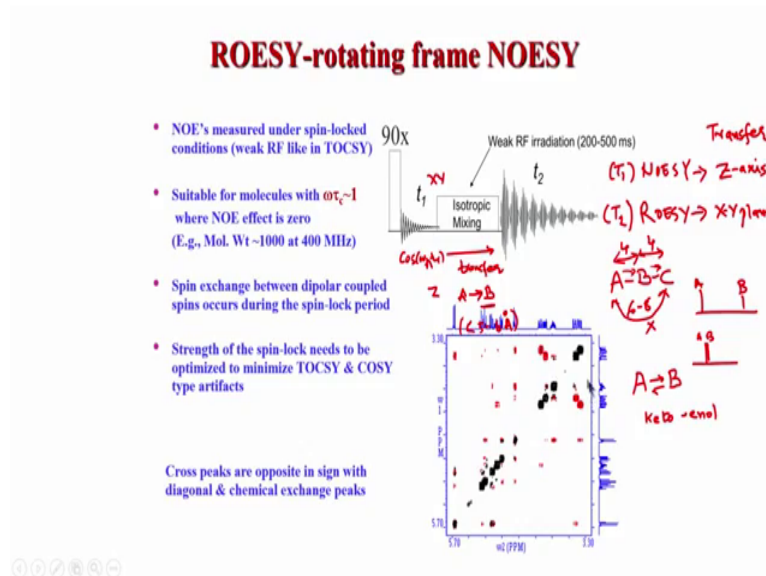

So, now as let us come to this second experiment which is alternative to NOESY and this is also very important experiment in case of small molecules and peptides. We saw that in the NOESY experiment does not work the NOE effect not the experiment as such, but the NOE effect itself fails if your omega into tau C is equal to 1. So, this was the problematic condition for certain molecules and as I showed you that it happens typically for peptides; peptides will come in this range that is 8 or 9 or 10 in amino acids for such kind of molecules at this kind of frequency. So, remember if I change the frequency this effect is not there.

So, it depends on both the frequency and the rotational correlation time. And the rotational correlation time we saw in the last class is depends on the molecular weight divided by 2 molecular weight in kilo Daltons divided by 2 is roughly the rotational correlation time in nanoseconds. So, if that happens, the NOE effect becomes 0 if this equation omega tau c is close to 1. It does not have to be exactly 1 even 1.1 or 0.9 it can the effect starts reducing the NOE is perfectly 0 at 1, but above and below 1 also it can be close to 0.

So, in such cases what do we do? So, there are 2 possibilities again which we discussed in the previous class. 1 is we can change the temperature. So, if you change the temperature you can actually go to a lower or higher tau c value. So, let us say if I change increase the temperature now tau c will decrease or if I decrease the temperature

tau c can increase. So, by just changing the temperature I can come out of this condition, if I am if it happens for a particular molecule or I can change the spectrometer frequency I can go to an 800 megahertz or a 200 megahertz I can go down also, but normally and we go to higher frequencies because of sensitivity reasons.

And therefore, higher spectrometer frequency can also help us to come out of this condition. So, now, during this experiment so, in this particular NOESY experiment how do we perform the experiment? This is now different from NOESY completely different in fact. In a NOESY we use three pulse experiment that is 90 90 90 here there is only 1 pulse and a set of pulses which is called as isotropic. This is actually the same experiment like TOCSY. So, if you recollect the TOCSY in the class where we did TOCSY experiment we discussed the TOCSY part we saw the pulse sequence was exactly like this.

So, the TOCSY and the ROESY you might think actually are similar experiments, but there are small differences in terms of the power levels and the mixing time. So, in a TOCSY experiment the mixing time is typically as I mentioned in that class is about 80 to 100 milliseconds or 60 to 100 milliseconds and that depends on the strength of the J coupling between the protons. But in a ROESY experiment we use much longer mixing time you can see here 200 to 500 milliseconds for small molecules. But if I use such a long mixing time and I use the same power level as a TOCSY is there its a problem it can create a heating in the sample. So, the radiation is actually not very strong is much weaker compared to a TOCSY. In a TOCSY we use stronger RF intensity because of what is called as mixing we want to have a stronger mixing, but in a in a ROESY the condition that condition is not necessary and we actually use a very low power compared to TOCSY. And that is called as a spin lock we also use the word spin lock this is similar to TOCSY again. So, all the terminologies which you see in TOCSY and ROESY are same. So, this is called as spin lock period.

So, during the spin lock the magnetization again are transferred between each other, but this is not from J coupling because we are not using the same J interaction the strength of the isotropic coupling mixing is not strong. So, the TOCSY effect is not so, high here although the experiment is same, but we have changed the parameters. So, here now because of a longer and a weaker mixing, there is a through space interaction which happens. So, the spin actually is in x y plane. So, we remember we are not in the z axis.

So, like in the NOESY. So, first we excite the RF the spin with an RF pulse. So, this is like any other experiment the spin is; now, in the x y plane when it comes to the x y plane it starts evolving because of the frequency.

