One and Two dimensional NMR Spectroscopy: Concepts and Spectral Analysis
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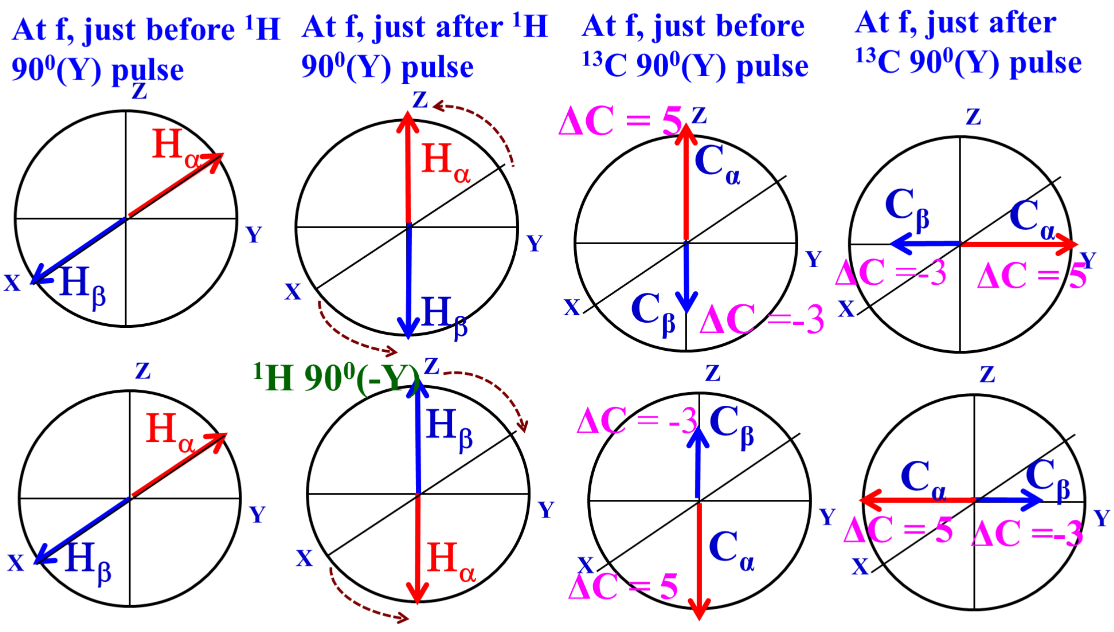
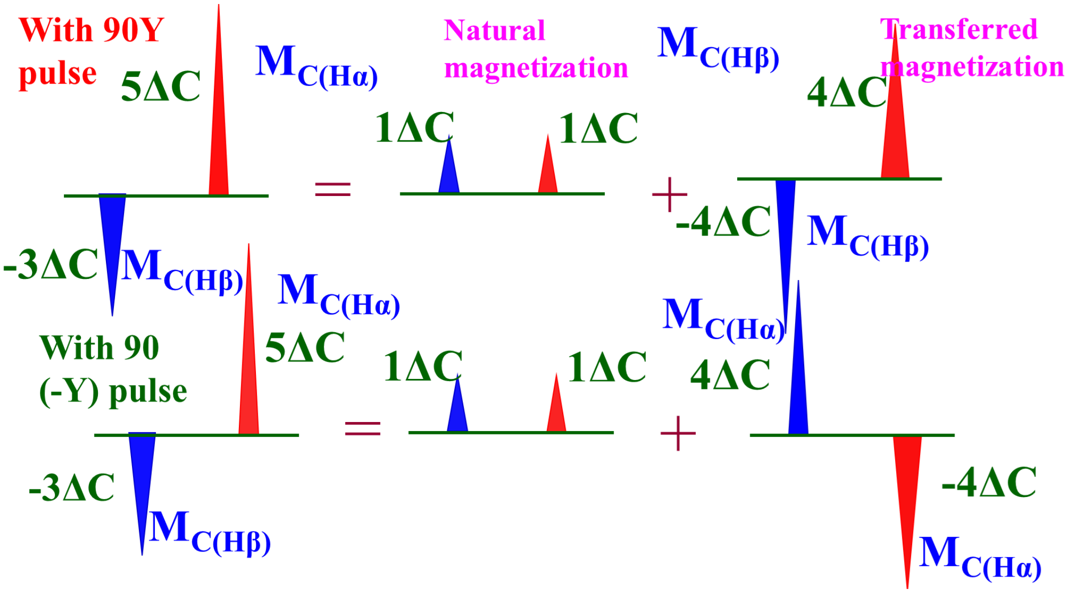
Lecture 36: INEPT

