Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 18
3D HNCA and 3D HNCO

Welcome back to the course. We started looking at 3D NMR spectroscopy and in the last few classes. So, we will continue with that. So, we looked at different examples how 3D NMR is actually constructed, how it does it look, how do we actually analyse. So, most important thing to understand is a 3D NMR as we saw can be divided into 2 parts as shown here. It can be a proton detection part or it can consist of carbon detection.

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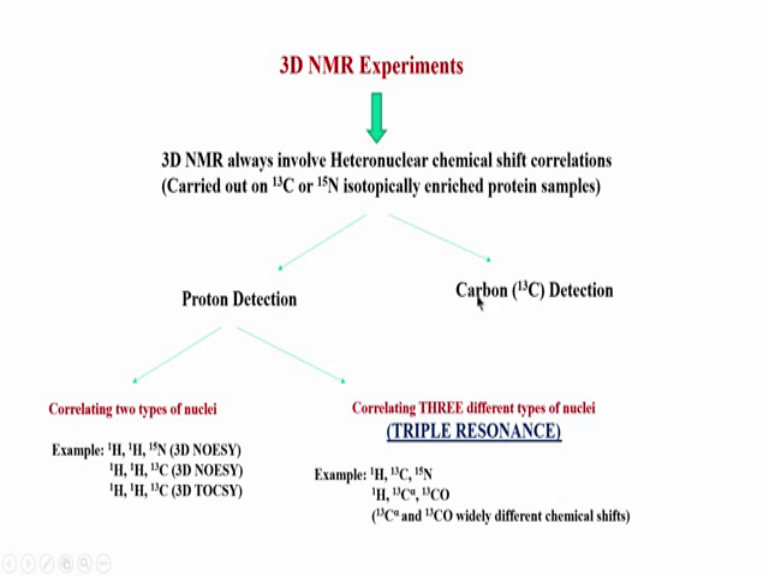

So, most of the experiments which we normally do colours belongs to the proton detection category. We will not study carbon detection in this course we will only look at proton detection; that means, the final detection the final part of this experiment which we detect the signal physically by using the inductor is actually protons. But in the indirect dimension remember in a 2 D and 3D NMR we have 2 die indirect dimension in 3D and one direct dimension.

So, when I say proton detection, what actually I mean is the direct detection, but the indirect dimension can be C 13 or N 15 and this is very important to know that this can only be achieved, if you have an isotopically enriched protein with us. So; that means,

we have to record we have to under we have to subject the protein to isotope labelling by some biological methods which will deal later in the course. So, this that once we have an isotopically enriched protein sample, then we can do proton detection.

So, in a proton detection if we can have further 2 categories of 3D NMR experiments one is we correlate 2 types of nuclei and third is we have another category where there are 3 types of nuclei. So, this is again something we saw towards the end of the last class essentially what it means is, if you have a 2 D 3D experiment with 2 nuclei, it could be experiment such as 3D NOESY HSQC which I have not added here, but it is 3D NOESY HSQC which consists of proton proton and has 2 dimensions and one dimension will be a nitrogen.

You can also have a 3D carbon NOESY, where 2 dimensions are proton proton and one dimension is carbon 13 or a 3D HSQC TOCSY this is something which we have seen in the last the previous class, there we have 2 dimensions proton and one will be carbon. Now you can see here every time we have basically only 2 types of nuclei either proton and nitrogen or proton and carbon. In the triple resonance experiment 3D experiment we have 3 different nuclei. So, which is proton, carbon and nitrogen.

We can also consider C alpha and CO as 2 separate nuclei because they are really well separated in chemical shift. So, we can apply it a completely different set of pulses to this and this and they can actually literally be separated from each other and therefore, they become as 2 different nuclei. So, that is a triple resonance experiment. So, some books and sometime we consider this also as triple resonance.

