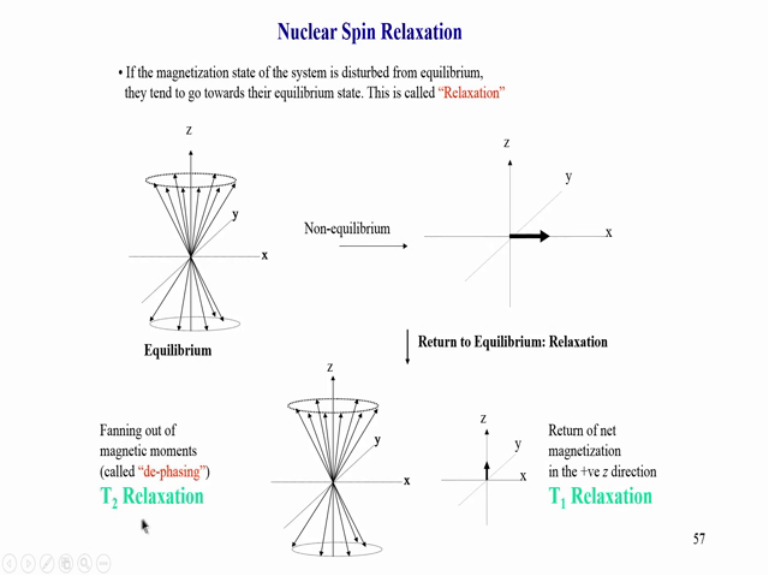
Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
Prof. Hanudatta S. Atreya
Department of Chemistry
Indian Institute of Science, Bangalore

Lecture – 05
Basic concepts in 1D NMR: Nuclear Spin Relaxation, 1H NMR and 13C NMR

Welcome back to the course. In the last class, we looked at the concept of J coupling, how the different J coupling values vary between hydrogen and hydrogen, and hydrogen and carbon. And these are very important values, because in biomolecules we exploit this J coupling to design new NMR experiments design 2D and 3D NMR experiments which we will see as we go along.

(Refer Slide Time: 00:57)



And another concept very important in NMR is nucleus spin relaxation which what is what we will briefly look at it today. So, what happens this is something which we already covered in earlier few classes. So, typically we have this is the classical picture of NMR in which the spins are represented by the vectors the arrows, and they are fanned along the x, y plane that is they are found along a cone and the cone is pointing in the z axis. This is for the alpha state; the spins which are in the alpha state. And similarly the spins which are in the beta state are also processing around the magnetic field ah, but they are pointing now in minus z direction. And they are also fanned out in form of a

cone and each vector is again represents one atom one particular atom in the molecule or one type of atom and these are different molecules.

So, now, if you combine these two we saw that you end up with what is called as net magnetization vector, which points along the z axis, so that is what we saw this is called the equilibrium magnetization. So, this is the equilibrium state. And this is what the spin has to spins have to achieve when you then they comeback to equilibrium. So, you have to keep this picture in mind that this is how it starts with the whole process starts like this, and we apply a pulse and we bring the magnetization to a x or y axis depending on the phase of the pulse. So, now, this is a non-equilibrium situation. The spins have been brought from z axis to x y plane. So, this now if I remove the pulse it has to go back to this situation.

So, what does it have to achieve? It has to achieve two things. It has to now this black this vector has to come back to z axis that is the net magnetization which is along z axis at equilibrium, not only that the individual spins what happens is when you apply a pulse they are all bunched together, they are all phased together. So, we use a word coherence. So, we create a coherence that is a same phase situation for the spins. Now, when I apply this, when I remove the pulse the phases have to go back into this random phase situation around the cone, so that is that is a second thing one has to achieve that is this spins have to now become decoherent or de phase.

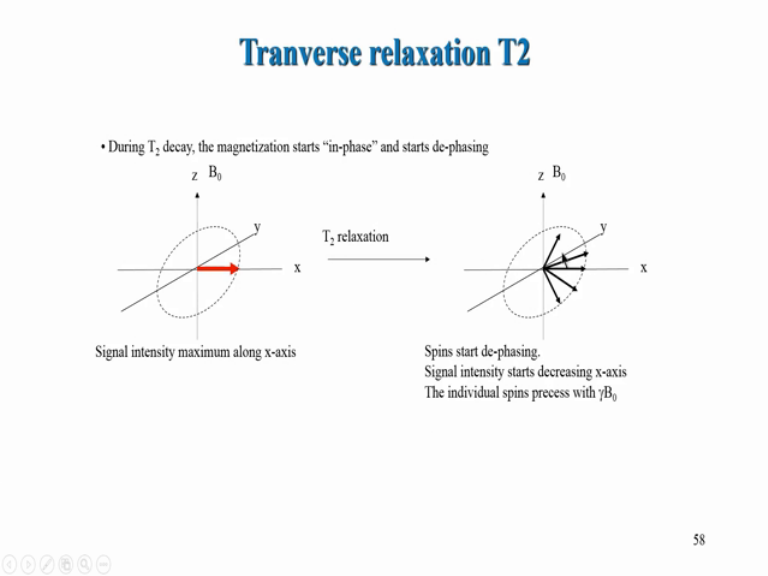
So, these are the two things that is one coming back along z axis and one de phasing in the x y plane, because you see this phase dephasing this vectors are actually not dephase in the z axis they all come together in the z axis, but in the x y direction they are all phased down they are randomised. So, therefore, that is the second thing which they have to achieve and the first thing along the z axis to recover along the z axis.

