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

Lecture – 13
Heteronuclear multiple quantum coherence (HMQC) and single quantum coherence (HSQC) – Part I

Welcome back to the course, we were looking at 2D heteronuclear NMR spectroscopy in the last class. We started with looking at HSQC which is very basic 2D NMR experiment and it can correlate either proton with carbon or proton with nitrogen. So, we looked at the pulse sequence a brief, now let us look again continue to look at that.

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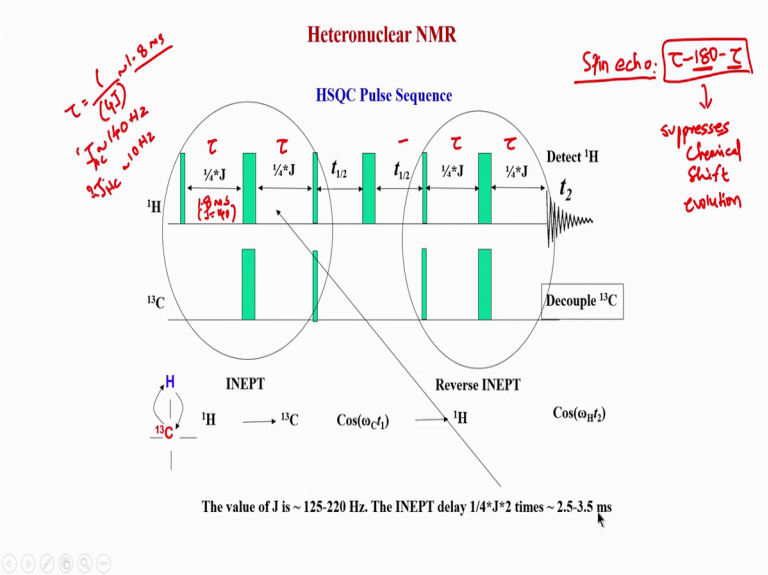

Variant of HSQC is HMQC which we will see next. So, we saw in this HSQC you have basically 3 building blocks or 3 important parts. Number 1 is this called INEPT. INEPT is the part of the sequence where the magnetization is transferred or the polarization is transferred from hydrogen or proton to carbon. It can be also nitrogen, it can be phosphorus, it can be fluorine, it can be any hetero nucleus, but in biomolecules we will mainly focus on carbon and nitrogen.

So, we will see that this INEPT is basically the important part, it does the work of polarization transfer which we saw in the previous class why is it very important. So, this is the first part of the sequence. Once you transfer the polarization to carbon from proton,

a carbon is now excited and the carbon chemical shift evolves during this period. So, this is this evolution which is shown here as cosine omega c into t 1. So, you can see this period here t 1 is shown and it is divided into 2 parts; half this side half on that side and the centre there is a 180 degree pulse on proton. And what is the function of this 180 degree pulse? 180 degree pulse here is there so, that the proton is decoupled from carbon.

So, this is similar to this kind of decoupling which we have seen in 1D NMR or in carbon NMR, that we decouple carbon or proton during detection, but here there is no detection that this is called a indirect evolution period. So therefore, in this particular period we do not apply a continuous decoupling as shown here we apply simply a 180 degree pulse and this 180 degree pulse has to be applied at the centre of the evolution. It should be exactly at the centre, it should not be displaced on this side or that side then the decoupling will not be complete.

So, this is a second important part in this pulse program, that is the evolution of carbon chemical shift during t 1. Then after this is period is over you apply another 90 degree pulse. So, the carbon magnetization which was along the x or y plane is taken to z axis by this pulse and the proton which was along z axis because of this pulse now comes back to x or y plane. So, you see this is exactly the reverse of this. So, this is a reverse of this particular step. So, we use the word reverse INEPT, but otherwise both are INEPT they are actually polarization transfer sequences, they are transferring magnetization from one spin to another spin, but the way the function of this is to confirm proton to carbon here it is back to proton.

So, now during this period again there is a evolution due to chemical shift and coupling. Chemical shift is suppressed because I mentioned in the last class that if we have a something like this, this is called a spin echo sequence here you have a delay or a tau period some delay which can be any duration 180 degree pulse and then another delay. So, if there is a sequence element like this, it supresses chemical shift evolution. So, this is for that nucleus where the 180 is applied. So, in this case the 180 is applied on both proton and carbon. So, there is no question of chemical shift on proton and carbon, but the coupling is not suppressed because of this, the coupling continues to happen and that happens during this total period that is 2 times tau.

