Principles and Applications of NMR spectroscopy
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Module 1
Lecture No 05

In the last class we looked at the experiment 2 D NOESY that is nuclear Overhauser effect spectroscopy and how it is useful in determining structures of molecules and we also saw some practical matters related to this experiment, such as how the intensity varies with distance and the mixing time how it should be chosen.

One problem in 2 D NOESY is that it does not work for many molecules in the particular size range and what do we do in such cases if NOESY cannot be used how can we determine the structures of those molecules. So, there is an alternative experiment which we can use and that is what we are going to see in this class and that is known as ROESY.

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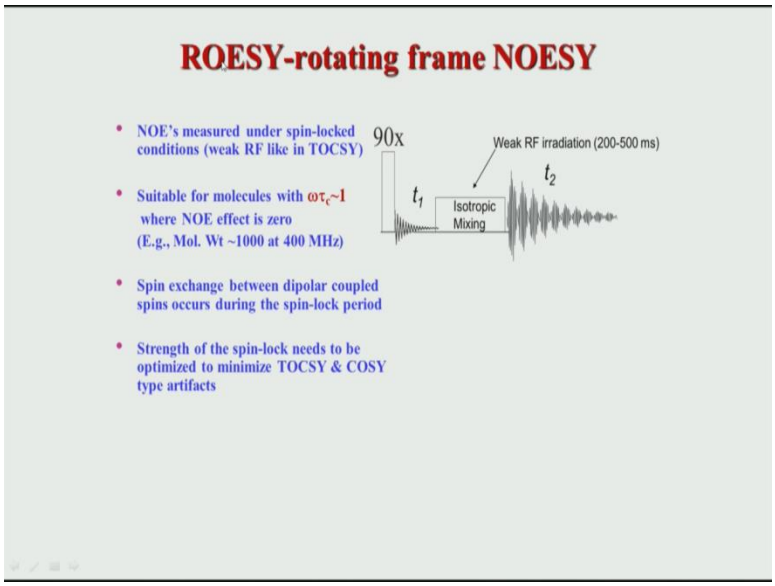

So, as you can see in this slide the 2 D ROESY means it is rotating frame NOESY. So, the word is just if the word rotating frame is there a Rotating Frame Overhauser Effect spectroscopy. So, this is an alternative to 2 D NOESY experiment and so let us see how this works and why is it required. So, in this experiment so if you recollect the previous class we looked at haw the NOESY experiment is done and that is done by applying 3 pulses.

So, what fun was 90 degree pulse then we had T 1 evolution, then we applied one more 90 degree pulse, brought the magnetization to minus Z and then during the mixing period it was allowed to interact with the another spin and after this transfer the second spin was detected by applying one more 90 degree pulse and then it (())(2:00).

So, in this experiment ROESY we do it in a different manner. So, here what we do is we apply a 90 degree pulse, then allow the spin A to evolve it is the same approach what we saw in the case of TOCSY, then you mixed the magnetization by applying what is called as a spin lock. So, this is nothing but a weak irradiation applied for a duration of this time 200 to 500 mili seconds.

And after that during this period again similar to what happens in NOESY the magnetization from one spin is transferred of polarization from one spin is transferred to the second spin which carries with it if the information of the chemical shift during T 1. So, the second spin when it is now comes to the X Y plane remember here if you look carefully we have started from X axis pulse. So, the magnetization is in the Y direction here and it remains Y throughout, there is no need for another pulse to bring the magnetization to Y.

So, in NMR remember we always apply one 90 degree pulse to bring a magnetization from Z axis to Y axis, but if the magnetization is already along X or Y we do not need to do anything we just need to directly start detecting that signal by using the detector and that is called FID.

So, we do not detect here we just allow the spins to evolve in chemical shift and they start moving according to the chemical shift frequency and when the intensity at the any point here is modulated means connected or related to the chemical shift frequency value and that intensity is transferred to the next spin based on it is because of the spin lock here, and that is because of the through space interaction and after that interaction is over, then the second spin now which has got the energy of magnetization from the first spin starts or moving and that is gives the second spin chemical shifts.

So, that say 2 spins are connected to each other correlated by this manner. So, now question is if you look again, if you recall this is exactly the same as the TOCSY spectrum TOSCY pulse sequence. So, the question is what was different here why is it similar to a TOSCY? The reason is yes it is very similar to TOSCY in fact it is like a TOSCY itself but only difference is the

power levels are slightly lower than a TOSCY and the time duration is very long compare to TOSCY.