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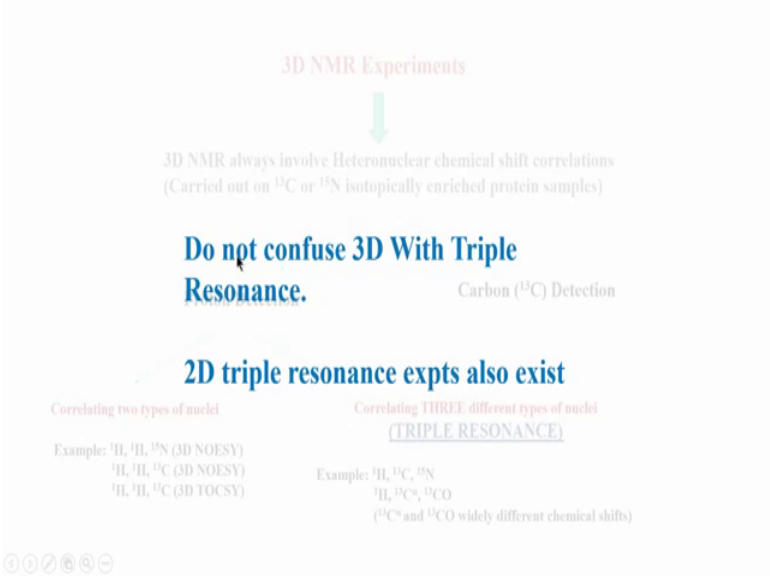

So, one thing one has to most important is this that do not confuse a 3D experiment with triple resonance. Which means every 3D experiment need not be triple resonance; triple resonance specifically applies only to 3 nuclei 3 different nuclei whereas, 3D and 2 D has nothing to do with the number of nuclei, it is to do with how many evolution periods indirect evolution periods are there in an experiment; that means, if I have 2 indirect evolution period then we use we that experiment spectrum comes out as 3D.

If I have one indirect evolution period, then that comes out as a one 2 D experiment. So, 2 D and 3D have no correlation with triple resonance versus double resonance or versus triple resonance can also be 2 D experiment, it does not have to be 3D yet always. So, this is a point of confusion which several people have, they think 3D automatically means triple resonance or they think triple resonance can never be 2 D so, that convince that confusion should not arise.

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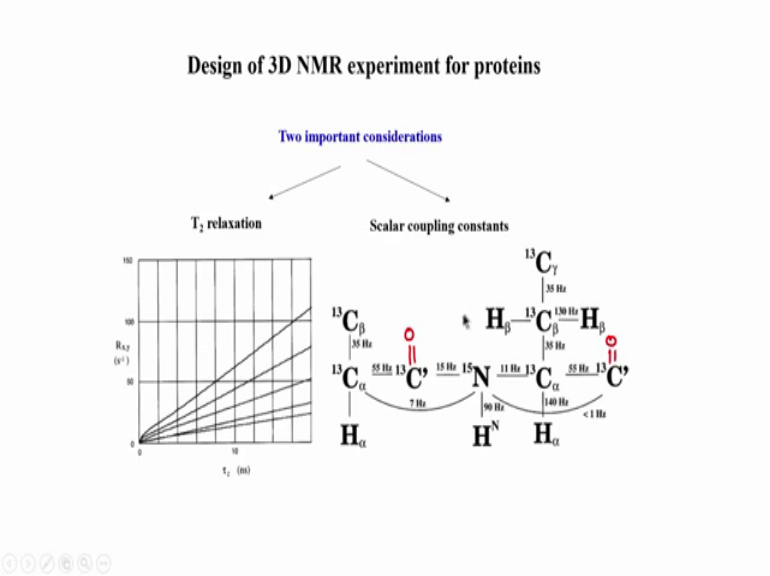

So, now, let us move at to look at what are the considerations which we have to take into account when we look designing or when we are looking at a 3D NMR. So, as the dimension of NMR increases like from 1 D to 2 D to 3D to 4 D and so, on what makes the experiment less and less sensitive the sensitivity goes down is the T 2 relaxation. So, T 2 relaxation basically is the transfers relaxation which we have seen, it causes the signal to decay very high rapidly especially this happens in case of large proteins, there you can see here the plot is showing the T 2 relaxation or R 2 R 2 basically is 1 over T 2.

So, if T 2 decreases R 2 will increase this as R 2 increases as the size of the protein increases. So, if you recall we I showed you how to convert tau c into a molecular weight or molecular weight into tau c. So, based on that formula 10 nanoseconds tau c roughly approximately corresponds to 20 kilo Daltons. At 20 nanosecond tau c roughly corresponds to 40 kilo Daltons.

So, you can see here for a 20 kilo Dalton molecule versus 40 the the rise in T 2 or rise in R 2 is very sharp very high. Especially for these kind of protons, you see this is a proton which is attached to a carbon 13 or a carbon nucleus C H c. This is for the carbon atom of any amino acid. So, we are looking at proteins here especially for proteins, amide protons also get affected with size and the nitrogen proton and the nitrogen is not so, much ok.

