Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 11
Principles of 2D Heteronuclear NMR

Welcome to the welcome back to the course. We were looking we started looking or about 2D Heteronuclear NMR spectroscopy. So, we covered 2D homonuclear in the last week, now we look at how heteronulcear NMR spectroscopy works. Before we go into the details of the 2D NMR experiments, let us look at briefly what are the problems we face with heteronucleus ah. So, this is given shown here in this table.

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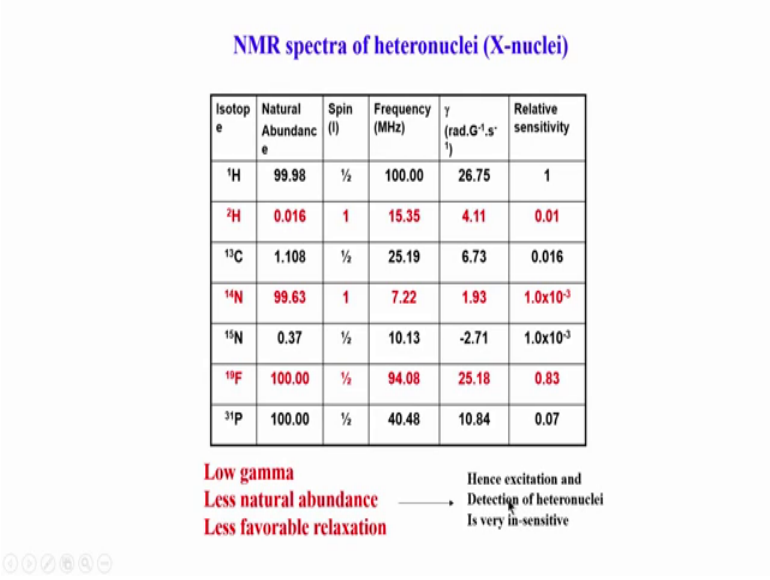

You can see that different heteronuclei have different properties. So, one thing is, these are the nuclei which we are going to look in as I said in proteins and nucleic acids here you can see that there is this hydrogen of course, which is the more sensitive nucleus and fortunately for us it has the natural abundance of 100 percent so; that means, every hydrogen in this universe almost every hydrogen is hydro proton 1 it is 1 spin half.

It has an isotope deuterium, which is very very less in abundance and also it does not have the spin half it is spin 1 nucleus. So, normally we do not do deuterium NMR in case of biomolecules, but we definitely use deuterium which is incorporated into the

molecule, which we will see in the in the part when we deal with isotope labelling; their the sensitivity is much lower ah in case if you want to directly look at deuterium signals ah, but we can use this for some other properties which we will see later.

Carbon 13 is the second most for popular nucleus after proton. For carbon 13 also as we saw in the previous class previous week, we have this problem of sensitivity and natural abundance. So, you can see the natural abundance of carbon is only 1 percent that is carbon 13. So, 99 percent of carbon is about C 12 which is NMR inactive. So, we cannot study carbon 12, but we can study carbon 13 ah, but if you want to study then you have to the 2 problems facing this number one is this in natural abundance and the second thing is a gyromagnetic ratio. So, this is another parameter which you have to look for.

Ah. So, you can see this is about 4 times the carbon 13 is 4 times less compared to proton. So, this is 4 times less and the second factor is this is about 100 times less compared to proton. So, you see about 400 times less and therefore, it (Refer Time: 02:55) is about the square of that is comes out to be about 1.6 percent. So, the relative sensitive is very low in proton as for in carbon mainly because of these two factors that is the gyromagnetic ratio is being small compared to proton and the natural abundance.

But we can actually improve the at least this part the natural abundance part can be improved by enriching or labelling or isotope labelling the molecule with 100 percent carbon 13. So, in such situation cases your carbon 13 abundance will go from 1 percent to 100 percent because we will be specifically designing molecules or labelling the molecules with carbon 13. So, this is what is routinely done in the case of biomolecules and how we do it will be the part of the second part of the course where we will see in detail.

Nitrogen 14 is again 100 percent abundance, but again it is a spin 1 nucleus. So, similar to deuterium we do not normally study this in biomolecules because they are special nuclei, they have a different additional properties which results in a broad lines and therefore, for sensitivity reasons we and resolution reasons we do not study this directly in NMR so, by in for protein NMR in liquid state.

