Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 12
2D Heteronuclear NMR: HSQC

In the last class we looked at the carbon 13 direct detection in a 2D experiment and we made this remark that direct detection of carbon has some problems especially with regard to sensitivity and resolution in case there is a carbon-carbon coupling. Although people have this has been dealt with in different ways in recent times, still the carbon direct detection is not so, popular compared to a proton. Now if you consider in fact, nitrogen it is even more going to be less sensitive for detecting nitrogen directly.

So, therefore, indirect detection that is first going from proton to carbon and coming back from carbon to proton is the most preferred root option in 2D and multidimensional heteronuclear NMR. So, let us have a look at why the sensitivity is bad when it comes to direct detection. So, this is shown in this slide here. So, the sensitivity of NMR is given by this formula here, that it is directly proportional to the gamma of the excited nucleus multiplied by the gamma of the detected nucleus to the power 3 by 2. So, this formula is very important to know that we can see from this formula 3 it matters what you detect and what you excite.

So, for example, proton has the highest gamma value. So, we can see that if I put gamma value of proton here and gamma value of proton here that combination will give me the best sensitivity. But if I put the least gamma value here in this formula and the least gamma value of in this formula that combination will give me the least sensitivity. So, therefore, it is very important to know that the 2 which gamma value I am using.

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Heteronuclear NMR

Inverse detection

- Sensitivity of NMR experiment ∝ $\gamma_{excited} * \gamma_{detected}^{3/2}$
- Sensitivity of ^1H excited and detected expt ∝ 32* ^{13}C excited and detected $\gamma(^1H) = 4 * \gamma(^{13}C)$
- Sensitivity of ^1H excited and detected expt ∝ √(time)
- To get same sensitivity in ^{13}C excited and detected experiment as ^1H One would need 32*32 ~ 1000 times more measurement time compared to ^1H excited and detected expt

Direct detection of X-nucleus may take 15-20 hours, whereas HMQC may take just 2 hours

- Note that this has no relation to natural abundance of ^{13}C, which is another independent parameter for determining sensitivity

So, now if you refer to the previous lecture the what we looked at is that, if the gamma of the excited was proton because we started exciting the proton first if you recall the last the experiment we looked at and we require. So, this part was taken care by applying by using the proton. But what happened was we detected carbon and because of that we ended up with 4 times less sensitivity for the carbon; reason the carbon is detected not only that the scales by 3 to the power 2 3 by 2.

So, that even further creates sensitivity loss. So therefore, instead of detecting carbon, if I can detect proton then that would have been the ideal situation. But if I excite proton and detect proton then what can, how do I get the carbon information? So, that information that is so, what here this formula is only telling us what matters is what you excite in the beginning and finally, what you detect in the end.

But now if I do both protons I need to still do a 2D experiment, where I need to go to get the information or connection or correlation to carbon. So therefore, we have what we use is that, we transfer it first to proton to carbon and we come back to proton for detection. So, we can see these some numbers are calculated here. So, sensitivity of proton excited suppose, I have put gamma of proton and I put gamma of carbon here. Now, compared to that if I put carbon 13 here and here; that means, I take carbon 13 I excite a carbon 13 and also detect carbon 13, that would have been 32 times less sensitive compared to a proton excitation in proton detection.

So, one thing we have if you recollect sensitivity in NMR is proportional to square root of measurement time. So, this is something which I mentioned in the very beginning part of this course, sensitivity is proportional to square root of measurement time ok. So, if 32 times if my sensitivity is going down by 32 times, I need to increase the time to improve my sensitivity. So, if I want to improve this then I have to square the time I need to get the same sensitivity back, I need the time the new time has to be now proportional to 32 square times the old time.

So, suppose old time was 1 hour which was I was using earlier, but now my sensitivity has dropped by 32 times. So, I need to increase my measurement time; I need to increase this to improve my sensitivity. And how much should I increase the time? I should increase by the square because of this square root if I take T square root of square of sensitivity, I need to increase that is close to 1000 times ok. So, what this basically the whole thing is telling us is, is telling us that essentially we need to improve the if you want to get back the sensitivity, we need to increase this time of measurement by a square of the sensitivity requirement.

