NMR Spectroscopy for Chemists and Biologists
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Lecture 53
2D Heteronuclear Experiments 1

So far we discussed the two-dimensional experiments, which correlated same kind of spins or which we call them as homonuclear correlation experiments. So we are now going to go into another class of experiments, which are called as heteronuclear correlation experiments.

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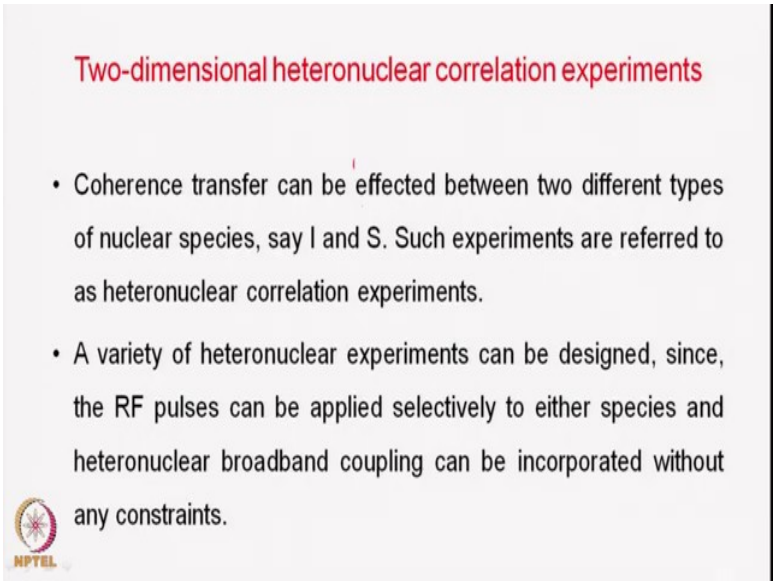

Coherence transfer can be affected between two different types of nuclear species, *I* and *S*. *I* can be a proton, *S* can be something else carbon, nitrogen whatever. Such experiments are referred to as heteronuclear correlation experiments. Now you can design a variety of heteronuclear experiments because the *RF* pulses can be applied selectively to either species, both proton and carbon, they are very widely separated in terms of the frequencies. Therefore application of the pulses is not a problem.

And you can also do heteronuclear broadband de-coupling can be incorporated without any constrains. So during the, indirect detection period or the evolution period where there is no acquisition going on. We can also do various kinds of de-coupling tricks. And even during the acquisition, when you are doing, if one particular kind of nucleus is being detected, the other nucleus can be decoupled because those pulses can be applied without interfering with the detection of the signal.

So therefore these are particularly useful and these are actually revolutionised the applications in biology. Structural biology came to a long way with the application of heteronuclear experiments and some of those experiment we will show in the coming classes.

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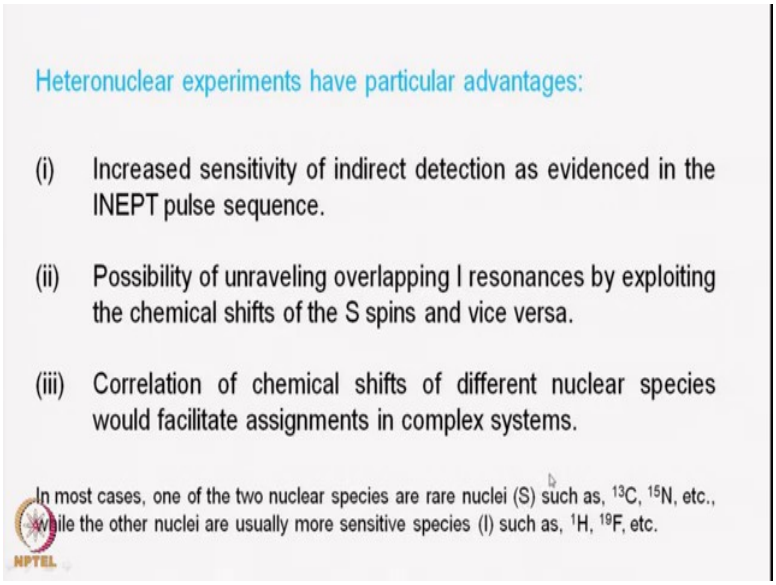

Now, what are the specific advantages? Say increase sensitivity of indirect detection as evidence in the INEPT pulse sequence. So in the INEPT gave a significant advantage to the insensitive nucleus, because you transferred polarization from the most sensitive nucleus like proton, you transfer proton to the carbon or you can do transfer from the proton to the nitrogen, so which are insensitive.

So nitrogen is insensitive, it is gyromagnetic ratio is one-tenth of that of proton and carbons gyromagnetic ration is one-fourth of that of proton, therefore if you transfer polarization from the proton to the carbon or the nitrogen, the X nucleus will have a much greater sensitivity and that is one significant particular advantage.

Secondly, possibility of unravelling overlapping I resonances by exploiting the chemical shifts of the S spins and vice versa. So, since you are using two different kinds of nuclei here, so in the correlation experiments, the overlap of chemical shifts of a particular type of protons, in the case of protons for example, can be resolved by making use of the chemical shifts of the S spins or the carbon spins or the nitrogen spins.