Welcome all of you. In the last class, in fact in the last one or two classes, we discussed a lot about Spin echoes and the polarization transfer techniques. In the polarization transfer technique, I told you about possible ways of transferring the magnetization. One is selective population transfer and the other is selective population inversion. Both are possible. I showed that by taking examples both in the homonuclear case and in the heteronuclear case. Two coupled spin system we took. And when I took example of proton with carbon, I showed that when I saturate one of the proton signals, one of the proton peaks, one of the transitions corresponding to proton, then for the carbon two transitions there was an enhancement in the signal intensity. Of course, they were anti-phase with +3 and -5 intensities. And we also understood after this thing how we can do the polarization transfer. We can apply it by bringing in another factor what is called spin echo. In the sense, we put a 180 pulse at the center of this INEPT sequence and we wanted to ensure that chemical shift is also refocused. At the same time, transfer is also going to take place. There is the enhancement in signal intensity. So, this is one of the biggest advantages of the INEPT I told you. Always INEPT takes place only due to the coupled spins. And the magnetization transfer takes place from anti-phase coherence to anti-phase coherence. So, we created anti-phase coherence for the proton after applying a 90 pulse. After exactly 1/2J, it became anti-phase and then we apply simultaneously 90 pulses on both proton and carbon. The coherence from proton jumped into carbon-13. That is what we observed. And we calculated the intensity and we know that how it works out, understood diagrammatically and by a vector diagram, we tried to understand everything. And afterwards, when we understood there is a need to do the decoupling of protons. We also wanted to give a delay after this thing so that the anti-phase coherence become in phase in character. So, minus 3 and plus 5 should become plus 3 and plus 5, so that we can do the decoupling. This also we discussed. But however, there are two points which need to be addressed. One is the suppression of the natural abundance signal and converting anti-phase coherence to in phase for decoupling. In fact, we did this, we understood this, how to do this when we were trying to understand the spin echo. We can adapt that here. Two points we have to address suppression of natural abundance and converting anti-phase to this thing, in phase signal. Both of these can be addressed individually. There is no issue at all.



For suppression of the natural abundance carbon 13 signal, we have to carry out two experiments. The phase of the last proton pulse, the detection pulse we apply, 90 degree pulse for coherence transfer from proton to carbon, is changed. You have to do two experiments, the last proton pulse phase should be once +φ, and the other is -φ. The two experiments you have to do. And what happens when you do these two experiments, we will see how it works. We saw the pulse sequence, how it is working, everything upto here. Already we discussed quite a bit. Up to this we have discussed. At this point, what is going to happen, we will see. At that point f, just before applying a 90 degree pulse, the proton two vectors are anti-phase to each other. Alpha and beta components are anti-phase with respect to each other. Alright, just after 90 pulse, I told you, they were in the anti-phase to each other in the Y axis and applying a 90 degree pulse takes them back to z axis. One alpha component goes to plus z and beta component comes to minus z. That we saw. This is as far as the proton is concerned. And then at f, what happens to carbon 13 signal? Just before 90 degree pulse, one is plus 5, other is minus 3. That is the carbon signal. We saw that. Signal intensity for C alpha is plus 5, C beta is minus 3. Just immediately after 90 pulse, what happens to carbon 13 signal? and then again what will happen is, till now when you are looking at the proton, the coherence was along the y axis or x axis, whatever the axis you have chosen. Whereas carbon 13 still was in the z axis. Now, what is going to happen? After applying a 90 degree pulse, you brought the spin vectors of carbon 13 into y axis. Now, again they are anti-phase because coherence has transfer has taken place from proton to carbon and C beta is minus 3, C alpha is plus 5. From z axis and minus z axis, you brought it to minus 3 and plus 5, then minus 3 and plus 5 are the components. All right. This is one experiment with 90 degree plus y. What happens if you do the identical experiment with proton pulse 90 degree minus y? So, what is happening is, this is 90 plus y, as usual at the point f, just before application 90 degree pulse, we have the signal like this. That is exactly similar to before. Now, we are applying a 90 pulse minus y. When you apply 90 degree minus y, remember, in the previous case, H alpha was along z axis, H beta was along minus z axis. Now they get reversed. H beta is along z axis, H alpha has come to minus z axis. That is what happened. So, magnetization got inverted because we are applying a minus y pulse. Further, what is going to happen for the carbon 13, just before 90 pulse, it was like this. Because we have not touched it yet, you know. So, now what we are going to do is, because of this, it is happening like this. As soon as we apply 90 degree pulse for the carbon 13, what is happening is, the C alpha which was on plus y became, came to minus y and C beta which was along minus y came to plus y. This is what happens. See, when you apply 90 degree pulse, I will do the two experiments, once with plus y and once with minus y 90 degree pulse. This is what is going to happen. When you apply simultaneous 90 degree pulses, we are changing the magnetization from C beta and C alpha. The two experiments, once C alpha is along plus y and other time it is minus y. Similarly, C beta also once along minus y, second time it will be on plus y. Now, with plus y 90 degree pulse, this is the magnetization of carbon 13 and with minus y, this is what it is. Now, in both the experiments, the natural abundance carbon 13 is detected with constant phase. That we are not touching. We are only transferring magnetization to this thing, from proton to carbon 13. The natural abundance carbon 13 still continues with the constant phase. The first FID and the second FID, we are going to do the experiment recorded through two phases of 0 degree and 180 degree for the receiver. That is what we have to do. What we have to do after doing this experiment, subtract experiment 1 from experiment 2. The 90 degree y pulse experiment 1 with experiment 2, we will subtract. See what is going to happen. This is 90 degree y pulse, subtract this with this one. What is that you are going to get when you subtract this one and the net intensity of each of them, you calculate here minus 3 and minus of plus 5. So, this is going to be minus 8. Similarly, with other one, plus 5 minus of minus 3, it is going to be 8. So, minus 8 and plus 8 is the intensity. The two transitions have intensity minus 8 and plus 8, gone up 8 times. So, that is okay. I mean, it was 2, now 4 times intensity has gone up. The natural abundance carbon 13 will give a constant phase signal in both the experiments. This is a transferred magnetization. Carbon 13 natural abundance is not transferred. It remains same. For the elimination of natural abundance carbon 13 signal, you have to change the receiver phase by 180 degree to eliminate it. How do we do that? First, we do one experiment 90y. With 90y pulse, we have signal intensity 5 and minus 3 for magnetization which correspond to H alpha, carbon vector corresponding to H alpha orientation and carbon vector corresponding to H beta orientation, they have plus 5 and minus 3 intensities, with plus 90y pulse. And this is natural abundance magnetization, of carbon 13, which is not transferred. So, it is intensity 1:1 because it is a doublet, it is 0.5 and o.5, we will take 1:1. And then transferred magnetization is, of course, anti-phase minus 4 and plus 4.