And therefore, you see this is a big difference; the square makes its a very big difference or a big factor to be taken into account. So, essentially I have to almost go 1000 times more if I want so, suppose if I do proton excitation and detection and compare to that if I do carbon 13 excitation and detection, my carbon 13 spectrum has to be recorded with 1000 times more number of scans to get the same sensitivity as proton. So, that is a big thing because if a proton spectrum takes 2 minutes, I need to record 2000 minutes for a carbon this is for 1 D where we excite carbon and detect carbon.

So therefore, it is very important this is what is shown this is very important to keep in mind that we need to be very careful about sensitivity. Especially in biomolecular NMR the entire effort, the entire effort of making a sample of recording data goes into how best we can improve the sensitivity. So, there are many other factors of sensitivity we already looked at in the very beginning of this course, there are factors like the concentration of the sample and temperature and so, a number of scans and so on. But, what here you have looking at is the gamma factor and that is coming up in this manner.

So therefore, is very important the entire 2D NMR is basically the most of it is what based on what is called as inverse detection. An inverse detection means we are not

directly detecting the carbon, we are detecting it indirectly; we are starting from proton we go to carbon come back to proton and detect that proton. So, this is accomplished by what is called as inept. The INEPT is something which I started referring to in the previous class and we will see that in more detail today in this class.

So, direct detection of therefore, X nucleus takes a long time whereas; a HMQC or heteronuclear 2D experiment takes a very short time about few hours. So, that is why it is preferable. Now one thing we have to keep in mind all this factors we have taken here is only related to gamma, we have not taken into account the abundance. Abundance is something different that is already as I said 1 percent abundance of carbon in natural abundance. So, that is 100 times less compared to proton. So, again you have to factor in 100 into 100 10,000 times again for if you have a natural abundance ok.

So, we can see that how much time extra is needed just because the carbon is four times less in terms of the aeromagnetic and it is 100 times less in terms of natural abundance. So, we therefore, in biomolecules we have to necessarily enrich the molecule with carbon 13, it is virtually impossible to do carbon 13 natural abundance experiments on biomolecules, which are already low in sensitivity and they have low in concentration. And therefore, it is very imperative to do isotope labelling.

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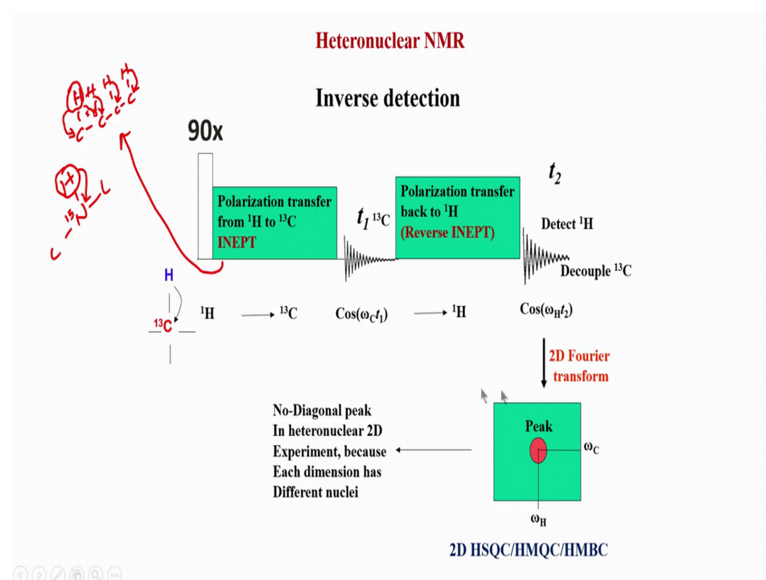

So, now let us look at this inverse detection which I have already told you in the previous slide how do we carry out that experiment. So, this is shown here. So, first let me give

you a broad overview of this experiment because it is very very important in this course to have a grasp of how this works. So, I would suggest you to pay a lot of attention on this particular type of experiments, which we will have which we will continue in this course in this class and the next class when we when we also will take this up when we go to 3D NMR. But without understanding these ideas it will be very difficult to appreciate how heteronuclear NMR can be used for protein structure determination.

So, first again we as I said we always start with a proton detection proton excitation. So, this pulse shown here is excited exciting proton nucleus a proton. So, that is what it brings a proton to the xy plane, then it we use we transfer the polarization to carbon in this period. This is using the block or a building block called as inept. We will show this in more detail as we go along. So, this block is essentially a transfer element meaning a part of a pulse sequence, which is transferring the magnetization or polarization from proton to carbon.