So, 2 times tau is 1 by 2 J and we were we did some calculation and showed that you have to adjust this tau value so, to the J value. So, this J is in the denominator remember like here it is not in the numerator as it appears here. So, 1 by 4 J in the denominator 2 times is 1 by 2 J and that turns out to be about 1.8 in plus 1.8, total is about 3.6 milliseconds from here to here assuming a coupling value of 140 hertz. So, remember we are tuning it to a 1 bond coupling. So, 1 bond is shown here this exam. So, therefore, the transfer will take place only from this proton to this carbon, no other carbon or no other proton.

So, that is important to keep in mind because this is very specific to a directly attached carbon. So, this experiment now at the end of this here we start detecting whatever is magnetization is present on proton. And what is that present on proton? The 1 which we started from here; is the same magnetization which has gone to carbon come back to proton is available now for detection and that is detected as cosine omega h t 2 because this is during t 2. And during that period we decouple proton from carbon because J coupling is present. So, this should not split into 2 because of J coupling.

So, now you can see here mathematically what has happened is, we have correlated the carbon chemical shift evolution which happened during this period with the proton chemical shift evolution during t 2. So, therefore, it is a 2D correlation information 2 dimensional information because, every carbon is connected now to its directly attached partner proton ok. So, that is a important point here in this experiment. Now coming to about delay values which already we discussed last time a bit more I can tell you today is that this value of J varies a lot in proton to carbon. So, this is roughly for methyl protons is of the order of 125 hertz and when you go to the aromatic spin system which is often the case in protein we have 3 or 4 amino acids which are aromatic in nature, they will have a coupling of this.

So, you cannot satisfy 1 delay period for all the carbons or all the carbon proton pairs. So, if you satisfy this delay or adjust this delay to 125 hertz then this will be way off. So, this is called missed tuning of the INEPT delays, but that is bound to happen because we cannot have a single number. So, one of the areas of research in NMR has been to have what is called as a broadband INEPT. Meaning given a single delay value can a still get optimal transfer between proton and carbon all the way for methyls as well as aromatics with a single delay in a single delay value and that there are several papers published on

that. For typically what we do is we record 2 different HSAQCs, 1 HSQC we record for the aliphatic part that is for methyl methylene and methine part and 1 HSQC we record separately for the aromatic part in proteins.

So, we have aromatic HSQC which detects only the aromatic portion of the thus, in the molecule and aromatic amino acids such as phenylalanine tyrosine tryptophan histidine etcetera and 1 experiment only for the other part of the protein that is aliphatic the hydrophobic amino acids or the non-polar amino acids. So, this delay period is something which I we already discussed. So, this varies now like this depending on the J values if I use a very short, suppose I use 220 hertz here and you can calculate 1 by 220 times 4 is almost close to 1.1 milliseconds that is for 1 tau value.

So, you now if I multiply with 2 times I will get about 2.2 to 2.5 so, that is the typical value delay period we can use if you are studying the larger coupling. If you are carrying if you are system if you are molecule has larger coupling value and for smaller a large smaller couplings like methyl, we go to higher delay periods. So, this is the very important part in HSQC to understand what are the different components in this pulse program, what are these thing doing ok.

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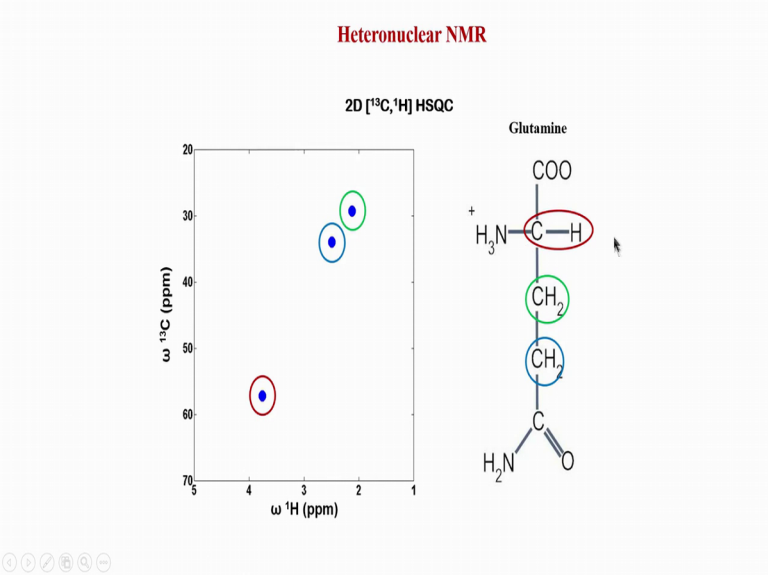

