NMR spectroscopy for Structural Biology NS
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Lecture: 20
Introduction to NOESY and HSQC 1

So, till now we considered 2 dimensional NMR experiments of different types J correlated spectroscopy. We discussed a couple of those examples COSY double quantum filtered COSY, TOCSY, J scaling ω_1 decoupled COSY or also called constant time COSY all of them were based on transfer of information on the basis of J coupling. The mixing used the J coupling information to transfer information from one spin to another spin.

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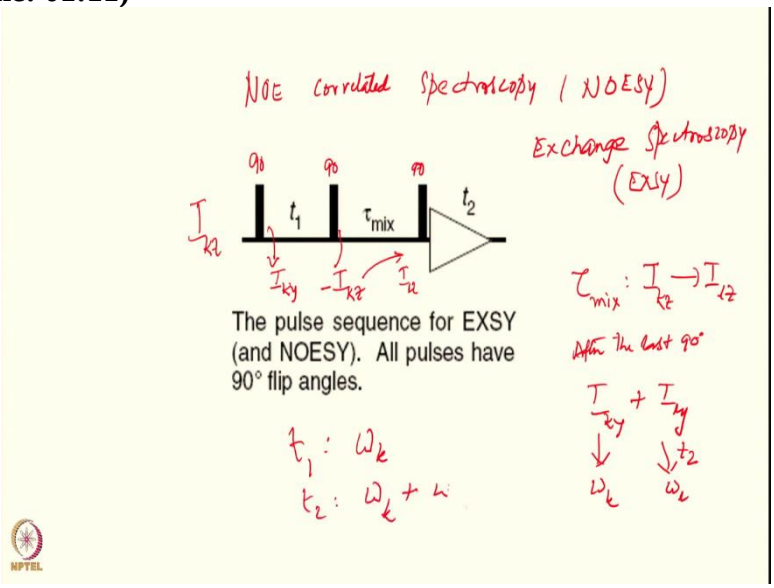

So, now we will look at another kind of transfer process which is based on the dipolar interaction. The dipolar interaction leads to what we call as nuclear overhauser effect and that is the NOE, NOE correlated spectroscopy or briefly this is written as NOESY it is also called as exchange spectroscopy it is also called as exchange spectroscopy because the mechanism of transfer although it is through exchange.

The appearance of the peaks will be the same exchange spectroscopy and this is called as EXCY the spectra appear in the same manner the pulse sequence is the same for both and you will can both kinds of information in this particular in this spectrum by and large it is the NOESY which is used for structural purposes for large molecules or even small molecules for identification and exchange spectroscopy is limited to those situations where there is actually chemical exchange chemical exchange between two confirmations that happens.

And you will see peaks occurring because of the exchange process. Now so, how does this work? This is a pulse sequence indicated here you have the first a 90 degree pulse here another 90 and a third 90. There are three 90 degree pulses here just like in the COSY we had two 90 degree pulses 90 β or something double quantum filtered COSY also has three we had three pulses but now we have a particular time period called as the mixing period.

You start the pulse sequence with a 90 degree pulse which creates a transverse magnetization and after the transverse magnetization evolves during the period t1 it is frequency labelled with the particular frequency whatever is the frequency that is present. So, for example if I want to start with the magnetization of the k spin the I_{kz}. So, then if I apply 90 degree pulse I will generate transverse magnetization of the k spin here.

And this will be I will call it as I_{ky} or something and then this will evolve during the period t_1 with this characteristic frequency which is ω_k. Now the second 90 degree pulse what it does it is adjusted in such a way that you select from the various kinds of coherences that will come we discuss the various types of coherences in the last class previous classes. That we had the z magnetization double quantum filtered coherences transverse magnetization single quantum coherences all of those things we discussed.

But what we will do here is we will adjust these 2 90 degree pulses their phases in such a way that at this point I will have the I_{kz} back again but with a negative sign. So, it is an inverted z magnetization of the k spin. We start with the equilibrium magnetization of the k spin and. Now I will at the end of this I will second 90 degree pulse I will have the z magnetization of the k spin but now it carries with it the frequency level because of the evolution during the period t_1.

Now during this period tau mix there is relaxation happening the relaxation what does it do it transfers magnetization between 2 spins. Now if there is a l spin which is close to the k spin in space. So, that it can interact through dipolar interactions between the spins then it will transfer part of the magnetization here to the l spin. So, we will get some amount of l spin here to the z component I will get little bit of the lz.