Why did I take 4? Because gamma is 4 times smaller, so we know it has to be 4 times gain. So, I have written because the transferred magnetization has to be minus 4 and plus 4 anti-phase. Natural magnetization is identical 1:1. This is with 90 pulse. Do another experiment with 90 minus Y pulse. Then what is going to happen? This is natural magnetization, here this gets reversed now. Because we are applying 90 minus Y pulse here. Natural magnetization of the carbon 13 will continue with the similar phase, constant phase, that phase would not get changed. Whereas for these two signals, the phases get reversed. Once this is negative, this is positive, second time, this is positive and this is negative. Now, you already got the idea. What we can do? We take the difference of the two experiments. When we take the difference of the two experiment, what is going to happen? The signal coming from the natural abundance carbon 13 gets cancelled out because they are of the constant phase. Only the transferred magnetization is retained. This is what happens. Because when you take the difference here, this and this gets nullified, cancels out. Whereas this will get added up. When you take the difference minus of this one, minus of minus plus 4, this is minus 8 and this is going to be plus 8. This is what we are going to see. Minus 8 and plus 8. Here, the advantages is we have eliminated the natural abundance signal from the carbon 13 and we have retained only transferred magnetization from proton to carbon 13.

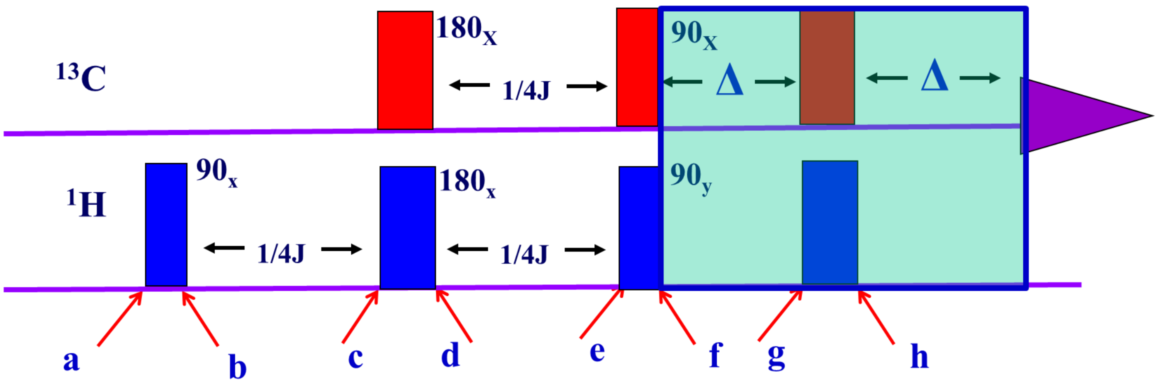
Now, some interesting things do happen here. What will happen to signal intensities for CH2, CH3, etc., in INEPT experiment? So far, we were dealing with only CH carbon and carbon 13 when it is coupled to single proton will be a doublet. So, we were dealing with only doublet, we got minus 3, plus 3, minus 3, plus 5, all signal intensities, anti-phase. We knew how to convert this into, give a delay and afterwards convert it into in-phase. We can do the decoupling, everything we understood.