In the TOSCY we used typically around 60 to 80 mili seconds and this is about 3, 4 times bigger longer, you can go all the way up to 500 mili seconds. So, that is the difference as for as NOESY and TOCSY is concerned but the picture, the pulse program like this sequence looks exactly same. So, now question is why are we doing this ROESY experiment at all why reason the ROESY help always. So, what happens is there is the theory which will not go into detail in this course but the theory says that suppose by accidently you are molecule is such that the product the multiplication of Omega into tau C.

What is Omega? Omega is comes from a spectrometer frequency and tau C is the rotational correlation time of the molecule and if you recollect we saw in the previous classes that it is related to molecular way divided by 2 kilodaltons and the value is typically Nano seconds. So, this product if it happens to be closed to 1 then the NOE effect it goes to zero, that means even if the (molecule) atoms are close in space you will not get any cross peak between them because there is no way that they can transfer to each other and the reason is that transfer efficiency has becomes zero because of this spurious or strange phenomenon.

Which is well explained in theory of course, it is not that strange but it is interesting that at this point you were get a zero? So, typically these will happen if your molecular weight let us says is thousands, the molecule which you are analyzing and if you are working at this frequency 400 megahertz okay. So, at that frequency omega is how much is 2 into pie 2 pie into frequency.

So, just not directly frequency here you have to use a 2 pie and multiply with the tau C for this and if you recollect the math the tau C for this is going to be 0$5 Nano seconds. So, 0$5 Nano seconds into 2 into pie into 400 if you calculate at room temperature. So, remember this is very important point because a temperature matters here we saw that earlier that if tau C temperature goes up that tau C changes the temperature goes down the tau C will change.

So, based on this that temperature you see that for this particular case you may get around 1, 1$2 and that means the efficiency of NOESY for this molecule at that frequency at that temperature is very not very good. So, if you are forced to work at under those conditions that spectrometer, in that molecule you will have to try an alternative experiment and that is why we used ROESY.

So, ROESY is the information wise is very similar or same as NOESY which means that similar to NOESY the intensity of the cross peaks is proportional or inversely proportional to the R to the power 6. Where R is the distance between two atoms. So, similar to what we do in NOESY we choose a mixing time and we look at the intensity and we measure or calculate the intensity of the cross peaks from the spectrum and based on that we calculate the distance.

So, for example thousand if you say one more thing in (())(8:07) look at it. A typical Amino acid residue in a protein and Amino acid is typically we will say the average is 100 or 110. So, that means this will correspond to a 9 Amino acid protein peptide. So, is in peptide is 9, 10 Amino acid and if you are working a 400 megahertz at room temperature, then there is highly likely that you would not get good NOESY or you may be nothing at all so therefore you should use this experiment.

So, that means if how will the spectrum look if the NOESY effect is zero. So, if you think about it. So, imagine that in that experiment which we saw in the last class you have, we tried will except a transfer of polarization from spin A to spin B but spin A does not transfer completely to B what we saw is we get what is called as diagonal peak and diagonal peak is a peak which comes because of incomplete magnetization transfer.

So, therefore if you have a zero transfer a thing is transfer. So, all the peaks will be in the diagonal because nothing is transferred to anything else. So, therefore in your NOESY spectrum if you see such only a diagonal peak and you do not see any cross peak it means that your your problem is because of this particular reason. Okay.

So, therefore one should now concentrate look at either change the magnetic field or that means I should go to let us say 600 megahertz if I have in my laboratory or you change the temperature because remember temperature also will help you to come out of this condition by changing temperature will affect the tau C or you must basically look at some other molecular weight. So, this is a way why ROESY is important because it our comes all this problem and you do not have to worry about NOESY indirectly record a ROSEY spectrum.

Now in a ROESY spectrum the spin exchange means the polarization transfer takes place during the period known as spin lock period. So, this word spin lock period is coming because of this there is an isotropic mixing and so what we saw in TOCSY so during this period there is a

transfer moving on. So, therefore remember if you recollect we I just now as we discuss the TOCSY experiment is also exactly same as a ROESY.

So, therefore one has to optimize spin lock. So, that you do not shut see in TOCSY peaks of artifacts or TOCSY type of problems in NOESY. So, these are practical aspects which are important to know because TOCSY and ROESY have same pulse program. So, whatever but remembers as I said in TOCSY we of course use very short mixing time where as in ROESY it is will longer.