So, these two different thing pathways we use the word relaxation. So, relaxation is a process in which the magnetization starting from this non-equilibrium position goes back into the equilibrium scenario. And that is now the two phenomena as I said two different pathways or two different mechanisms, one is the z axis recovery which we use the word t 1 relaxation. And the second is a de phasing or de coherence of the vectors, because when you create vectors like this they are actually coherent they are together and that is

not an equilibrium situation, it has to recover back into the z axis and de phase out completely and that is T 2 relaxation.

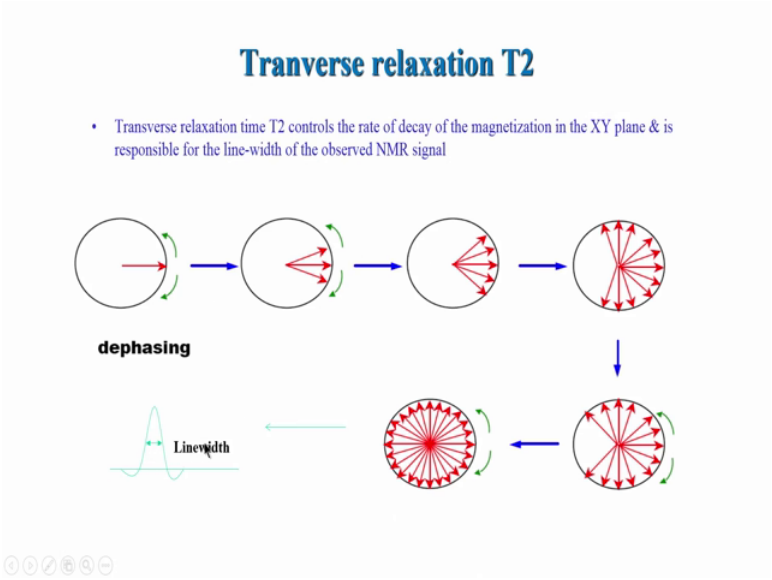
Now, these two parameters T 1 and T 2 play a very important role as far as the sensitivity of NMR is concerned as per as the line width of a spectrum or the peak in the spectrum is concerned. So, one has to therefore very carefully pay attention to this two mechanisms in say specially in biomolecules. They play a very important role and any wrong understanding of T 1 and T 2 can result in a not a very good data or NMR spectra.

(Refer Slide Time: 04:59)



So, let us look at this T 2 relaxation again a little bit more, because this is a very important phenomenon for large molecules like proteins. Here you can see again what is shown here is the non-equilibrium situation, the signal is maximum along the x axis, and now the spins start dephasing. So, they are dephasing in the x, y plane. And this is a picture we also saw in a few classes ago.

(Refer Slide Time: 05:21)



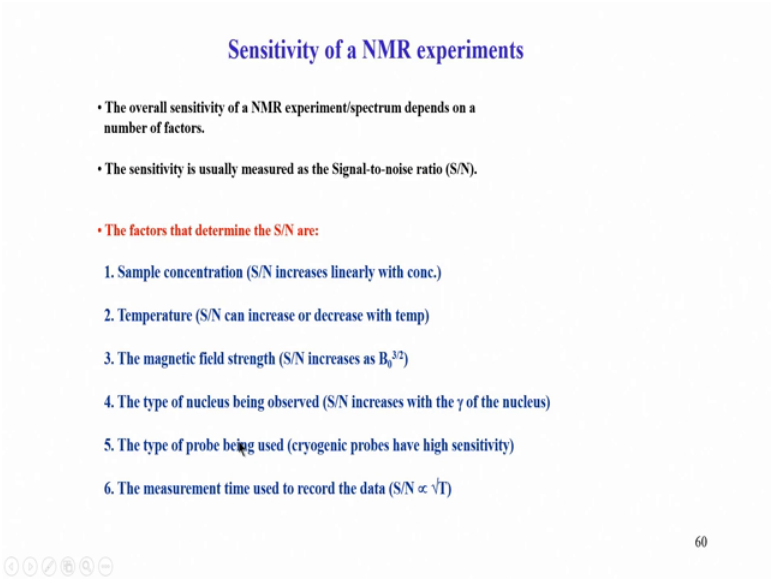
And now this dephasing is shown further in this manner. So, you are looking from the z axis and you are looking in x, y plane. So, this is a x, y plane this circle is in the x, y direction. So, this you can think of this as a x axis, and you can think of this as a y axis. So, you can see the spins are dephasing. And after some period of time even, you are looking from the z axis, they are completely dephased. This is the end of the T 2 process. So, the T 2 relaxation is essentially if you look at it is an is an exponential decay, exponentially goes back or goes starts from here at T equal to 0 and exponentially goes to at a long after a long time it goes into this stage. So, this typically happens about 5 times the T 2 values.

So, if you know the T 2 value of your if we are of your molecule of your proton, then after 3 to 5 times the T 2 value this is the scenario. And this as I said is very important, because this T 2 value this how fast or how slow this happens is directly related to the width of the lines. So, if you look at a NMR peak, NMR peak looks something schematically like this, and then the width which we measure at half the height. So, we use the word full width at half maximum. So, full with at have the maximum that is called a line width and liner width is directly related to the inversely proportional to T 2. So, longer the T 2 that means, the longer the time it takes to go from here to here the narrower the smaller is a value of the line width. So, it is a inverse proportionality.

If the T 2 is very short that means from here to here it happens in a very short duration then this line will be broad. So, what is the consequence of a broad or a narrow, narrow line in NMR spectrum? See, if the line is very broad the height will go down, because the area has to be constant. Let us say this represents an one proton whether the T 2 is high or low the area should not change. So, therefore, if the line width goes high or becomes more, more bigger, because of short T 2. The height has to go down to compensate to make, the area to keep the area constant this results in a very broad line and then signal to noise goes down.