For CH2 groups, what is going to happen? The signal intensity will be like this. One dimensional carbon 13 signal will be in the ratio of 1: 2:1. That is correct. There is no difference. In the INEPT experiment, what happens, central peak do not precess and there is no transfer of magnetization for that. That is not going to precess, that would not get transferred. Now the magnetization gets transferred only for outer components, outer components which are in anti-phase, there is a signal. That is where, as I told you, the transfer of magnetization takes place from anti-phase to anti-phase. Only these two will get the signal and how much is the gain in the signal? You can find out from this.

This is the gain in the signal intensity. So, if you put that value, the intensity of the CH2 peak are minus 8, 0 and plus 8. Central peak will not get any magnetization transfer, whereas outer components will have minus 8 and plus 8 intensity. This is what happened for a CH2 carbon. What about the CH3 carbon? CH3 carbon intensity is 1:3:3:1 in a normal carbon 13 spectrum coupled to 3 equivalent protons. That we know, intensity is 1:3:3:1. Now, in INEPT experiment, it is analogous to CH carbon where it is attached to a single proton. Instead of doublet, now we have a quartet with four lines, 1:3:3:1 ratio. They are precessing in two orientations, one clockwise and the anti-clockwise, fast moving and slow moving will be there. Two will be fast moving and two will be slow moving. Again, they are anti-phase like this. And if you calculate the intensity ratio for INEPT experiment, it turns out to be like this for the carbon 13 four peaks. And if you look at the intensity, it is minus 12, minus 12, 12, 12, very easy. This is the intensity, you are going to get minus 12, minus 12, plus 12 and plus 12. These two are minus 12, minus 12, these two are plus 12, so it is very easily you can understand for this one. The signal gain should be 4 times. When there are 2 carbons, it became 8 times. When there are 3 carbons, it is 12, that is all. So, this is very easy, gain depends upon the number of protons attached to carbons. That is how CH3 group will come.

Now, the coupled INEPT, an experiment, if you see, it looks like this. An example of a molecule called coumarin, this is a molecule. We have only CHs here, no CH2 or anything. But of course, there are quaternary carbons here also. We need to coupled INEPT, that means we are not removing the coupling; that means after the INEPT, there is no delay is given. No delay is given and we are not going to acquire the signal with decoupling. And of course, in which case, if you do the inept, the coupled INEPT, this is what it is, because they will be anti-phase in character. We do not need to worry about decoupling means anti-phasing is still okay because there is no question of getting signal nullified. Only when you want to decouple, then this will get nullified. All the opposite, negative, positive, gets cancelled out. But that is not going to happen because we are looking at the coupled INEPT. This is the same thing with gated decoupling, where it is coupled, but still get there is gain in the intensity because of the gated decoupling. Look at this one. This is what the comparison of intensity ratio. There is small gain in the intensity; but if you look at this thing, there is small gain in the intensity. Of course, you have to look at the signal to noise ratio. And then with this, alright, so far we have not done the decoupling. We have been doing only to suppress the natural abundance carbon signal. We can do what is called a refocused INEPT. The same INEPT experiment with a 180 pulse in the middle as a spin echo for refocusing of the chemical shifts. We can extend further.