And therefore, this is a specifically important from in complex molecules where there are larger number of protons or larger number of signals of a particular species and they are very invariably they will overlap and therefore you need a second nucleus to separate this into

multiple distinct peaks. Then correlation of chemical shifts of different nuclear species would facilitate assignments in complex systems. And this is because you improve the sensitivity on one hand and improve the resolution or the separation of the peaks on the other.

And this is typically used for proton-carbon correlations, proton-nitrogen correlations or sometimes proton-^{19}F correlations and all of these are particularly useful in many complex systems.

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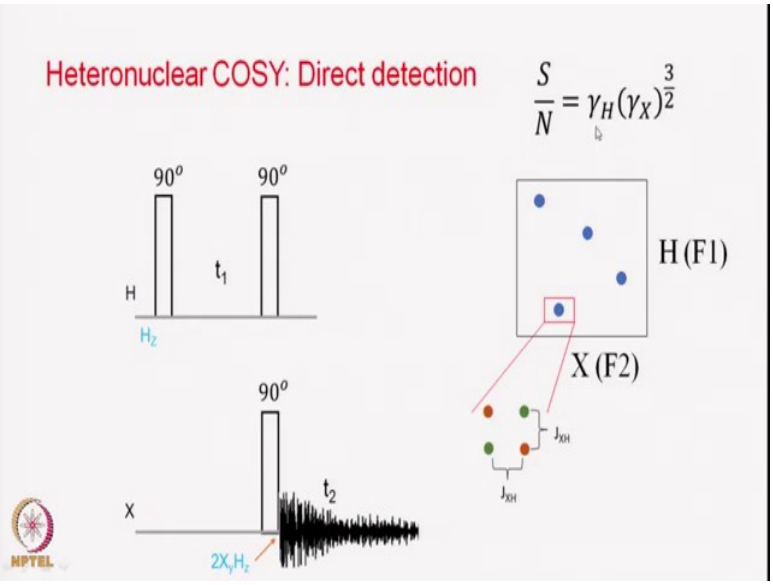

Okay, let us look at some of the standard correlation experiments here, heteronuclear COSY to understand the principles and how do they work. Now this is the simplest correlation experiment. This is heteronuclear COSY by direct detection here. So this is very similar to the COSY, homonuclear COSY what we have studied earlier, okay. Except that we have two separate channels here, this is the *X* channel and this is the proton channel and we can apply pulses selectively to this channel or to this channel and that is the big advantage as I mentioned to you before.

So, in this case we apply a pulse to the proton, so therefore I create here only the transverse magnetization of the protons at this point okay and the proton magnetization then evolves during the t_1 period and this is the 90˚ simultaneous pulses on both, the proton as the *X* nucleus. And then the transfer of magnetization from proton to the *X* nucleus happens as the result of this mixing, this is the mixing pulse right.

So this is the mixing sequence here, so there will be transfer of magnetization from here proton to the carbon or to the nitrogen, whatever the *X* nucleus is and then after that you

detect the signal of this nucleus, so detect the *X* nucleus during the t_2 period. And let me repeat here. So during the t_1 period I have the proton magnetization evolving with it is characteristic frequencies and with this pair of 90° pulses applied simultaneously, you transfer the magnetization, transfer coherence from the protons to the *X* nucleus and the *X* nucleus is detected during the t_2 period.

So therefore what do you expect? You expect after the two-dimensional Fourier transformation I should have the *X* frequencies along the F_2 axis and the proton frequencies along the F_1 axis. So a typical correlation spectrum will therefore look like this, so this is schematic here, you are taken four peaks here. Now, each of these peaks will have the fine structure, why do they have fine structure?

Because there is also coupling evolution here, the proton *X* nucleus coupling evolution happens here. And during this period as well there is also the *X* nucleus coupling evolution happens with the proton, so because *X* is coupled to the proton. Therefore, the structure will be very similar to that in the COSY cross peak, so in other words we are recording just the cross peaks here, there are no diagonal peaks in such a kind of spectrum.

We are only recording the cross peaks that is the correlation peaks between the proton and the *X* nucleus and each of those cross peaks has the fine structure as in the normal COSY, okay. So it will have ¿ structure and the separation between them is the coupling constant as we have discussed before, okay. So along both the axis we have this coupling information present. And notice here, by enlarge these are one bond couplings, so if I am talking about proton to carbon, it is a proton attached directly to the carbon.

So typically we are talking about one bond couplings and the transfer is happening on the basis of the one bond coupling. Then these coupling constants are usually very large, so 140 hertz and they do not vary too much, so therefore one bond coupling is nearly same in all kinds of species. Therefore, this separation will be always be the same in every molecule. So generally this information may not require for you okay, so that we will see separately later.