And that can be actually written as I can show you here we can write it as cosine omega A t 1, this is same as NOESY and during this period there is a transfer of magnetisation from A to B and that where B is a spin which is close again close in space to A. So, again the same idea applies it has to be less than 5 to 6 angstrom even in a ROESY experiment. So, ROESY and NOESY are not different in terms of the distance information that we can obtain it is similar, but the main difference is here you can see here it is in the x y plane.

So, here it is in the x y plane where as in the NOESY we transfer the magnetization to z axis. So, I can write here NOESY is a transfer of magnetization; transfer happens along z axis when the magnetization is along z axis and in a ROESY it happens when it is in the x y plane. So, this is the major difference between the 2 experiments 1 is along z and another. So, therefore, in a NOESY what matters is a T 1 relaxation because remember T 1 is what is governs the relaxation along z axis and T 2 in case of ROESY because T 2 is the relaxation is what dominates in the x y plane. So, when you are in the x y plane and the T 2 relaxation matters whereas when an in a z axis T 1 matters

So, in a NOESY experiment we saw that our T 1 should be long, we cannot we cannot use t relaxation the mixing time cannot be chosen arbitrarily it depends on the T 1 of the sample and consideration such as pin diffusion. Same thing happens in ROESY. In a ROESY also you are now the mixing time. So, this is a mixing time here which I wrote as 200 and 500 milliseconds 200 to 500 milliseconds it depends on the T 2 of your sample if the T 2 is short then ROESY experiment will not be very sensitive if T 2 is long which happens in the case of small molecules and definitely we are working with small molecules in case of ROESY we do not use ROESY for proteins.

We use ROESY in mainly for peptides and small molecules. And in such cases the T 2 of the molecule will be rough sufficiently long. So, that condition is not a big critical factor at the same time what we want to do is, we want to transfer we do not want the spin diffusion effect which I discussed we will discuss that late again where the transfer happens beyond the 2 spins which are more than 5 am angstrom strong, but still transfer

happens because of the intermediate nucleus and that can be avoided by choosing a appropriately short mixing time. So, that is something which one has to optimise and this number is something which is not a priory known for any given molecule.

So, 1 typically does what does is he records for a series of mixing time and based on that we can decide whether which particulars mixing time is the most appropriate. So, we will see that when you come to the practical aspects of structure determination in proteins and biomolecules. So, this is a spectrum a spectrum example for a ROESY. So, we can see here the point main point to take home is the cross peaks are opposite in sign with respect to the diagonal peak, we will look at this chemical exchange part little bit later. So, now, if this is the important point which we do not see NOESY.

So, in a NOESY experiment the cross peak and the diagonal peak they have the same colour they have the same phase. So, if this is black the NOESY its cross peak also would be black, but in a ROESY the cross peak is opposite and why is this so? And this is a little technically involved answer for this and that that is the cross relaxation sign. So, what happens typically when you have omega tau c less than 1 which is in the case of small molecules, your cross relaxation or in NOESY typically what happens is the cross relaxation is I can be positive or negative.

But in a ROESY the cross relaxation is always positive. So, because of that there is a change in the sign which we cannot go in to detail unfortunately it in was mathematically solving some matrices, and that is not the object of the course or the objective of the course is basically where you get an idea how the NOESY experiment and NOESY experiment works what is the type of peak patterns we get and how do you analyse it. So, in a case of ROESY, that of diagonal peak and cross peak are opposite. What is one more thing is the spin diffusion peak. So, this is something which I mentioned that suppose I have A going B and C.

Let us say I have three spins and this is less this is between let us say 5 angstrom or 4 and let us say this is about 4. So, this to this probably is about 6 to 8 depending on the geometry. So obviously, between A to B I cannot A to C, I cannot expect any cross relaxation or any NOE effect or ROE effect. Because ROE or let us say ROESY and NOESY both have this constraint of this distance. But still I may end up seeing a peak between A to C and this is something which I call or referred to as the spin diffusion

effect which basically says that the magnetization has gone from A to B and then it has gone from B to C during the mixing period.

So, it looks as if the nitration actually has come from A to C. So, this is what typically mistake happens in interpretation in NOESY is that I may see a cross peak between A and C, but actually A and C are not really close and why do I get that peak? That my peak comes because A to B is there is an intermediate at middle nucleus B here which can transfer the relay the magnetization from A to C through B and that happens because if I choose a long mixing time and that affect can happen and therefore, you may end up interpreting or analysing the seek and A cross peak are saying that they are 2 are closer than 6 to 8 closer than 5 angstrom. And which can be a wrong structure now because actually in reality they are not less than 5 angstrom there are actually far away.

So, this is called a spin diffusion effect and nosey is very difficult to find out where one can do some experiment for example, one can reduce the mixing time slowly gradually and systematically and see if that cross peak of C disappears because the cross peak of C it depends on how long you choose a mixing time. So, that is that is why we are as I said one has to optimise the mixing time, in a protein typically if in general we use about 60 to 80 milliseconds in a NOESY, where as in a peptide in ROESY is we typically use about 200 to 300 milliseconds 500 is for a smaller peptide very small tri peptide or for a small molecule we can go up to 500.

