NMR spectroscopy for Structural Biology NS
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Lecture: 21
Introduction to NOESY and HSQC 2

So, we continue with the discussion of the heteronuclear correlations as I mentioned the most common experiment here is the heteronuclear single quantum coherence which is also called as HSQC.

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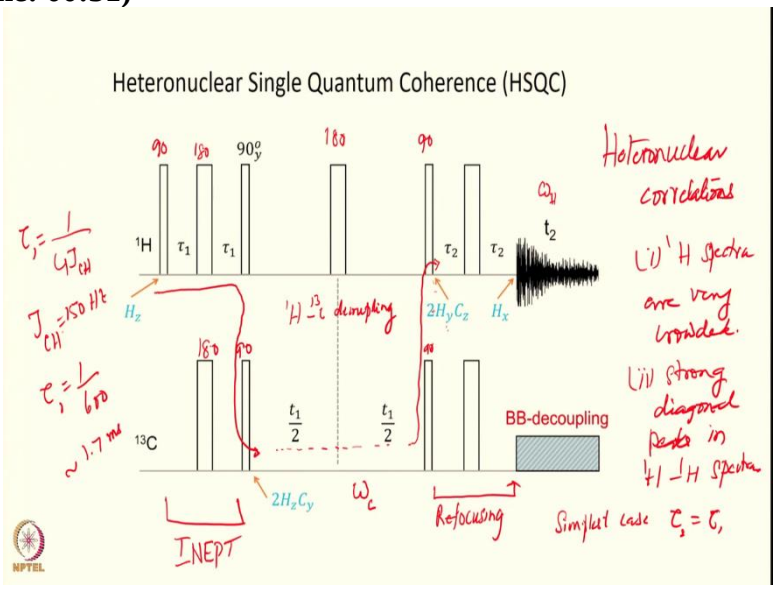

Now how does this experiment go let us look at the pulse sequence of course there are two channels here. Now you apply the pulses on one channel these are called as the proton channel you selectively apply pulses on the proton these are not selective pulses they are non selective pulses are hard pulses then you can also apply pulses on the carbon channel. So, we have the carbon here.

So, you require 2 different coils in your spectrometer you have a carbon coil and a proton coil. So, pulses on proton coil are applied with using the proton coil this channel and pulses to the carbon 13 are applied using this channel here and there will be no interference between these 2 because these are. So, widely different frequencies if this are 500 megahertz this is 125 megahertz if it is 600 megahertz this is 150 megahertz.

Because one fourth and if it were nitrogen 15 here this is one tenth of the proton if it is 500 here it is 50 there for nitrogen and like that. So, now how does this experiment work. So, here we start with the proton we start with the proton magnetization that is we represent it as H z and then this sequence is already what we have seen before this is called as the INEPT sequence. So, you have proton mechanization 90 τ 180 τ and there is a 180 on the external as well here let me write that as 190 and 180 we write it as 180 and this is also 90.

So, now when I do this so this is called as the INEPT experiment. So, during this period the proton is. Now transferred from the z magnetization it comes to the transverse magnetization and this will evolve during this period with the coupling to the X nucleus carbon 13 proton

coupling and from here these two, 90° pulses the magnetization is transferred to the carbon. So, this is the INEPT sequence.

Let me write here this portion is your INEPT, INEPT transfer. So, therefore the magnetization flow how does the magnetization flow? It starts here comes here and then it moves to this. Now this is. Now the magnetization here is on carbon how do we know that is what is represented by $2H_z\ C_y$. So, this C_y means it is a carbon magnetization here because it is on the carbon 13.

So, the carbon magnetization which is transverse this will evolve during the period t_1 and in the middle of the t_1 period I apply a 180° pulse onto the proton channel. So, when I apply 180 pulse to the proton channel what does it do it actually decouples proton carbon coupling. So, this is a spin echo period from here to here t_1 half 180 t_1 half this is the kind of spin echo period and during this period there is proton carbon decoupling.

So, we can write here proton carbon decoupling that happens and the carbon frequencies will evolve with the carbon frequencies. What frequencies will be present here let us say the frequencies which are present elevated as ω_C carbon frequencies are present whatever carbon is attached to the proton and that from whichever proton magnetization is coming to the particular carbon and that is what is evolving with the frequency of carbon.

So, this will continue like this carbon magnetization evolves then it is transferred back to the proton magnetization goes back to the proton by these two 90° degree pulses this is 90 here this is 90 here by these two 90° pulses magnetization goes back to proton this is again an INEPT. Now here it is a reverse INEPT this is the reverse inner transfer this is the refocusing we have got the magnetization on the proton once more but this is $2H_y\ C_z$.