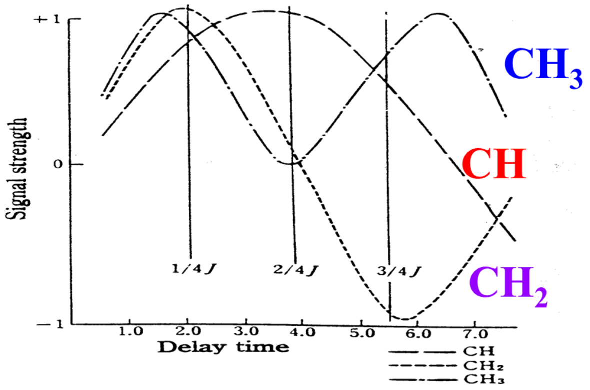
Refocused INEPT



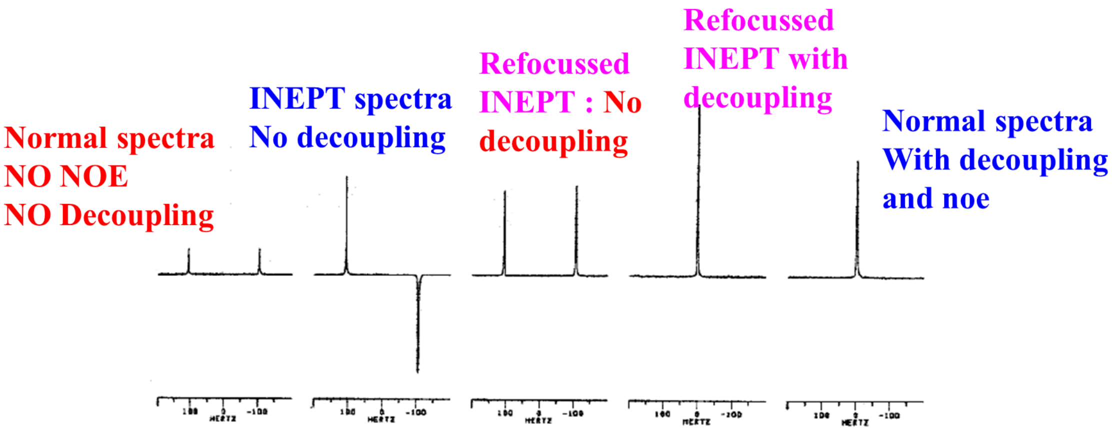
This is a refocused INEPT. Refocused INEPTwill circumvent the problem of decoupling due to anti-phase character. This is what one of our experiment, which we already discussed in the spin echo. There is nothing new in this. All we have to do is we have to give a delay after the last pulse, which is equal to 1/2J. Then you are going to see what is going to happen here. Up to this is known. This is up to f is regular INEPT. For the refocused INEPT, we are going to give like a spin echo sequence. There are two delays in between with 180 pulse on both proton and carbon. What is going to happen for this now? Let us see what is going to happen, especially for this part. Up to this we know, we have already understood. At the end of the first delay, especially here, then the doublet component of CH spin system would have acquired a relative phase difference. That is true. They would have acquired some relative phase difference. 180 pulse on the X nucleus refocuses the chemical shift. 180 pulse and proton inverts the alpha and beta spin states of X vectors. It will invert it ecause it is coupled to that. So, it is a doublet. As I told you already, applying 180 pulse will invert alpha and beta labels. That is what is going to happen. Now, what is going to happen? The magnetization corresponding to C alpha, which is coming because of the H alpha, becomes now magnetization corresponding to H beta. Magnetization of carbon coming because of H beta now becomes magnetization vector coming because of H alpha. This is what the 180 pulse has done. It just inverted the spin states. That is all it is done. This is what I said. It inverts the alpha and beta labels. That is what it did. During the last delay, what will happen? In the last delay, two carbon 13 vectors continue to precess clockwise and anticlockwise, as always. There is a delay you give and then they continue to process. One is fast moving and the other is slow moving. One goes clockwise, other goes anticlockwise. And after certain delay, they again come back to the x-axis. This is what you understood in spin echo. You understood, it gets refocused again with this delay and this is what we have to understand how it happens. At f, at the end of the regular INEPT, we understood carbon 13 vectors are anti-phase, minus 3 and plus 5. That we know. After the first delay, they started evolving, you know evolving like this. The fast and slow moving components are moved by some angle for some delay. You apply a 180 pulse now, what is going to happen? The labels get interchanged. H alpha and H beta label gets interchanged and after second delay, what is going to happen? They will move and then come back and then get refocused along the plus x-axis, after another delay. So, again it is delay everything is given. First of course, 180 pulse is to invert the alpha and beta spin states and then second delay, we ensure these vectors continues to move after getting inverted and then get refocused along the x-axis. This is what is going to happen after f, all right. Now, we can do the decoupling of proton with carbon 13. Why? Because you can see both are in-phase vectors. If they are in-phase vectors, decoupling is possible. Only if it is anti-phase vector, there is an issue. Then signal gets nullified. We can do the decoupling. You may ask me a question. We said there is a delay, but what is the refocusing delay we have to use? Of course, we have already discussed this when we were trying to do the spin echo with decoupling. Even a simple INEPT experiment we saw and we saw that when the signal is anti-phase, what is going to happen? If we do not give the delay, it gets nullified. We saw that. And then how did you overcome this issue? By giving an extra delay. Exactly. We also know how much delay we have to give everything, but we will understand again. What is the length of the refocusing delay? That depends upon, you have to choose the optimum value that depends upon the J coupling between carbon and proton, one bond. We are not worried about long range coupling. This period has to be calculated based on one bond coupling. And the delay of 1/2J gives the maximum refocusing. Maximum signal only for system like IX where two heteronuclear coupled like carbon and proton. In such a situation, if you consider CH, if you put delta is equal to 1/2J, you get maximum refocusing. That is what is going to happen. If you put the delay equal to 1/2J there is a refocusing for this type of things. For CH2, when do you get the maximum refocusing? The CH J coupling is between these two vectors. In fact, it is half J, one is this j, one is this j and your on resonance is at the center. For CH2, central peak is here and other two peaks are equal to J, moved apart. For fast moving, slow moving component already not half Jj, it is J. For CH2 systems, refocusing is maximum when delta is equal to1/4J, the delay has to be 1/4J. And if all the multiplicities are there, CH2, CH3, etc., and you know all these different carbons attached to different protons will have different J couplings. And you cannot keep on varying the delay and accordingly for different CH, CH2, CH3 carbons, what we have to do is we have to arrive at the compromise in setting of the value. Delta approximately equal to 1/3J is an appropriate value, more or less. There is no preferred value, but approximately $^1J_{CH}$ is 150 Hz is taken and one third of that value is given as the delay. That is appropriate for all the multiplicities of CH, CH2 and CH3 will be refocused. So, refocusing you can use this to edit what is called the spectrum, based on the multiplicity of the carbons. How? We already discussed. CH carbons, if you put delta is equal to 1/4J, all the carbons CH, CH2, CH3 carbon appear as positive signals. Exactly at 1/4J, all carbons are positive signal. The 1/2J, if you give, only CH signals are seen, others are not seen. If delta equal to 3/4J, interesting thing is CH and CH3 signals are positive and CH2 is negative. very interesting thing is going to happen. At 1/4J, all are positive, 1/2J only CH signal is seen and at delta equal to 3/4J, we get positive signal for CH and CH3 and negative signal for CH2. So, now what you are going to do, based on the phases of the signal, you can identify different carbons. Compared to, in fact, APT we did that, we saw that in the APT, when exactly equal to 1/2J, we found that all signal CH, CH2, CH3 will be 0 and then only quaternary carbons will be seen and at 1/J, we found that only two of them are positive and other is negative. We saw that CH2 is negative, CH and CH3 are positive. But between CH and CH3, we are unable to distinguish which is which, but here we can utilize this to find out very easily. See, these are the things now. Signal intensity,