So, z magnetization of the l spin I will get. So, therefore during the mixed time what happens I will have I_{kz} transferred to I lz partly not entirely partly it is transparent there will also be little bit of the kz at the left. So, then when I apply the last 90 degree pulse then both this I_{kz} and I_{lz} will get converted into transverse magnetization. So, I will get after the after the last 90 after the last 90 degree pulse I will have both $I_{ky} + I_{ly}$.

Both the transfer magnetization components will be present and these ones this will evolve in t_2 with the characteristic frequency omega l this will evolve with the characteristic frequency omega k during the t_2 period. Therefore I_{kz} in the in the t_1 period I have the frequency ω_k therefore what I will have then the t_1 period I have ω_k and in the t_2 period I have mixture of $\omega_k + \omega_l$.

And this transfer has happened through the dipolar interaction or the exchange process. So, therefore the 2 dimensional spectrum therefore will represent the transfer process.

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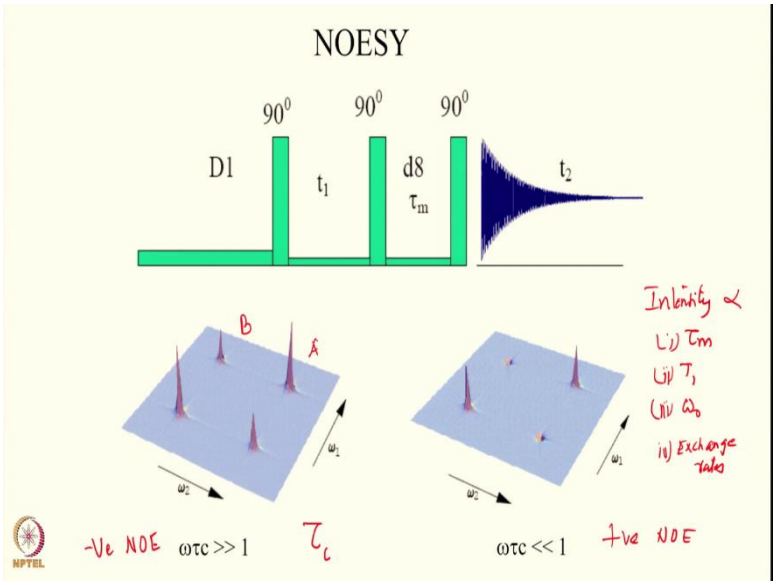

So, here this is indicated in the more explicit manner. So, we have the NOESY you start with the relaxation delay here. So, you may do something saturation whatever something here that is for proton saturation or whatever same thing in the preparation period. So, you have this 90 t_1 90 and this mixing time tau m is indicated by d8 and the third 90 degree pulse and after that you collect the data as a function of time t_2.

Therefore two-dimensional Fourier transformation will produce peaks with a diagonal this is the diagonal here in this spectrum and we have this cross peaks here these are the cross peaks and these ones will appear either because of the dipolar interaction or because of the exchange process between the two sides. So, suppose these two sides are A and B suppose this where this site is called as A and this site is called as B.

And if there is an exchange between A and B then you will see a cross peak between these 2 you have the diagonal and also you will have the cross peak. Now depending upon whether the motion inside the solution is fast or slow this kind of a NOE which is there will have a positive and negative signs this we have seen before the NOE can be positive or negative when it is positive when when $\omega \tau_C$ is much smaller than one when $\omega \tau_C < 1$ then I will have a positive NOE.

And when it is much larger than one it will be negative energy and what is ω is the spectrometer frequency and τ_C is the correlation time. So, notice this is τ_C here τ_C this is correlation time if this is very, very large which is the case in case of larger molecules then you will have $\omega \tau_C$ much larger than one but in small molecules where the τ_C is very small of the order of 10^{-10} to 10^{-12}

Whereas in large molecules it is of the order of 10^{-8} 10^{-9} in such situations you will have two different signs for the NOEs this we have seen. So, here you will have positive noise positive NOE and here we will have negative NOE and how does this reflect in the 2D spectrum? In the 2D spectrum you see here you will have both the cross peaks and the diagonal peaks appear with the same sign.