So, this is anti phase proton magnetization and here it was the carbon magnetization which is antiphase with respect to the proton that remains like that only when I apply the two, 90° pulses it is converted to antiphase proton magnetization and then during the refocusing period I will get here the in phase proton magnetization. So, H_x represents in phase proton magnetization. So, the in phase meaning then when I detect the signal during the period t_2 I can decouple carbon all the proton carbon coupling is removed during the t_2 period all right.

So, what we have now only the proton frequencies are present during the t_2 period there is no coupling during the t_1 period what do I have I have only the carbon frequencies I do not have the proton frequencies. So, let me repeat this here we start with the proton magnetization which is the z magnetization and during the first INEPT sequence we transfer the magnetization to the carbon it comes as anti phase magnetization.

Now what does this tau one depend upon tau one depends upon the time required for this INEPT transfer typically in the case of proton carbon this will be typically τ one will be of the order of 1.7 millisecond that is approximately $\frac{1}{4}J$, $\tau_1 = \frac{1}{4}J_{CH}$. So, if let us say your proton carbon come these are one bond couplings. So, the one bond coupling if it is let us say 150 hertz assume 100 is in the higher side if it is 150 hertz.

So, $\frac{1}{4}J$ is approximately $\frac{1}{600}$ therefore approximately 1.7 milliseconds. So, if J_{CH} is equal to 150 hertz one bond coupling. So, tau one will be 1 by 600 and that will be approximately 1.7 milliseconds. So, therefore it is extremely small period very small period you apply this and

then you have the 180 degree pulse and you have the transfer coming here the magnetization remains is 2 H_z C_y.

And then it goes back to after frequency labelling with the frequency of the carbon during the t_1 period it goes back to the proton and this refocusing period also τ_2 should be roughly equal to t_1 τ_2 is it need not be exactly equal there are other advantages of having $t_2 \neq t_1$ but by and large in the simplest case τ_2 can be equal to simplest case τ_2 can be equal to τ_1 there is a refocusing.

So, you get H_x there and you have the then the decoupling happening from the on the carbon channel. So, proton carbon coupling is removed during the t_2 period. So, therefore you have in the t_2 period only the proton frequencies and the t_1 period we have only the carbon frequencies. Now let us see what happens how does, the spectrum look see this is what it is.

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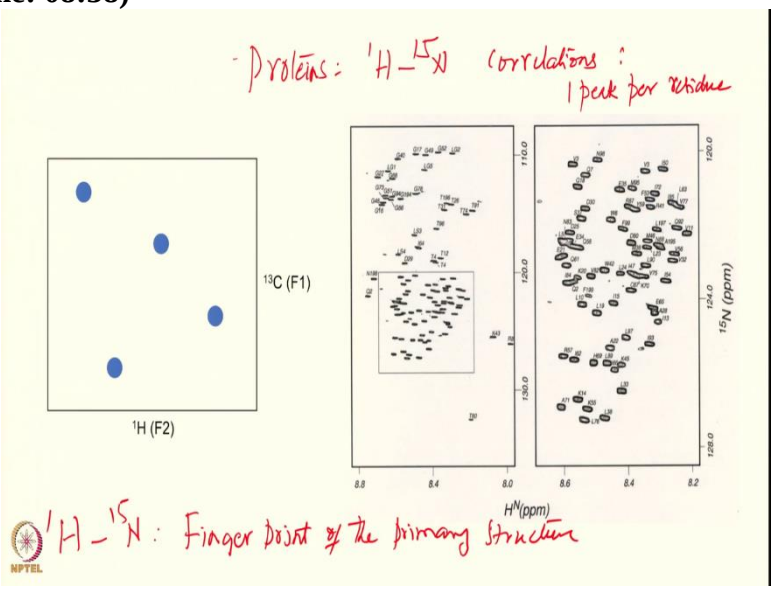

Along the F_1 along the F_1 as I said or the ω_1 the t_1 evolution I have only the carbon frequencies along the F_2 I have only proton frequencies therefore this is like cross peaks only right the entire spectrum has only cross peaks there is nothing as a diagonal here. We are only cross peaks you have the proton carbon correlation spectrum. So, if there are 4 different carbons here you will have single peak each one, one peak each for this.

