Advanced NMR Techniques in Solution and Solid - State Prof. N. Suryaprakash Professor and Chairman (Retd) NMR Research Centre Indian Institute of Science – Bengaluru

Module-25 Coherence Transfer Pathway Lecture – 25

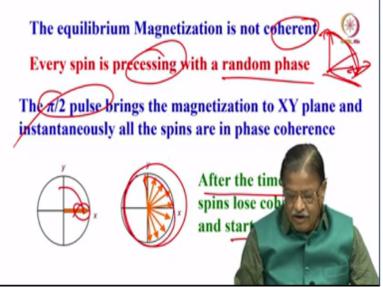
Welcome all of you. In the last one or 2 classes, we discussed a lot about polarization transfer, what is a polarization transfer, how we can enhance the signal intensity by taking the magnetization from the abundance spin like proton and give it to the dilute spin like carbon 13. And conceptually we can visualize this by selective population inversion or selective saturation of a particular transition, this is what we saw.

And especially in the case of heteronuclei, the signal enhancement goes by the ratio of the gamma, we also saw that. And then of course, when we understood how the polarization transfer takes place, also with a vector drawing we understood how the component of the doublet of the proton coupled carbon is changing as a function of delay, as a function of different pulses when we apply. It was antiphase character, how we made it to refocus and became inphase character, so that there will not be nullification in the intensity or the reduction in the intensity, so, that immediately after the collecting the signal, we can do the decoupling also. So, we did understood lots of tricks and titbits of how we can get the polarization transfer. And of course insensitive nuclei enhancement of polarization transfer, which is called the INEPT is a common thing which is always used in all the 2 dimensional experiments, especially heteronuclear 2 dimensional experiments lie, HSQC, HMBC, etcetera, where the polarization transfer is very, very important to enhance the signal intensity. And of course, in the one dimensional way, we can also use it for identification of different carbons by spectral editing. Depending upon the delay that we are going to give in the last delta delay in the INEPT sequence, then we know that we can identify carbons; CH carbon, CH2 carbon, CH3 carbon, based on the intensity and the phase of the signal, whether is positive or negative. Similarly, we saw the DEPT sequence, DEPT sequence is also similar to the INEPT. The last pulse we are going to have a different flip angle pulse, 45 degree 90 135 DEPT pulse sequences. Again we can use this as a polarization transfer technique, but we can also use this to identify different carbons. We can use this for carbon 13 editing. These are all one dimensional experiments, of course, DEPT is not combined with 2d very

often, but INEPT is combined, so, we will come to that when we come to 2D later. With this we will now jump over to a different topic.

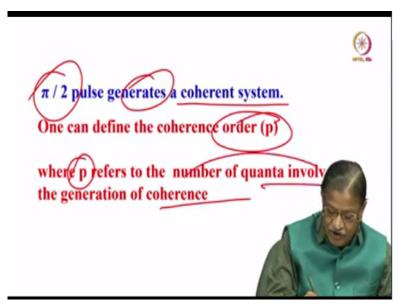
So far we discussed about coherence transfer everything in the INEPT, we will discuss more about these things. Now, I am going to touch up on what is called the coherence transfer pathway today; what is the coherence transfer pathway? We will see that.

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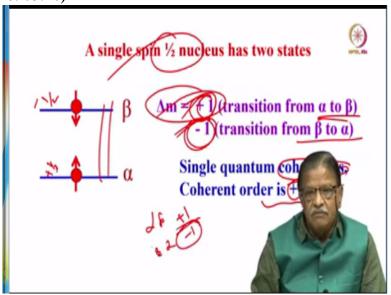
The equilibrium magnetization you know it is along Z axis, is it coherence? Coherence means all the spins should be in phase; it is not there. They are not coherent, every spin is precessing with a random phase. As a consequence, the Z magnetization or the equilibrium magnetization is not coherent. Whereas, if we apply pi / 2 pulse and bring the Z magnetization to XY plane, then what is going to happen? All the spins instantaneously have phase coherence; immediately they have a phase coherence; after some time they start undergoing decoherence. That is what we discussed, when we discussed about the T1 other things, especially during FID decay, we discuss that. So, after a certain time what will happen, the spins will completely loose coherence, and become completely dephased. So, immediate after 90 degree of pulse, instantaneously there is phase coherence; and then phase coherence get completely destroyed with time, and the spins will get completely dephased.