Δ	**CH Carbon**	**CH_2 Carbon**	**CH_3 Carbon**
¼ J_{CH}	+	+	+
½ J_{CH}	+	**0**	**0**
¾ J_{CH}	+	**-**	+

I have made a table here for different carbons during refocusing. Put a delta value is equal to 1/4J, CH is positive, CH2 is positive, CH3 is positive. Put delta is equal to 1/2J instead of 1 over 4J, you will see only CH carbon, other two will be disappearing. Put 3/4J, this is positive, this is positive and this is negative. This is the intensity pattern and phases of different signal depending upon the delay delta. All right, this what you will see variation of the 13C signal intensity during refocusing as a function of delta. This has been plotted as a function of delay type.



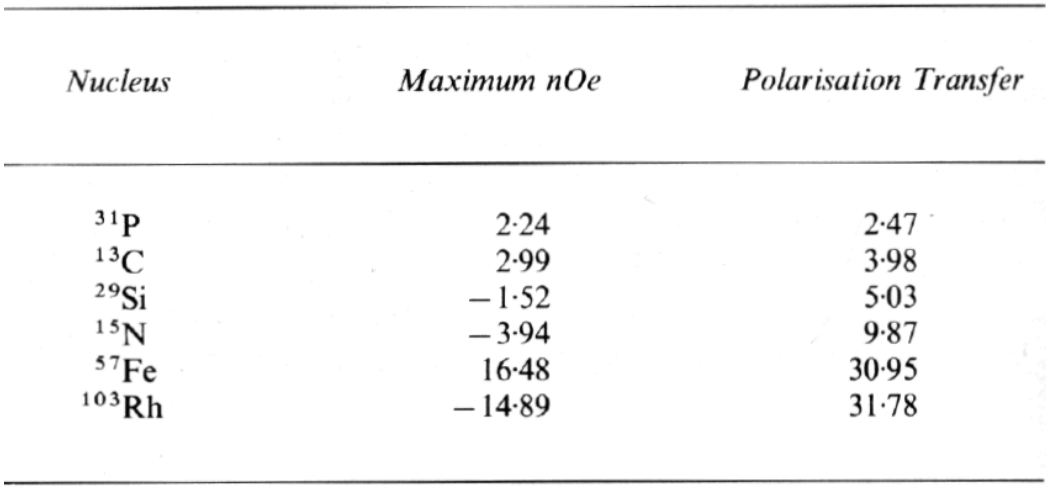
I have chosen only three for the different values, but here continuously the value is varied, the delay delta and intensity for different carbons are plotted. Look at it at exactly equal to 1/4J, all are positive. At 1/2J, then what is going to happen? This CH carbon and CH3 carbon both are 0, it is going to 0, exactly at 0; whereas CH carbon is positive for 1/2J. Go to 3/4J, now CH2 is negative, both CH and CH3 are positive. So, using this, you can identify different carbons, you can also get a decoupled spectrum, you can do the decoupling by using this refocused INEPT. At the same time, by manipulating the delay, you will be able to identify different carbons attached to different number of protons, this is what it is. You can compare the intensity, how beautifully it works is being compared in a simple example for a molecule like this, this is called chloroform.