Because nitrogen aero magnetic ratio is small, so, its relaxation also is not is relatively shallow or increase compared to the size. But carbon and proton you can see here are maximally affected by increasing the size of the protein. So, therefore, it is very important to understand that I cannot design a 3D experiment arbitrarily for any protein. I have to take into consideration what is the size of the protein. So, this matters a lot in designing and doing 3D NMR not only 3D even for 4D and so, on. So, that is one important consideration in designing, the second one is a coupling constant. So, you can see here this is for a labelled protein and you can see the backbone shown here of a peptide.

So, this is C prime C prime means C O. So, I can write it more explicitly here basically this is carbonyl, this is also carbonyl. So, when you are looking at carbonyl this is a peptide bond here. So, if you look at all this one bond couplings you can see the values shown here. So, carbon carbon C 13 C 13 coupling is 35 hertz. Again remember we are labelled this protein with C 13 so; that means, it is not natural abundance it is an enriched system. So, in a enriched system you will have these couplings present nearby carbon carbon direct one bond coupling that is roughly around 35 hertz.

Carbon to proton is which I have mentioned in the 2 D part is around 125 to 140 hertz again depends on whether it is a methyl or a methylene. So, in this case it is shown for a methyl methylene group here. Now if you look at the nitrogen; nitrogen to proton is 90 hertz. So, these numbers one should actually try to memorize it should be in your mind, because whenever we design NMR experiments 3D or when we try to study or understand 3D NMR experiments, coupling constant play a big role. Especially in modules or units in this 3D called inept in a if you recall in the 2 D part HSQC we looked at 2 D in the inept module.

And there we said the inept depends on the J coupling and J coupling should be accordingly known. So, 90 proton to nitrogen is 90 hertz now if you look here nine nitrogen to carbonyl which is neighbour one bond is 15 hertz, and you see nitrogen also has a coupling to all the way here say 2 bond coupling 7 hertz. And nitrogen has a 2 bond coupling here, but that is pretty week less than 1 hertz and nitrogen and then this carbonyl C alpha and this is a important also for us to know and this coupling constant is 55 hertz. So, we have to know all these numbers in our mind and in our.

So, that we are able to quickly know what kind of delays should be done in used in a experiment. And why this numbers are so, different? That something we cannot go into detail in this course and they can be use waste calculated based on various parameters which we are not going to, but this number should be kept in mind. So, these are the 2 important parameters that is relaxation, how fast it will relax the system relaxes and what are the coupling constants which are needed for the 3D. So, we will see now how we can use this coupling values to transfer the magnetization from one nucleus to another nucleus.

So, essentially when we transfer magnetization we need J coupling and this is something very similar to what we saw in the 2 D homonuclear TOCSY, homonuclear COSY and HSQC and so, on all those 2 D experiments we had used the coupling between 2 neighbouring atoms or nuclei to transfer the magnetization. Without J coupling it is not possible ok. So, this is all J coupling based experiments which we will look at.

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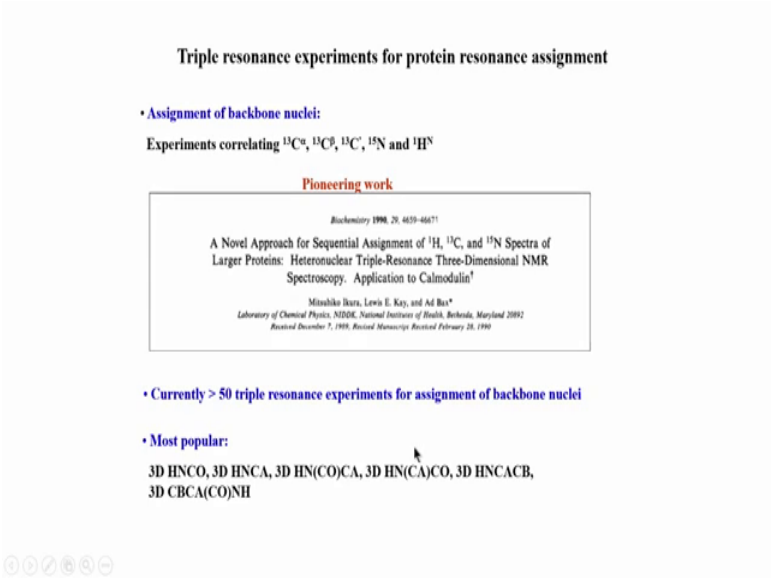