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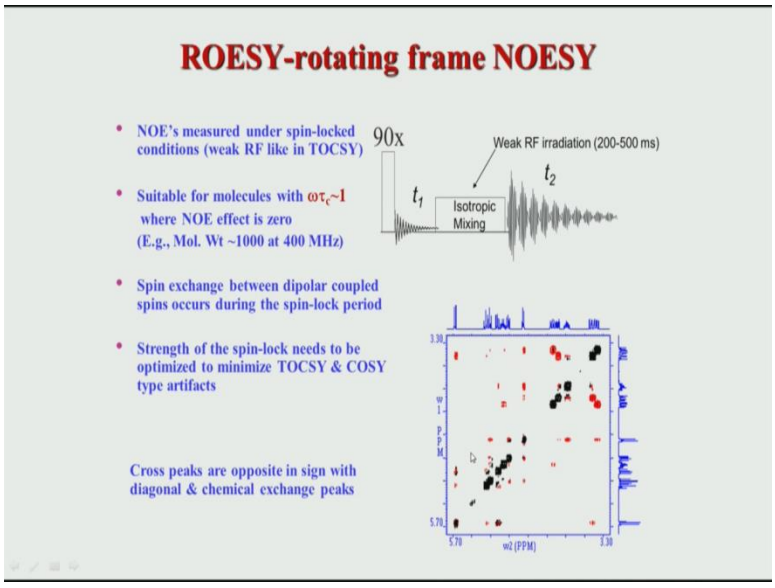

So, this is an experimental spectrum, how it looks so you can see here that there is a cross peaks and we can see there is a diagonal peak and there is this cross peaks so red color. So, you see one thing if you notice immediately the cross peaks are opposite in sign compare to diagonal and the chemical exchange.

So, what is chemical exchange will worry about it little latter and come to the advanced NMR topics we will cover chemical exchange but what is currently we have to worry about is this peak the diagonal. So, you see the diagonal is black in color that means it is let us say positive and the red is apposite to diagonal peak means apposite in color because it is negative.

So, the cross peak and diagonal peak have a negative phase relationship between each other and that is why we say that it is very nice because you can immediately figure out any peak which is

apposite to diagonal is the actual cross peak which we need to worry about. It is another very beneficial or useful feature in ROESY which is not present in NOESY is as follows that remember we discuss what is called as spin diffusion peak. A spin diffusion peak arises because two spins A and B are not closer than or further than 6 Armstrongs or 7 Armstrongs that means it could be 10 Armstrongs.

But still I may end up seeing a cross peak in the spectrum between A and B which is not correct I do not expect A and B to show a cross peak but it is showing a cross peak. If you look at it in a other way if a person does not know the structure that is should be 5 or 10 Armstrongs or so the person may think that A and B are now close to 5 to 6 or less than 5 to 6 Armstrongs because I am seeing a peak in the NOESY spectrum.

So, this will lead to a wrong structure wrong structure because in reality they are about 8 Armstrongs but in practice using my equation and using my the idea that intensity is proportional to power 6, one by R power 6. I may end up interpreting that these 2 peaks these 2 atoms are less than 5 to 6 Armstrongs okay. So, this is a misinterpretation and can very much lead to a wrong structure.

So, why did it happened this happened because A and B you are not close in space but there was (such) third spin suppose we call it has a prime or C which connected A and B because first that magnetization went from A to C and from C to B during the long mixing time. So, if the mixing time is not chosen appropriately I may end up with this kind of artifacts or this kind of problem.

So, in a NOESY there is no way that a given peak we can say whether it is a direct NOESY peak because of less than 6 Armstrongs distance or it is coming because of spin diffusion very difficult to say unless of course the one option we use is to vary the mixing time. So, let us say you record your spectrum at different mixing times. So, if you record a spectrum at different mixing times remember we saw that the intensity is proportional to the mixing time I mean it increase with mixing time to some extent.

So, therefore if the intensity of a cross peak is keeps on increasing as I vary the mixing time then that particular peak I can say is coming from direct NOESY effect. But, if the intensity of peak does not vary with mixing time it decreases then I will say it is an indirect NOESY effect that is spin diffusion peak. So, for a spin diffusion peak the intensity does not increase proportionately

with mixing time. There is one way in NOESY to rule out weather the peak is coming because of direct NOESY or through spin diffusion.

But, the disadvantage now it is that you need to record a several 2 dimensional NOESY spectrum with different mixing time and remember each 2 D spectrum takes about hours to record a few hours and therefore if you want to do several 2 D experiments you may end up spending days a 1 day or 2 days just for this purpose. But in case of ROESY what happens is the ROESY your red colure which is shown here is red colure is a direct cross peak and this is diagonal peak.

So, if you a have a spin diffusion peak, then that will come as black color which will be the same as the diagonal. So, interestingly that diffusion peak will now can be distinguish from the (the) a direct cross peak because there will be a change in the sign or change in the color of the peak. So, based on that one can now distinguish and say confidently that look this is a spin diffusion peak and something else and the some other peak is a actual cross peaks.