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So, that means pi / 2 pulse generate coherent system; and decoherenc taking place, is a different question; but we can say pi / 2 pulse generates a coherent system. Once we know that, we can also define what is called a coherent order, that is something new term which will tell you, I will discuss. P is a term which is generally always used to refer the number of quanta involved in the generation of coherence. The coherence depends upon number of quant, p = 1 means single quantum, that is coherence order 1, P = 2 is coherence order 2. Depending upon the quantum of the coherence, we can define the coherent order.

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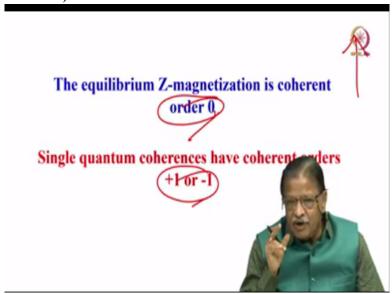


Now consider a single spin half nucleus, it has 2 states that we all know. For spin half nuclei alpha and beta states. Now, this is of course, minus half, this is plus half. Now, delta m = +1, it is the transition from alpha to beta, the changes spin angular momentum is from alpha to beta; that is plus half to minus half, the difference is plus 1; that is delta m = +1, the transition

from alpha to beta. Similarly, delta m = -1 is the transition from beta to alph;a these are called single quantum coherences that is what we discussed long back.

When I come to MQT, multiple quantum NMR, I will discuss this. These are called coherent orders +1 and -1. Remember, these are single quantum coherences; and I call this as coherent order 1; +1 is the coherent order this one, and -1 is the coherent order this one. So, the transition from alpha to beta is called coherent order +1; transition beta to alpha is coherent order -1. This is important to remember, what is the coherence order.

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Now, if we consider also, starting with the Z magnetization, which is in thermal equilibrium; this is not coherent, but we still call coherent order 0, there is no coherence order for that. The Z magnetization is not coherent. All nuclear spins are undergoing precession with different frequencies and different phases are there. So, single quantum coherences are coherent order +1 or -1, remember Z magnetization is coherent order 0; single quantum coherences are coherent order +1 or -1.

Similarly extend the logic, the double quantum coherences are coherent order +2 and -2; like that you can have different coherent orders, depending upon the spin system you are dealing with.

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Phase cycling is a way to choose the proper coherence pathway in order to acquire the NMR signal of particular interaction

Phase cycling also eliminates artifacts that can appear in the NMR spectrum

The basic requirement is to define a coherent order for each spin. We can also define the overall coherence order for an operator product

Now, if I want to select a particular coherent order, I want to detect let us say -1 coherent orders signal, is it possible to do that? yes there is a way to do that, that is what is called coherence pathway. If I know how the signal is going, how the coherency is getting transferred, from one path to another path, if I know the way it is going, I can choose the proper coherence pathway to acquire the signal of my particular interaction.

If I want to get a particular interaction to detect signal for that interaction, if I want to choose a particular pathway for that, we can do that, if I know the coherence pathway. So, phase cycling is one thing which eliminates all the artifacts that can appear in the NMR spectrum. And we can do that by using what is called phase cycling. That is why I introduced about pulse phase and receiver phase earlier.

The basic requirement to define a coherent order for each spin system, there is a requirement, that is we have to define a coherent order for each spin, then we can also define the overall coherence order for an operator product. These 2 things are important actually.