Now notice the carbon frequency range is quite high almost like 100 ppm you can have forget the carbonyl and if you take the aromatics it can go up to 120 – 130 people the whole range a wide range of frequencies here and the proton frequencies can go to from the all the way from 0 ppm all the way to 10 ppm. So, you will have all of these proton frequencies here so, one peak each for one carbon.

So, this is very, very simple quite a substantial enhancement in the information content. Now here I have shown you the schematic with regard to the carbon but you can also have the nitrogen here and typically in the case of proteins we do proton ^{15}N correlations. So, for proteins we have proton nitrogen 15 correlations these are all one bond couplings every amide group every amine group of an amino acid residue produces one correlation peak.

So, one peak per residue so, this is the; and this produces one peak per residue along the backbone I am looking at the backbone. So, it will produce one peak per residue. Now this is

an experimental spectrum of a particular protein. So, it does not matter which protein it is all labelled there you can see here this is the ^{15}N axis the F_1 axis is the ^{15}N axis that is indicated here goes from 110 ppm to almost about 30 ppm range.

And here you see such a wide distribution of peaks and these are all correlation peaks only nothing else and on the top you have the glycines then you have the threonines here then you have various other ones asparagines alanines. And things like that this is the little crowded region by and large this area is more crowded this contains all the aliphatic amino acid residues the leucines and the alanines isoleucines glutamine everything will come here all the aliphatic ones.

And this is the blow up here you can see this portion is blown up here in this the right side spectrum. All of them are very distinctly seen and you can actually identify all of these peaks this is the spectrum of something like about 130 amino acids and in a proton spectrum it is impossible to get this kind of a separation. Impossible to get this kind of separation proton-proton correlation spectrum and in the N15 it is easy to get the N15 lines are also quite sharp.

Therefore you can resolve these ones very well and since there is only one peak per residue one peak per residue you can simply count to the number of peaks here and see whether you have got all the peaks or not. So, if you have a protein of 100 residues you should get hundred peaks barring prolines. The prolines do not have the amide proton. So, the prolines will not produce a peak here.

All other all other residues will produce a peak therefore by simply count the peaks here and see whether you got the proper spectrum. Therefore this experiment is called as the fingerprint of the primary sequence. The primary structure essentially it is a amino acid sequence this is which one you are talking about now proton ^{15}N. Proton ^{15}N correlation spectrum is a fingerprint of the primary structure.

This is I here it looks like a J. So, this is I fingerprint of the primary structure. So, this for this is an extremely useful experiment to perform. Now can we do something more than this we got the HSQC spectrum we got the NOESY spectra 2D correlation spectrum. Now we can; we combine these 2 combine these two information's well you can do that.

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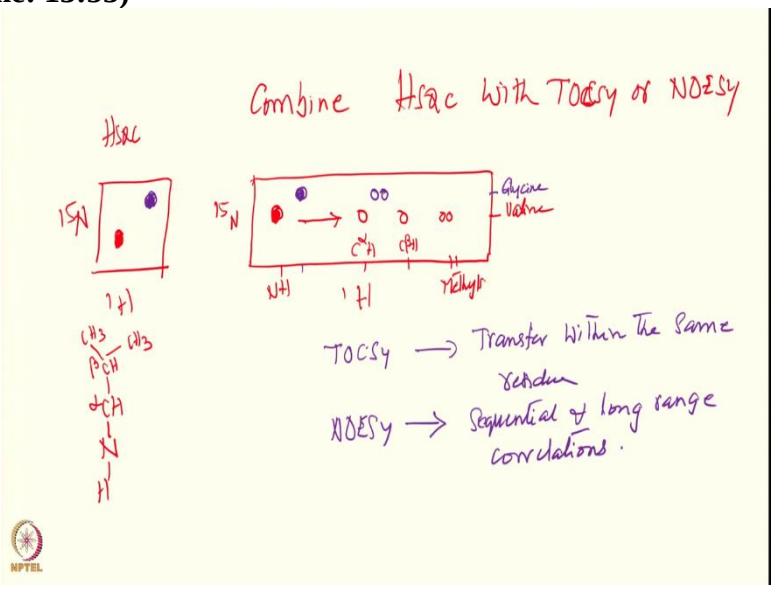

So, these are called as then you can combine HSQC with TOCSY or NOESY the pulse sequence can be designed approximately that way and what is what are you going to get here the HSQC spectrum let us say we talk about the N15 proton. So, I will let us say I have here N15 and I have here the proton assuming that I have one peak here which belongs to a valine residue let us consider a valine residue.