So, they will appear on both they're like this is up this is also up on the same side of the plane whereas if this is positive NOE you will see that these ones are appearing on the negative side these are going down they are going below the plane these peaks are going below the plane

that is why it is looking like this. These are the diagonal peaks and these ones going down the plane and those that is an indication that whether it a molecule is a small molecule or a large molecule.

This is extremely important because it directly you can distinguish where there is very rapid motion in a small molecule or when the large molecule also there is a certain segment which is extremely rapidly moving almost behaving like a small molecule then you can distinguish these different kinds of NOEs in the same spectrum. So, this is an extremely important parameter and now what does this intensity of the cross peak depend upon here?

The intensity of the cross peak first of all depends on the mixing time here intensity, intensity will depend upon is proportional to 1 mixing time this is the tau m and it will depend upon the t_1 which is the spin lattice relaxation time longitudinal relaxation time of the molecule or the particular spin which you are considering and then it will also depend upon the spectrometer frequency omega naught because ω_0 τ_C can be larger or smaller depending upon the spectrometer frequency as well.

And then in the case of exchange then you will have the exchange phenomena exchange rates. Dipolar interaction will be will be the driving factor for the NOE and for the in the where there is exchange happening there is a transfer magnetization through the exchange process which results in the cross peaks. So, from the point of view of structural biology both are important and by large for structure determination of large molecules it is the NOE which is extremely useful.

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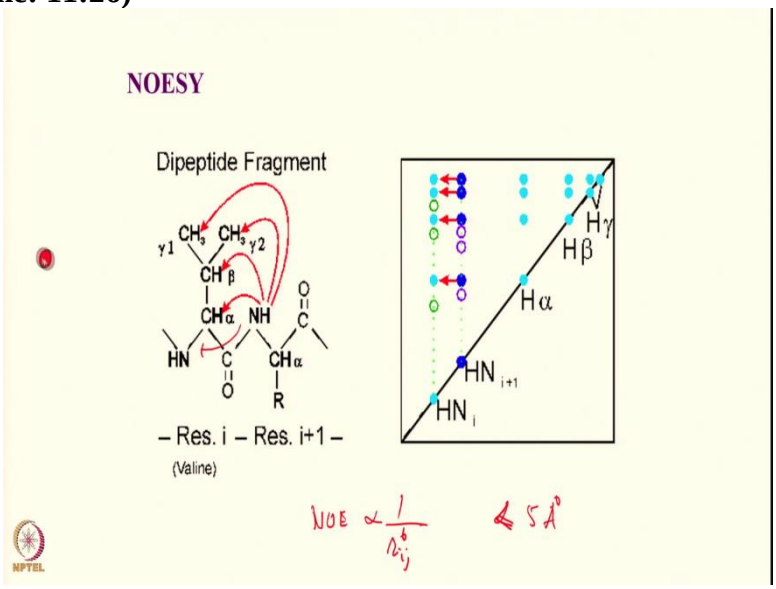

Here I will give a little bit of an example of taking a small dipeptide segment what do we have here. So, I have here a peptide this is an H C alpha CO this is one residue called as a residue I and this is the residue valine see here CHα CHβ and the 2γ methyls. So, this is the valine residue and then the next residue starts here this is the NH of the next residue then the C alpha of the i + 1 and CO.

So, NH Cα CO this is residue i + 1 and H Cα CO this is residue i. Now the dipolar interaction as we said that depends upon the in or the NOE, $NOE \propto \frac{1}{R_{ij}^6}$

So, this is R_{ij} is the distance between the two protons. So, wherever the distance is small you expect to see a cross peak that is indicated here. So, if you see here you see this is a short distance from one residue to the previous residue α proton.

And amide proton to the beta proton of the previous residue and also from the amide proton to the CH_3 protons of the previous residue in the case of this is the valine and these ones are less than 5 angstroms. So, you will see NOE if the distance is less than 5 angstroms so therefore less than 5 angstroms is typically what one observes. If the distance is 2.5 angstroms of course the NOE will be stronger because this transfer process will be more and the interaction will be more and therefore the intensity will be more.

Therefore the intensity is proportional to the inverse 6 power of the distance which means the shorter the distance stronger is the peak. Now of course it cannot go beyond below 2.4 angstroms or something like that because why then there is a steric clash no two atoms can come low and closer than 2.4, 2.5 angstrom depending upon the atom times.