So, this is what happens in biomolecules. We always deal with very low signal to noise, because there the molecule size is so large that the T 2 is very short. Now, this size and T 2 dependence we will take it up later, we also we have to use the word rotational correlation time and so on that we will introduce at a later point, but for large biomolecules the two is short for small molecules remember T 2 is long. And if T 2 is long, the line width is sharp; and if T 2 is short, the line width is high.

(Refer Slide Time: 08:15)



So, these are the different factors which govern the sensitivity of NMR. So, this is kind of a summary of one d NMR, how are what are the different factors, which you have to keep in mind. When we want to increase, again remember from a point of view of biomolecules, biomolecules are very low in sensitivity. So, therefore, any factor which improve the sensitivity is always welcome. So, starting from the very important the first

thing is a concentration, the signal to noise or that is that is the sensitivity increases linearly with concentration. So, if I double the concentration, I double the sensitivity of the spectrum; or if I half the concentration, I reduce it by half. So, the sample concentration is a very important parameter typically for biomolecules, we go up to about one millimolar, but many biomolecules cannot sustain at 1 millimolar, they start precipitating in NMR tube.

So, therefore, we go down we dilute the sample to 100 micro molar 50 micro molar and so on, but as you go below 100 micro molar the sensitivity drops dramatically. And therefore, unless you do some kind of a special NMR technique, if you do not use special NMR techniques, the signal to noise will be very low and the spectrum is hardly analyzable.

So, signal sample concentration is a very important parameter therefore, its rank number 1 as far as sensitivity is concerned temperature is a not. So, great a parameter, but yes it, is a important parameter, it is it also determine sensitivity. So, what happens is typically if you are a bio molecule, if you increase the temperature, the sensitivity goes up that is because the molecule now starts rotating or tumbling very fast faster relatively and that improves the line width meaning the line width reduces.

So, if the line width reduces, your signal to noise goes up. So, temperature can be a helpful parameter at times, but again temperature increase in temperature is not always possible, this biomolecule may start degrading at even at room temperature many biomolecules are not stable. So, we have to decrease the temperature go below room temperature. But if you go below room temperature, the problem is the sample starts the signal starts little bit broadening.

So, if you go to very low temperatures like 5 degrees or 4 degrees, the signal to noise drops. So, if your sample is stable at room temperature that is ideal situation, if it is stable in a higher temperature that is very good. But if it is not, then at least at room temperature or near room temperature what would be and as good condition for as well as temperature is concerned.

Magnetic field strength plays again a very important role. Now, you can see here the signal to noise goes up as B 0 or magnetic field to the power 3 by 2. So, it is not just linear, it is 3 to the power. So, if I go from 400 to 800 megahertz I do not double just

double the, the sensitivity, the sensitivity goes more than double, because it is to the power 3 by 2. So, therefore, it is one of the reason why a lot of research has gone into building higher and higher magnetic field. And you can see you can see in literature or you can see in, in, in industry or there are you now 1.2 gigahertz NMR spectrometers getting ready.

And this is one of the highest, this is the highest field strength as of today and that is because of this factor that any increase in magnetic field brings about a very high increase in sensitivity. Not only sensitivity, it also brings about improvement in resolution which is not listed this is not the factors, I mean resolution is not been discussed in this slide. But that is also a very important gain, which happens with for a high field magnet have high magnetic field also has some negative drawbacks some drawbacks, which we will not discuss now we will see that as we go to the bio molecular part.

Now, what is the important is what type of nucleus use you are observing. Now, proton is always the best as nucleus it has the maximum sensitivity as and carbon is not so great it is less 3 times nitrogen. In fact, nitrogen 15 is 10 times less. So, proton is always the best. But in biomolecules you cannot just rely on hydrogen proton unlike inorganic molecules, so you need carbon and nitrogen. So, as we see we will see how the improve the sensitivity if we are using carbon, and nitrogen that we will we will covered under hetero nuclear NMR spectroscopy.

The probe this is something which is hardware related, we had we are not gone into the hardware aspect in this course. I would suggest or recommend to go through the books or the previous course which was in which we covered the hardware part in detail. So, probe is essentially the part of the NMR system or spectrometer, where actually the sample is place and it receives the energy, and it emits the signal back to the probe.

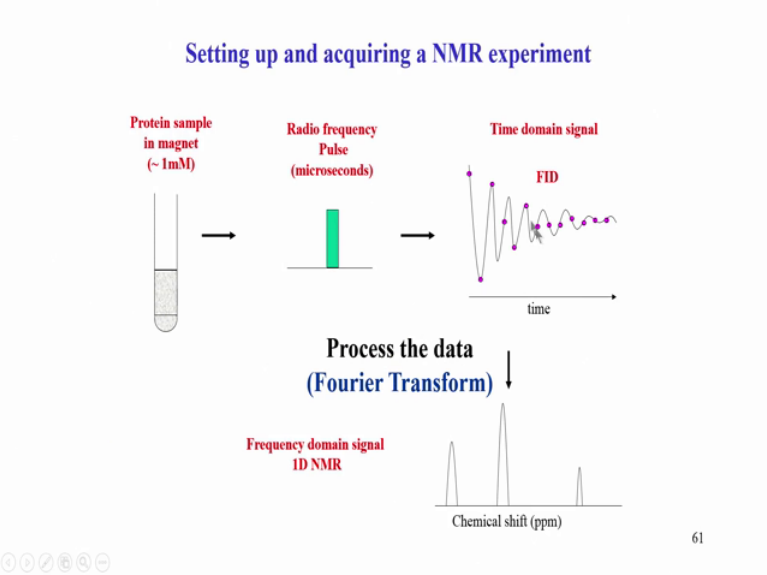
So, this special type of probes which are available today, we use cryogenic probes and they have high sensitivity. And for biomolecules these days it is by default at high magnetic field combination of your high magnetic field and cryogenic probe is what is used for bimolecular studies. So, typically people use 600, 700, 800 megahertz NMR that is higher magnetic field with cryogenic probes.