So, what is the valine residue NH, C, CH this is the CαH here then I have the CβH then I have 2 methyls. These are the γ ones and the γ twos in this case what will I see I will see only one peak here in the HSQC in the HSQC. But now what I want to do is I want to transfer the information what we have transferred here we initially started with the proton transfer to the nitrogen and back to the proton to measure it.

Now what I want to do is once it comes back to the proton i want to transfer this to the alpha proton to the beta protons and the gamma protons I want to transfer this I can do by the TOCSY or the NOESY. So, therefore what will I do I do I combine this with either the NOESY or the TOCSY this is the proton axis here and this is my NH proton and here I have the CαH I have the CβH and here I have the 2 methyls I have this peak here this is my HSQC spectrum NH to the ^{15}N on this axis I have the ^{15}N.

But now I want to relay this information once it is on the proton I will relay this to the other protons. So, what will I get I will get a peak here I will get a peak here and I will get peaks here. So, therefore this is my TOCSY HSQC. So, I am using the full proton range here while I will have only the correlation peak from the proton to the nitrogen 15 by combining this with the TOCSY mixing sequence or a NOE mixing sequence.

I can generate this information from NH proton to the α proton or to the β proton or to the γ protons and if I have another residue some other residue let us say. So, let us say I have only the glycine suppose I have a glycine this is for the valine this was for the valine case. This was the valine case suppose I have the glycine. Now let me use a different colour for that suppose I have a glycine here which is usually on the top of the HSQC spectrum and this is at a different NH chemical shift.

And let us say now that NH chemical shift is here I will have this peak here then I will have two to the alpha protons of the glycine. If the 2 glycine, glycine has 2 alpha protons if the 2 are non equivalent then I will see 2 peaks here. Now this will be for the glycine. So, by doing this you can actually identify all the peaks of the amino acid residues individual spin systems as well from a combination of these sequences.

Now if you do a TOCSY this will happen within the transfer within the same amino acid residue but if I do it NOESY this also will have sequential and long range correlations see here there is no diagonal you will only have cross peaks therefore in either case when you combine this making use of the dispersion of the ^{15}N chemical shifts then you will be able to separate out and identify the individual amino acid residues by looking at this relays it has also enormous structural information.

So, in the next slide I will simply give you the pulse sequence how it is done we will not go into the details of that one there.

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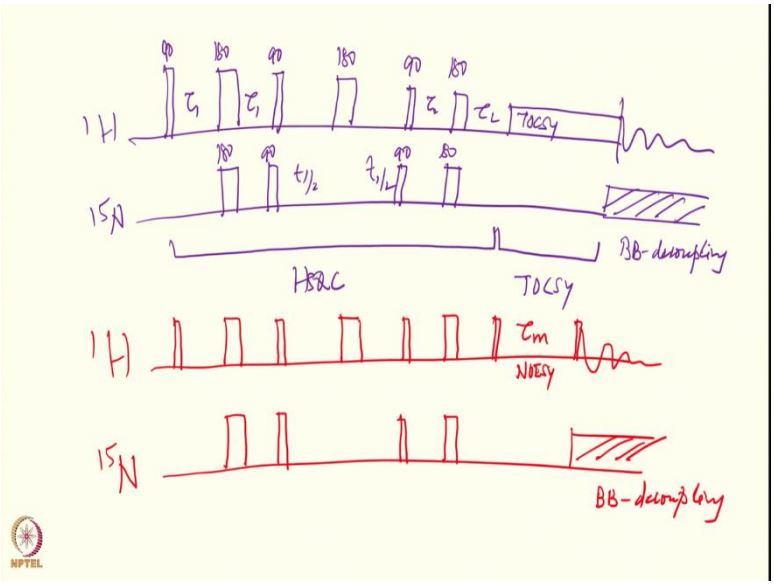

But I will just indicate to you the pulse sequences. So, here I have the HSQC TOCSY. So, it starts in the same manner I have two channels here. Let us say I call this as ^{15}N and this is 180° pulses on both the channels this is the proton channel this is τ_1 then I have the 90° there the 90° here this is a nitrogen 15 and this is $\frac{t_1}{2}$ then I have a one hundred eighty degree pulse here no pulse here then this is again another $\frac{t_1}{2}$.

Then I have two, 90° pulses as in the HSQC and after this I have this τ_2 this is now; these are all 90 180 90 180 this is 90 this is 180 and this is 180 here this is 90 then this is 90 this is 180 and then I have another τ_2. So, then I come to this point here this is the refocused point this is again done. Here I introduce what we call as a TOCSY, TOCSY mixing. TOCSY mixing is used and after that I collect the FID and when I collect the FID here.