So, historically the triple resonance that is the 3D triple resonance experiment, remember which correlate these kind of nuclei. The first paper which appeared in literature was in 1990 and this started the whole game of 3D triple resonance NMR. 3D NMR was known prior to this, but 3D triple resonance again 3 different nuclei was done first by these group and they showed it for a protein and you can see this are all N 15 labelled protein so; that means, it is all isotopically enriched. Currently as of today there are more than 50

triple resonance experiments which have been which are known in literature, and it keeps evolving it keeps developing this 50 is a number which was about a few years ago, but if you count all the triple resonance experiments they are many many in literature. But we will not go through all of them in this course, because actually the most important one most popular ones are listed here HNCO HNCA and so on. So, we will look at these experiments in detail one by one and see how these experiments what kind of information we get and how they are actually constructed and the its utility or its use in a assignment we will not look at in this part of the course, we will look at the second part of the course after we finish isotope labelling there that time we will come to use of these experiments for assignment of a protein. So, remember we are actually using all these experiments for assignment of backbone nuclei.

And assignment basically means, identify the chemical shift value of these atoms for every amino acid. Not just one amino acid if there are 200 amino acid, each and every amino acid whichever you can you should be able to get the values of these chemical shift for each and every amino acid. So, let us begin with the most basic experiment that is HNCO and slowly we will build our knowledge for the other experiments.

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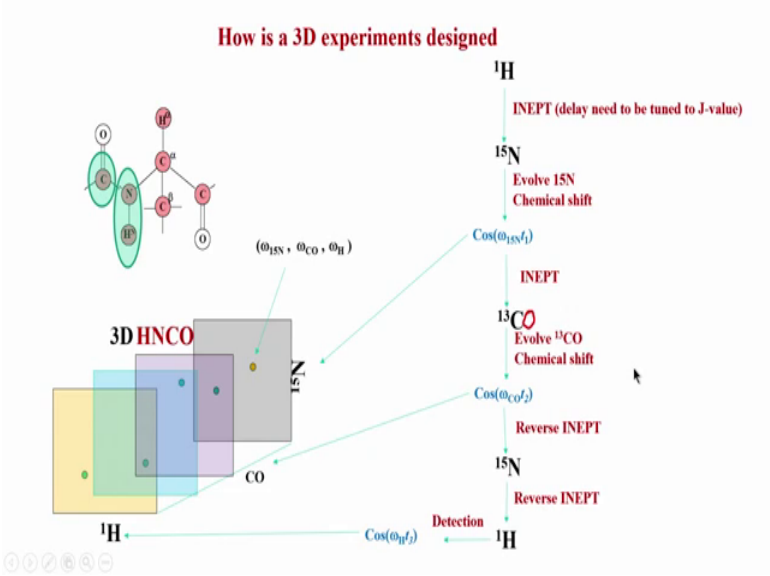

So, now before we go into in 3D experiments. So, this is again example of HNCO the most basic experiment, how do we design it means how does it how is it constructed, how is it made, how does it work.

So, these are the questions we can try to look at now. So, 3D experiment which already I showed you is represented like this. So, you can see it is a 3D experiment, 3 and you have number of slices in between right and each slice or each slice you can call it as a plane. So, each plane corresponds to a one particular chemical shift value of CO, and normally we can use this axis as CO or we can tilt the box we can change the box and make N 15 come here and CO go on this axis. So, which is just a matter of rotating the 3D box and each peak here now gives us these 3 coordinates.

So, it is a 3D experiment, it is a 3D triple resonance experiment. So, therefore, it will correlate or give us information of 3 different chemical shifts and those 3 different nuclei chemical shifts are nitrogen, carbonyl and proton ok. So, how do we actually make this construct this experiment? How does it is made we will show you this one sequence hm. So, 3D HNCO. So, what you do you start from amide proton, you excite the amide proton with a pulse I will show you the pulse program sequence shortly. So, you start from amide proton, you transfer the polarization by inept to nitrogen and for that one has to now keep in mind this J value.

So, if you do not know the J value then this transfer will not be correctly done. So, once you should know it correctly. So, that this transfer is complete and efficient. So, that is a first step you transfer the magnetization from hydrogen or proton to nitrogen. Now this here is shown in this picture, this hydrogen to this nitrogen. Now remember this peptide, this protein has many amino acid in the chain. So, when I say hydrogen to amide for one amino acid, for every amino acid parallelly the same thing is going on. So, you should not think that this is happening only for one amino acid, we are doing this for all the amino acid at the same time in the protein chain.

So, it is simultaneously all amides are transferring the polarization to their respective partner N 15 at the same time ok. Now this N 15 which has now got the magnetization from hydrogen now we can evolve the chemical shift of nitrogen. This is similar to what we do in HSQC, there also we did the same thing and you can get the chemical shift of nitrogen. So, evolution is during t 1 period for a short time then you transfer. So, this is the information this is a chemical shift which is shown here.