The, the last point which is again a important point is that it your sensitivity how strong is your signal or spectrum depends on how much time was used to record the data. So, if you use a very short duration and that is the number of time averaging or scans, if you use less, your signal to noise will be low. If you increase the number of scans, your signal to noise will go up. So, signal to noise is proportional to the square root of time. So, this is a important point here, it should be noted.

Suppose I double my or if I increase the measurement time from 1 hour to 4 hours, my signal to noise will not go up four times, it will go up by square root of 4, 4 by 1, which is 4 and square root of 4 is 2. So, if I quadruple, if I increase my signal as measurement by 4 times, signal to noise goes up only by 2 times. So, you can see the square root is important.

So, if I increase the measurement time 10 times, so if I record a spectrum instead of 1 hour I record for 10 hours my signal to noise will go up only by square root of 10, which is 10 3.6 3 point something. So, this is basically the way to look at how the signal to noise can be improved, there are different parameters and which is very important to keep all these in mind as we go along.

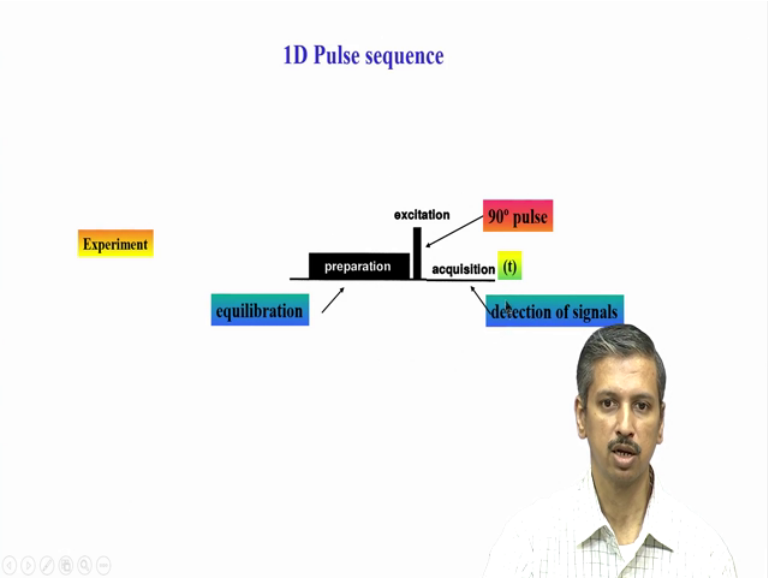
(Refer Slide Time: 15:07)



Now, this is again this is just a summary of how NMR 1D NMR spectrum is recorded. So, we looked at how the sample and the sample is then put it in the magnet. And we apply radio frequency pulse this is the resonance part which is applied in the frequency

with appropriate frequency. And then the signal which comes out from this molecules the sample is collected and as and as an FID, which decays with time and it is digitised. And after digitisation it is processed with Fourier transform and that gives you the final spectrum. So, the all the signals which are present in this signal, there are many different frequencies which could be present are now presented or separated in the frequency domain. And this domain is a frequency domain signal or a spectrum and that is converted into a ppm scale or represented in a ppm scale for analysis.

(Refer Slide Time: 16:03)



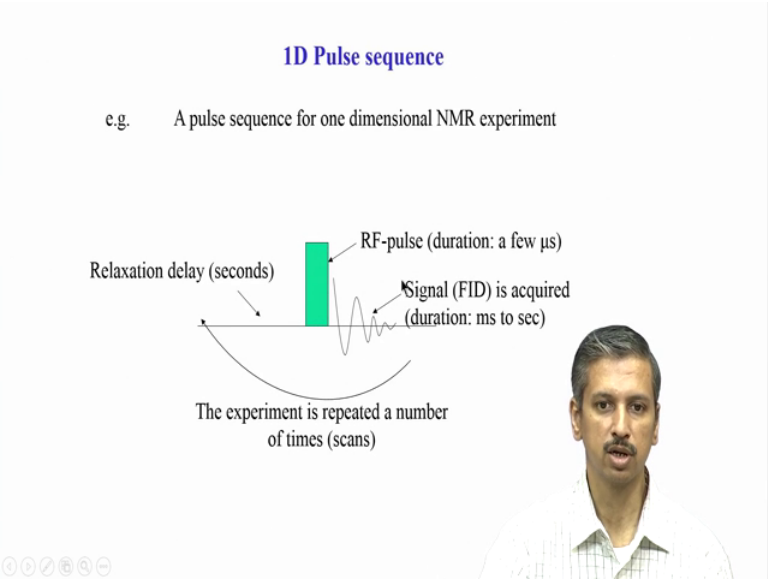
So, when we look at higher NMR 2D and 3D as we go long we have to understand how is an experiment and 1D NMR experiment and 2D NMR experiment are build. So, this is what is called as a pulse sequence. A pulse sequence is like a blueprint. It tells you that what are the things parameters which are important in a NMR experiment. So, all though we will not go into detail of each and every parameter here, we have we need to understand what comes first, what are the different words or the technologies which are used because when you go to 2D NMR shortly we will be referring to these terminologies.

