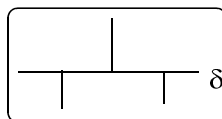


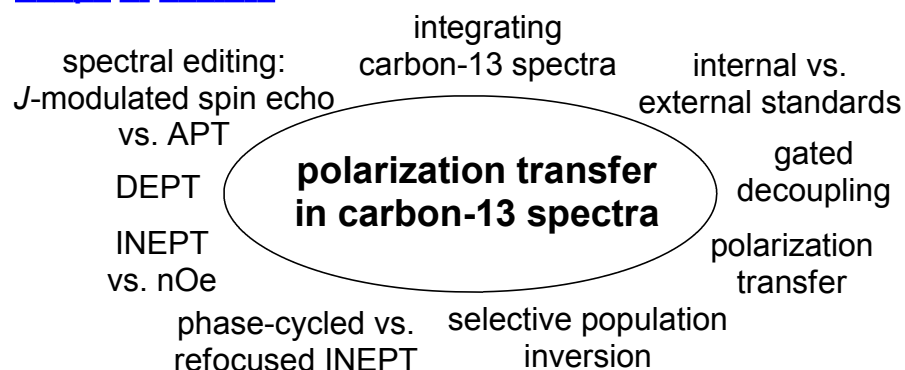
Polarization Transfer in Carbon-13 Spectra

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February 19, 2012.



Scope of Lecture



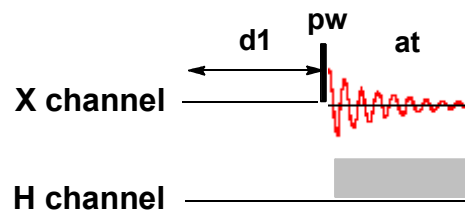
Key References

1. Understanding NMR Spectroscopy (2nd Ed.) Keeler, J. Wiley, **2010**.
2. NMR Spectroscopy Explained: Simplified Theory... Jacobsen, N.E. Wiley, **2007**.
3. The ABCs of FT-NMR Roberts, J.D. University Science Books, **2000**. (**Chapter 7**)
4. High-Resolution NMR Techniques in Organic Chemistry (2nd Ed.) Claridge, T.D.W. Elsevier, **2009**. (**Chapter 4**)

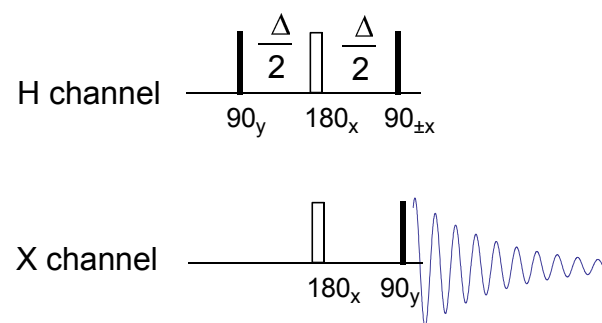
This lecture follows the treatment in references 3 and 4.

Key Questions

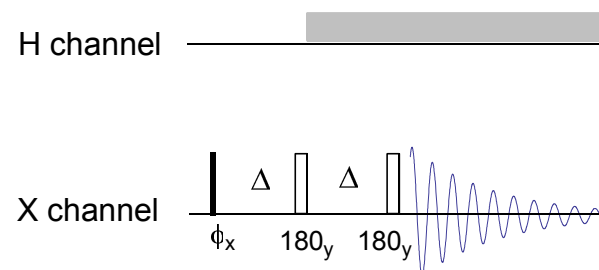
(1) Can carbon-13 spectra be integrated?



(2) How can S/N be enhanced in carbon-13 spectra?



(3) How can the multiplicities of carbon atoms be distinguished?



Quantitative Integrations

ref: This lecture follows Chapter 4 of Claridge. Chapter 7 of Roberts may also be a useful reference.

Under normal conditions, without special calibrations,

proton integrals are accurate to within about 10% while **carbon integrals** are only useful for qualitative purposes.

Q: What affects integral accuracy?

(1) **delay time:** relaxation of z-magnetization between scans

According to the Ernst equation, a delay time d_1 of $1.3T_1$ is optimal for 90° pulses. However, this is not nearly enough time for all the z-magnetization to recover between scans. Thus, if two protons have *different* T_1 times, the proton with the longer relaxation time will have a smaller integral.

(2) **digital resolution:** Hz/point

Integration is done by summing up the areas of rectangles. If too few rectangles are used, the integrals will not be accurate.

(3) **filtering, pulses:** weak intensities at edges of spectrum

To prevent the noise beyond the spectral window from aliasing into the spectrum, a combination of analog and digital filters are used. However, the "cutoffs" for these filters are not perfect, and can reduce the intensity at the edges.

Later, we'll see that a "hard 90° " pulse doesn't excite all the resonances in the spectrum equally. These off-resonance effects can affect signal intensity.