In the chloroform, we have only two spin half nuclei, heteronuclei, carbon 13 and proton. So, if you look at carbon 13, obviously, it is going to be doublet because of its coupling with proton. I will take a normal carbon 13 spectrum, no decoupling, no NOE, nothing. NOE I am going to discuss after another one or two classes. Remember, I told you earlier NOE gives you signal enhancement, one way of transfer of magnetization like INEPT.

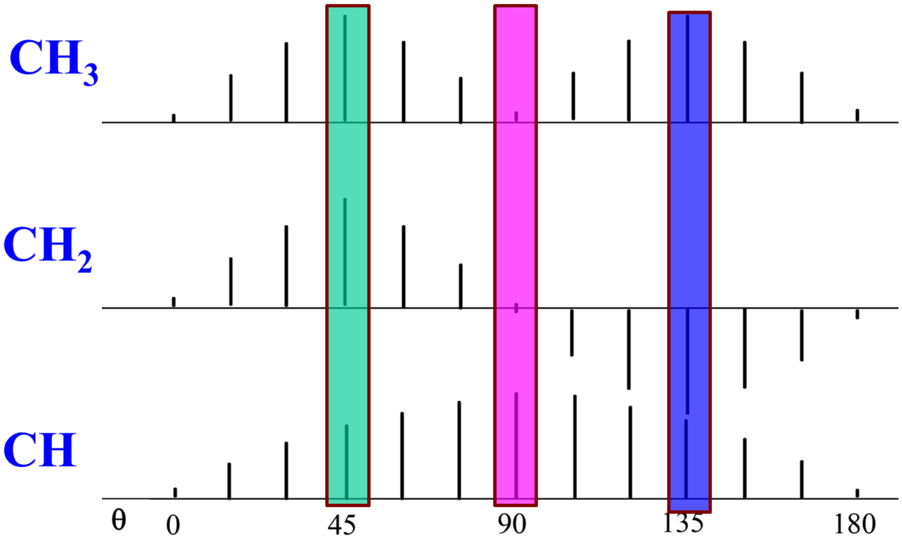
Here, you use J coupling, In NOE there is no j coupling, it is only spatial proximity, that is what I said. We will discuss more about it later. If you take a normal carbon 13 spectrum of CHCl3, no NOE, no decoupling, you get a doublet like this, of this intensity. Same thing you do INEPT experiment, not refocused INEPT, just INEPT experiment in which we saw there is opposite phase, we get doublet, one is positive intensity, other is negative intensity. We do not do the decoupling. If you do decoupling, it gets nullified, it is a coupled spectrum, no decoupling. Same thing you do refocused INEPT. What does refocused INEPT does? It will convert this anti-phase into in-phase, that is all. That is what happened. Again, no decoupling. As a consequence, you still get a doublet, that is also fine. What will this do? we have to see, this is the refocused INEPT. What is the refocused INEPT? This we are going to make anti-phase as in-phase plus we will do the decoupling. The collapsing of the multiplicity, doublet into a singlet and we are going to get a single peak like this. And this is a normal spectra with decoupling and NOE. Here without decoupling, without NOE, here with decoupling and NOE. Look at it, there are different types of experiments have been done here to make a comparison. Where do you see the better signal? We want to identify decoupled spectrum attached to different carbons, attached to different protons. At the same time, there must be gain in the signal intensity. Obviously, you will see refocused INEPT with the decoupling gives you a better spectrum. The intensity is positive and much better compared to conventional experiments. This is a conventional experiment with decoupling and NOE. Here no NOE at all. And this is what we have the advantage. There is a gain in the signal intensity which is coming because of refocused INEPT.