Now, once we arrive on carbon by polarization transfer, we excite the carbon. Remember here we have excited the proton and only transfer it to carbon we have not excited the carbon yet. So, we need to apply a pulse here which is not shown we will see that when we will see the actual pulse sequence, you will see that there will be a carbon pulse a pulse on carbon 13 which will now excite the carbon and the carbon will evolve with its frequency during this period labelled as t 1 the carbon will evolve with its chemical shift. Then carbon is back transferred the whatever polarization we received from proton is transferred back to the proton and that is done using this reverse inept.

Now, reverse INEPT where INEPT are very same they are similar its just that in one case we are coming from proton to carbon, second case we are back to proton. So, the pulse sequence will look exactly same whether it is forward INEPT like this here or it is reverse inept. Then once you transfer it back to the proton, you can now excite the proton or proton is already excited if it is excited, we can start detecting that proton. So, this is the direct detection of proton magnetization.

During that period now we have to decouple the carbon. The reason now you may think of is this a reverse approach. So, if you recollect the previous class we have detected carbon and we decoupled proton and here we are decoupling carbon and detecting proton. So, what is happened here? You have started from proton magnetization was

transferred to its nearest neighbour carbon. So, this is something you have to be very careful and remember in mind; when I say I am transferring a polarization from proton to carbon I am doing it for the next very nearest carbon which is available and that is the directly attached carbon ok. So, that is not some carbon which is far away.

So, if you have let us say let me show this again very clearly with a figure, let us say I have a molecule like this which is a long chain molecule what we are doing in this INEPT here in this blog, what we are doing? We are transferring magnetization or polarization from this proton to this carbon, this proton to this carbon, this proton to this carbon, this proton to this carbon. So, each of the partners each of the protons are transferring the magnetization from themselves to their respective partner meaning directly attached carbon. We do not go from here to here this is not done. This transfer from 1 proton to a little far away that is 2 bond is not done. That can be done and that is also using inept, but we can now very specifically design a INEPT which only goes to 1 carbon we will see that subsequently.

So, this is the standard approach which we do it in a 2D NMR heteronuclear NMR. So, this is something which now we shown schematically which I told you qualitatively, let us look at it a little bit in mathematical way. So, we can see we have excited the proton transfer to carbon, carbon now has evolved with its chemical shift ok. So, proton has not evolved anywhere. So, during INEPT there is no evolution of proton with its chemical shift whereas, the carbon evolves ok. So, when the carbon evolves with its chemical shift, it is the label we use the word frequency labelling and that is during this t 1 evolution t 1 evolution period.

Now, the transfer is back to proton and that proton now evolves with the carbon or the proton chemical shift, but whatever has come to proton has come from this carbon. So, this chemical shift this modulation; modulation meaning the change or modulation happening because of this cosine frequency cosine modulation is actually carried over. This is similar to any 2D NMR experiment, but now it is a carbon to proton correlation. And that if you do a Fourier transform now we can see here compared to what I showed in the previous class, here the second the direct dimension that is the detection dimension which was that we are detecting proton. So, that is now proton that is t 2. Whereas, the t 1 dimension that is in direct dimension is carbon that is here which is coming from here.

So, by doing this transfer back and forth, I am correlating the chemical shifts of carbon and proton. But I have reversed the axis compared to what I showed you previously here its indirect dimension. So, that is why it is called inverse detection, this is called inverse detection is basically we are inverting we are not directly detecting the carbon, we are indirectly detecting the carbon and therefore, we call it as a inverse detection. So, this is the most popular way today to do the experiment that is go from proton to carbon or nitrogen. In case of nitrogen also you can do the same thing. Let us say we have a biomolecule or a molecule, where we have a proton attached to nitrogen, but remember it has to be N 15 we do not study nitrogen 14 and if this is carbon this side is carbon. So, we take this proton transfer the polarization to this nitrogen 15.

So, if it is not nitrogen 15 if it is 14, then we have to look at natural abundance of nitrogen 15. But in biomolecules as I said we isotropically label this nitrogen and make it deliberately nitrogen 15 and that is done for every nitrogen in this molecule in the biomolecule. So, this is becomes a nitrogen 15 to proton correlation. So, whatever I discussed about carbon and proton can be same idea can be used for proton and nitrogen. Except the INEPT will slightly be different now, I mean the INEPT block will not change, but the delay values etcetera the J coupling etcetera will be different for proton to nitrogen compared to proton to carbon. So, we will also look at that as we go along.