So, therefore we can we, we can then when we analyze or interpret the intensities one can then ignore those black peaks and focus only on the red peaks. So, this is one way to look at spin diffusion but of course there is another thing in ROESY is that exchange peak that you and also in NOESY make change we happens is also in NOESY. So, NOESY and ROESY both show a very interesting feature which is the very important dynamics information in many systems in organic in chemistry we look at it as you said in the as I said in the advanced section but let us ignore.

So, keep in mind that in diffusion in ROESY there are spin diffusion peaks that is multi step ROESY peaks are apposite compare to direct one step NOESY peak and therefore it is very easy to distinguish. But then the questions you may ask is why do not we then do ROESY every time, why do we ever have to do it NOESY the reason is because of this factor here. If you see this diagram picture here the magnetization is in the Y axis here X or Y and then it remains in X or Y for a long duration up to 500 mili seconds and then you detect.

So, during this portion of the time it decease decreases because of T 2 relaxation. So, remember T 2 relaxation is all happens in the X Y plane and T 1 relaxation is happens in the Z axis, when it magnetization is go into Z axis. So, T 2 relaxation starts playing a big role in this case. So, if a T 2 is short which is typically the case for a larger molecules if you let us say a peptide which is 15

Amino acids or 20 Amino acids as you go increase the number of Amino acids or if look at larger molecules the T 2 effect T 2 is very short.

So, by that time you apply this isotropic mixing it is what is called spin lock or a weak RF radiation in the spin starts relaxing towards Z axis very quickly. So, therefore the signal intensity when you reach here is very less. So, the overall sensitivity of ROESY is less compared to NOESY. So, only if there is no option for NOESY, ROESY is resorted to this people used ROESY if in case you are not able to get a good NOESY but otherwise NOESY is still preferred because as far as I control my mixing time properly the spin diffusion is not such a big problem.

And so from one if you are experienced then you will know what mixing time one should use and therefore you do not have to worry about this negative positive and you can analyze a NOESY which is more sensitive compared to ROESY. So, this is brings end to the ROESY part that I ROESY or NOESY we look at one last two dimensional experiment before we move on to the heteronuclear.

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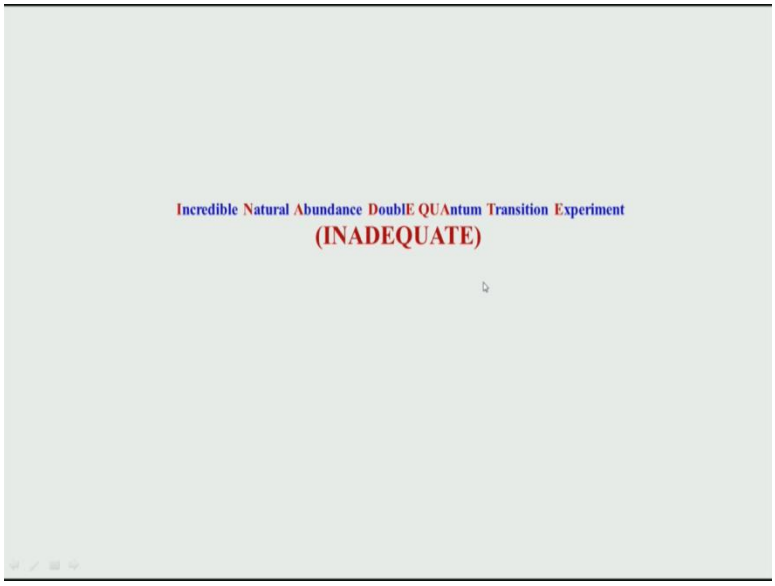

So, we look at one more homonuclear experiment 2 D homonuclear which is known as inadequate again a very interesting acronym and that is stands for this particular word this is incredible natural abundance double Compton transitional experiment. So, now the name sounds very complicated but actually the experiment is a very simple experiment it what it means is it, it

is what it tries to do is, it tries to correlate like any other 2 D the chemical shift of carbon to carbon okay. So, remember our focus is now carbon to carbon we have come out of hydrogen.

So, this is a typically a carbon-carbon correlation experiment and used a very frequently in chemistry not so much in biological molecules because the sensitivity is of course very low. So, here what are you trying to do we are trying to correlate or connect two carbon atoms. So, let us see how this happens.

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So, this I said it is a very useful for establishing carbon-carbon correlation that means two like a COSY remember in a COSY we saw proton to proton correlation. Similarly, here we try to do carbon to carbon correlation again using J coupling. So, J coupling is like common feature everywhere only through J coupling we can a transfer polarization if you want to study like COSY experiment.