So, let us continue now and look at the peak pattern how does a peak pattern of this comes in this spectrum. So, what kind of peaks you expect to see. So, here is an example of a simple amino acid glutamine is one amino acid. So, you can see now: what are the

correlations we can expect to observe in this spectrum. So, the first peak which is here is about is downfield meaning is close to 60 may cause of the deshielding from this groups. So, because of that this is deshielded and this proton or this carbon proton pair, remember this is we are all detecting this pairs here carbon on this axis and proton on this axis. So, this pair comes around at a 60 ppm. Now the next pair which we will detect is methylene. Now you can expect you may think that these 2 are equivalent, but actually they are not because there is a chiral carbon here. So, there is asymmetry in this molecule molecule is not symmetrical as we discussed for chemical equivalence earlier.

So, therefore, in principle these are not chemical equivalent they are called diastereotopic or prochiral protons. So, there chemicals shifts are (Refer Time: 10:20) proton chemicals shifts will be different which is not shown here, but if you record a spectrum with very good resolution it will come as 2 peaks here. Now in sometime they can be well separated. So, they will come far apart. So, when we look at a real carbon proton spectrum of a protein down the line you will notice this difference. So, now, here you can see this proton there are 2 protons now, but the carbon is attached the same they are attached to the same carbon. So that means, they would not be any shift difference in the carbon, carbon is only single carbon which will becomes around 30. But the protons will have 2 different values if they are have.

In this case we are I have not shown it like that. Now the next 1 is the remaining carbon proton pair which will come slightly down now and why is it again down compared to this green colour here? Because of this amide group to which it is attached; so, there is a deshielding effect because of that and therefore, it comes downfield or lower or higher in chemical shift compared to the other proton. So, this is example of a simple glutamine spectrum. Now you can see there is no other carbon proton pair in this left in this system. Carbonyl does not have a proton attached to it and so on so forth. So, only 3 peaks are expected in HSQC spectrum for this molecule.

So, like this you with this we can call it as a family of protons and carbons in a amino acids. So, we use a word spin system. So, we can think of this molecule where the whole molecule as one family and in the whole molecule there is a spin system carbon proton spin system consists of this 3 groups; methyl 2 methyls and alpha beta gamma ok. So, this is the this is the way how we have to think about spin systems in amino acids and

proteins, because that will be very useful concept when we go for further assignment of proteins and structure determination.

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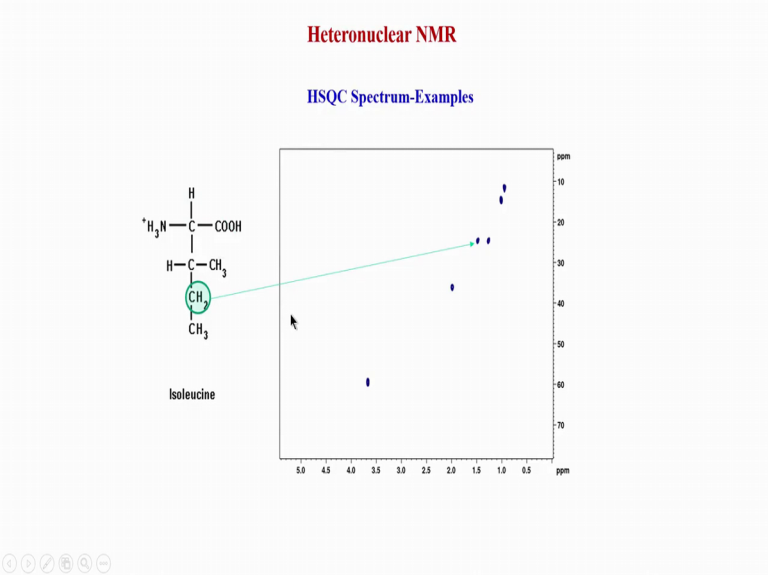

So, let us look at some more examples this is isoleucine and you can see in isoleucine again we have different carbon proton pairs and let us see: what are the ones which we see. So, the methyl group this is called the delta, because you can start from here alpha beta gamma delta. So, the delta proton and carbon of isoleucine are most up field shifted and why is this so? Because they are very hydrophobic and deshield shielded from all the other functional groups; so, they are the most up field shifted then you have this other methyl also here. So, this is called the gamma 2 of isoleucine. So, this is gamma 1, the 1 which is shown here is gamma 1 carbon proton and this is gamma 2. So, for gamma 2 you will get again close to the other delta proton delta carbon.