Now let us look at some of the signal to noise considerations here. Typically the signal to noise in any experiment is dependent on the gyromagnetic ratios of the exited nucleus and the detected nucleus. It is proportional directly to the gamma of the exited nucleus with regard to the detected nucleus it is proportional to the three-half power of the gyromagnetic ratio of that nucleus. Therefore in this case,

$$\frac{S}{N} = \gamma_H (\gamma_X)^{\frac{3}{2}}$$

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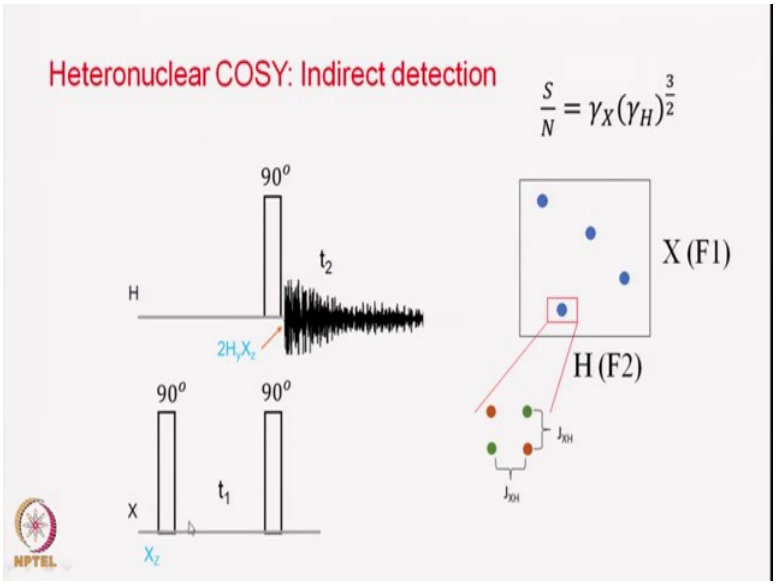

Now let us look at the an alternative sequence, here is an indirect detection, there it was carbon *x* magnetization one was directly detected. In this case, we will record the *x* magnetization in an indirect manner, how do we do it? Okay, we start with the *x* magnetization here, so we start with the *X* spin, apply 90*x* pulse to the *X* spin and the *x* magnetization evolves during the t_1 period okay.

So *x* magnetization evolve during the t_1 period and with these pair of pulses here you transfer the polarization to the proton okay, the relevant density product operators are indicated here, you have the *x z* here and at this point you create proton magnetization which is anti-phase to *X* and you have the $2H_yX_z$.

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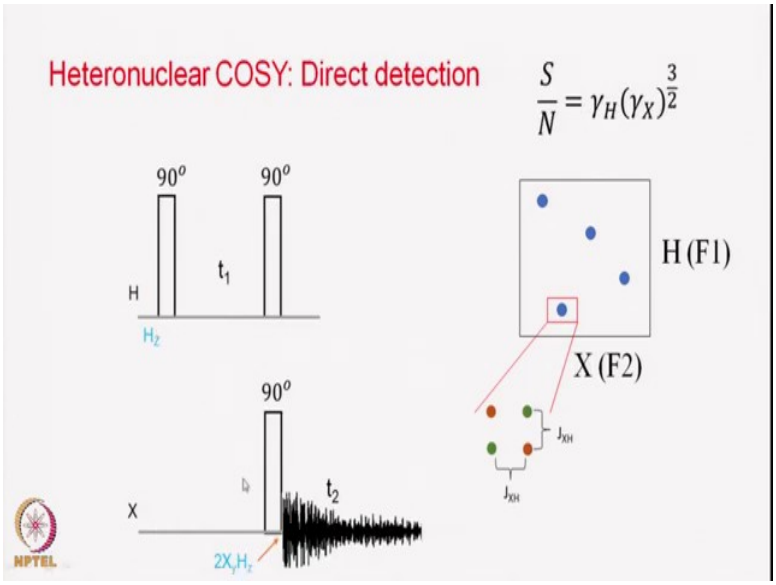

In the previous case also, it was the same, you started with H_z here and you ended up with anti-phase magnetization of the x spin to $2X_y H_z$ is the product operator term and that results in the anti-phase nature of the fine structure in the cross peaks.

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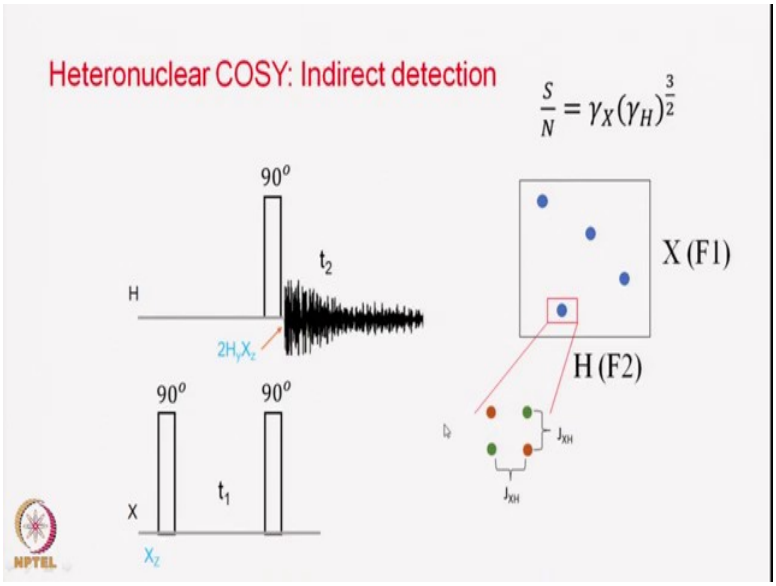

So here also it is the similar, you start with X and you end up with $2H_yX_z$ and now we detect the proton because it is anti-phase proton magnetization, we detect the proton, therefore during the t_2 period you will have the proton evolution going on and coupling evolution also will be going on.