Now, if you look at nitrogen 15 this is another favourite or more popular nucleus after carbon 13. So, carbon 13 and nitrogen 15 are most popular in biomolecules. Now here again the problem of sensitivity is there coming from the same 2 factors that is 1 that

natural abundance is very low. In fact, is 3 times lower compared to carbon 13 and the gyromagnetic ratio is shown here is even ten times smaller than proton. So, compared to carbon nitrogen is even more lower in sensitivity. So, the total sensitivity we can see is thousand times less relatively to proton.

Ah, But again nitrogen 15 is a very abundant atom in biomolecules. So, it is important to study this atom and use it for biomolecular structures. So, what we do again there is we enrich or we isotope label the protein molecule with 100 percent nitrogen. So, in such cases the nitrogen sensitivity goes up because this will become 100 percent like proton. So, we jack up this number by basically the isotope labelling. This factor cannot be touched this is something which is natural to this nucleus. So, we cannot do much with this particular number similarly for carbon for any of the nucleus, they are fixed and therefore, this factor we have to leave with it whereas, this factor can be modified by labelling.

And if you look at nitrogen sorry 19 fluorine it and phosphorus as I said we will 19 F is something we will not study in this course because it is not part of biomolecules ah, but that has also very sensitive nucleus compare and it is almost similar to proton 100 percent in abundance and look at the gyromagnetic it is almost same. So, you see the the sensitivity of fluorine is almost similar to proton close to one. So, fluorine and hydrogens you can think of has similar type of nuclei, but fluorine has many other properties and that is useful for studying the this nucleus ah, but we will not look it in this particular course.

For phosphorous 31 is something we will try to look at when it comes nucleic acids and there you can see again the sensitivity is not so, bad it is about 100 percent abundance and gyromagnetic ratio is about 2.5 times less compared to proton. The sensitivity is still much better than carbon and nitrogen, but the problem with phosphorous there are several other issues one thing is it is a very high chemical shift anisotropy in short it is called CSA which we will not go in to detail the theoretical aspects of CSA, but that actually results in very broad lines. So, if you go to and relaxation first relaxation.

So, typically into phosphorous we do not do beyond 400 or 500 megahertz spectrometer because CSA scales with the square of the magnetic field. Now because of this squaring factor it rises very fast the factor contributing to the relaxation of phosphorous and

similarly same with fluorine it contributes rapidly when you increase the magnetic field. So, therefore, one has to take into account that factor. The CSA in nitrogen 15 carbon is also high, but not so, high as carbon 13 and proton. So, carbon proton are very low in terms relatively lower in terms of CSA.

So, that factor therefore, carbon and proton remain always the best nuclei to study at higher magnetic field ah, but carbonyl we will see that also has a very high CSA. So, not all functional groups of carbon are easy to study in biomolecules about methylene methyl and methane and those kind of systems are not so, big problem. Aromatic carbons also have CSA ah, but again not so, prohibitive as in the case of phosphorus and fluorine.

So, these are the different parameters which are shown in this table as I said most of these has been half nuclei and that is what is favourable in liquid state NMR. I have a spin 1 nucleus nuclei we do not normally study in liquid state, but solid state NMR is done before these 2 nucleus. So, let us now look at the factors. So, this is the factor which I mentioned you have low gamma is the first issue, the gamma value matters a lot that decides the sensitivity what other thing we decide sensitivity is, the natural abundance and as I said relaxation. Relaxation due to CSA or it can be due to quarter polar interactions which happens in case of spin 1. So, spin 1 the reason we do not use it in liquid state is because it has very rapid relaxation and in some of the cases like fluorine and phosphorus, you have carbon chemical shift anisotropy which contributes relaxation.

So, these are the factors which are important to consider when we look at hetero nucleus other than proton which nucleus to take and so, on. So, hence what we do in hetero nuclear NMR? Because all the heteronuclei are less sensitive compared to proton we excite. So, what is shown here we excite and detect protons in a the selective manner. So, we will see that how we do that we do not directly look at heteronucleus, we actually indirectly look at them by transferring energy or magnetization from proton to carbon or proton to nitrogen and then come back to proton to study it. So, this is called as indirect detection of heteronuclei this is sensitive. So, let us go a little bit into that.