So, these 2 come here the next is the gamma 1 carbon proton pair. So, here you can see now there are 2 peaks and this is because these 2 protons are not equivalent chemically. Because as I have mentioned in amino acids we have a chiral centre, and also there are they are called diastereotropic or prochiral protons and they are not actually equivalent. They may have accidently the same chemical shift value, but they will not be equal because they are having a different chemical environment around them and there is no plane of symmetry which can be drawn to show their equivalent. Then comes the beta proton there is a single proton single peak and this comes around 39 ppm.

And the carbon is 39 and the proton is around 2 ppm. And the alpha proton again is shifted down because, around 60 ppm mainly because of these 2 groups which are deshielding effect which have deshielding effect. So, in proteins one thing which I should mention at this stage although we will again repeat this are later on is a C beta. This chemical shifts of c beta are very very significant, are very they are signature of amino acids. If I go back in this spectra slide if you look at this here this C this is C beta here and this C beta were somewhere around 29 or 30 ppm. So, that is a signature value for glutamine. So, one can actually try to identify glutamine based on this chemical shift.

Of course, its not that it is unique for glutamine some other amino acids can also have the same chemical nearby chemical shifts, but in general C beta chemical shift of amino acid helps in identifying that amino acid ok. It is like a unique identification for that particular amino acid similarly for isoleucine, it is always comes around 38 to 40 ppm, independent of the structure. There is small variations will be there based on the structure of the protein, but normally it does not vary much. So, again it helps to identify an amino acid based on its C beta chemical shift. So, we will use this idea later on to see how proteins are assigned in chemical shift. So, this will be very useful.

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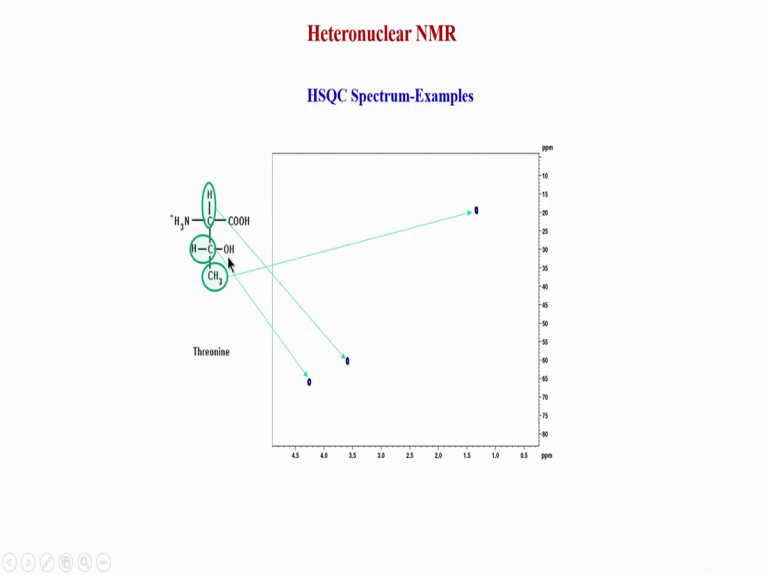

So, this is another example of another amino acid called threonine. So, in threonine this is a very interesting amino acid, because at C beta there is a OH group. So, it will be nice to see: what is effect of this on the carbon and proton chemical shift. So, if you look at

this now again starting from methyl as always methyls are half filled. Half filled meaning they come around 10 to 30 ppm. This is something which we have seen the range of carbon chemical shifts was covered in the part 1D, where we looked at the range of the dispersion of carbon shifts. So, that is we saw that methyls always come between 10 to 30 ppm. So, you can see that is what is shown here.