So, this experiment which I is shown this correlation, where you have a carbon in the indirect dimension and we have a proton in the direct dimension is called as a HSQC or an so, HSQC stands for Heteronuclear Single Quantum Correlation. HMQC is Heteronuclear Multiple Quantum Correlation and this is for multiple bond correlation. So, HMBC is a very standard experiment which organic chemist use because that gives them a very long range information. So, there is basic related to this proton to a longer range carbon-carbon transfer.

But as I said in a biomolecule like protein NMR, we do not look at HMBC so; much the main experiment which we do is either HSQC or HMQC. Now, these 2 experiments give the same information wise there is not much difference, but sensitivity wise and resolution wise there are lot of differences which we will have to look into 1 by one. So, we look at first HSQC how it works and what is advantages disadvantages then we will go to HMQC, but we will not focus on HMBC in this part of the in this course because

that was covered extensively in the previous course which was more focused on organic molecules.

So, one thing which I should mention and already we discussed this in the previous a few classes ago, that in a homonuclear experiment if you recall we always had a diagonal peak. But in this experiment like HSQC or HMQC or even HMBC, there are no diagonal peaks, why is it so? Because each of the dimension you will see here 1 is hydrogen 1 is carbon. And the reason we got diagonal peak in the 2D homonuclear experiments was because we had proton and proton. So, the proton did not transferred completely to another proton and what was remaining on the same proton was again frequency labelled.

So, 2 times the same proton that frequency labelled in a homonuclear NMR experiment and that results in a diagonal peak. And that possibility does not arise here because we are transferring completely to carbon there is no proton left. Even if there was a proton magnetization left it would not be detected here because we are detecting carbon 13, we are capturing the carbon 13 (Refer Time: 18:24). So, therefore, a proton frequency does not evolved during t 1; t 1 is only left exclusively reserved for carbon. So, there is no chance of having 2 proton chemical shifts correlated during t 1 and t 2. So, if that is the case there is no chance of having a diagonal peak because, frequencies are completely different.

So, therefore, that is a very important come difference between a heteronuclear NMR experiment and it is devoid, heteronuclear NMR experiments are devoid of diagonal peak and that is a very big bonus which we do not appreciate much. But, is a very big bonus for us compared to proton because there the diagonal peak as I told you is sometimes dominates the spectrum the diagonal peak will be about 20 30 time as compared to across p, and that suddenly can cause a lot of other problems and that is not there in case of heteronuclear NMR.

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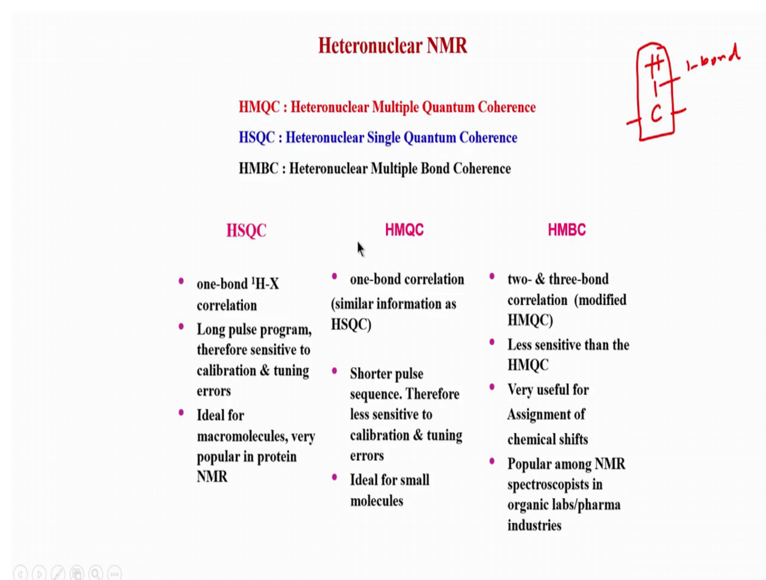

So, these are the differences between the 3 experiments which I just now mentioned, we will not go into details of all the experiments. As I said HMBC is something we not at all look at it, but let us quickly have a look at the differences between these HSQC experiment which stands for heteronuclear single quantum when HMQC is multiple quantum what are the differences we look at the pulse sequence also in the next slides. So, HSQC and HMQC both of them are giving us direct 1 bond proton and heteronuclear correlation. And this is something which I have been emphasising it is basically if you have a molecule you are only looking at this pair 1 bond this is 1 bond coupling ok.