So, this axis is the nitrogen chemical shift, which is coming from this term here cosine omega t 1. Now I do reverse I mean further go is I further go from nitrogen to carbon

through inept. Now this carbon is this carbon here. So, I can write explicitly here as CO. So, this is inept to C O 13 C O and this 13 C O is this one. Now if you notice there is a 13 C O or C O here also, but this to this transfer now you recall the previous slide, there we saw that it is only one hertz very small and this is 15 hertz. So, if I tune my inept J value like I written here.

If I tune it to a J value of N C O coupling which is 15 hertz; that means, I should use one over 2 J. So, one over 15 into 2 1 over 30. So, typically that is about 25 to 30 milliseconds if I use then my transfer can be selectively done to carbonyl from nitrogen. So, that is the next step we do after the evolution of nitrogen, we can do a inept to carbonyl. Now we have got the magnetization on carbonyl, we can evolve that magnetization and we can capture the chemical shift of carbonyl t 2 when t 2 evaluation. So, this is this chemical shift here.

So, this chemical shift axis is coming because we have captured the chemical shift of carbonyl by evolution and that evaluation period is t 2. So, this is t 1 t 2 and finally, this will be t 3 ok. So, once the evaluation of carbonyl is over here we have to come back to proton remember this is a proton detection experiment. That means, I have to come back to this hydrogen. So, how do I come back? Again I do a reverse inept ok. So, you should recollect what we did in HSQC, even from proton to nitrogen that was forward inept we did a reverse inept to come back.

So, same thing will have to do here, you have to do a reverse inept to go come back from my carbonyl to nitrogen and this inept is same as this inept, same delays in just that it is coming back to nitrogen the magnetization and then we again go back to proton because we started from proton and then this is finally, detected physically. So, that is why a proton detection experiment and that it during the detection the chemical shift of proton is captured as omega H into t 3 cosine. So, you can see this is how this axis comes about because of this. So, therefore, by having 3 chemical shifts we have got 3D experiment. So, a 3D experiments basically involves correlation of 3 different chemical shifts as shown here and what did we do in this entire experiment? Basically we use inept and evolution inept and evolution you can see everywhere is matter of just inept and evolution to understand it basically.

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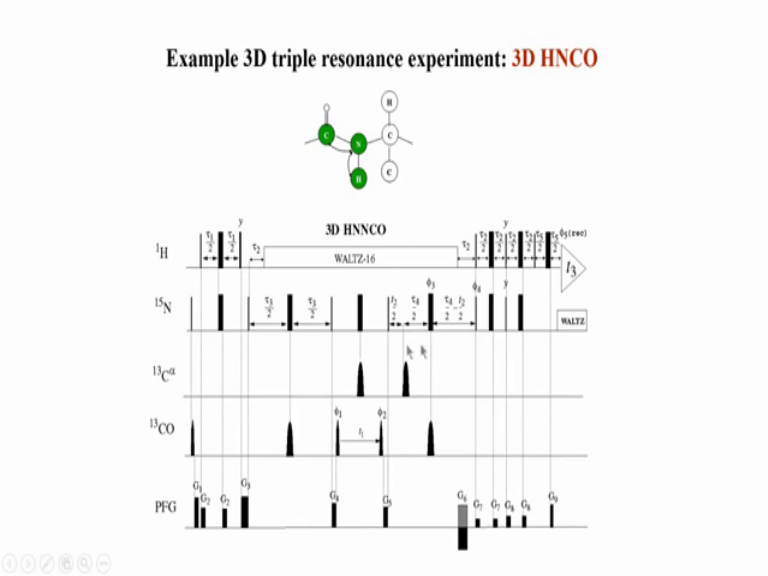

So, let us look at the actual pulse sequence of a 3D HNCO, it looks really complicated. So, in this course we will not be looking at any more pictures like this, because this is very difficult to explain each and everything here and there is also involves many a new concept called gradients which we have not dealt with.

So, the idea is just to give you a flavour of how this kind of experiments look in real life. So, this is what is actually implemented in a spectrometer in a NMR spectrometer instrument so, but what it is actually doing is implementing this design. So, remember what is important is to understand this design and this is just a matter of converting the design into the language of the pulse program and putting it in the spectrometer. So, this is a little advanced concept you do not need to worry about this. This is just shown for you to have a feel of how actually this things are put in its like a blueprint, like how we have architects in a construction companies they make drawings of their building and they show all the scales, similarly this is like a blueprint of a NMR experiment which has to be implemented on a computer, but we do not need to worry about this.