So, it is very useful for natural products and so on. But remember it is C 13 detector means everything is carbon 13 here there is no carbon involved, proton involved. So, the carbon 13 is a not a very sensitive nucleus because it is natural abundance is very low number one 100 times less compared to proton, number two it is 4 times less Gyromagnetic ratio it has 4 times smaller Gyromagnetic ratio compared to proton.

So, these two factors really reduced the sensitivity of carbon if you are studying at natural awareness. So, therefore this is a very insensitive experiment, it requires very long time to record and more sample. Remember one of the slides we saw long back in the theory part. Where we saw first part it was known of theory we will saw that that the sensitivity of NMR is proportional to square root of measurement time and directly proportional to concentration.

So, if the sensitivity is very less like this you either need long time or you need more sample or you can use both we will take longer time with more sample.

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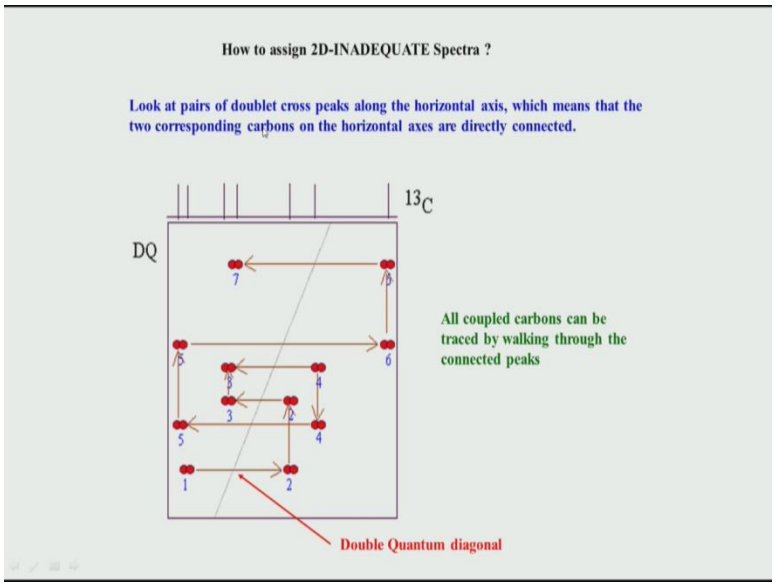

So, this is one class of experiments which falls under that. So, let us see how this work, so this is basically as it says here how how what information do you get from inadequate. Inadequate looks at pairs of doublet cross peaks along the horizontal axis which means that two corresponding carbons are directly connected.

So, let us say example suppose this is a carbon atom in may this is what is shown here and this X axis here this lines are strict or lines are nothing but peaks of a carbon spectrum. So, remember if you recollect we saw that carbon spectrum looks like single sharp lines because we decouple the hydrogens. So, there is no J coupling to hydrogen which can we seen in a (proton) carbons 1 D spectrum plus carbon spectrum do not have any carbon-carbon interaction.

So, there is no homonuclear J coupling because neighboring two carbons if very low probabilities that both of them will be C 13, we saw that is 1% of 1%. So, therefore in a carbon spectrum you will typically see single line this is not a real spectrum here but in a reality you will also you not get very much different from this. So, let us say I have recorded a sample hypothetical we will see a real example in the next slide but let us say we have a molecule which has spectrum like this a carbon spectrum what I am trying to do now is look at the close next connected carbon atom that means neighboring carbon atom. So, let us say lets me draw this example.

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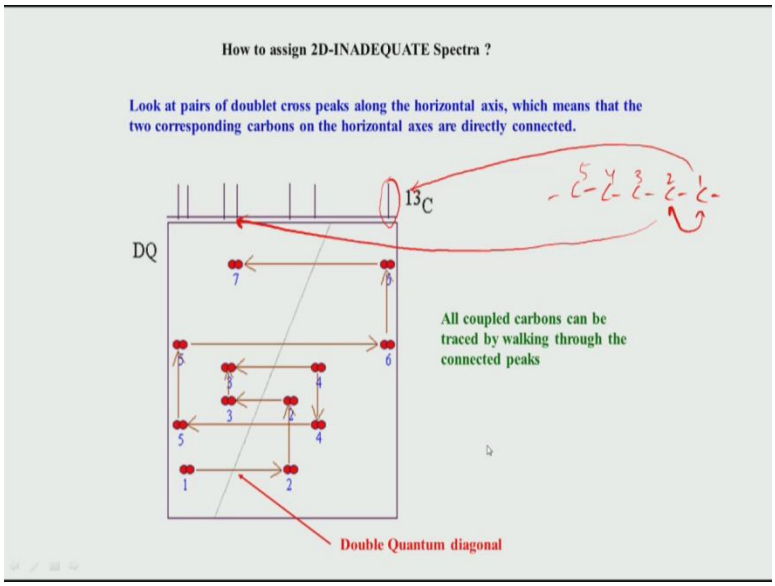

So let us say I have something like this going on in the molecule. So, I have 1, 2, 3, 4, 5 so what is this inadequate experiment doing is trying to connect the neighboring carbons or trying to tell you which peak here this carbon is connected which carbon this carbon is connected to which other carbon.