So therefore in the 2D spectrum you have here, the proton along the F_2 axis and the X nucleus along the F_1 axis, therefore we call it as the indirect detection of the X nucleus. Once again,

the fine structure will be the same as in the previous case we will have the ¿ character in the individual cross peaks here. The coupling constant appearing as a separation between the two peaks in the fine structure.

Now, the signal to noise will be different, because we are exiting the *X* nucleus, therefore it is proportional to the γ_X but we are detecting the H nucleus. And now, therefore it is proportional to the three-half power of the proton.

$$\frac{S}{N} = \gamma_X (\gamma_H)^{\frac{3}{2}}$$

So, it will appear therefore that this might have a better signal to noise in the spectrum because this is $(\gamma_H)^{\frac{3}{2}}$. However, when you are detecting *X* nucleus indirectly, then along the F_1 axis you have the spectral width of the *X* nucleus. Notice the spectral width of the *X* nucleus is very large, especially if it is carbon you have a 200-ppm chemical shift range.

And therefore, it will be very difficult to cover this entire spectral range with a good resolution, therefore resolution along the *F* axis will suffer. So because you cannot give a such a large spectral width, if you such that width your increment will be very small, you would not be able to excite all your frequencies by this pulses, special tricks will have to be used. But generally, because the large spectral width here, you suffer from the resolution problem in the indirect dimension.

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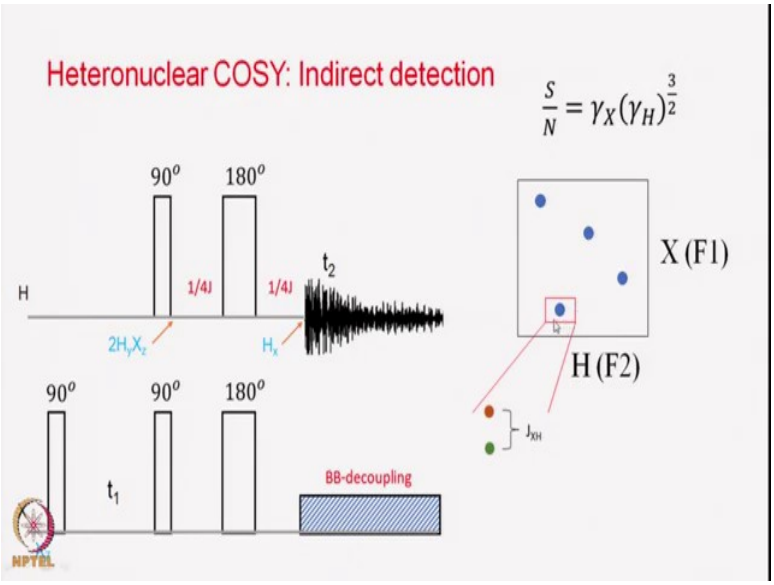

Now, so what shall we do to remove this coupling constant? Because we say that the coupling constant is the same in all the molecules for all the carbons, why do we need that information here? We are looking for correlation between the two nuclei. So what we need is the correlation between the chemical shifts, coupling constant is not necessarily required here, because we are not going to, since there is no variation in the coupling constant, there is no need to measure it either here.

So, we simply want the correlation information, so but we cannot remove it unless we can decouple them, if you decouple the two species then only, we can remove the coupling information. So what we will have to do for that? So therefore let us look at the indirect detection experiment here, so we started from here and we came to the proton at this point. Now this was anti-phase magnetization of the proton at this point.

Now we have to refocus this into in-phase magnetization of the proton then only we can decouple the *X* nucleus. Because if you decoupled here, what happens? The ¿ components will merge and then they will cancel each other, therefore the peak will vanish. Therefore, we cannot afford to do a decoupling in the previous experiments because of the anti-phase nature of the cross peaks. So therefore we will have to re-focus it. To for refocusing we adopt this INEPT type of sequence you remember this is the INEPT sequence basically.

So you have $\frac{1}{4J}$ evolution for $\frac{1}{4J}$, 180 pulse again $\frac{1}{4J}$, so during this period the one bond coupling evolution happens and you can exactly match it with these delays. Taking this *J*, you can exactly calculate how much this delay should be and you put that here. Then there will be complete transfer of anti-phase magnetization into in-phase magnetization.

So, you have your, at this point, the only operated term will be H_x and you do not have anything from the anti-phase components at all here. So once it is H_x, now then you can decouple these, so this is a broad band decoupling. Across the entire spectral width, you can do decoupling, so all the protons, all the *X* nuclei will be decoupled, okay. You are detecting the proton and every coupling with proton, *X* nucleus coupling will be removed.

So, therefore what happens? The F_2 axis and this is F_1 axis, along the F_2 axis the coupling has been removed right, this is the F_2 axis here, detection here gives you the F_2 frequencies and there we have decoupled it, therefore the coupling along the F_2 axis have been removed. However, it still remains along the F_1 axis because here we did not do anything to remove the coupling evolution.