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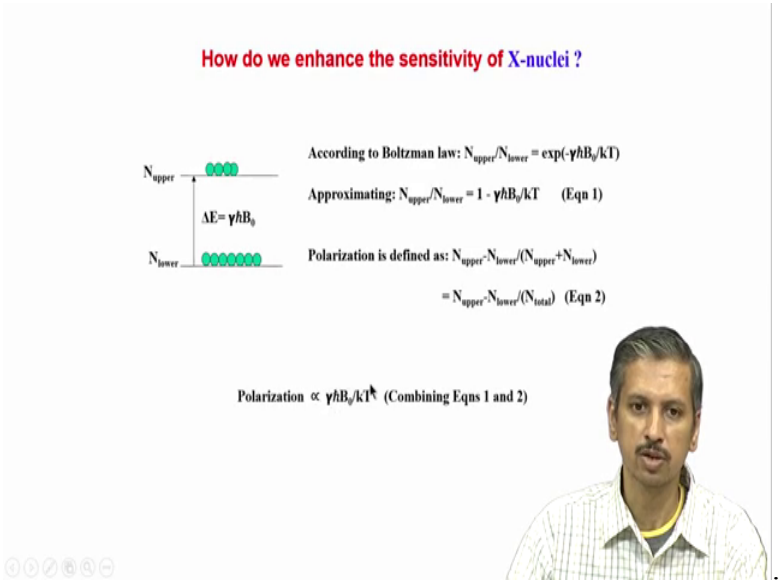

So, first let us again come back to this picture of the Boltzmann distribution of spins. So, you can see this is what we saw in the very beginning of the course, that when you apply a magnetic field the any spin NMR active nucleus especially in case of spin half nucleus they get split into 2 possible energy states and that energies levels are now the population of the spins are not equal. They are distributed according to this famous Boltzmann law and you can see the factors which contribute to this distribution are temperature magnetic field and gamma the other 2 are constants.

So, we can approximate again this is a slide approximation because what happens is, we consider in NMR an approximation called high temperature approximation high temperature means not temperature which is very high. The idea is that at a given temperature whatever temperature normally we study that is room temperature or close to zero degrees and in that range of temperature, this factor this that is T is in kelvin this factor becomes very small number this entire exponent which is on then here shown here.

So, in mathematics whenever you have an exponent which is very small we can approximate like this. So, this is something coming from basic mathematical principles that when exponential of something a number is very small e raise to minus x is where x is small can be written as 1 minus x. So, the x is this factor here.

Now, further you can define a term called as polarization now polarization what is polarization? Polarization is a difference in the population between the 2 levels. So, that is shown here the difference divided by the total population. Total population is nothing, but just the total number of spins in the sample. So, that is basically related to the total number of molecules which are present in the sample or depending on your concentration. So, that is just a number which remains constant, this is does not change as long as you do not change the concentration.

But what can change is this difference and that difference depends on these factors. So, for a polarization; therefore, is proportional to this factor now which is mentioned here. So; that means, if I have a lower gamma which happens in the case of heteronucleus my polarization also will be small which means my difference in the population also will be small. So, this difference in the population is now going to be maximum highest for proton, because that has the highest gamma value and it has it is going to be very small or relatively small for nitrogen for example or carbon, because of this lower gamma all other factors are same for proton and carbon.

For example temperature is the same because you are looking at the same sample. So, temperature is not going to be different between proton and carbon and magnetic field is going to be the same because you are not looking at difference in the magnetic field magnetic field is external magnetic field whether we are studying carbon or proton or nitrogen that value cannot change, but what changes is this gamma.