But this has to be optimised for spin diffusion problem, but in ROESY one one strength of ROESY is, that the cross peak is opposite number 1 which already we saw not only that the spin diffusion peak that is from A to C will be positive again; that means, it will be same as diagonal. So, diagonal and spin diffusion peak is not shown in this graph I do not see any spin diffusion peak, but if there was a spin diffusion peak, it would have come that cross peak would have come and the same colour sign as a diagonal peak. So, the spin diffusion peak also becomes opposite to the cross peak. So, this colour changes based on whether it is a direct peak direct peak meaning A to B is a direct peak because they are close than 5 4 5 angstrom strong.

B to C is also a direct peak because there are less than 5 angstrom, but A to C is a indirect peak there is no direct dipolar interaction between A to C. So, that is a spin diffusion peak and interestingly in ROESY the a the cross speak between A and C cross

peak I am not talking about diagonal peak cross peak between A and C will be the same colour as the diagonal peak of A and C or any diagonal peak. So, that way by changing the sign that phase that use technically the correct way to say the phase of the peak now tells us whether it is a cross peak or a diffusion peak and that information is not there in NOESY.

So, unfortunately NOESY although it is very useful for proteins does not carry that information of spin diffusion which very routinely happens when we make a mistake in choosing the right mixing time, but in case of a ROESY it very beautifully brings it out. And there is another effect call chemical exchange which was mentioned here and this is something which is I will right now I will explain in the brief manner, but we will take it up later towards the end of the course, when we study protein ligand interaction. So, exchange is a very simple idea exchange is a molecule A is undergoing an exchange or conformational or a structural change to another state.

So, will same molecule is undergoing a conversion to another molecule it is like for example, the Keto enol in in organic chemistry Keto enol transformation is a kind of a structural change or conformational exchange between 2 different structures, but the same molecule is undergoing. So, this can happen slowly the exchange can be slow or exchange can be fast.

Some this fast and slow depends on how the chemical shift difference between A and B and the rate of this reaction. So, whenever in remember when we say fast or slow exchange all depends on how close the peaks of this peak spectrum. If A and B are very close, then it is very difficult to figure out the exchange the exchange will always look very fast. But if the A and B far away if there A is well separated from B then we can decide whether the exchange is slow or exchange is fast or exchange is somewhere in between that is calling intermediate.

So, there are the levels of exchange we use the words like fast exchange, slow exchange intermediate exchange and all of this is very important in biomolecules because biomolecules are constantly always in exchange with different states of the same molecule ok. So, there is nothing like a single structure of a bio molecule. Biomolecule always has multiple structures and each of the structures are now exchanging with themselves through different exchange rates and therefore, it is a dynamic system and

there that depends now very well now that will be reflected in all your experimental for example, in NOESY especially, if the molecule has 2 distinct states and the exchange is slow.

Suppose a chemical shift difference is very high and the exchange is slow, then you will start seeing a cross peak between those 2 states A and B ok. So, that cues a information about that there is an exchange going on between the 2 structures of the same molecule the exchange is in the slow or fast that depends on as I said the chemical shift difference and that the exchange rate. Now this also depends on the temperature if I slower that the lower the temperature I cool down the sample the exchange rate will slow down if I increase the temperature the exchange rate will go up.

So, by changing temperature one can actually analyse the exchange rates and that is how it is used, but ROESY is not the only experiment, ROESY chemical a simple NOE NOESY experiment can also be used, but here the point is that the cross peaks or the exchange peaks will look same as a diffusion peak. So, exchange and diffusion again can be confusing if I see 2 black peaks here suppose I see a black peak here where the arrow is pointing, the black peak can denote either in exchange peak between some other species or it can denote a spin diffusion between 2 peaks. So, we will not go into many more details of this experiment ROESY basically we will stop here because this is essentially for small molecules and peptides.

Our focus in this course is going to be on larger systems such as proteins where we have about 50 to 100 amino acids and such larger protein systems we do not need to do ROESY we will be only doing NOESY experiments. But the idea here was to show you that this is an alternative to NOESY that is the ROESY effect and it is very similar to TOCSY. So, there has lot of been papers published where they have actually shown how the TOCSY effect can be removed from the ROESY spectrum. So, as I said in ROESY you can also end up getting TOCSY peaks because TOCSY also is a same experiment although the parameters or the pulse levels I mean the power levels are different, but still there can be a leakage problem.

The TOCSY peaks can show up similarly ROESY peaks show up in TOCSY, but that that has been lot of developments and lot of research papers which have gone into this on addressing this point. So, in the next class we will look at the hetero nuclear NMR now,

we have completed that homo nuclear NMR experiments we looked at 2D cosy and we looked at 2D TOCSY, 2D NOESY, 2D ROESY and these are the main 4 experiments which are very typically used in homo nuclear experiment molecules.

But when it comes to biomolecules where we have carbon 13 and nitrogen 15 definitely we need to use hetero nuclear NMR experiments that is has much powerful or more information from there we can actually built 3D NMR which also we will see in this course. So, we will start looking at 2D hetero nuclear NMR experiments from the next class.