So, a 1D NMR essentially begins with a preparation period, this period is what is called as a equilibration period. So, you have put the sample and you wait for sometime for it to equilibrate. And this is of the order of the t 1 of the sample. Once the sample has equilibrated, then you apply the pulse. When you apply the pulse the signal the

molecules are excited, so that is why we use the word excitation pulse. This is a 90 degree pulse. 90 degree pulse causes excitation remember and the 180 degree causes inversion. So, we are now looking a excitation.

Once the signal the excitation is pulse is over the FID the signal now starts getting collected in the detector and that is a free induction decay and that portion of time when we collect the signal, we use the word acquisition time. So, acquisition is the duration for which the NMR signal, the FID is collected this is why we call detection of signal. And once the signal detection is over, we repeat this whole experiment again by going back to this point here and that this whole process is repeated n number of times and that is called as scans. This is what is shown in the next slide here.

(Refer Slide Time: 17:55)



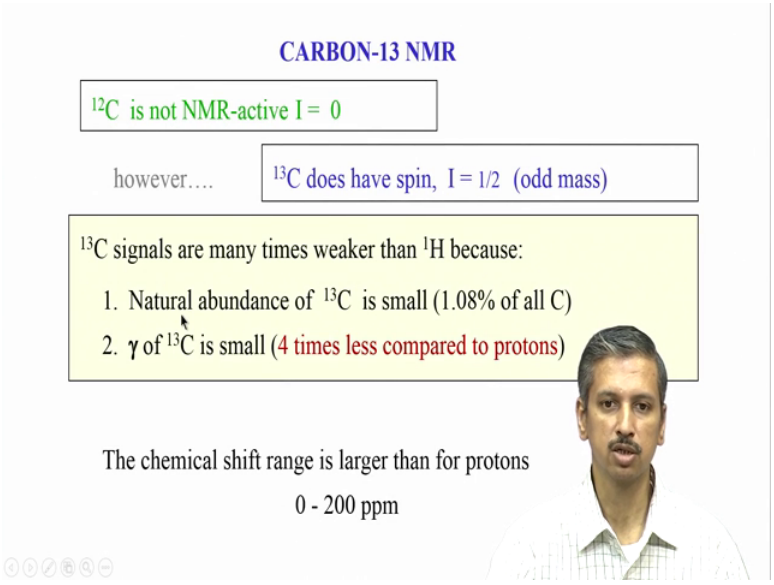
So, this is what I showed the pulse sequence for a 1D experiment. So, you have before you apply a pulse you have an equilibration time that is called relaxation delay and that is of the order of few seconds, and this depends on the t 1 of the sample remember. So, if the t 1 is long you need a longer time. If t 1 is short you need shorter time, you do not need to wait long time. And then you apply the pulse which is again duration of few microseconds, it can be also few milliseconds depending on the selectivity which we will go into detail later.

So, once our pulse is applied and removed the signal which is the FID, it is collected and that FID is now the acquisition time, the time it takes for the FID to go to 0. So, this time

which is about 100 milliseconds to a few seconds. Now, this depends on the t 2 of the sample. So, the FID depends on the t 2, the length of the FID or the duration for which it is collected. And the relaxation delay is before the pulse that depends on the t 1. So, t 1 and t 2 play a very important role in a signal this acquisition.

Now, once the signal is detected as I mentioned you go back to this and repeat this n number of times and that is basically called as scans. So, if you do a single scan, the experiment is over at this point; if you do multiple scans, you do multiple times. But each time you have to go back to here not just before the pulse you have to wait again for the equilibration to take place before you apply a pulse. So, this is this process of a 1D NMR experiment and we will see as we go to 2D NMR, this will this kind of a picture or diagram will be repeated.

(Refer Slide Time: 19:49)



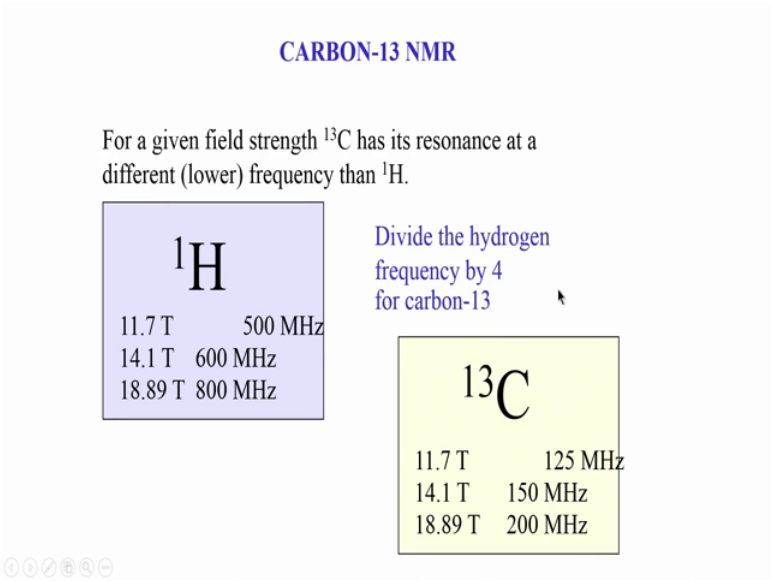
So, therefore, it is important to understand this concept which was covered also in detail in the previous course and also is covered nicely in many different text books. So, before we end 1D NMR important nucleus which it comes in bio molecular NMR is carbon-13. So, let us look at carbon-13 NMR spectroscopy now, what is special about carbon-13. Carbon-13 is carbon-12 as we know does not have a spin and this I is equal to 0, because it has even atomic number, and even atomic mass. Or carbon-13 which is isotope of carbon has a spin value half, because it is odd mass.