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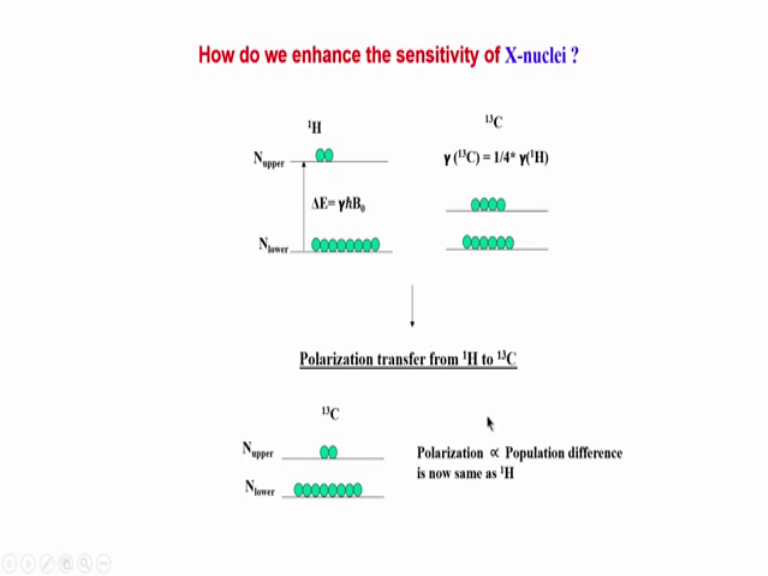

So, essentially we are looking at the gamma difference between in the case of heteronucleus. So, you can see here that is shown for case of proton it is 4 times more or carbon is 4 times less 1 by 4 of hydrogen. So, pictorially again this is only a schematic you can see the population difference between the 2 that is n lower minus n upper in this case is shown as 8, then the population difference between carbon and proton will be 1 between for the proton for the carbon will be one fourth of 8 ok.

So, if you let me go back one slide here, we can see here this this particular number n upper minus n lower the difference that is the polarization is directly proportional to the gamma. So, if the polarization of proton that is a difference between the upper and lower state of proton is 8, then for carbon it will be the same difference will be scaled down by factor of 4 because of gamma of carbon is 4 times less.

So, that is what is depicted here in this picture that if you consider the difference between proton as population as 8 for carbon it will turn out to be 2. So, that is why now what we do is that we can enhance this polarization; that means, I can enhance the difference if I can somehow transferred the difference from here to here ok. So, this is called polarization transfer. So, polarization transfer essentially in a layman terms you can think of it as a bank balance. So, let us say you have 50 rupees with you and somebody has 200 rupees. So, you urgently need that money the 200 rupees bank balance can be transferred to your account and you become you get now 50 plus another 150 and you

can have as much as this person had earlier and you can return it back once your job is done. So, this is a very simple idea which can be thought of the same thing happens in the case of NMR, you can think of this polarization as the bank balance as a as an asset as a positive point for proton because the difference matters and higher the difference more is the energy more is the sensitivity of this of the nucleus. So, hydrogen is 8 times or 4 times more than carbon. So, hydrogen can donate its population difference to proton to carbon or nitrogen in case of nitrogen heteronucleus.

So, this this is mechanism of transferring the difference of population from one nucleus to another nucleus is known as polarization transfer. And this boost now the carbon 13 we will have same polarization after transfer it will become the same polarization as proton. Although naturally it cannot have that it is artificially done that. So, we have to keep in mind this is something we are very artificially doing this to enhance the sensitivity of carbon because that is the only way we can improve the sensitivity we cannot change the natural abundance. For example, let us say we are looking at natural abundance molecule that 1 percent is something which we cannot do anything we have to live with that, but polarization is something which we can change by doing this idea.

So, how do we transfer polarization? There are varieties of methods when we will look at one of them which is very standard very popular, it is called inept which shortly we will look at that. So, the point here is to understand what do you mean by polarization transfer. It is nothing, but the transfer of the magnetization or the difference in the population from 1 nucleus to the heteronucleus. So, this is what is shown here once I do the transfer the polarization now becomes the same as proton ok. So, this is artificial this is not natural what is what does it mean when I say it is artificial? It means that if I now allow if I allow this carbon 13 and I leave it alone after the transfer, it will go back to this original situation original population difference.

So, this population difference is something what is a equilibrium value for carbon whereas this is something we artificially created. So, by T 1 relaxation there is a mechanism the T 1 relaxation it will go back to its original population. But before it goes back completely to back to its original, we can play around or we can use that now the newly created higher polarization to do the experiment. So, this is what we use in heteronuclear NMR we first transfer the polarization from proton to carbon by some mechanism which we will see shortly. And once we have created this new polarization

on carbon we use that difference to do the experiment on carbon. Once that experiment is over we give it back to hydrogen by reverse polarization transfer. And then detect the hydrogen we do not detect carbon we have done this indirectly on carbon, but the final detection happens back in proton.