So, for example let us say this particular carbon may be this particular peak, then you are this particular carbon will become this peak here. So, basically we are trying to form a connection between two neighboring carbon atoms okay. So, this is what is shown here, so let us say we start from one some arbitrary peak we are using the word double quantum here. So, let us not worry about what is double quantum for the time being this is more mathematical or that is why

we will not be able to cover this in this course but for us what matters is just the appearance of peaks.

So, you have a carbon peak which is let us say position one remember, this both axis okay. Both sides this horizontal vertical axis and horizontal is carbon to carbon. So, both dimensions are carbon, so but the only thing is in this axis we do not get direct carbon chemical shifts, we say that it is a combination of two chemical shifts okay.

So, what happens is it is basically is a chemical shift here is the chemical shift of one some peak one plus the chemical shift of it is connected peaks. So, both these peaks are connected to each other. So, both the chemical shifts you add them together, some of the chemical shift is what you will observe here okay. So, let us say we if this is directly connected to peak number 2, so it will be in the same line okay.

So, then what you do next is you go vertical in this line okay. So, this is basically if you go vertical this position will be 2 plus 3. 2 plus 3 means chemical shift of two this 2 plus chemical shift of the next peak 3. So, that is what comes at this position, so if you go in the horizontal line it is 3, 2 plus 3 is this peak and this will be 3 plus 2, so both are equal so they are in the same line. So, 3 plus 2 comes here but 3 plus 4 comes in the vertical because I have to add this is 3 and I have to add a chemical shift 4.

So, there will be addition to that we will come here and then again 3 plus 4 and 4 plus 3 is same. Then come 4 plus 5, so 4 plus 5 is comes down why does it is come down it comes down because 5 is on this side and 3 was on that side. So, whenever you go to down field depends in it can either increase or decrease or it is always apposite. So, whatever happens so this chemical shift here is more than this chemical shift or I can reverse the approach I can say this side is less and this side is more it does not matter what a matter is that when you go this side it is on one direction and this side is opposite direction.

So, from 4 when I go to this peak to 5 it is coming down because the chemical shift of 4 plus 5 is less compared to chemical shift of 3 plus 4. So, 4 plus 5 comes here and if you go horizontal this is 5 plus 4. So, 5 plus 4, 4 plus 5 is same but now 5 is connected to 6. So, this 5 is connected to 6. So, 5 plus 6 is different now and goes up and this is 6 plus 5. So, 6 plus 5 and 5 plus 6 are same,

then the 6 is connected to 7, we will go up and see the same the same line the next peak and then go horizontal that is the last peak 7, okay.

So, basically what is happening here is we are going from horizontal vertical, horizontal vertical. So, when you go on the horizontal line from one atom one peak to another peak we get the neighboring connection information. So, we get you two atoms connected to each other directly through one bond.

Then we go up we get second connection, we go left we get another connection like that we can walk like this and keep connecting two neighboring carbon atoms and this this vertical will match with this peaks here. So, you have to place your carbon 1 D spectrum on the top and then along this line you start looking at those chemical shifts. So, this corresponds to this, this corresponds to this and so on okay.

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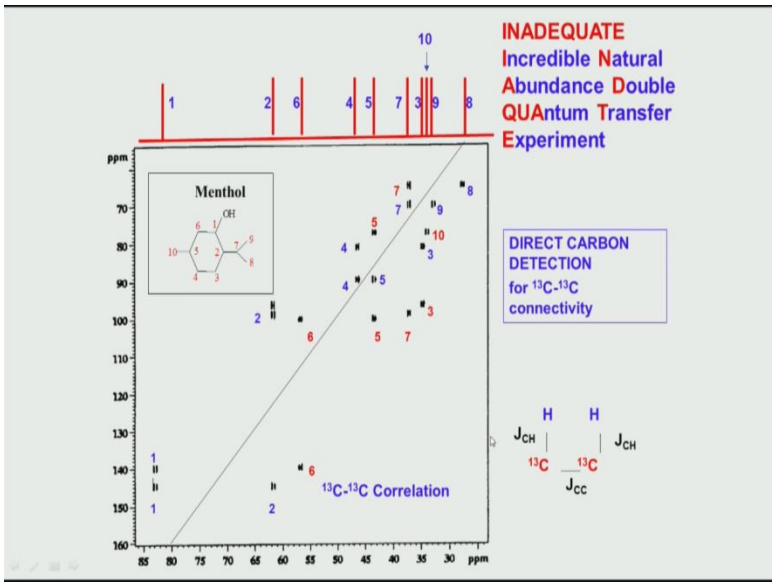