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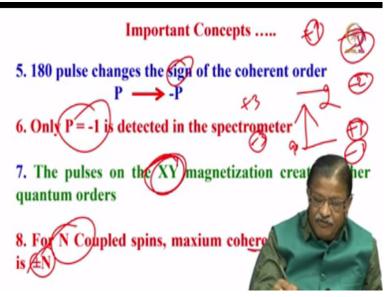
Important Concepts Free precession (Delays) will not change the coherence order Only the pulses change the coherence order A 90 pulse on equilibrium magnetization creates only +1 and -1 coherent orders, by equal amount By convention only P= -1 coherence is associated with +ω₀ and P=+1 coherence is associated with -ω₀

Now we will go to the important concepts, these are all titbits, these are the points you have to remember; and then we use these things in understanding coherence transfer pathway. Free precession, for example delay, you are apply radiofrequency pulse and give some delay and then apply another pulse no problem. During this delay, we discussed a lot about chemical shift evolution, J coupling evolution etcetera fine, this free precision will not change the coherence order, that will not change during free precession.

Only RF pulses which are apply, that can change the coherence order. So a 90 degree pulse on equilibrium magnetization creates only +1 and -1. With this logic now, the equilibrium magnetization along Z axes, we are going to apply on 90 degree pulse here, and then bring the magnetization to other axes. Then you are going to create +1 and -1 coherent orders, 90 degree pulse applied on thermal equilibrium, when you are applying 90 degree pulse, you create +1 and -1 coherence orders; we already discussed what is +1 and -1? Plus 1 is transition from alpha to beta, and minus 1 is transition from beta to alpha. So, beauty is we can select a particular transition pathway. So, for particular coherence pathway we can select. Now by convention P = -1 is what is the coherence which is associated plus omega naught and P = +1 is associated minus omega naught; that is because, the sense of precession and we always nu = -1 gamma into B naught whole 2pi.

This minus is coming to picture, it is simple thing do not worry; please remember P = 1 and P = -1 are depending upon plus omega and minus omega.

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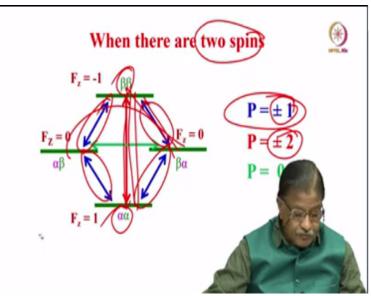
And important concept is , the 180 degree pulse changes the sign of the coherence order. If I have a coherence order +1 in a particular path, I apply 180 pulse immediately after that, this will change the path to -1; the 180 pulse will change the sign of the coherent order; the +P become -P. Let us say, I have chosen a coherent order 2, apply 180 pulse; immediately I will go to coherence order -2. And P = -1 is always detected in the spectrometer.

All the spectrometers are designed in such a way you cannot detect P = +1, you detect only P = -1; that is the coherent path is always chosen. And the pulses on the XY magnetization, creates higher quantum order. This is an important point. Remember, when I have a thermal equilibrium magnetization, you apply 90 degree pulse in the XY plane you create +1 and -1 coherent orders; that means you are already in the XY plane.

Now, we apply another pulse, or some more pulses, 1 or 2 or 3 whatever you want, then what you do, is you apply the pulses on the XY magnetization. Then you create higher quantum orders like +1, -1 become +2, +3, +4; you can go to higher and higher quantum orders. That is possible; For that the magnetization must be the XY plane, you cannot create that from the Z magnetization.

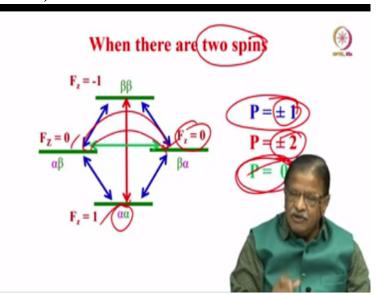
Similarly, for the N coupled spin system, the maximum coherent order that you can create is +N or -N. If I have 3 coupled spin system, maximum coherent order is +3 or -3, I have the 5 coupled spins system, the maximum is +5N, -5N. This is the important concept you have to remember.