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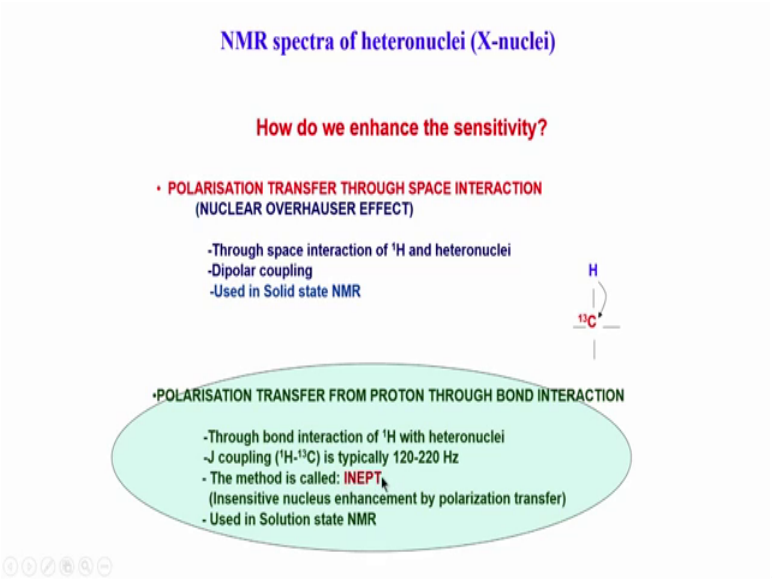

So, this is what again is depicted here in its we do can do this both in solid state and in liquid state. In solid state NMR generally this is done using what is called as the CP or di cost polarization and that happens with through dipolar interaction. So, remember a proton is always attached to a carbon in a organic molecule or in a proteins or in biomolecules. So, they are very close by this distance is about 1.5 angstroms or 1.2 to 1.3 angstroms. So, this difference this distance is very small. So, therefore, I can actually the dipolar interaction between hydrogen and carbon is very high and therefore, I can use that interaction to transfer the polarization from this proton on to this carbon. This is something very similar to what we saw in NOE and this is actually the same nuclear overhauser effect where we saw that if we have 2 protons which are less than 5 angstroms, we can actually transfer the population or polarization from one proton to the other proton.

So, if the same concept is applicable here except that we are now using instead of 2 protons, we are using 2 1 proton and 1 carbon. Because both are now spin half nucleus and therefore, this is possible. But in liquid state we cannot use this dipolar coupling

because in liquid state the dipolar coupling averages to 0 because the molecule rotates or tumbles in all directions. So, because of that the dipolar coupling is average to 0. So, we cannot use dipolar coupling very efficiently in liquid state. So, we have to rely not on dipolar interaction, but on J coupling between these 2 head these 2 nuclei.

Now, what is the value of J coupling between these? If you recall the slide I showed in in the J coupling part it is about 120 to 140 hertz. In fact, it can go up to 200 hertz also depending on whether it is aromatic or it is it is depending on the hybridization of carbon in case of methyls it is about 125 hertz; in case of methine is about 140 and in goes up to 160 and 180 in aromatics. So, we will assume average value which is about 140 hertz for 1 bond. So, we are now considering remember the directly bonded proton and carbon.

So, this is what is shown here that we use hydrogen J coupling between proton and carbon which as I said has a wide range, an average being around 140 we use this and that method which is done which I show you is known as inept. This is using J coupling to transfer the polarization from proton to carbon an inept is an acronym for this statement or this particular mechanism that is called insensitive nucleus enhancement by polarization transfer.

So, this is basically what is depicting here in this in this word is essentially telling that the insensitive nucleus carbon is enhanced in its polarization by transfer from proton. So, this is what we use always in solution state NMR. So, all over 2D NMR experiments 3D NMR experiments are built based on this is experiment this building block. So, inept is basically like a building block its a basic unit of an 2D and 3D and 4D NMR experiments or multidimensional NMR heteronuclear NMR experiments. So, we will have to now carefully understand how this works, how does it transfer the polarization.