So, let us look at a real concrete example in this case menthol is shown. So, menthol has this structure here we will see on this side okay. And then you can see that these are the numbers we have put on the carbon atoms. So, let us say you start from which through this assignment means which carbon is which chemical shift as already will known in this molecule. So, we are for this is just an example.

So, suppose you have one here atom number one remember atom number one is directly connected to 2 these also connected to 6 okay. So, for let us follow one path now let us go from 1 to 2, then 2 next is here, now 2 is connected to both 3 as well as 7. So, you see there are 2 peaks here 2 is connected to 3 and we will see later how it is connected to 7.

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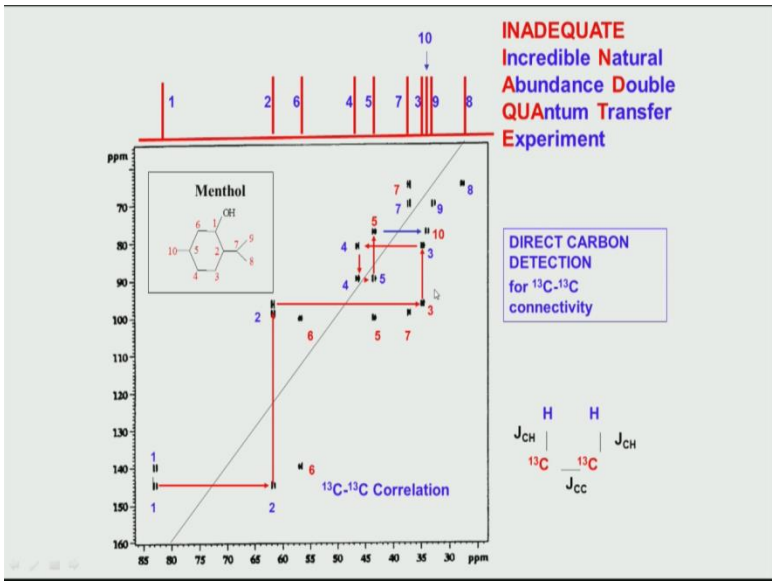

So, first let us see is connection to 3 which has comes here then from 3 if you go vertical it is 3 again because it is same line 3. But now if you go horizontal you get 4, when you go again vertical along 4 to remain on 4 but next horizontal gives 5, then you go along vertical to get 5 and then gives you 10, remember 5 to 10.

So, we have completed one path, so along 10 you do not see any vertical if you see carefully along 10 I do not see any other peak. So, therefore 10 is a last atom, so it is stops there okay. So, it is like a train moving one station to another station and it is stop at the last station.

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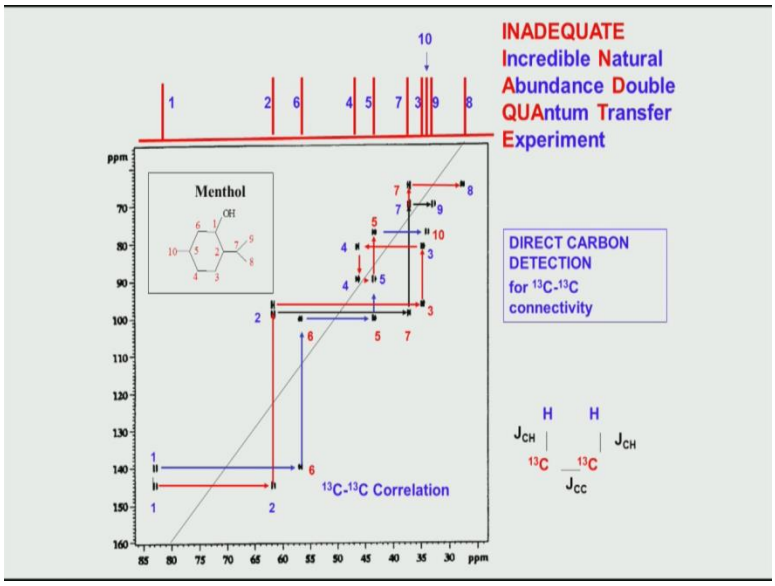

Now, this was one path way means one direction. Now I can also go through 1 to 6 to 5 to 10 so let us starts from there. So, this is from 2 okay. So, let us look from 2 onwards, so 2 from 7, 7 to 9 and 7 should also to 8. So, if you see here we started from this 2, there are 2 peaks earlier we looked at 2 to 3 connection. Now I am looking at 2 to 7 connection, then 7 to 7 vertically up, then it will branch out into two possibilities one is 7 to 9 and other is 7 to 8 that is what we will see here.