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Now, consider a situation we have 2 spins, how many coherent orderz we can think of? see We can have coherent order, we can see this, this transition is allowed. And this transition is allowed, this is single quantum, this is single quantum, this is single quantum. These correspond to P plus 1 or minus 1. This is also allowed, double quantum 2 spin simultaneously flipping from alpha alpha to beta beta, alpha alpha, to beta beta simultaneously flipping, this is order P; plus minus 2. We are going from here to here is +2 coming from here to here is -2. Similarly, P plus minus 1; there are 4 possibilities you know here to here you are going from here to here, alpha going to beta is +1, here beta coming to alpha is -1, here 4 single quantum; you have 4 such plus P and +1 and -1, but +2 or -2 is only one +1. Similarly, you can think of transition from here to here, I will show you here.

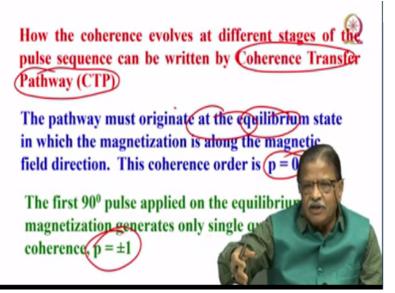
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See now you can think of transition from this state to this state that is alpha beta to beta alpha that is 0 to 0, this is 0 quantum; this is P = 0. Then you might get confused, I told you the Z

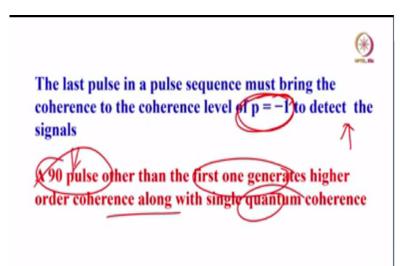
magnetization order is 0, coherent order is 0, but this also coherent order is 0. We will wait there is a way to distinguish that, both are 0 order, I agree. But if you have to detect only this zero quantum transition, then we can do something.

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But now, how the coherences evolve at different stages pulse sequences. This is a way we can understand by doing what is called coherence transfer pathway. CTP, it is called. Using this coherence transfer pathway drawing, I can understand how the coherences are evolving at different stages of the pulse sequence. Now, let us look at this one; how we do; The pathway always starts with equilibrium. If you start with somewhere else, then already transfer magnetization in the XY plane, you have created a higher quantum order; but you do not know where to start. You always start with you know equilibrium magnetization, that is P = 0, you are starting with P = 0 quantum order; and then first pulse I told you always creates plus 1 and minus 1, then subsequent pulses will create higher quantum coherent orders.

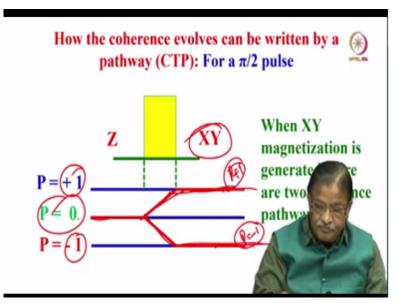
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The last pulse always should create P = -1 order, because I told you, only P = -1 coherent order is going to be detected by the spectrometer. Any other coherent order, will be rejected, you cannot detect it. So, whatever you do between the pulses, start with 0 in the thermal equilibrium, apply a 90 pulse create +1 or -1 coherent orders; in between you apply many pulses. Finally, you have to choose the coherent pathway in such a way, the last pulse should create only P = -1, then only we can detect the signal, that is an important point.

Now, 90 pulse other than the first pulse always generates higher quantum coherence, that is what I told you. Other than the first 90 pulse others generates higher quantum; first one, you are applying 90 pulse, first time it creates +1 or -1. Apart from that any pulse you are applying, second 90, third 90 pulse, they create only other higher quantum orders; and only this one creates the single quantum order.

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Now, how this coherence evolves, you can write by coherence transfer pathway diagram; that is what I have been telling you. Now, how do you write that? First, let us take a simple example for a pi / 2 pulse. What does a pi / 2 pulse do? You know that; the magnetization is along the Z axis, it will bring it to XY plane. If I apply a pulse, the magnetisation is along Z axis, if you apply X pulse, you bring the magnetization to Y axis, that is what you are doing.