So, but the problem with carbon-13 that there are two issues, one is the gamma that is the gyro magnetic ratio is 4 times less compared to protons. Not only that the second problem is also compounding the whole issue is that the natural abundance means how many it carbon-13 atoms are present in a molecule is small is about 1 percent, which mean 1 percent out of 100 or 1 out of 100 atom in a molecule or in a collection of molecules will be C 13.

But then why do we have to use carbon-13 at all that is because of this single factor that is this range chemical shift range is very large. You can see it goes up to 200 ppm in biomolecules or it can go even up to 300 in case of inorganic or organic molecules, but 200 ppm is a very large dispersion or resolution which is achievable with carbon-13. And also it carries lot of interesting structural features which can be useful for biomolecules. So, carbon-13 although it these are the two problems it is still used very popularly.

And the first problem here which is an abundance can be dealt with using isotope labelling which is what we will cover in this course for biomolecules, we can make a molecule fully carbon-13 labelled in which case it will be 100 percent of all carbons will become C 13, no longer 1 percent. So, 1 percent is a natural abundance case. It taken any molecule from nature, but I cannot artificially enrich the molecule v C 13 using special techniques especially biomolecules, we use biological techniques and that will result in 100 percent carbon-13. So, the first problem is completely taken care by using this method of isotope labelling. Whereas, the problem remains this is again a difficult to handle, but this also can be removed by hetero nuclear NMR techniques which we will see when we come to 2D NMR. So, both this problem can be tackled and the reason for the motivation for doing that is this use range.

(Refer Slide Time: 22:29)

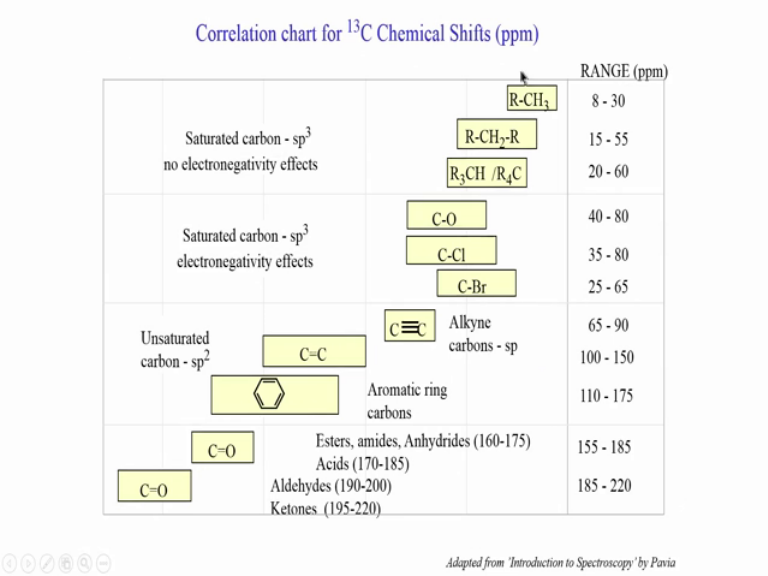


So, let us see what are the different frequencies at which carbon resonates for a given proton frequency. So, this is very simple to calculate. So, this is a magnetic field shown here for hydrogen, I mean for a for a magnet. And for hydrogen the frequency corresponding to that magnetic field is shown here. So, this comes from the equation omega equal to gamma into b 0. So, b 0 is given; gamma we know for protons, so we can calculate omega. So, these are the different values for different magnetic field.

For carbon it is just 4 times less because carbon also has a same equation omega equal to gamma into be 0, but now the gamma is 4 times less compared to proton, so therefore, its gyro magnetic ratio is 4 times less, so its frequency will become 4 times less. So, you can see for a 500 megahertz in a spectrometer, your carbon-13 will come at 125 megahertz frequency ok. So, similarly for 600, it becomes 150; and for 800 it is 200. So, we essentially divide the hydrogen frequency by 4 times to obtain the frequency of carbon-13.

So, what is the implication of this? The implication of this is on the sensitivity. Remember gamma I we showed in the very first or second class is a factor which determines the sensitivity by through Boltzmann equation because of 4 times less gamma that the sensitivity of carbon and the polarization or the population difference of carbon is also less.

(Refer Slide Time: 23:57)



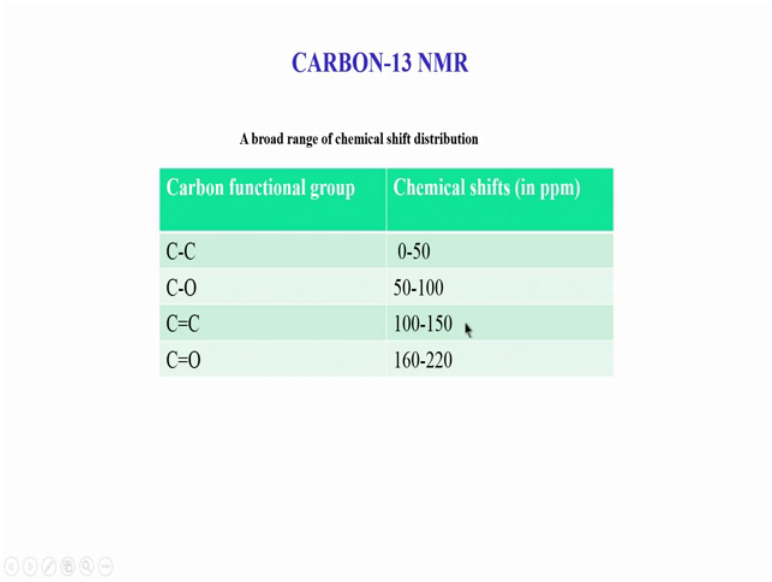
But this is where the advantage of carbon comes in, you can see the different the range the huge range of chemical shift variations possible in carbon-13. So, it varies from 8 almost 0 ppm, where the initial the first 20-30 ppm belong to this methyl groups and methyls are very strong NMR they give a very very strong NMR signals both in carbon-13 and proton. And they are very easy to identify and they come around this region then comes the methylene in amino acids and proteins.