So, in the t_1 period the coupling evolution still happens and therefore we do have the pleading due to this J coupling in the F_1 dimension okay. The signal to noise remains the same as the previous case.

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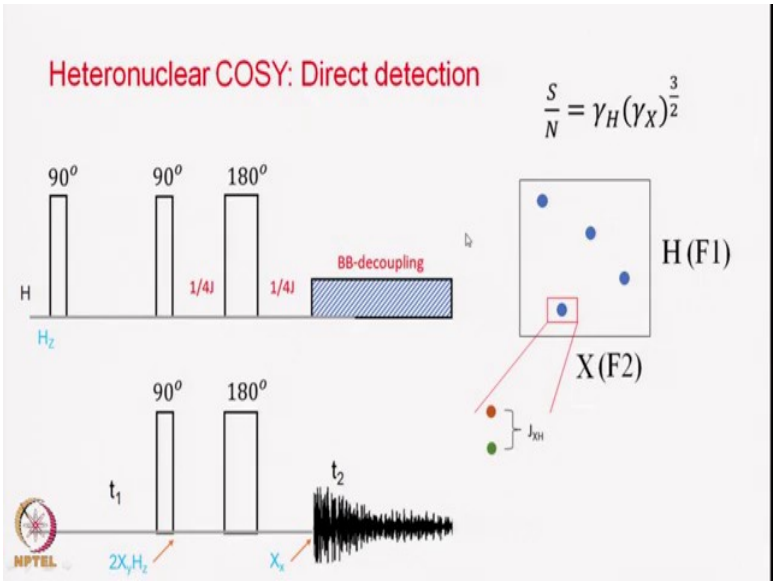

Okay, now if you do that for the heteronuclear direct detection, you start from here and you come to this anti-phase x magnetization here. The x magnetization is anti-phase with respect to the proton. Now, you do the same trick, you put the same $\frac{1}{4J}$, $\frac{1}{4J}$INEPT kind of a sequence called the refocusing sequence, refocusing element here. So you need you apply 180 pulses simultaneously on both, X nucleus as well as the proton.

So, at this point you will have the in-phase magnetization of X nucleus, okay. Now the X nucleus evolves during the t_2 period, therefore so this is the decoupling has been effected along the t_2 dimension. Therefore, there will be no splitting along the F_2 axis but there will be splitting along the F_1 axis. So this portion will remain the same as in the previous case, okay notice so this will be [+ -] here along the F_1 axis and not in this manner.

So, now we have here X here and proton here and this coupling appears along the t_1 and along the t_2 axis there is no coupling evolution. Therefore, in the F_2 axis there will be no splitting, there will be splitting only along the F_1 axis.

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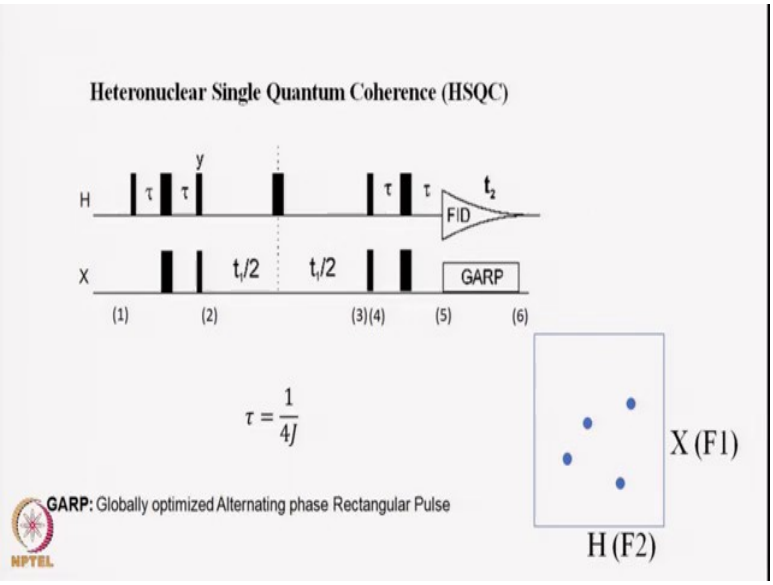

So, therefore we want to remove this as well and that leads us to the so called heteronuclear single quantum coherence and typically known as HSQC. And this goes in the following manner. So, you have 2 channels once more, proton and *X*.

First of all, there is an inept sequence here, you start with the proton magnetization here at this point and you do an inept sequence here, this INEPT sequence is 90-τ-180-τ-90 here and there is a 180 here and a 90 here, these are 90˚pulses okay and if this is the *x* pulse, this has to be a *y* pulse and this can be anything, *x* or *y* does not matter. So we start here, we select $\tau = \frac{1}{4J}$, when we do that this is an exact inept sequence, therefore there will be a total transfer of magnetization from the proton to the *X* nucleus at this point.

So here I will have anti-phase magnetization of the *X* and then during this next t_1 period, I have the evolution of the *X* spins and there is in the middle I have put a 180 pulse and at the end of the t_1 period there is magnetization which transfer back to the proton here and then during the next refocusing period, this is the τ-180-τ, coupling evolution happens and there is refocusing here and then you generate in-phase. We will look at the product operators little bit more explicitly here.