Now, if you look at the beta proton and carbon here, you can see that there is a very downfield shift this is coming around 70 ppm and why is that? That is because of this OH. The OH has a deshielding electron withdrawing effect on both the carbon and protons. So, the proton is also coming around 4.5. So, you can see if you recollect the previous slides we had the beta protons were not so, down field they were coming around 2 ppm or 2.1 2.5, but here its coming around 4 point to 4.5. So, that is why I just said that is why I said that amino acids C beta are very interesting a very peculiar and for many of them they can be very easily used to identify what amino acid we are looking at.

And this C alpha it comes around again down field shifted around 60 ppm. This is because now it has also it has also this effect and this affect now also it has effect from OH. So, you can see there are 3 different although this is OH is not really close to it, even then there is deshielding effect happening for this more compared to other amino acids. So, this is threonines alpha is also coming down compared to other amino acids other amino acids we saw comes around 60 whereas, this one comes. So, little bit down around 65.

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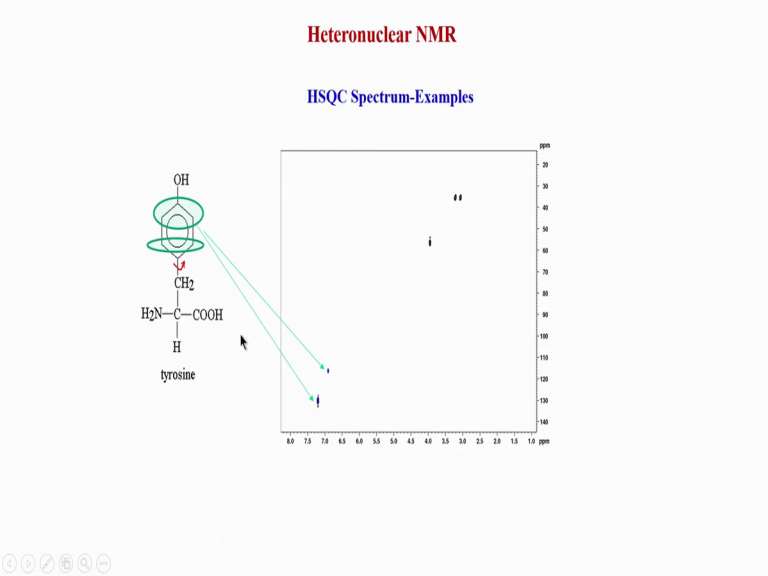

So, this is the examples of different amino acids that we are looking at because in this course we are mainly interested in bio molecular NMR. So, we are looking at all the amino acids as much as possible not all of them, but representative examples. So, example now we are come to the aromatic amino acid. Now aromatic amino acid is very interesting structure. So, you can see for all aromatic typically in proteins you will have this alpha carbon this is amino acids. You have amino group acid group of course, in in proteins you will have a peptide bond.

So, it would not be COOH or NH 2, it will be attached to another neighbouring amino acid this side and a neighbouring amino acid this side and you have now this is the beta there is there are CH 2 group and which is case for all the aromatic amino acids, then you have this rotation. So, what is shown here is this aromatic part and what this red arrow indicates is at there is this aromatic along with OH, they flip in the horizontal axis on this axis very fast. So, this is called ring flipping.

And which happens very high is high speed at room temperature. So, it this aromatic ring is not actually setting static, this is rotating very fast like the methyl groups around this bond ok. So, they have been again a research area on how to how can we slow down this motion of this aromatic ring flipping and several papers have been published on at how to go down in temperature. So, one problem is if you go down in temperature even at 0 if that the flipping is still very high. So, I have to go below 0, but if I go below 0 the

molecules will freeze I mean the sample will freeze because these are typically made in water.

So, how do I prevent freezing and still go to lower temperature is the art which is has been published. So, that something will not go into details now, it was just digression to show you that yes this in flipping is actually very interesting field research on its own. So, in a tyrosine now let us look at the chemical shifts, the CH comes around 60 ppm which is as same as all other amino acids because this has nothing special it is similar to other amino acids. The CH 2 comes around 40 ppm 30 to 35 36 actually between 36 and 40. And again you can see there are 2 peaks here and that is because these 2 protons are not equivalent they are not equivalent, but they are attached to the same carbon.

So, the carbon is a same chemical shift, but the protons are different slightly different. So, about this is about 0.2 to 0.3 chemical shift difference ppm then comes aromatic system. Now if you look at the romantic, you can think of some symmetry here see this 2 as I said they flip around the axis. So, they actually average out which means these 2 protons are almost equivalent, they are not they are exactly equivalent because their flipping so, fast they see the chemical environment similar to each other. Similarly these 2 protons will have a flipping around because of the flipping they will be equivalent in chemical shift.