If you are taking protons of course you can't have it less than 2.3, 2.4 if that is because there will be steric clash such conformations will not occur. So, and then you start calculating which are the distances which are small in the given particular dipeptide segment. Of course you will have the short distances between here as well this NH proton to this α this NH proton to this beta.

Also you will see but these ones you will see in the COSY spectrum as well in the COSY spectrum these are all J coupled therefore we will see an NH to the Hα of course you do not see an H to the Hβ in the COSY spectrum you will see Hα to the Hβ and Hβ to the CH_3 and CH_3 this C3 will see in the COSY spectrum in the NOESY spectrum you will see from NH to this Cα alpha H this is also a short distance this will be approximately 3 through 3.5 angstroms you will see this.

Now if you look at these distances calculate these distances of course it will depend upon the dihedral angles various dihedral angles in the dipeptide. So, you will have the either the helical protein or a β sheet protein these dihedral angles can be different. So, but there is a certain range of variation of all these the distances. In every case this distance no matter what the dihedral angle combinations are the ideal angles are the phi and the psi of the Ramachandran plot phi psi are the Ramachandran plots.

And those ones are responsible for the variations in the conformations of the peptides or the dipeptide. So, they can range from 3.5 to 5 angstroms these distances can vary and so, therefore these this you will always see these peaks but with more or more intensity or less intensity depending upon whether it is α kind of a structure or a β kind of a structure you will see these peaks.

And there will be intensity variations which will be extremely useful for structure calculations. Let me see how this can be used for your sequence specific assignments of the individual protons. This is an extremely important experiment you use the COSY and the NOESY together or the double counter filtered COSY and the NOESY together to obtain resonance assignments in proteins.

This is a segment which will continue like this the COSY let me repeat here COSY will show you cross peak from the NH to the α and then from α to the β and β to the 2 methyls and the

COSY will also and the NOESY will show you from the NH to this it may show from here to here it may also show from here to here it can show those in addition all these are all within the same residue all these are within the same residue.

Now in addition in the nose you will also see correlations from the NH of i + 1 residue to the α to the β and the γ methyls of the i^{th} residue the previous residue notice you don't see to the next residue not to the i + 2. Therefore there is a directionality involved here. So, you will always go residue to the previous residue energy amide proton to the alpha beta and also NH this also will be seen this distance also will be available that is typically in the alpha helices you will see that distance as well.

So, you have all these distances available in the in the NOESY spectrum therefore if you look at this spectrum schematic spectrum here I have here the diagonal the diagonal has the NH proton of residue i and the NH of residue i + 1 at this point and then have here the alpha proton of residue i because you look at the colour look at the colours. So, I am talking about this valence residue this cyan colour is reflecting the valine residue.

So, all these cyan ones are the correlations from the valine residue. So, you will see the NH proton of residue i to the α proton of residue I that is this peak here this will also be seen in the COSY. Now from this NH proton you will see to this proton which is the β of the same residue and also to the 2 methyls of the residue those ones are this to this and these to this you will see those peaks are all field cyan peaks.

Now let us see from the NH of i + 1, NH of i + 1 I see to this same fellow here. That is this to this that is this peak here and I will also see to this one here you see from this to this and then to the 2 methyls from these 2, these 2 methyls γ one and γ two. So, these are extremely useful sequential information's sequential connectivity we call the sequential connectivities. Now what are these here.

Now you also have some other things here what are these here un field peaks this obviously will belong to the alpha beta of the of i + 1 residue of i + 1 plus these ones belong to the alpha of the i + 1 residue these ones belong to the betas of the i + 1 residue they will also show up and what are the green ones here these green ones are the sequential peaks from the residue i to residue i - 1 they will go to residue i - 1 this is the alpha of i - 1 and these are the beta or the gamma or whatever of residue i – 1.

Therefore you see you can start from the NH of i + 1 you go to the alpha and then you come to the NH of I then from this NH of i you go to the alpha of i - 1 go horizontal find out where its own self peak is present therefore we can walk along the polypeptide chain in this manner. You can do that using the alpha proton or you can also do that using the beta protons from the NH of i + 1 you go here you go to the beta proton of residue i from the NH of i + 1 to residue I that is the beta then you go here.

So, it is the self peak we call this as the self peak of residue and Hi to its own β then from here you come down to the β of residue i - 1 either here or the top then from here you can go further to the residue i - 1 either it will be this way or this way wherever that NH proton of i - 1 is present. So, therefore in this manner you will see sequential connectivities from the NH of i to all of those.