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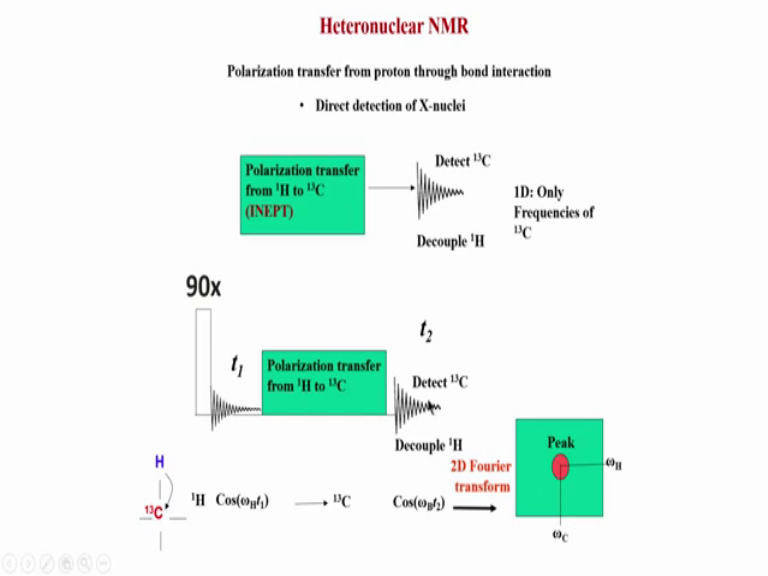

So, this is schematically shown here in a case of a simple heteronuclear NMR. So, let us start from simple 1D heteronuclear NMR. So, what we do is we directly detect the carbon. So, as I said normally this is not what is done in 2D and 3D NMR, but to understand let us look at it as very basic first that, first let us say you transfer the polarization from proton to carbon by in that inept concept mechanism which I have not showing here, we will see that shortly it is shown as a box here.

Now, this inept transfer the magnetization from proton to carbon. So, what happens is the carbon sensitivity goes up by a factor of 4, this is because of this difference in the gyromagnetic ratio. So, therefore, now if I detect the carbon spectrum or record a carbon spectrum, I will get 4 times more sensitivity compared to what I would have got if I directly gone for carbon. If I directly detected the carbon without doing this polarization transfer, I would have got 4 times less sensitivity because now the transfer has helped me to detect. So, that is theoretically the limit or 4 time practically it is not always 4 times which we will see shortly.

Now, we will get in the 1D only frequencies of carbon. So, as I showed you that in the previous class when we look at 2D, NMR generally why do we need 2D NMR we saw that 1 of the reasons we need 2D NMR is you want to correlate 2 chemical shifts right. So, you want to connect one proton with the corresponding carbon we do not want to only look at carbon alone this is something which is done in organic chemistry

experiments where you are characterizing simple molecules, but when it comes to biomolecules 1D carbon NMR has no not much applicability not much use we have to go for 2D NMR 2D heteronuclear NMR.

So, now let us look at how 2D heteronulear NMR works. So, this is a schematic here again look at this here we are detecting carbon, but this is again a not a very popular experiment in solution state NMR. In solids yes it can be it is sometime done like this, but in solution state NMR we do not do this solution state NMR as I will show you we have a little additional steps because we come back to proton we do not detect carbon in many cases. But the recent years sensitivity of carbon has improved and there are what is called direct detection of carbon. So, its not that carbon 13 is never detected directly in protein NMR, that in fact, protein NMR carbon 13 detection is evolving rapidly and is used in many cases ah, but the most popular what remains still sensitivity wise is detecting (Refer Time: 24:24) proton. So, we will see that as we go along.

So, we start from a simple 90 degree pulse that it excites the proton ok. So, we are looking at proton evolution now not carbon proton pulse not carbon pulse now proton pulse after you apply a pulse on proton it is on the x y plane and then it starts moving in the x y plane because of the Larmor precession. This is something we have been talking about extensively in the homonuclear NMR part. So, I will not go into details again for this.

So, when it is evolving, it evolves with its own frequency of proton and that evolution period is known as we call it as a t 1. Then we use polarization transfer element that is inept to transfer it to carbon. So, whatever proton magnetization has come up to this point, it has got transferred to carbon and then the carbon is detected the immediately.