And then you decouple, now you can decouple with the detecting proton, it would detect the *X* nucleus. Now I have proton along the F_2 axis and *X* along the F_1 axis. How does this work?

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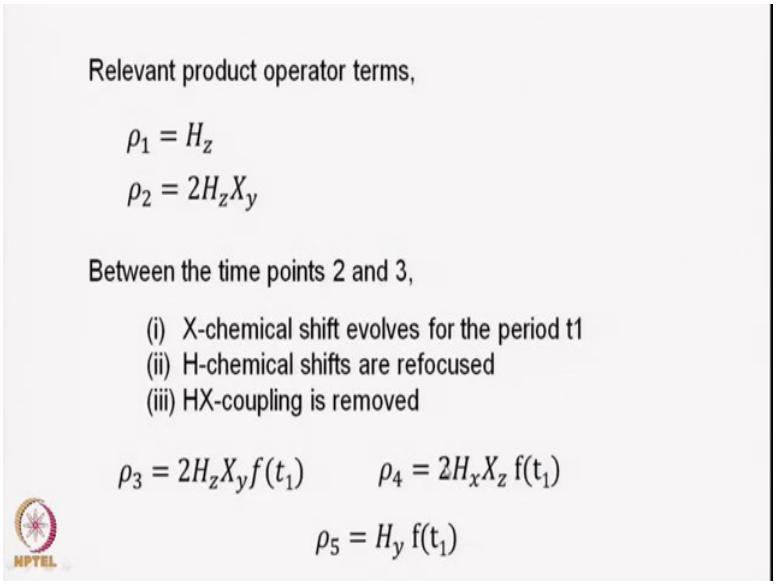

Let us look at this little bit more explicitly using product operator terms, $\rho_1 = H_z$, proton magnetization, z magnetization of the proton and $\rho_2 = 2H_zX_y$. Between the time points 2 and 3 the following things happen, X chemical shift evolves for the period t_1. Protons chemical shifts are refocused, because I apply 180˚ pulse on the proton.

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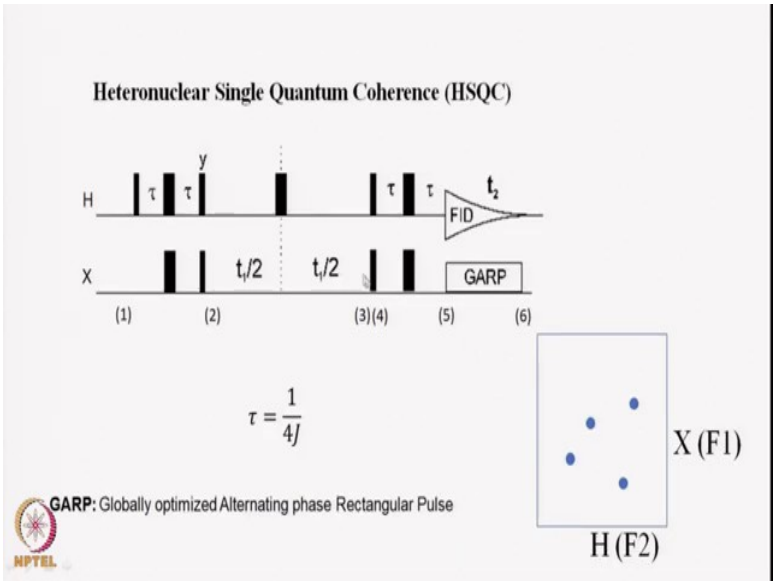

We recall whether is a 180-pulse applied on the thing and therefore there is no proton chemical shift evolution happening in the t_1 period.

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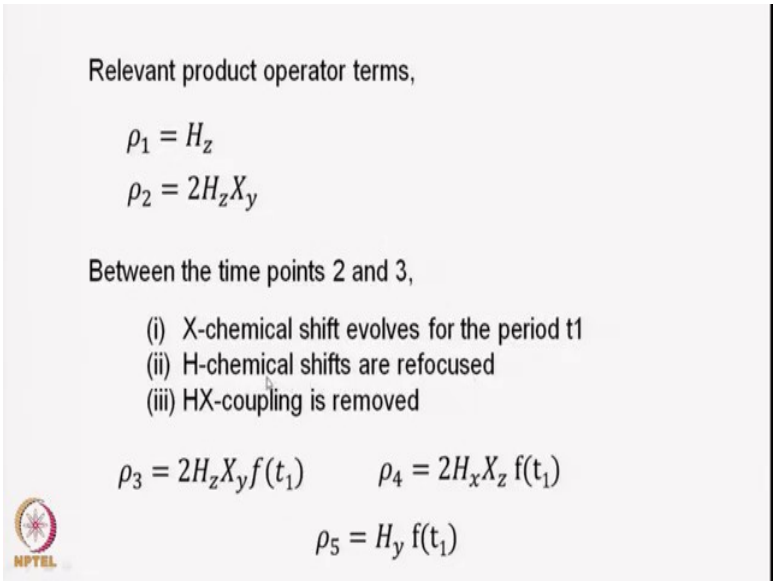

H chemical shifts are refocused. *HX* coupling is removed okay. Now *HX* coupling is removed why? Because I have applied a180 pulse to only proton, I have not applied to the *X* nucleus. Therefore, when you apply a 180° pulse to only one of the species, you have seen before that it refocuses the coupling evolution as well. Therefore, there is no coupling evolution during this real period t_1.