You also will see here the α to the β which is the peak which is present in the COSY this peak is present in the COSY. And now you will see in addition to this peak you will also see α to the γ and the γ_1 and γ_2. Therefore the NOESY has lot more information than the COSY however the COSY helps you to figure out which are the peas which are belonging to the same amino acid residue and which are not sequential residues.

You can go from one residue to another residue using the NOESY you cannot go from one residue to another residue using the COSY spectrum. So, therefore this is an extremely important experiment for from the structural biology point of view. So, this is so, far as the near neighbour interaction let us look at a typical spectrum of a protein.

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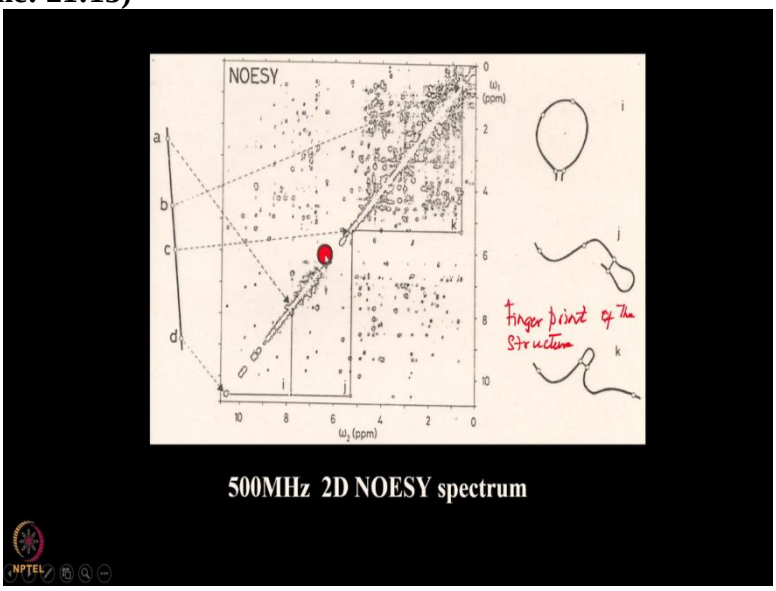

But this is one of the very early experiment of on a protein this is another was such a wonderful great experiment but nevertheless it is such a very early experiment and therefore it is extremely important to show this one and today's spectra of course they are much more crowded we will see some of those examples as you go further. Now what do you see here this is a spectrum of a protein.

And you see here the diagonal which is the one dimensional spectrum and you see a whole lot of peaks all around and these ones belong to those some of those examples which I explained to you i to i – 1, i + 1 to i to i - 1 and so, though the sequential peaks but in addition you will also see some peaks which are not sequential, sequential means not the near neighbour why does that happen?

Consider a polypeptide chain which is like this suppose you have a polypeptide chain which is like this and you are considering the protons A B C and D. The A proton has a diagonal peak here the B proton has a diagonal peak there the C proton has a diagonal peak here and the D proton has a diagonal peak here. Now suppose the polypeptide chain this is the big polypeptide chain suppose the polypeptide chain folds like this if the polypeptide chain falls like this what happens now.

You see the two end ones have come close that is the A and the D have come close here. This is a short distance therefore then I will see a cross peak between A and D between A and D I will see a cross peak these are long range peaks suppose the polypeptide chain folds like this

then which ones are coming close let us say this is A B C and D. Now c and d are coming close if C and D are coming close less than 5 angstroms.

Then you will see a cross peak between these 2 that is the C and D, C and you may see a cross peak between these two on the other hand if it folds like this then it is the 2 central ones the B and C are coming close and that will show up in this here as a peak therefore you see in not only the sequential peaks from one residue to the neighbouring residue you will also see long range peaks depending upon the nature of the fold of the polypeptide chain this is an enormously important information.

So, therefore the NOESY spectrum is called as the fingerprint of the structure of the molecule. So, therefore this is called fingerprint of the structure and now all of these peaks which you are seeing here these ones do not have the same intensities as you can see these ones having different intensities some are weak and some are strong and why does that happen? Because the distances between the various pairs of protons are varying some are 2 to 2.5 angstrom.