So, I would recommend very strongly to understand these steps, which we will repeat again and again now as we go to other experiments, you will see it will become slightly more complicated, but the ideas are the idea is basically you transfer from one to another by inept and evolve then again transfer inept and so, on.

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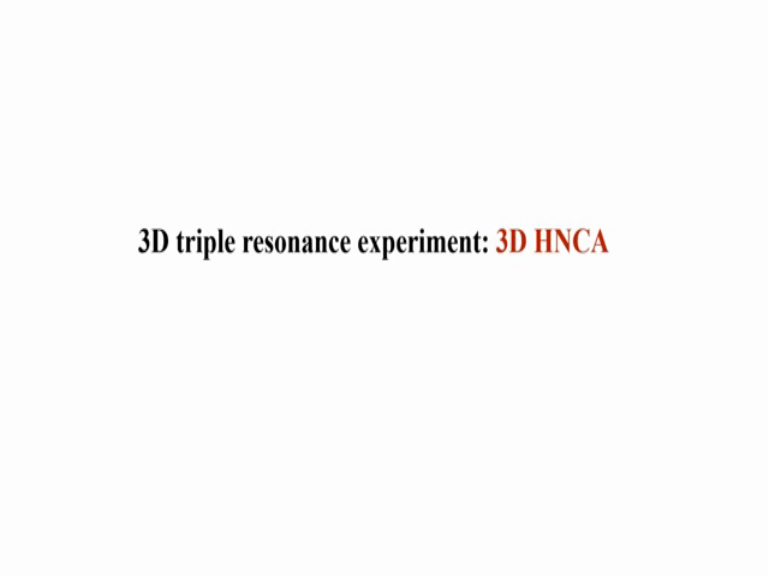

So, now let us look at the peak pattern of another experiment, which is 3D HNCA. So, this HNCO is something which we have covered and we have seen in the previous class also what kind of analysis we do.

How do we analyse a basically look at the slices and take a strip out of the slice remember the strip plot concept. So, that is what we will do in all the experiments. So, let us now look at 3D HNCA.

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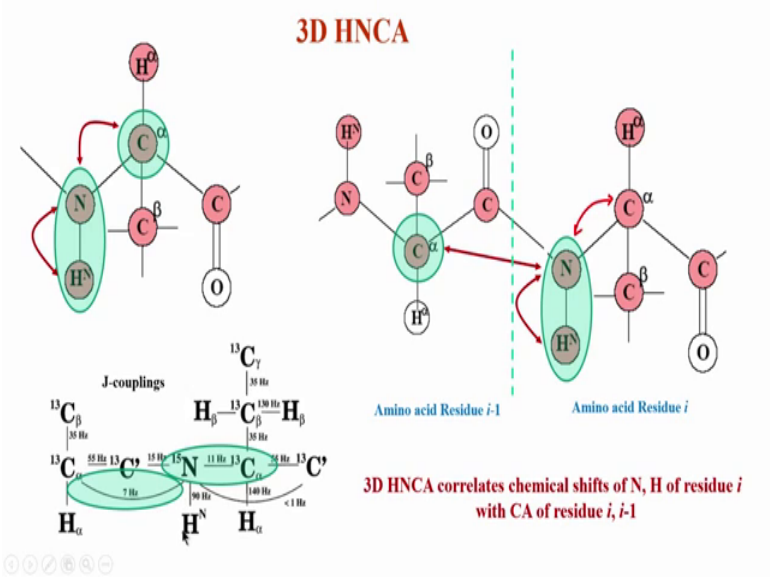

So, 3D HNCA is an experiment in which we correlate or connect the chemical shift of nitrogen to amide proton to nitrogen to C alpha of the same amino acid you can see here. But there is another additional feature in this experiment is that you cannot simply do it to its own amino acid which is residue i, the nitrogen also gets transferred to the neighbouring amino acid which is i minus 1.

So, in an HNCA experiment you transfer the magnetization from amide proton to nitrogen to C alpha which is this side shown here, and also to this side which is shown here. So, these 2 actually happened together. So, you can also add here this arrow ok. So, why is it a double headed arrow? This is a double headed arrow because remember we are transferring the magnetization from proton to nitrogen to carbon and then we come back to nitrogen and then come back to amide. So, that is why it is arrows are on both sides ok. So, this is important to understand why this arrows are like this.