Therefore, there is only chemical shift evolution of the *X* nucleus. Therefore, at the point ρ_3 which $\rho_3 = 2H_zX_y f(t)$

the *f(t)* is the function of t_1, because this has happened as the result of the chemical shift evolution of the *X* spin. So this will contain coefficients arising from the evolution of the *X* chemical shift. Now when I apply next 90° pulse on both, the *X* as well as the proton, I convert this x magnetization into proton magnetization.

Now this will be $2H_xX_z$ which is proton magnetization anti-phase to *X* and this ofcourse remains the same.

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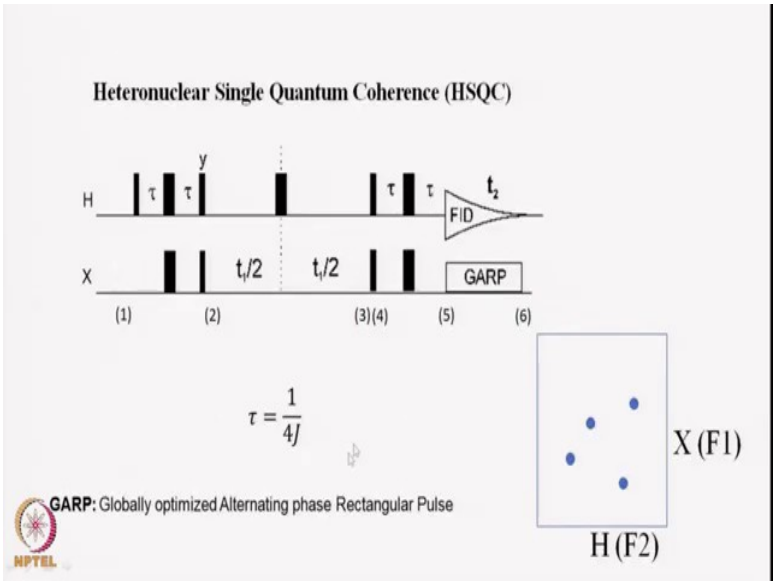

During this next refocusing period, that is here, that in this period here τ-180-τ and then I come to the density operator term time 0.5 here.

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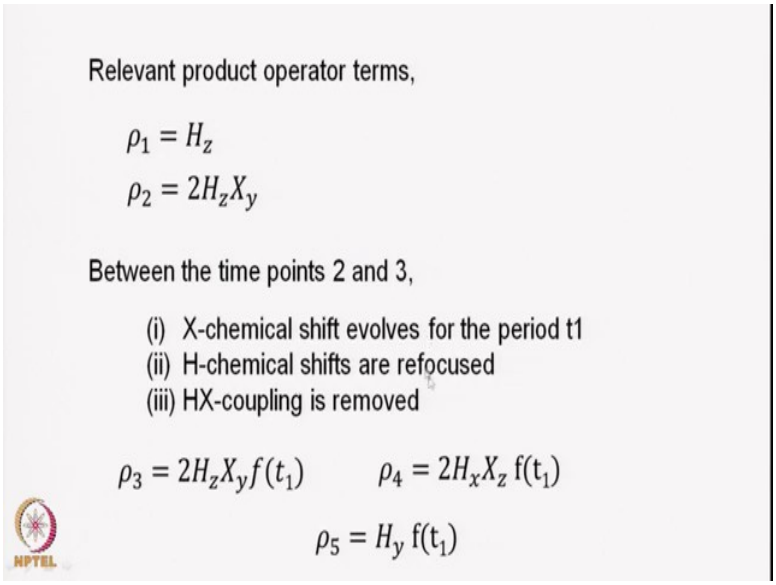

When at that time point, I have the in-phase magnetization, these $2H_xX_z$ evolves under the coupling to produce $\rho_5 = H_y f(t)$ ofcourse remains the same. Now, you see this is in phase magnetization of proton and which can be detected with X nucleus decoupling.

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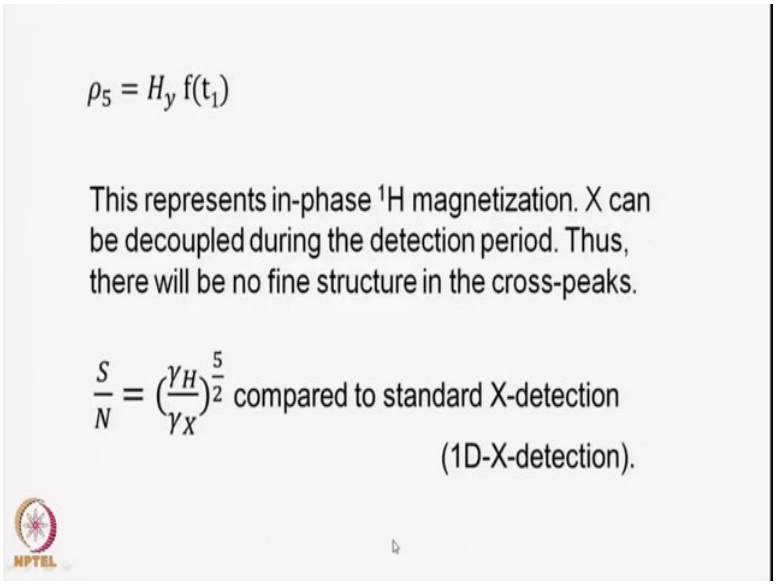

So this represents in-phase proton magnetization X can be decoupled during the detection period. Thus there will be no fine structure in the cross- peaks. So you see during the t_1 period there was no coupling evolution and therefore there is no coupling along the F_1 axis. And during the t_2 period you have decoupled the x nucleus, therefore again there is no coupling in the F_2 axis, therefore finally you will only have one peak. You will not have any fine structure in the cross peaks at all.