So, this is the most important point we are getting 1 bond for connectivity information in both HSQC and HMQC whereas, HMBC is 2 bond or 3 bond away which we will not look at it. So, the other advantage or disadvantage of HSQC compared to HMQC that you will see shortly is that it has a longer pulse sequence slightly longer it has more number of pulses etcetera. So, sensitivity wise HSQC is slightly therefore, less sensitive compared to HMQC where. In fact, HMQC is ideal if your sensitivity is a big issue. But resolution wise it is much better HSQC than HMQC.

So, as we will see that the popular experiment which people do in case of protein is HSQC, but these days in recent times there are lot of advantages have come up for HMQC for very large systems; there is a mechanism there is a phenomenon called TROSY or experiment called TROSY which can be implemented on HMQC. So, HMQC

as is also very sensitive when it comes to some of the systems; similarly HMQC also can be optimized to do rapidly means fast. So, remember 2D NMR experiments are generally slow and 3 D is even slower than 2 D.

So, to make this experiments faster this is an area of research in NMR, a lot of groups are working on that. So, one of the things they have done is to make an HMQC very fast; so, that you can record it in a few seconds whereas, HSQC takes at least a few minutes. So therefore, there are many advantages and disadvantages of these 2 experiments now we will see as we go along. So, let us look at HSQC let us start from looking at HSQC experiment.

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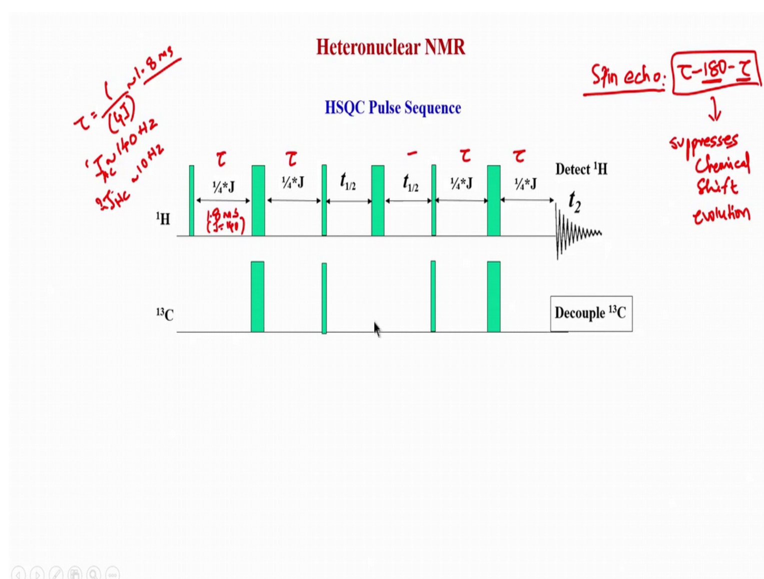

So, that is the pulse sequence shown here now. So, this pulse sequence remember is nothing, but a sequence of pulses applied with a specific gaps in between. So, this is what we had seen in the very beginning was only a 1D experiment, we also saw for 2D COSY and TOCSY and they were relatively simple and in fact, NOESY had only ninety degree pulses, but if you look at HSQC now it is looking pretty complicated. But once you analyse this once you start practicing how to analyse these type of pulse sequence it will become easy.

So, this is something which we cannot learn within a day or 2, I would advise or recommend you to go through this in many different books which are been recommended, also you can go through think about this and every time you look at this

pulse sequence and slowly it will emerge out what exactly happens how it works. So, that requires some practice. So, let us start theoretically how this works. So, this is first thing is you excite a proton with a 90 degree pulse, again the standard thing every time remember we excite a proton first now this part here is a INEPT block.

So, there is a delay, delay meaning sometime before you apply 180. So, in NMR literature or in books in general remember, if you see a pulse which is fat or a broad it is typically denoting an 180 degree pulse means a pulse which rotates the magnetization by 180 degrees. So, it is twice in terms of the period time compared to 1 D. Although in the picture it looks more than twice, but in general a 1 D or a sorry 90 degree pulse is shown as a very short pulse and a broad pulse is referred to as a 180 degrees.