So, now we are correlating the amide and nitrogen chemical shift of one amino acid i with the C alpha of i minus 1. And this is something you know has to be very carefully understood what is going on here. So, that is written in the words now that is now why is. So, here is what is written the 3D HNCA correlates chemical shifts of the N and H of a residue i that is any amino acid i with the C alpha chemical shift of residue i and i minus 1 i and i minus 1.

Now why just both the transfer takes place i and i minus 1 is depicted here? So, this is the same picture which I showed you a few slides ago, these are the values of all J couplings. So, you can see that N to C alpha of the same amino acid that is this side here e what is it is actually 11 hertz, but if you go this side that is also almost close not very different. So, when I tune my J coupling for this value, my J coupling is also getting slightly inept is also getting slightly tuned to this also. So, it is not possible for me to only go to this carbon or this carbon. When I go from nitrogen to C alpha it will go in both the directions because that inept which I will design cannot be tuned specifically for only this transfer.

So, therefore, I will get magnetization going both sides, and this is very useful for us as we will see for assignment of the protein. This information that I get both I and I minus 1 C alpha chemical shifts for a given nitrogen proton pair is very useful we will see that as shortly.

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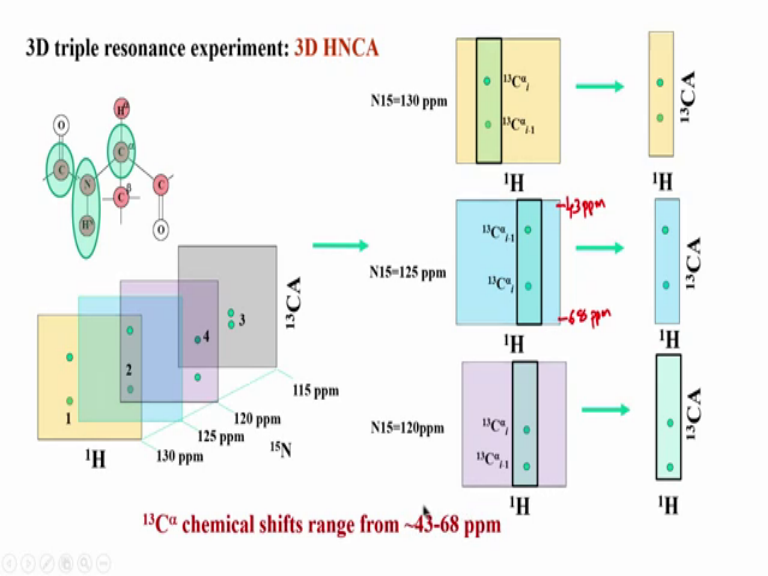

So, how does the peak pattern now come about for this experiment? It is shown here. So, you can see here for a 3D experiment like this now for every plane here, you look at this plane ok. So, for every plane now this is nitrogen dimension this axis and this is carbon alpha. So, for a given plane let us see here for a given plane which is taken at N 15 value of 130 ppm means I am taking this slice this slice here.

See if I take this slice in a for a given amide proton means if I draw a line here, for a given amide proton I am seeing 2 chemical shifts 2 peaks one peak corresponds to the i that is this amino acid and one corresponds to i minus 1 which is this amino acid ok. So, you can see in this case the i chemical shift is up and the i minus 1 is down, but that is not really true for every amino acid. If you look at some other plane of the same experiment which I have taken at 125 ppm, you can see here that I am actually getting i as down and i minus 1 is up ok.

So, this is the important thing to understand that chemical shifts all i and i minus 1 do not have any particular pattern, they can either come depending on that value. See i minus 1 is up field. So, this is chemical shift is a up field here, up field means lower value here and down value is down field. So, I will shortly give show you the range where it comes this chemical shifts. So, you can see typically this is from although we will write it slow shortly, I will still give you the typical range. So, typical range for C alpha chemical shift is from 43 ppm to 68 ppm.

So, you can see here that if the C alpha is close to this 43 or 45 it will come there and C alpha of I may be something else. So, it will come down, but here it may be other way round the C alpha may be close to 50. So, it is coming up C alpha i minus 1 may be 55 or 60 and it is coming down. So, this order does not is this no particular order they come in any order depending on their chemical shift value. But for every amide proton for every amide proton you see here, I am getting a corresponding 2 peaks here and that is one is i and other is i minus 1. Similarly here if you look at another example from the same spectrum, I have again shown 3 out of the four slice for the space restriction problem.