Some are 3.5 round some are 4, 5, 6 things like that and then you will see strong peaks when the distance is short and you will see weak peak when the distance is large. Therefore now we can use this information you can use this information quantitatively you give a certain range of the distance let us say a particular peak on the basis of the intensity you classify the intensity the distance range to be 2.5 to 3.5 or 3.5 to 4.5 or 4 to 5.

So, you can classify the peak intensities and in terms of the distances and then you try to build a model till to build a model of the protein and see that you get a structure which is consistent with all those peak intensities which you are giving. This has been the basis of structure determination of biological macromolecules whether it is protein or nucleic acids or carbohydrates or whatever it is it does not matter.

So, in every case you will see such kind of picks of course the information is quite enormous not easy to extract all of this information because there are things which are very close to the diagonal and they are not easy to extract. Once which are far away from the diagonal and this typically will happen when you are considering from the amide protons to the aliphatic protons. Also from the aromatic protons you also have the aromatic protons in this area from 6 to 7.5 you will have the aromatic protons aromatic ring protons will show cross peaks to.

So, many other protons along the polypeptide chain and you will see those cross picks as well. So, typically you may get like 1000 to 2000 such kind of peaks in the protein spectrum and you will be able to calculate the structure of the molecule on the basis of this distance information all right. So, that is. So, far as proton-proton correlations are concerned. We talked about proton-proton correlations we have the diagonal and we have the cross peaks.

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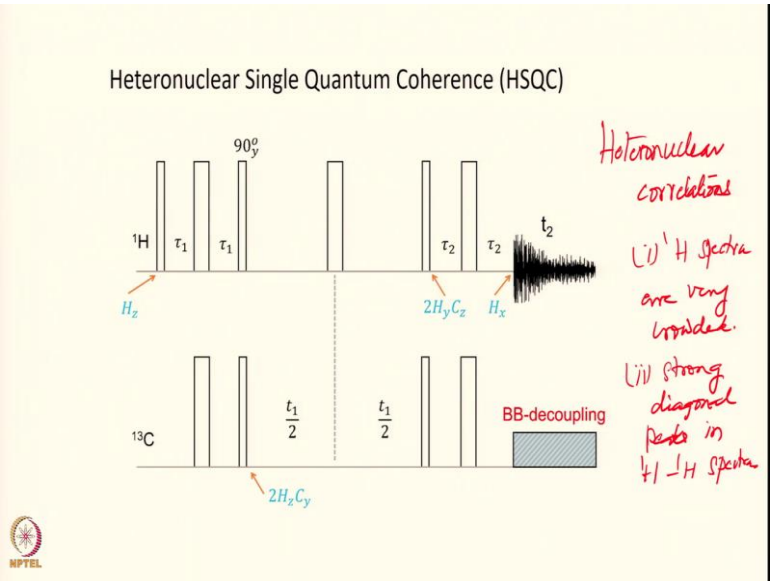

Now we go over to a further class of experiments which are called as heteronuclear experiments. Heteronuclear experiments let me write here heteronuclear correlations. Heteronuclear correlations why are these important these are important because the proton spectra can become very crowded when the protons spectral are very crowded proton spectra are very crowded. And secondly we have strong diagonal peaks in proton spectra in proton proton spectra.

But information which you are using is only the cross peak information the cross peak information is what we want so therefore can we limit this only to the cross peaks surely you can limit it in the in the proton-proton spectroscopy also it is possible to do it but it is much more easier to do it in the case of heteronuclear correlations because you have information between proton and some X nucleus correlation.

And this is one of the most important experiment which is called as heteronuclear single quantum coherence. There are several other methods several other possibilities but the most commonly used is the heteronuclear single quantum coherence and this can have the can the heteronucleus can be the carbon 13 or the nitrogen 15 whatever. So, depending upon the need one can do these different kinds of experiments.

Of course if you are working with proteins typically it may require the enrichment of this X nucleus typically you have ^{12}C. Now if you want to get a spectrum of such kind of correlations you need to produce ^{13}C labelled proteins or ^{15}N labelled proteins and fortunately this thing is now easily possible thanks to molecular biologists. So, we have possibilities of producing the proteins and other things inside the cells where you can the cell itself will incorporate ^{13}C and nitrogen 15 in your protein chains.

So, these bacteria can do this job for you and then you actually isolate the protein and do the correlation experiments using proton and X nucleus. So, will continue this in the in the next class.