So, 7 to 9 connection is so, essentially we are connecting all the peaks which are each next to each other direct one bond. So, this is complete this side, now we are still left with 1 to 6 this path way which we did not see. So, that what comes here. So, we start from 1 again remember one we had 2 peaks one was this path way other is now this path way.

1 to 6 and 6 will go vertically up you get this peak which is again 6 only because, we are in the line of 6 but then if we go horizontal here it is a connection from 6 to 5, 6 to 5 and then now I expect 5 to 10. So, first I will go vertically on 5 and see wherever 5 is there, so I can see that there is 5 here and then saw we will see a 5 here, from here to here and then 5 to 10.

So, 5 to 10 path way is common. So, these 5 to these 5 to these 10 is same and these 5 to these 5 to these 10 is same. So, because 5, 10 are finally a single connection whether you come like this or you come from other side there is no distribution. So, this is how this inadequate experiment

works again if it is will look at a application of this later on and real system where we will used this two calculate or will use this two get the connection information.

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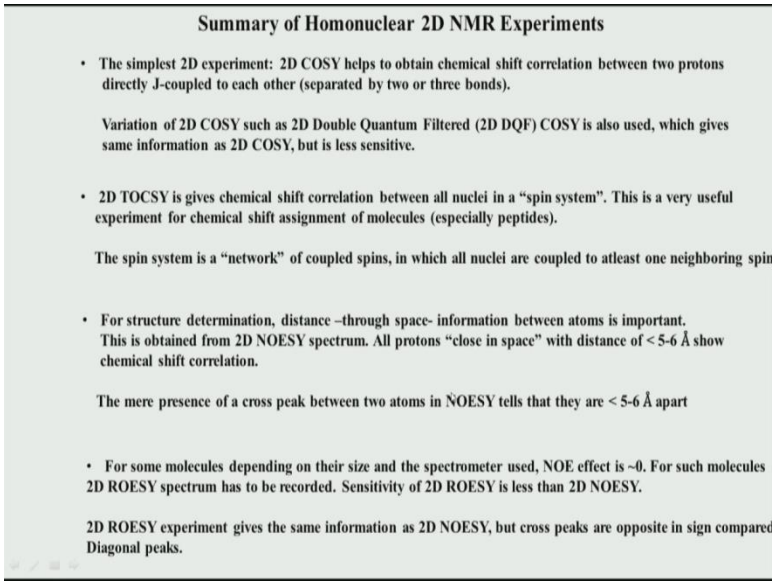

So, we will now we (())(31:57) actually to the end of all 2 D experiments that we have looked at and will just quickly summarized. So, what we looked at is homo nuclear 2 D experiments. So, we saw that the simplest experiment is 2 D COSY which helps us to get information between two protons which are directly J coupled to each other.

Usually, that is coupling is either 2 bonds or 3 bonds and in literature in text books you will see many variants of 2 D COSY the very important covariant is called 2 D DQF COSY Double Compton Filter which is exactly same or same information as COSY but is much better then COSY. So, normally now a day people do not do 2 D COSY as such they do what is called DQF COSY information wise they are same. But the problem is 2 D DQF COSY is not sensitive it is less sensitive. It is similar to like ROESY and NOESY which we saw depending on the sensitivity you can choose.

TOCSY is a very important experiment you will saw there it gives all the information about a spin system which is very useful in the spin system was we saw is a network of couples spin means which are coupled to each other at least one neighboring spin and for structure it determination we saw the important experiment of 2 D NOESY where all protons which comes

close in space within 5 to 6 Armstrong will show chemical shift correlation and interestingly as I said just a mere presence of a cross peak between two atoms tells us that they are less than 6 Armstrongs.

Just a presence is enough to give some distance information and then we saw that in the case of ROESY, NOESY does not work many times depending on your molecule and spectrometer frequency and in such cases we have to use ROESY. So, this brings us to the end of 2 D homonuclear NMR experiments and now we will move on to heteronuclear 2 D experiments that is where we see chemical shift correlation between carbon and proton and then that next that will be in a next peak and when we will see how these two homonuclear and heteronuclear together can you combine to get a complete structure determination end of molecules.