So, in terms of time it is basically double of this if it is both are rectangular pulses. If it is a shape pulse means it is not rectangle, then it will have a different duration that we can we will not go into details of shape pulses in this course. They have more for the advance part if we if we required we will touch upon this as we go along. So, now we excite this proton and we give a very specific delay and this delay is equal to 1 over 4 times J. This J again remember this is J is in the denominator it is not in the numerator. So, this I should write it like this 1 over 4 times J in the denominator ok.

So, now if the J value which we will see later shortly, if it is if I look at consider it as 140 hertz, then my time this time tau let me call it as tau sorry here the tau is equal to 1 by 4 J. So, if you calculate if you do with this is mathematic just simple calculation 1 by 4 into 140 it is 1 by 560. So, this is roughly 1.8 milliseconds ok. So, this value here is 1.8 milliseconds if J is considered as 140. So, remember if this J value is something which is we can change according to our requirement this is not something which is fixed in NMR, it depends on what you are studying for example, this is for this experiment maybe for detecting the heteronuclear correlation in aliphatic system.

Aliphatic meaning methyl methine methylene, but if I go to aromatic spin systems there my J value will be higher it will be a close to 180 or 200. So, I should not use this value I can use a higher value, then in that case the delay will become shorter it will reduce. So, this delay or this period this tau is simply dependent on how much is your J coupling. For example, in HMBC if you remember which we are not going to do in this, but this was done in the previous course in HMBC we are looking at a longer proton to carbon.

So, this is basically a 1 bond J coupling 1 bond carbon proton J, but if I go to 2 bond carbon proton, then that will be much smaller suppose it is let us say a 10 hertz, then I need to use much longer delay.

A tau will now become 1 over 20 1 over 40 milliseconds and that is much longer compared to 560 which we got because of J was 1. So, it is about 15 times longer because a coupling is also about 15 times smaller. So, this tau value completely depends always entirely depends on the J value. So, let us assume that right now for this example that we are looking at proton to carbon 1 bond, in which case we will use 140 which is for aliphatic systems. So, this delay is applied we will apply then a 180 degree pulse, that 180 degree pulse inverts the magnetization of proton as well as carbon.

So, now, you can see here we are applying this pulse both on proton and carbon its not just applied on proton is applied to both proton and carbon. And then after this application we will see that what happens shortly later, we apply another delay again a symmetric delay symmetry you see this is tau, this is also tau. The same delay is applied again and during this portion of the time nothing is going on there is no pulse or any other experiment. So, therefore, this is an empty delay period nothing is happening during this period. But of course, the chemical shift of proton or coupling of proton to carbon keeps happening during this period, but chemical shift is suppressed by doing this being in a centre.

So, whenever you apply a 180 degrees a equal 1 delay before and after equally, that removes any chemical shift evolution and that is called spin echo. Spin echo is basically any experiment like this tau 180 tau degree. So, if you apply a delay a simple delay which is any value you want anything, this can be microsecond, milliseconds, seconds and you apply 180 degree pulse after that, then again you wait for the same amount of delay this is called spin echo. So, what is the advantages of spin echo? It suppresses chemical shift evolution chemical shift evolution or removes chemical shift evolution.

Why that happens we will not go into detail? This is given in many textbook this is a most very basic aspect of NMR it is in fact, spin echo was discovered very early on in NMR and has become very popular in every NMR experiment, the MRI completely depends on this an INEPT is of course, dependent on this and many experiments all use the spin echo. So, spin echo suppresses the chemical shift evolution there is no evolution

which happens for proton and or carbon. And of course, there is J coupling definitely continues, that is not suppressed because the we are applying pulse on both proton and carbon. And that is basically till here the total period now has become 2 tau tau and tau is 2 tau. So, the coupling evolves a coupling happens during the full period of 2 tau.

Then after this period is over we now take the proton which was along the xy plane. So, this was coming in the xy plane we take the proton into z axis. So, after this pulse which is shown here this proton pulse, the magnetization of the proton becomes goes along z axis, but the carbon is excited now and it comes into the xy plane during this period. So therefore, you see this is what we I showed you in the previous slide, that we have a INEPT block. So, this delay 180 delay followed by this pulses, we call this as a INEPT experiment this entire block here ok. And that is INEPT because now that has brought the magnetization from proton to carbon using the J coupling, the J coupling was a crucial factor here.

So, after it has come here it evolves and then it is transferred back. So, what we will do is in the next class, we will continue with this and we will see how the carbon evolution takes place and what is this reverse concept reverse INEPT concept which I showed you and then take it up to the next experiment HMQC which, also has similar elements. We will see that in the next class.