Then there is broadband decoupling. So, this portion is HSQC from here to here and then I have combined that with the TOCSY. So, this is the HSQC TOCSY spectrum I can do the same thing for the NOESY, HSQC NOESY let me use the different colour for that. So, this portion is the same and then I will again this also is the same here. Now at the end of this what we will do is the ones different the NOESY mixing I have to introduce.

For the NOESY mixing I will put here a 90° pulse which creates the z magnetization and from here onwards it is a mixing time there is a tau's, τ_m this is the NOESY and at the end of this I have another 90 and we collect the FID and when we do that then we decouple this, this is broadband decoupling. So, therefore it goes exactly the same way except when you want to do the NOESY mixing.

Then you will have to use this sort of a modification in your pulse sequence and you have depending upon you can adjust your tau and do it in the same way as you do in the normal 2D experiment in the normal NOESY or simply in the one-dimensional NOEs this depends upon the t_1 period the same conditions. Same criteria are applicable here as well. So, this is proton and this is nitrogen 15 and you get the relay information in the NOESY as well and also the sequential connections.

So, this is the 2 dimensional experiments. Now let us see we can go forward in the little bit more complicated situation here we extend it.

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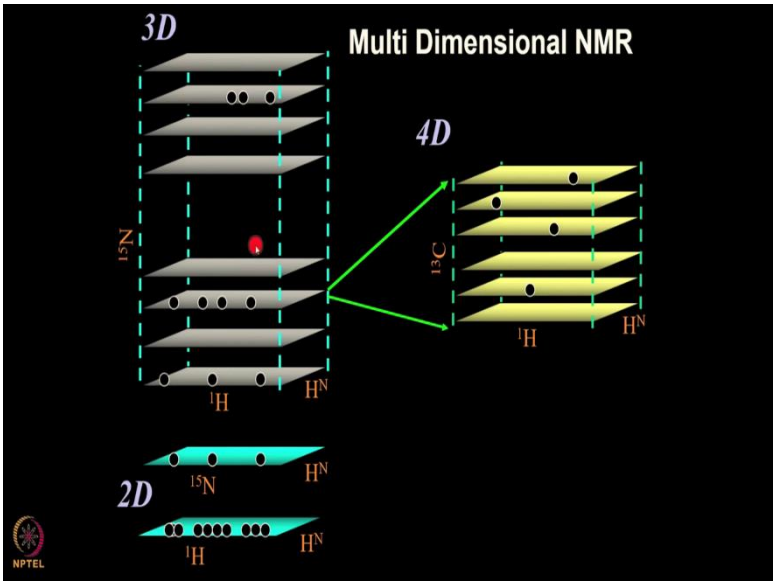

Now how do we use this combine this it to improve the experimental resolutions okay. Now you combine 2D, two kinds of 2D spectra and generate a three dimensional experimental sequence. A three dimensional experiment how is it produced a three dimensional experiment is produced by doing a 3D type of time data collection. So, 3D experiments so, this is typically as with the schematic you have a period here the t_1.

Then you have a mixing this is a cordless m one then you have the t_2 then you have a mixing which you call as m_2 then you have the data collection which is t_3. So, here we will generate one frequency axis F_1 F_2 and F_3. So, we can have different combinations. So, m_1 is a mixing which will produce one kind of a correlation m_2 is mixing which can be another kind of a correlation and these can be heteronuclear experiments as well the combination of heteronuclear experiments as well.

I will illustrate this to you how this is going to be useful in 3D or 4D. If you want to go to the 4D then of course you must introduce one more step there as mixing sequence m_3 and then the t_3 when I say make m_1 or m_2 it includes all of that what it described before for the HSQC what sort of mixing you use for the TOCSY what mixing you use for the NOESY what mixing you use and this is a the benefit of this is schematically indicated in the in this slide.

Let us see here you have a 2D proton-proton spectrum here proton-proton spectrum and at this point you have. So, many peaks here the one particular amide proton is producing those. So, many peaks at the same NH chemical shift it is producing. So, many peaks it is difficult to figure out they are where they are coming from. Now if I do on the same sample a proton nitrogen 15 correlation spectrum this is the HSQC spectrum as indicated to you then you will see at the same NH proton I am seeing three peaks here three and 15's.