Now, since here I have excited the proton and I also detected the proton, therefore

$$\frac{S}{N} = \left(\frac{\gamma_H}{\gamma_X}\right)^{\frac{5}{2}}$$

compare to standard X detection. You apply 90 pulse on carbon and detect the carbon or apply 90 pulse on X and detect X nucleus and decoupling the proton, compared to that you have a $\left(\frac{\gamma_H}{\gamma_X}\right)^{\frac{5}{2}}$. And this is the quiet a substantial an aspect in the sensitivity.

So you look at for example, if this way proton and nitrogen this is the factor of 10, 10 to the power 5 by 2, so huge, 10 to the power 5 is 100 thousand and the square root of that is almost 330. So, you gained a signal to noise of 330 in the case of proton nitrogen and that is the substantial saving in the experimental time and sensitivity enhancement is there for a big-big advantage in this HSQC experiment.

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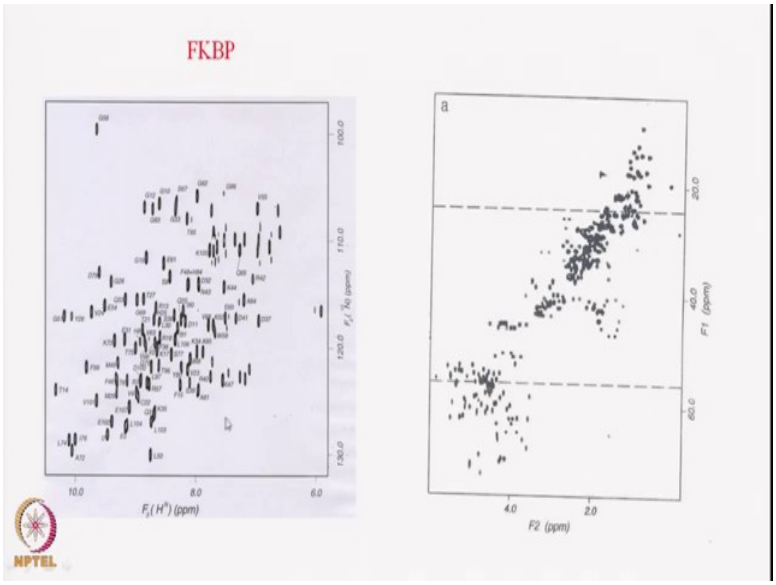

Okay, so let me show you some examples here, so here is the typical proton nitrogen correlation spectrum obtained large molecule. This shows you the advantage which I have mentioned in the very beginning, so even a complex molecule you will have the protons chemical shifts overlapping and then you will not be able to resolve them in the normal spectrum.

Now you see you make use of the n15 chemical shift then you see, you separate those peaks along the ^{15}N axis and you get a cross peak structure which is like this. These are all cross peaks and therefore in complex molecules such as proteins you can resolve this peaks in the 2-dimensional correlation spectrum, heteronuclear correlation spectrum, in fact you can count the peaks here, you can count the peaks and say how many residues are there in your protein?

Each amino acid produces 1 protein, which is 1 amide group. So 1 amide proton to it is own nitrogen will appear a correlation peak. So, you will count the number of peaks here and say okay this has so many residues in my protein. Therefore, this is typically called as the fingerprint of a protein. Now, similarly this is the proton-carbon correlation spectrum, you see so many peaks are present in the carbon proton correlation spectrum and these are pretty well resolved here compared and you could not have achieved this in the normal 1-dimensional spectrum at all.

Any separation of the chemical shifts here would be impossible in the normal 1-dimensional spectrum of proton and in any proton-proton correlation spectrum you would not be able to resolve these. And this advantage of *X* nucleus chemical shift, the *X* nucleus chemical shift is

a significant feature of this heteronuclear correlation experiments which are mentioned to you earlier.

Okay, so therefore, to repeat you have a significant signal to noise enhancement by this indirect detection schemes. The *X* nucleus is detected along the indirect dimension, and you detect the protons. You also excite the protons and detect the protons you have a significant advantage of the sensitivity in the spectrum.

However ofcourse when you decouple the *X* nucleus and it is also very demanding, because *X* nucleus chemical shift range is quite large therefore you need special tricks to decouple *X* nuclei and that is the broadband decoupling sequences, there are many broadband decoupling sequences designed to cover a wider range of spectral width along the t_2 dimension for the decoupling purposes, okay so I think with that I will stop here and we will continue with the heteronuclear correlations in the next class.