And because of these effect you will see in the aromatic part which is shown here, here which comes around 120 to 140 aromatic remember always come this the 110 120 to 140 to 145. So, this is the range of chemical shift for carbon where aromatic comes in terms of proton they come between 7 to 7.5. So, this is something which all of many of you in chemistry are aware of. So, that is aromatic part, but as I said because of the equivalence due to the ring flipping, not because of a symmetry in this system because of the ring flipping they are equivalent and therefore, you see only 2 peaks.

That is 1 peak from here and 1 peak from here. So, now, you can think of which is which the one which is up field is this peak and one which is downfield is other one why is it down field because of the OH. So, OH group essentially causes deshielding effect and it results in a down field shift similar to what we saw in the case of thyronine and also glutamine.

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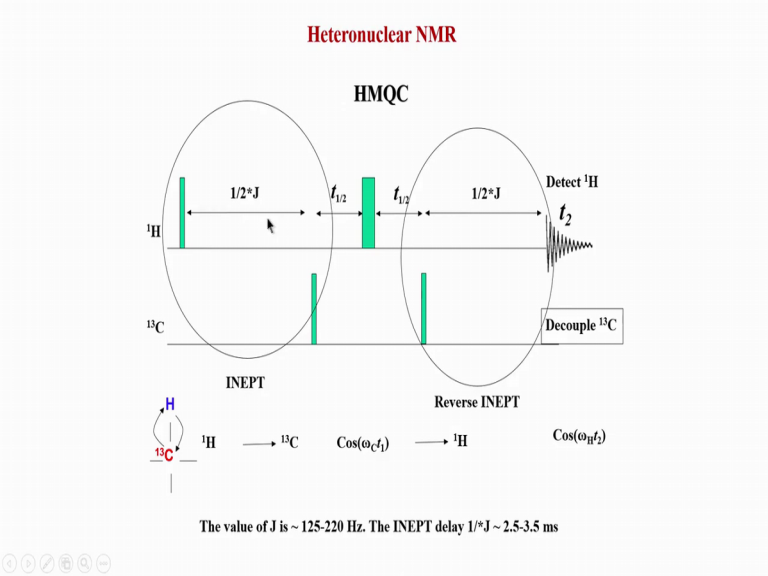

So, now this was example of HSQC we saw with some important amino acid examples, let us come to the next experiment which is very similar in terms of the information or same in terms of the information provided by HSQC but it is much simpler and more sensitive in nature compared to HSQC. So, it depends on what system you are looking at to study typically HSQC is very popularly used, because there are more versions in HSQC you can have sensitivity enhancement and so, on, but the problem is HSQC is a speed. And the speed here can be enhanced in HMQC by varieties of methods which is again a area of research and. So, there have been always equal usage HSQC is also used and HSQC is also used.

So, let us see how HMQC works and we can appreciate the differences between the 2 as we go along. So, again an any as in any pulse program again there are 3 parts here to this experiment one is this part here and the second is the this is INEPT part and there is a INEPT again here back. Although you do not see that 180 degree is here like you saw in the HSQC, but we still use the same idea of polarization transfer like we do in HSQC. So, the polarization is happening because of J coupling. So, that is one thing you have to keep in mind in heteronuclear NMR any polarization transfer between 2 nucleus happens using the INEPT concept INEPT block.

Now, INEPT can be implemented by adding a 180 here to suppress the chemical shift, but to keep the coupling active. Here that chemical shift revolution happens where there

is again suppression which is taken care by 180 here. So, we do not have to bother about 180 here. So, this block actually does the same job from here to here as what we saw in the case of HSQC. And the third important portion of this experiment is this here which is similar to what you saw in HSQC. So, if you recollect the HSQC part this this part is the same there is no difference and finally, you have detection and decoupling.

So, let us go through this again step by step. So, you excite the carbon proton first you excite the proton chemical shift. So, again remember we are in HMQC as well as same as HSQC, we look at only the directly bond proton to carbon pair. Now we correlate the proton chemical shift to its partner directly attached carbon chemical shift. You are not looking at proton to another carbon here neither we are looking for proton to another proton. So, there is only proton carbon correlation and that to specifically only 1 bond similar to HSQC. So, this therefore, the coupling the J value has to depend on this coupling value.