And now we will compare the sensitivity of the INEPT with the direct detection of low gamma nuclei, some low gamma nuclei, nitrogen 15. Nitrogen 15 spectra of Gramicidine S acquired with refocused INEPT. The normal nitrogen 15, no NOE. This is a INEPT experiment where you see the enhancement in signal intensity with decoupling, fantastic experiment. See beautiful, we can see the nitrogen 15. Of course, INEPT experiment can be done with any other nuclei, need not be carbon 13 or nitrogen 15. INEPT enhancement of rhodium 103 where the polarization is transferred from phosphorus in a molecule like this. Here there is no decoupling has been done because of its anti-phase character. That is why you look at the anti-phase. But here is the normal spectrum. See, this is signal. So weak, you will not be able to see anything. This is a simple INEPT anti-phase character, it is not a refocused INEPT. See already gain in the intensity. In case if you do refocused INEPT with decoupling, you get a better signal. See the advantage of INEPT. This is called a polarization transfer experiment where you take the magnetization from the abundant spin and give it to rare spin. In the previous example, we took from proton and give it to carbon and nitrogen. Here, phosphorus is abundant. Take the signal from phosphorus and give it to rhodium. This is what we do. And of course, you can compare the intensity of different nuclei. We can do the comparison and if you do the comparison, how much is the gain during polarization transfer and what is the maximum intensity gain you will get with NOE. They can be easily compared.



Nucleus	*Maximum nOe*	*Polarisation Transfer*
^{31}P	2·24	2·47
^{13}C	2·99	3·98
^{29}Si	−1·52	5·03
^{15}N	−3·94	9·87
^{57}Fe	16·48	30·95
^{103}Rh	−14·89	31·78

Polarization transfer from proton to heteronuclei like this. For example, in the case of rhodium, 30 times enhancement of the signal you get. For iron it is 30. For example, carbon to 4 times, silicon 5 times, nitrogen 10 times, approximately and this is the advantage of this. Ofcourse DEPT is another experiment called Distortionless Enhancement using Polarization Transfer. I already discussed this. This is one which is used to distinguish different carbons attached to different number of protons. This uses polarization transfer I told you when I explained carbon 13, but I did not explain polarization at that time. Now, with understanding of polarization transfer, you know what is DEPT. So, simply I will show you an example of the DEPT sequence. This is what we discussed and then intensity of the different carbons as a function of flip angle, especially the last flip angle here on the proton and if you consider, see how the signal is changing. At 45 degree, all the signal are positive CH, CH2 and CH3. At when it is 90 degree flip angle CH2 and CH3 are 0. And for 135 degree, what is going to happen? CH3 is positive, CH is positive, CH2 is negative. This is how we can do the identify different carbons attached to different protons using DEPT. This is what I already discussed during carbon 13. I just told you the advantage of polarization transfer. So, compared to APT, the advantage of DEPT is you can still identify different carbons attached to protons, but there is a gain in the intensity because of polarization transfer coming to it. So, this is the advantage of DEPT versus APT. Both the experiments are used to identify different carbons based on the multiplicities. DEPT requires less experimental time because of polarization transfer, but accurate phase of the decoupler channel is needed. APT is easy to set up, requires calibration of a perfect 90 degree pulse and no need to wait for carbon 13 T1 to repeat the experiment. But one disadvantage of DEPT is it cannot distinguish between CH and CH3. So, this is what it is. So, I just wanted to summarize this and with this polarization transfer experiments have completely covered. So, in this we discussed about the refocused INEPT. At the same time, how do you suppress the natural abundance carbon 13? We saw the gain in the signal intensity by doing two different types of experiments. Once with 90 degree pulse plus Y and minus Y, take the difference, you could suppress the natural abundance carbon signal. With the refocused INEPT, you are able to put a delay, convert anti phase into in phase. So, we can do the decoupling. So, we did that experiment and we saw there is a gain in the signal intensity and of course, depending upon the delay that you are going to use in the refocused part of the INEPT sequence, we can identify different carbons attached to different protons depending upon whether it is a 1/ 4J, 1/2J or 3/4J. And then we can identify CH, CH2 and CH3 carbons based on the phases. In some case, it is going to be 0 and in other two cases it is going to be positive or negative. Based on that we can identify everything.



I showed you the polarization transfer is used in DEPT also and I just show you one example what we discussed earlier about DEPT in carbon 13. DEPT takes the advantage of signal enhancement due to polarization transfer, but still you can utilize it to identify different carbons. There is a subtle difference between APT and DEPT. Both are used only for identification of different carbons attached to different protons. I hope you got the idea about polarization transfer DEPT and APT experiment, INEPT experiment, how we can use to identify different carbons. I told you about only proton carbon, but you can use it for any heteronuclei like nitrogen 15 any other thing you can utilize for polarization transfer. That is what also I said. So, with this I am going to stop from next time onwards we go to a different topic called two dimensional number. So, I will stop here. Thank you very much.