A pi / 2 pulse, simply brings the equilibrium magnetization from Z to transverse plane that is all. Now, let us write a diagram for that. How do we write a diagram? it is very important to know how you write the diagram for the coherence transfer pathway. So, now I am starting with a 90 degree pulse. And initially the magnetization is along Z axis in thermal equilibrium. So, this is what; 0 quantum order; zero coherent order, P = 0.

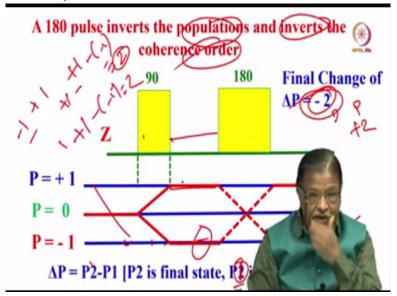
Now I am applying a RF pulse. When I am applying a RF pulse, what is going to happen? it will create 2 coherent orders like this. I have brought the magnetization the XY plane by applying on 90 pulse. Now, this was P = 0, we started with and the magnetization was along Z axis. Now as soon as we apply a 90 pulse, you have created 2 more coherent orders, +1 and -1, the 0 is always there. But in addition to that 0, you have created +1 and -1, 2 coherent orders you created just by the application of a 90 pulse.

So, simply what we did? to write the coherent diagram, it is very simple. What we have to do is, I wrote a path here before that the magnetization is along the Z axis. And then it is a P = 0 state, 0 coherent order. And then apply 90 pulse, bring the magnetization the XY plane, you

have already created P = +1 and P = -1 coherent orders. So, now this to this is called a pathway from 0 to +1.

The magnetization in the XY plane, we have generated 2 coherence pathways; from 0 we created a pathway 0 to +1; and we created a pathway from 0 to -1. These are the 2 coherent pathways we have created, just by application of a 90 pulse. That is why I said 90 pulse creates P = +1 and P = -1.

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What does the 180 pulse do? It inverts, I told you also, when I gave the titbits. The 180 pulse inverse the populations; that mean it also inverts the coherence orders. We can see that now this is a 90 pulse. Already I told you, after we apply a 90 pulse we have created coherence order, P = +1 and P = -1. Initially it was 0, along Z axis. Due to the delay, of course, nothing happens, I told you during delays, the free precision, the coherence order will not change. It remains the same during the delay; +1 and -1 remain same.

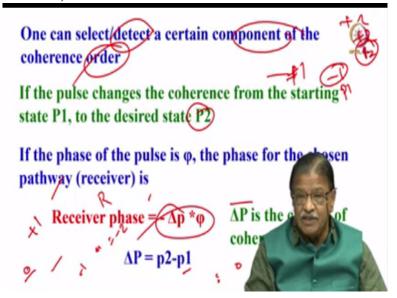
Now you are applying a 180 pulse, double this width. The 180 pulse interchanges the coherent order. This +1 comes to -1, -1 goes to +1. Now, you have interchanged here, after the 180 pulse. This is the important concept that 180 pulse inverts the coherence order. And finally, what is the change in the delta P now, if I go from here to here from -1, I went to +1. What do you mean by that, because of 180 pulse I changed the coherent order from -1 to +1 the difference is 2. You have to say this is P1 and this is P2. So, you always take P1 – P2 you take it. Now, this is the initial stage -1 and P2 is +1; minus of 1 plus this minus 1 it is -1. The change the quantum, the delta P, if you go from here to here is -2. If you come from here to

here, it is +2. Either way is possible. You can select from here to here, that is also possible, You can select from -1 to +1 also; both are possible. But the change is 2. Now we can say final change is -2, depending upon what I am choosing, So, P2 to P1; P2 is the final state and P1 is the initial state.