Now, as I we discussed it has a range of 125 to 220 hertz and therefore, you have to tune according to this chemical this values here. So now, once you have this delay here from 1 by 2 J up to this much, the magnetization from proton which was excited here by this pulse gets transferred to this carbon here, but the carbon is not excited yet. So, it has to be excited by applying this 90 degree pulse on carbon. Ones that happens the carbon chemical shift evolves in during this period and that is what is similar to HSQC we have a t 1 evolution and there is a 180 right at the centre and that 180 is because we want to decouple the proton from carbon.

So, then during that period here, the carbon chemical shift evolves by according to this and that is cosine omega c into t 1. Once the evolution period is over we have to transfer it back to proton for detection. So, that is why its called inverse detection. So, now, proton transfer back the back to proton takes place through again a INEPT element which is say called as reverse inept. Now that during that period carbon now has gone from carbon to polarization has gone from carbon to proton and proton, now is evolved during t 2 and detected during t 2 and decoupled decoupling on carbon is carried out during that period to avoid any J interaction or to suppress the J coupling.

And during that period you are evolving. So, you can see this part and this part is similar to HSQC there is no difference as per as the evolution is concerned, but the major

difference here is you do not have 180 to 180s here and 2 180s here. So, the 4 180s which you saw in HSQC were extra which are not used here. And what is the advantage? The advantage is in NMR technically which although we would not be able to go into detail in this course, this 180 has a very bad spin inversion effect it is not a broadband inversion a simple 180 if you take in general 180, it is does not have a broadband inversion and re focusing effect.

So therefore, you lose the magnetization because we want to invert because of the 180. The job of 180 is to invert or refocus a magnetization that is typically not very efficient in NMR 180. So, if you remove 180s like this it becomes more efficient because the transfer efficiency overall is improved or enhanced, but one problem here is because there is no during this entire sequence because the proton is along the x y plane. So, if you look at this here we do not see any other 90 degree on proton which means the proton is always in the x y plane although it is refocused here it is only 180. So, it is brings x to x minus x y to minus y, but it does not bring x to z or y to z. So, therefore, this proton remains in the transverse plane which means is relaxation is going on t 2 relaxation is going on throughout this sequence.

Whereas, in a in a HSQC we bring it along z axis at this point and we bring it. So, from here to here in HSQC it remained along z axis. So, its relaxation was not affecting during the chemical shift evolution of carbon, but here it is now affecting number 1. Number 2 is that the J coupling between proton to proton is active during this portion of the time and this is I am talking about this proton to another proton nearby. Suppose there is another proton which is 3 bond away that proton to proton coupling or if there is a proton which is 2 bond away a germinal proton, they will be couple to each other and their coupling is sufficiently strong.

And that coupling evolves or also is takes place here and mathematically you should add another term here which I have not shown I have ignored that, but technically there is another term which is cosine pi J t 1 means the J coupling also evolve. But that does not happen during the HSQC experiment. Because in HSQC we put the magnetization of hydrogen proton along z axis at this point this you should recollect from the previous slides; now when it is along z axis the coupling will not evolve. So, there is a theorem or a theory in NMR which says if you take a magnetization along z axis, it does not evolved to other with other protons due to J coupling.

So, that because of that there is no J coupling between proton and proton and similarly between proton and carbon anyway we had decoupling here and also in HSQC. So, that is not an issue the main thing is proton to proton coupling is active here which was not the case in HSQC. So, that its creates an extra dampening or reduction in the signal because of extra coupling which was not there in HSQC was suppressed. So, therefore, overall the HS impression QC reduces in sensitivity because of these factors the one is the proton relaxation second is the coupling which degrades the resolution also the spectrum.

So, it is therefore, sometimes not popularly used for bio molecules, for small molecules it is very popular, but as I said you can improve this sequence and make it faster with some latest technique development which has happened and that therefore, makes this popular if you want to record data very rapidly. So, this brings us to the end of the HSQC, HMQC we will look at more detail of HMQC in terms of the quality of the of the peak pattern again similar to what we saw in HSQC, but as I said HSQC peak pattern is same as HMQC there is not much difference. So, as far as the peak structure is concerned they all look same.

So, in the next class we look at some examples of HMQC. And, then from there go on to another 2D experiment which is again very popularly used and following that we will go onto 3 D NMR.