Then comes methane. And then there is kind of a gap normally you do not see anything up to 80, 70 to 80 and there is where the amino acids which are attached to oxygen, the carbons in amino acids attached to oxygen come in this range. In biomolecules we are not concerned with chlorine and bromine, as similarly in biomolecules we do not worry about alkines. So, this particular zone in biomolecules from 35 to 90 or sorry it is from 80 to about 100 or 120 is a kind of empty, we do not see anything in this zone because this species are not present in biomolecules

So, biomolecules typically we go up to 80, and then we next the region which is of interest is the aromatic because of amino acids which are aromatic in nature, their peak start appearing at between 110, 120 to 140. So, aromatic region is another region of importance then another very important region is a carbonyl. And this carbonyl in case of proteins comes from amides. So, we are looking somewhere like 170, 165 to 175 or 180 ppm in this range for the carbonyl. We will not normally get peaks so down field that is

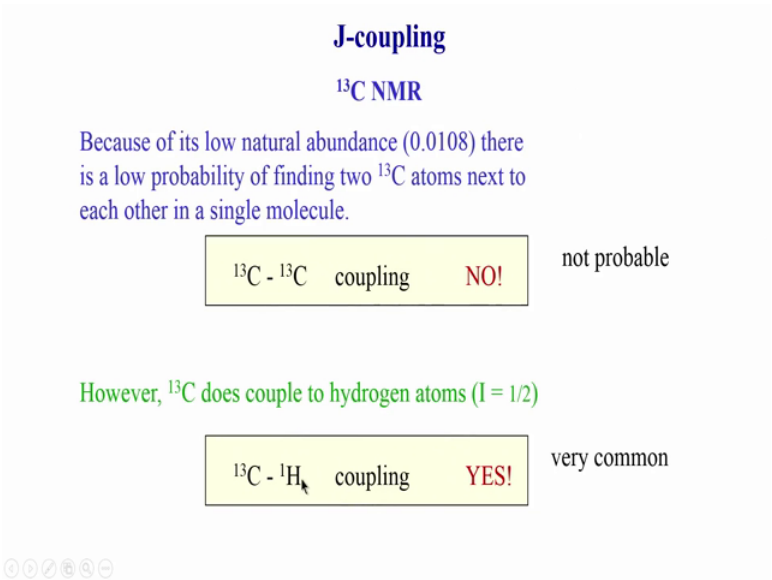
between 180, 200 in proteins, but for organic molecules this is very important zone. So, carbon-13 NMR spectrum is therefore, very useful it has a wide range. So, we can actually look at the spectrum and identify the different functional groups which are present in the system.

(Refer Slide Time: 26:05)



So, this is again a broad range of chemical shifts given for carbon. So, single bond is somewhere between 0 to 50; carbon oxygen the carbon attached to oxygen come around this range; double bond which is in aromatics, and carbonyl in this range.

(Refer Slide Time: 26:23)

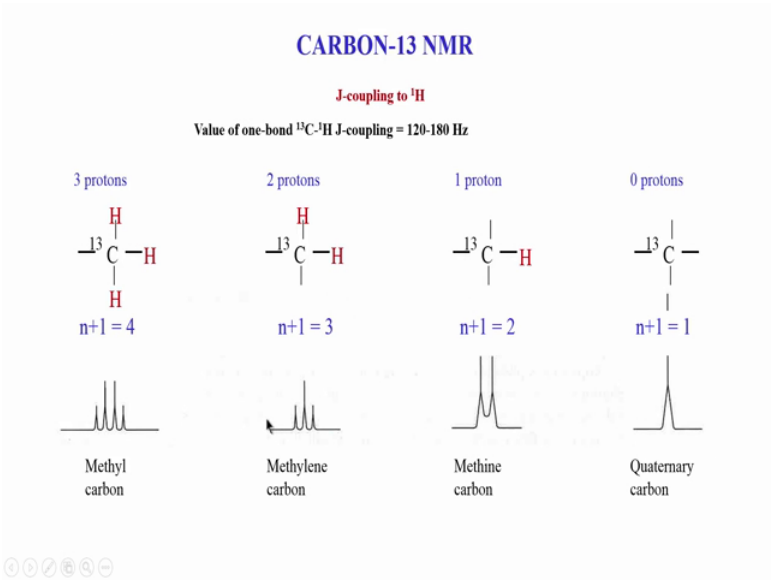


So, one thing very important in carbon-13 NMR is if there is a coupling between two carbons. So, naturally there is going to be a coupling because both are NMR active. So, if there are two carbons attached to each other, it is possible. But then the thing is there is the abundance of carbon and natural abundance is very low. So, if this is 1 percent abundance, this is also 1 percent. So, what is the chance that you will encounter two carbon-13 next to each other and that chance is 1 percent of 1 percent that is very very low. And therefore, carbon-13 carbon-13 coupling will never come in the spectrum unlike protons and whereas very conspicuous when a NMR spectrum of proton as we saw as shows very nice doublets, triplets and so on.