What is the final state for me? If a consider here to here; final state is +1 and the initial state is -1. The +1 minus of minus 1, it is = +2. If you go from here to here, delta P = +2 if you come here to here -1 minus of plus 1 = -2 here; or if you go from here to here it is -2 if you come from here to here it is +2. Let me make it clear for you. Ok now, P2 - P1 is what you have to consider, the P2 - P1, where P2 is the final state and P1 is the initial state.

So, now which is our final state in which is a initial state? Let us say I go from -1 to +1, -1 is my initial state, my initial state is P2. No this is wrong; I made a mistake. Here P2 is the final state, this must be P1. So, P1 is the initial state, that is -1, P 2 is the final state, it is +1. So, now, I have to consider P2 - P1; +1 minus of minus 1, which is equal to 2. So, if you go from -1 to +1 same, the delta P = +2. If I come from here to here, initial state +1, minus of -1 = 2. So, depending upon which one is your P1 and which is your P2 you can find out what is delta P. At this point you understand, as you go ahead we can find out more of these things.

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How to design the sequence of phases? One can select or detect a certain component of the coherent order, it is possible. If the pulse changes the coherence from starting state P1 to the desired state P2, let us say, I start with -1, and then I want to take it to +2, let us say some

state; starting with -1 to +2, it is possible now. This is the initial state, this is P1 and this is the

final state; starting state is P1, final state is P2.

If the phase of pulse is phi, and the phase of the chosen pathway of the receiver is this one,

see if you want to select the pathway, you should know what should be the receiver phase.

What is the receiver phase? The receiver phase is given by simple equation, minus of delta P

star of phi, phi is your phase. What is the phase of the pulse, we are applying? That is the

pulse phase, phi is your pulse phase.

Now minus delta P is what is the change of the pathway; from initial state to final state. If

you know this, simply you have to set the receiver phase which is calculated by -P delta P

into phi. Then you can automatically select the particular coherence pathway. For example, in

the previous example, 0 this is the -1, +1 is there. if I go from here to here -1 to +1, -1 is

initial state; minus of plus one, which is equal to -2.

So, now delta P = -2, if delta P = -2; you put it here, so minus of minus 2 into phi; so it is 2

times phi, if my receiver phase let us say is 90, then the transmitter has to be 180. You can

have a sequence of pulses; sequence of receiver phases, I can change the phase of the pulse,

let say 90 or 180, 270, 360 like that. Correspondingly receiver phase has to be doubled like

this. Then you can set the particular pathway, here first pathway, you get the point how to

choose the pathway. That is the simple equation, you should not forget this equation, this

equation is going to tell you. The receiver phase is equal to minus delta P into phi, phi is

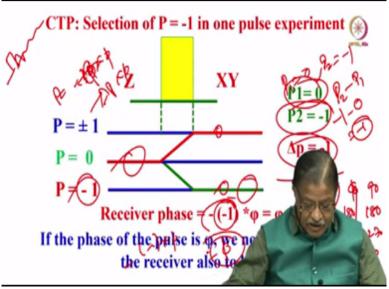
nothing but a pulse phase, phi is a pulse phase and this is the receiver phase. And you have to

remember the equation delta P is the change in the coherence pathway of initial to final stage.

If you know that, then you can work out receiver phases and the pulse phases for any

sequence of your choice, and you can have any particular pathway of your choice.

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Now, let us see in the CTP for 1 pulse experiment. What is the 1 pulse experiment? 1 pulse experiment is simply apply a 90 degree pulse and start collecting the signal. What does a 90 degree pulse will do? I told you a 90 degree pulse creates +1 and -1 quantum coherence order. +1 coherence order and -1 is coherence order, this is created. Now, I want to select only this one, -1. What is my delta P? Initial state is 0; P1 = 0, what is P2? 2 = -1, what is delta P? Delta P is always P2 - P1, P2 is -1 and P1 is -0; so, delta P is -1.