So, what does it do? It here this experiment is now we can see once you transfer the polarization you are actually connecting joining or correlating proton and carbon. So, this is a 2D NMR experiment which is telling me the correlation between the proton chemical shift and its partner carbon chemical shift. So, this polarization transfer only transferred to the nearest proto[n]- carbon and what is the nearest carbon possible? The directly attached carbon.

So, if you want to transfer it to a further carbon that is 2 bonds away or 3 bonds, there are also experiments which can be done that is what of this standard experiments which we

do, but in biomolecules normally we restrict the transfer to only one bond and this can easily be done by using appropriate inept experiment which I will show you when we come to more detail 2D heteronuclear NMR.

So, right now we will let us see only the correlation between proton and carbon, the only problem here is this experiment as I said is not very popular because we are detecting carbon. So, this is how it works. So, you have excited the proton first with this 90 degree pulse, it has evolved by its frequency that is protons frequency whatever is this proton chemical shift then when transfers to carbon the carbon evolves with its frequency now and therefore, what we have done is by connecting we are having this 2D experiment we have connected the 2 chemical shift.

So, if you look at the final 2D spectrum how it will appear? It will appear like this the proton will come in the t 1 axis; t 1 is nothing, but the indirect dimension if you recall our lectures on homonuclear NMR. So, this vertical axis is basically the indirect dimension and that is t 1 value t 1 axis or when you do Fourier transform you can call it as a f omega axis frequency axis.

The t 2 dimension is the horizontal axis or omega in the horizontal part in case of Fourier transform and in this case we are detecting carbon. So, our t 2 that is horizontal axis now consists of carbon frequencies not proton is along the y axis. So, if this experiment this spectrum now is giving us the information between the connectivity information between a proton and a carbon, and that is basically an experiment heteronuclear correlation in short this is actually called hetcor. So, this is an experiment which is very routine used to be done in the beginning of heteronuclear NMR, but as I said there are many problems with detecting carbon directly the sensitivity is not so, great although in recent years we have been improving this sensitivity part.

There are lot of favourable properties of detecting carbon for example, the chemical shift dispersion of carbon is very high and relaxation is also little slower compared to proton. So, the lines are sharper in case of carbon compared to proton, but the problem with other things in carbon is sensitivity although we the gyromagnetic ratio is slow. So, when I detect directly my polarization transfer has no effect. Polarization transfer only has effect for initial gain, but actually in the carbon detection yes here it helpful to us, but

generally when we do carbon detection experiments we have sensitivity goes down because of the gamma factor and we have to decouple carbon and carbon.

So, if you recall the lecture on carbon 13 NMR, we saw the 2 carbons neighbouring carbons can never be C 13 because of natural abundance of carbon is very low C 13 is very low. But if I do enrichment means I if I isotope weekly label a biomolecule like protein with C 13 all my carbon atoms in the protein will become C 13 in such a case my C 13 C 13 coupling will not be 0, it has to be taken care because every carbon 13s neighbour neighbouring carbon will also be C 13 because we have labelled the entire molecule deliberately with C 13.

So, in such scenarios the C 13 C 13 coupling J coupling will start affecting the spectrum and typically that J th C 13 C 13 J couplings are about 30 to 50 hertz. So, they are substantial value they are not something you can ignore. So, therefore, that is a big issue with C 13 detection when we will see how that actually that is also overcome these days ah, but that also is a concern.

So, 1 final thing is that you have to decouple proton from carbon when you are detecting and this is very important here because a proton is directly attached to carbon we had shown here in this picture. So, both are spin half nucleus. So, there will be a doublet this carbon will appear as doublet because of J coupling to this hydrogen.

So, unless we decouple this carbon from hydrogen, we cannot get a clean spectrum we will end up with all carbons peaks coming as doublets and that is very reduces the sensitivity and resolution. So, therefore, a carbon 13 spectrum when you record carbon 13, you have to always decouple it from proton whether it is indirect dimension or a direct dimension in this case or indirect in the case which we will say later you will have to decouple proton. So, this is something we have to be very very careful always remember to do this.

So, now in the next class we will go into the details of why carbon 13 detection is an direct detection of heteronucleus is not a great idea and why indirect detection is now the most popular route or method used in 2D and 3D NMR ah. So, and further we will take up in the inept in more detail.