But if you record a carbon-13 1D NMR, you will hardly notice any carbon-carbon coupling at natural abundance. But this is no longer the situation if you have a C-13 enriched biomolecules like in proteins when I will show go through the isotope labelling part we are essentially focusing on completely making all the carbon in the molecule has carbon-13. In such a scenario your coupling is going to be present. So, carbon-carbon coupling at natural abundance is not a problem, no not probable, but in carbon-13 enriched molecules it is definitely a problem which one has to consider when designing experiments, we will see that how we when we go along.

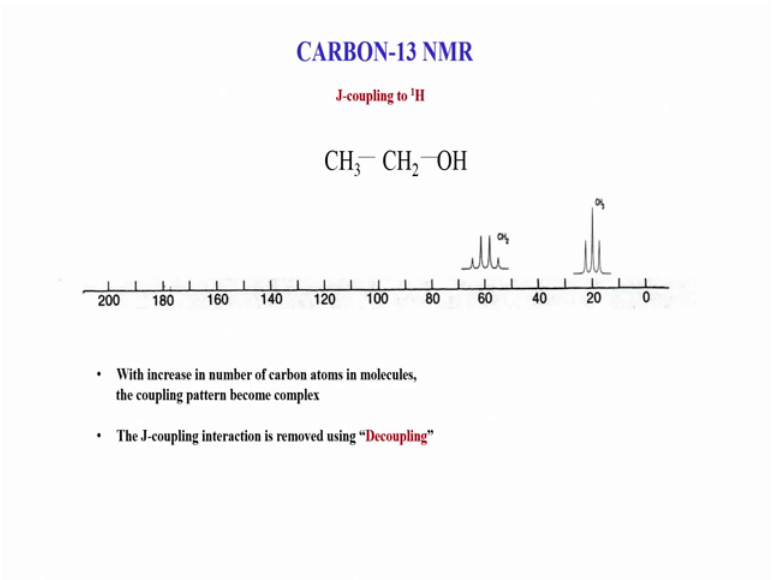
But carbon-13 definitely couples to hydrogen irrespective of whether it is enriched or not enriched because every carbon-13 will most likely we attached to a proton unless it is a quaternary, but you will have this carbon-13 attached to hydrogen and hydrogen is 100 percent NMR active their abundance. So, any these two are now magnet two NMR active nuclei, so they will start coupling nobody can stop it from coupling. So, this coupling now is strong coupling about 100, 200 hertz and that coupling will now possible you can see in the spectrum. So, if you record a carbon-13 spectrum, you may see a coupling to proton unless we remove that coupling, so that is what is important to understand.

(Refer Slide Time: 28:39)



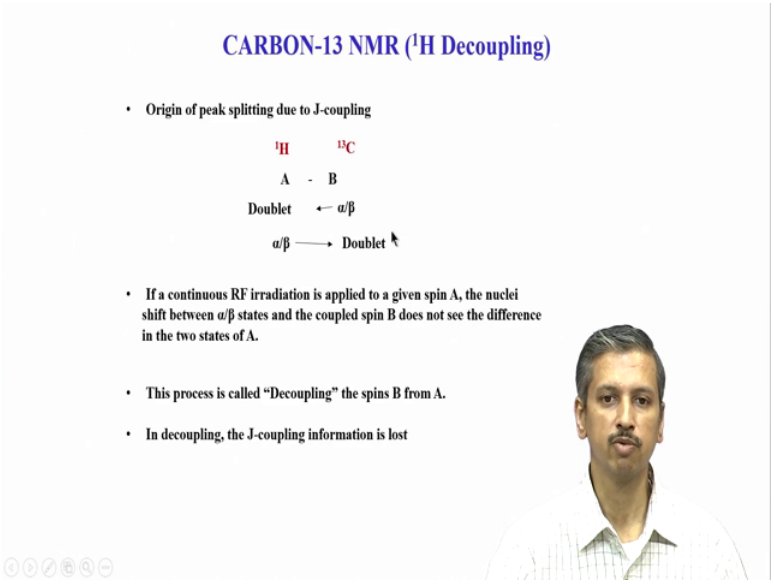
So, let us look at first how the patterns will come, this is similar to what you see in proton NMR. If you have a carbon-13 attached to 3 hydrogens, it will be n plus 1 4 that is that n plus 1 rule. Similarly, if it is has to 2, it will be triplet doublet and a singlet. So, this is similar to what we saw in the case of protons hydrogens that if a hydrogen is attached to one hydrogen, it shows a doublet if it is attached to two, triplet and so on. So, this can be a problem because you see the sensitivity goes down, each time you record a splitting or get splitting the sensitivity goes down by half.

(Refer Slide Time: 29:13)



So, that causes a problem and that increases the complexity also, because instead of one peak and now I start seeing many peaks. So, the very the standard technique which is used to remove such couplings between carbon and hydrogen is called as decoupling. So, decoupling is a technique in NMR to remove the J coupling between carbon and hydrogen in a carbon NMR experiment. Decoupling is a general word, it can be also used for carbon-carbon decoupling, carbon-nitrogen decoupling or any any two nuclei if you want to decouple we use the word decoupling.

(Refer Slide Time: 29:51)



So, in carbon-13 NMR, it is essentially a technique where we apply a continuous RF irradiation, a RF energy to a given spin A and then the nuclei starts jumping between the two because you are applying RF and that results in decoupling. So, we will not going to the details of decoupling in this course, but this has been covered in the many books. And the idea basically is which you have to understand and take home is the carbon-13 appears now as a singlet, because we have removed its coupling to the j coupling to proton. We can also do this with other nucleus carbon-13 with nitrogen or carbon-13 with another carbon-13 which we will see when we go to biomolecules. So, this brings us to the end of the 1D NMR spectroscopy. We will now move on to 2D NMR and we will see how now in the different homo nuclear and hetero nuclear 2D NMR experiments are conducted and analysed.