Delta P is always final phase minus the initial phase, that is what it is, which is the final path minus initial path, that you have to remember. So, delta P in this case is -1, we started with 0 we wanted to get -1. So, P1 = 0, P2 = -1, delta P = -1. So, what should be my receiver phase? minus of -1 because, the equation is this receiver phase is minus of delta P into phi. What is delta P here? Delta P is -1, so, minus of -1 into phi ;minus of -1 so, it is just phi.

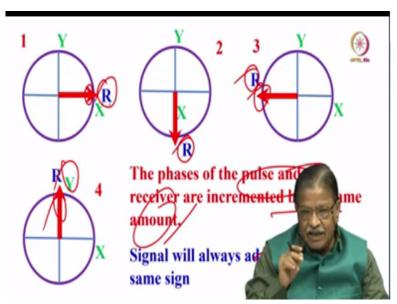
So, whatever may be pulse phase you apply, pulse phase is phi, that is a transmitter phase or pulse phase whatever you call that; pulse phase if it is phi, then the receiver phase also has to be phi, then you can select only this -1; delta P = -1 pathway. In a one pulse experiment, if you want to select delta P is going to -1 pathway you have to have a pulse phase which is 90 then receiver phase is 90. If the pulse phase is 180 if you increase the phases of the pulses, then this also correspondingly increase; 270, 270 and 360 and 360. So, this is how you are going to do that. So, if the phase of the pulse is phi, we need the receiver pulse also of the same phase.

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	Scan	Pulse Phase	Receiver Phase	
	1	0 (X)	0 (X)	
	2	90 (Y)	90 (Y)	
	3	180 (-X)	180 (-X)	
	4	270 (-Y)	270 (-Y	
Calle		OPS: The Phase ections in both the		

This is a table which is going to tell you; look at it, I am doing the experiment acquiring 4 scans. And each time I am changing the pulse phase, I am changing the transmitter phase, first I will use 0, the receiver also has to be 0, because that is what I calculate minus delta P into phi which is only just +phi; so it is same. If this is 0, this is 0; this is 90, this is 90. So, if I use this pulse phase and the receiver phase, then what is going to happen? you will select only particular pathway that is -1. This also has an advantage. I am moving the receiver, I am moving the pulse in different axes from +X, +Y, -X, -Y; accordingly, receiver also is moved. So every time we are co-adding the data and if there are any imperfection, that will be removed; and this is a phase cycling called Cyclops. Cyclops is the phase cycling, it is the 4 phase cycle; in which transmitter phase and receiver phase is identical. The transmitter phase is 0, at that is pulse phase and the receiver phase is also 0, and you move the pulse phase in each acquisition by 90 degree. Start with +X go to -Y, -X and Y; correspondingly you have to move the receiver also simultaneously along with the transmitter pulse; in which case, any pulse any imperfections which are there, can be removed. And this sequence is a phase cycling called Cyclops.

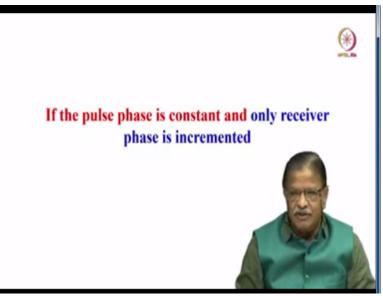
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Now how does it work we will see diagrammatically. Start with let us say, first you have a receiver phase here, and your brought the magnetization here and receiver is here. Now, I will move these 2 by 90 degree, I take the pulse to the -Y axis, the receiver also here, I will take it to -X axis, I put the receiver also -X. Now I will take to +Y, I will put the receiver as +Y, our pulse also +Y. What is happening you see?

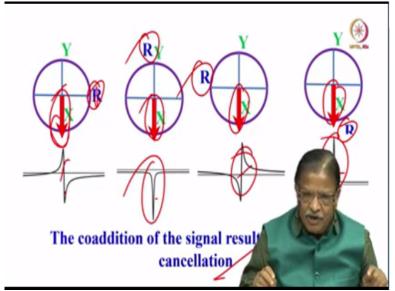
Receiver is following the transmitter phas, you know the receiver pulse is along the X axis receiver also kept along X axis. So, now for your –Y receiver is here, for -X receiver is here similarly, and all the four acquisitions, every time I change the pulse phase by 90 degree each time I am moving the receiver also by 90 degree along with the pulse phase, along with the transmitter phase. Then what is going to happen is the phases of the all the pulses and the receiver are incremented by the same amount. So, signal will add up by the same sign. In case if there are an imperfections that would be eliminated.