So, if you look at this slice which is taken at 120 ppm in this slice, for a given amide proton that is this line if I draw for a given amide proton chemical shift value I am waiting again 2 peaks and one corresponds to i and other corresponds to i minus 1. So, this is the same 2 peaks which are shown in the strip format this is a slice format this is the strip format. Now the question we will ask is will how do you distinguish whether this is i or this is i minus 1 we will look at that shortly. So, this is a chemical shift range which I was mentioning for a C alpha typically the range comes in this a 43 to 68 ppm.

So, that depends from protein to protein, but typically all proteins will be in roughly in this range.

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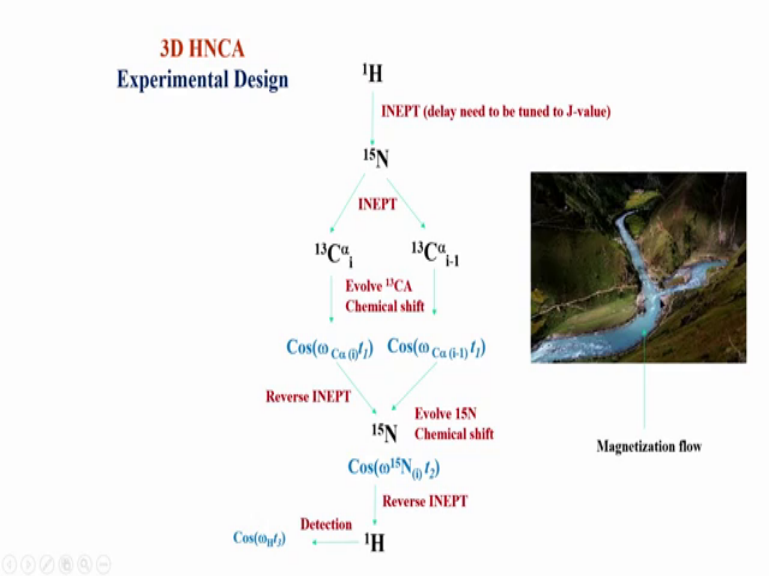

So, let us see how this C alpha actually experiment is designed means how does it work? So, it works like this you start from a proton amide proton, you transfer to nitrogen this is

same as we saw in HNCO. In fact, all h n experiments in a triple resonance HNCA, HNCO c a HNCO etcetera will be like this they will always start from the amide proton, you apply inept and go to nitrogen. Now here we apply inept and go to c carbon alpha, but as I said this inept will take the magnetization from here to here and it will also take it from here to here there is no selectivity you cannot say I want to go only this direction it will magnetization will flow also in this direction. So, the flow of magnetization is not controllable because the 2 couplings are almost similar.

So, therefore, this inept will work for both transfer. So, this is anyway good for us as we will see. So, this transfer is simultaneous then you evolve this chemical shift. Now if you notice nitrogen is not evolving here now compared to what I showed in HNCO now that is because the nitrogen can be evolved either here or here it does not matter, it can be done in either positions. So, practically we do it here in this part.

So, once you transferred to see carbon, you evolve the carbon alpha chemical shift. Now because both have been transferred they will both recaptured ok. So, both will recaptured as C alpha and C alpha i minus 1 into t 1. Now with reverse inept that is back 2 nitrogen. So, it is opposite of this. So, we are going back to nitrogen, then nitrogen chemical shift is captured evolved during t 2 and then you go back to proton and then detect the proton that is proton chemical shift. So, you can see now there are 2 correlations we have getting now one is through this pathway.

So, you can follow this arrow one is through this pathway ok. So, we are getting this chemical shift correlated with this chemical shift correlated with this chemical shift the other pathway is through this ok. So, that is second path way the second path way is giving us correlation of i minus 1 C alpha with nitrogen i with and proton i; i means is amino acid 1 any amino acid I. So, these 2 belong to the same amino acid, but either you will get correlation to this and this I mean not either it is both ok.

So, we can see that this pathway gives us one set of correlation, this path way gives us one set of correlation. So, this kind of a channelling or splitting of magnetization into 2 paths like this can be actually visualized like this like a river. So, you know many times a rivers they split it into 2 paths and they flow in the 2 paths, then again like this they will flow like this and again they will join back somewhere and they again they become a single flow like this.

So, this is what happens in nature all the time and we are also exploiting that in NMR that we are splitting the channel or magnetization into 2 channels or 2 paths evolving them and then bringing them back into the same and then detecting the signal. So, this is basically how HNCA is done, we will look in the next class we will continue with HNCA and see what is a problem is slight problem with this and how it can be circumvented with another experiment we will see that in the next class.