3 ^{15}N what does that mean it means at least there are three amino acid residues who have the same NH chemical shift three amino acid residues which have the same NH chemical shift and those ones are showing me these three nitrogen 15 peaks for the nitrogen 15 chemical shifts both those three amino acid residues are different. Now what I do is I take this spectrum pull this apart using the ^{15}N chemical shift in a 3D manner.

So, I put the ^{15}N chemical shift on the third axis and these two axis are proton-proton axis on the third axis I put the ^{15}N chemical shift in other words all these peaks which I am having the

10 peaks which are present here I am pulling this along the nitrogen 15 axis. So, what I get here is three peaks which is easy to understand. Now well this can be NH to the alpha to the beta to the gamma of a particular amino acid residue.

And then another amino acid residue this produces 4 peaks which is certainly possible you have the from the NH proton to the alpha to the beta the gamma and the delta of that amino acid residue and you have a third and fifteen third amino acid residue whose ^{15}N chemical shift is here that is this. So, we have this N15 chemical shift is this one here this ^{15}N chemical shift is this one here.

And this ^{15}N chemical shift is this one here along the ^{15}N chemical I have here three peaks this may well be the NH to the alpha and then to the 2 betas maybe the threonine or the serines and things like that. So, you have these three ones here. Now if there is any further ambiguity in these ones you can also use carbon chemical shift then you go to the 4D we will not go into that detail here.

So, we can use the carbon chemical shift to separate these ones out further on the basis of the carbon chemical shifts. So, then you will have all of these each plane is. Now separated into multiple planes here having different carbon chemical shifts here this becomes a 4D experiment. So, you have the 2D experiment proton-proton and you combine that with you combine that with a nitrogen 15 chemical shift you generated a 3D spectrum wherein the proton-proton correlation peaks are separated out on the ^{15}N axis.

And then if you have further complications arising which may not be maybe may not be if it happened then of course you need the 4D but of course all of this will increase your experimental time as well because the t_1, t_2, t_3 all of them will have to be independently incremented. So, that will take a quite a tall on the experimental time on the machine time. So, by and large one tries to minimize these number of dimensions you try to get as much information as possible from the 2D or the 3D experiment. So, therefore this is going to be extremely useful in all our future experiments.

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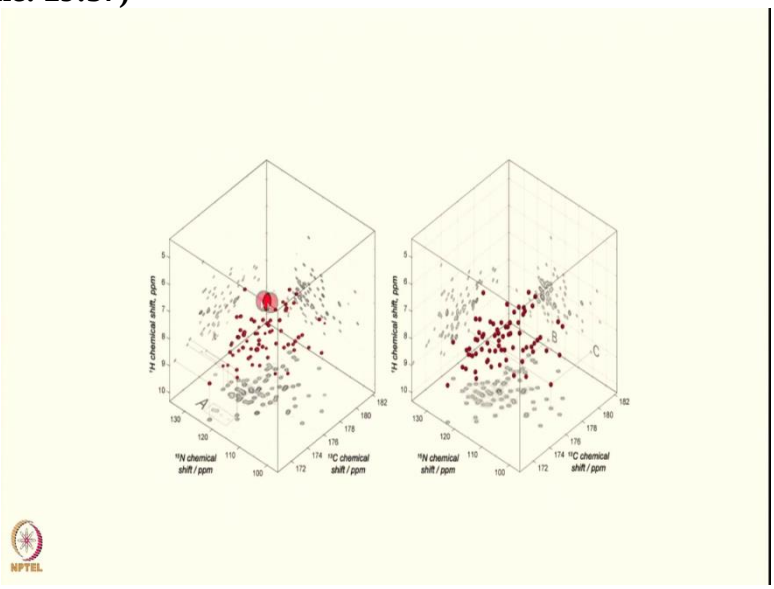

So, this is an experimental spectrum of a 3D spectrum see here you have proton chemical shifts on one axis the nitrogen chemical shifts on one axis and the three see that in chemical shifts on the third axis all these are different chemical shifts. So, the peaks are appearing in. Now in a

box and if you take projections of this on to these individual planes then if you take a projection on this bottom plane then you get nitrogen 15 ^{13}C correlations nitrogen 15 ^{13}C correlations.

You will get if you take a projection on this plane then you will get the nitrogen 15 proton correlations. So, therefore depending upon which projection you take you can get the different kinds of correlations there and. So, therefore this is how the 3D spectrum looks like. Now you see the instead of in a plane. Now you are putting the peaks in a box this is enormous visualization possible if you go to 4D it is not easy to visualize this but up to the 3D you can visualize this. So, I think we will stop here.