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So, if the pulse phase is constant, supposing you ask me a question, I was trying to do an experiment, identical experiment Cyclops where the pulse phase and receiver phase both are identical for all the 4 scans, keep incrementing pulse phase, identically increment the receiver phases, so it is very simple sequence. Now, you do change the pulse sequence pulse phase by 90 degree, first X then -X, -Y, +Y all those things. So, you are changing the phase of pulse by 90 degree. Let us you forgot to increment the phase of the receiver. What is going to happen?

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We will see this with a very simple drawing. Now, let us say this is pulse, it is along the X receiver is here. Now, what I can do? another thing your pulse is here and receiver is here, your pulse is here and the receiver here, you have pulse here and the receiver is here. So, what is happening here is, you are changing the receiver but pulses phase you are not changing. You are forgetting to change the pulse, then what will happen?

But of course we understood what is positive absorption? What is negative absorption? What is positive dispersion and what is negative dispersion and positive dispersion and everything? Now, in the same logic, if you have a signal here, if magnetization is here, and if a receiver is here, then you are going to get positive dispersive here, the negative dispersive here, here you get negative absorptive positive absorptive.

Now we are going to add up the signal; what is going to happen? This and this will nullify this and this will nullify. The co-addition of this thing here is complete cancellation of the signal. It is very bad, so that is what is going to happen. So, the phase cycling has to be properly chosen, you know ho2 find out the delta P, what do you want, you know what is your pulse phase, first define your pulse phase, that is your choice.

You can have 90, 0, 180, 270 whatever it is, once you know that once you know delta phi use it. the receiver phase is minus delta phi into phi; find out what should be the receiver phase accordingly keep changing that along with the pulse phase. Then you will choose the right pathway you want and every other unwanted thing we have completely removed, it will be discarded or if you make any mistake, you nullify the signal.

So, we will further understand and the time is up, we will see what will happen if I have to select a different pathway, or if I select one pathway will the other path way not get through; along with that? can I reject a particular pathway or when I select one particular pathway whether all other pathways are rejected or not, these things we need to understand, we will do that later.

So, in this class what we discussed today? we discussed about coherent transfer pathways, some titbits I told you; for 90 degree plus pulse if you apply 90 degree pulse for the magnetization along Z axis, whether it is +X pulse or -X pulse the +Y or -Y does not matter, you bring the magnetization to XY axes, you are creating coherent orders +1 and -1. coherent orders +1 is transition from alpha state to beta state; plus half to minus half; and the coherent order -1 is the transition from minus alpha to plus alpha, and it is from beta to alpha.

And the first pulse always creates coherent orders +1 and -1 from the equilibrium magnetization, which is in thermal equilibrium; and for that one the coherent order is 0. For the magnetization which is in thermal equilibrium the coherent order is 0. And once the

magnetization is in the XY plane, any pulse you apply will create higher quantum orders and we know how to define a particular pathway.

And once you know the pulse phase, I can calculate what should be the receiver phase for a particular pathway which I want to choose, I took the example of a single one pulse experiment, where one pulse creates +1 and -1 coherent orders, if I want to select a -1 pathway I showed if a pulse sequence is 0, pulse phases are 0, 90, 180, 270; exactly the receiver phase also has to be 0, 90, 180, 270.

Then what is going to happen, I can co-add the data. Without any problems, all the imperfections if there are any, get eliminated. And this sequence is called Cyclops. So, if you do not do it properly, if there is a mistake, I also showed you can nullify the signal. So these things do happen. So, we understand more about the phase cycling and how do you choose the correct pathway etcetera in the next class. So, I am going to stop it today. Thank you.