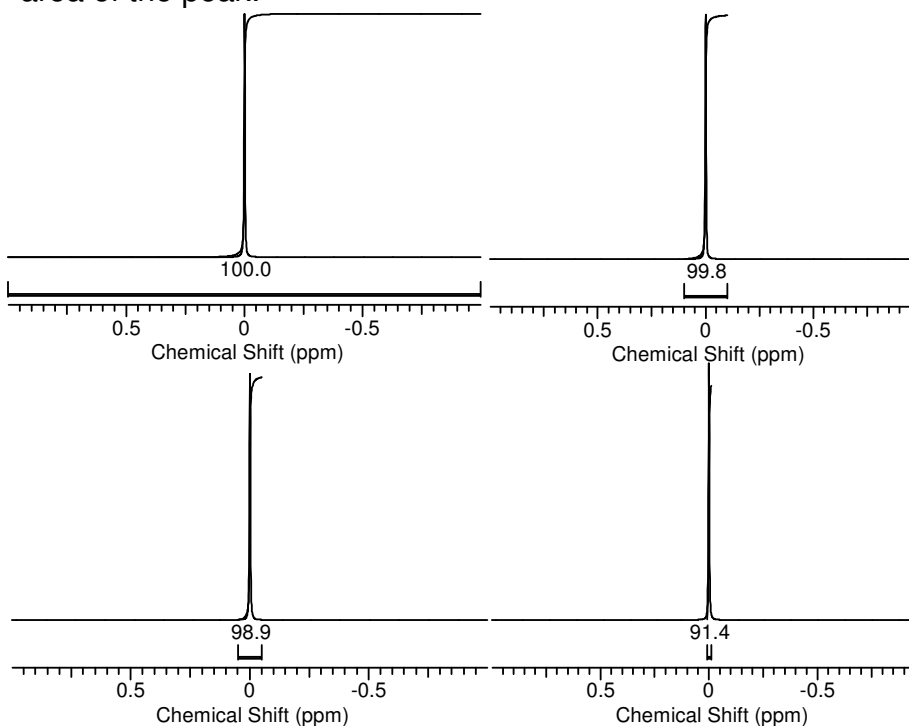
(4) **nOe:** through-space magnetization transfer

Carbon spectra don't have accurate integrals largely because

broadband proton decoupling is typically used. The protons are irradiated, which transfers magnetization to the carbons, increasing their intensity. This is good from an S/N standpoint, but bad from a quantitative integral standpoint.

(4) bounds of integration

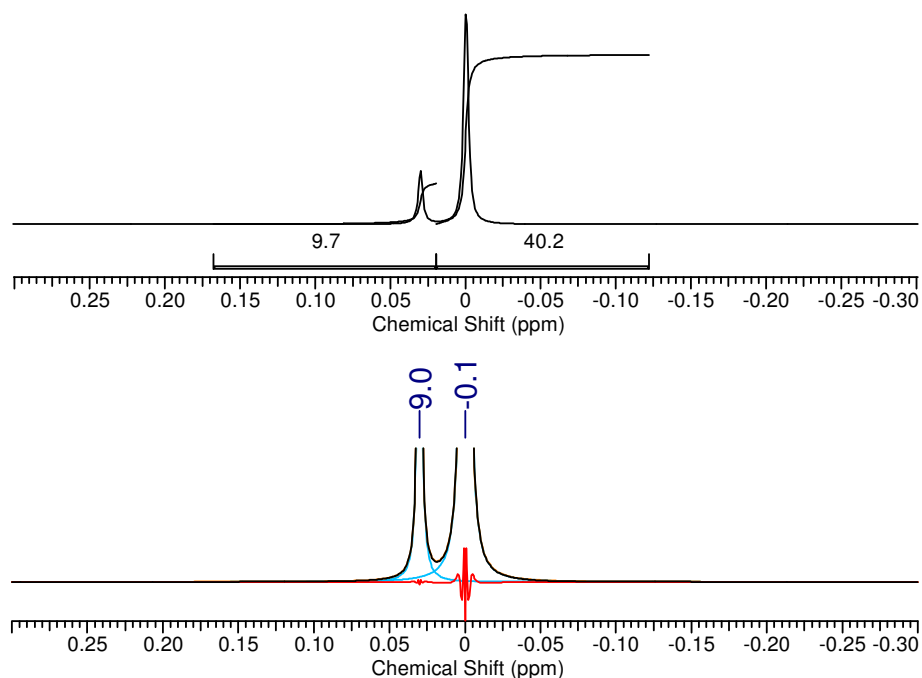
The Lorentzian nature of the peaks means that one has to integrate a fair bit on either side of the peak to "get" all of the area of the peak:



This is a computed peak with a 1 Hz linewidth, which is what you would get in a nice, well-shimmed spectrum without any impurities on the baseline (another common source of errors). (The spectrum is computed at 300 MHz.) **Integrating 20 Hz on either side of the peak is necessary to get accurate integrals.** By default, many more decimal places are given for integrals than are actually significant.

Quantitative Integrations

If two peaks are next to each other, spectral deconvolution methods will be necessary for accurate integrals:



The red line is the residual of the least squares fit.

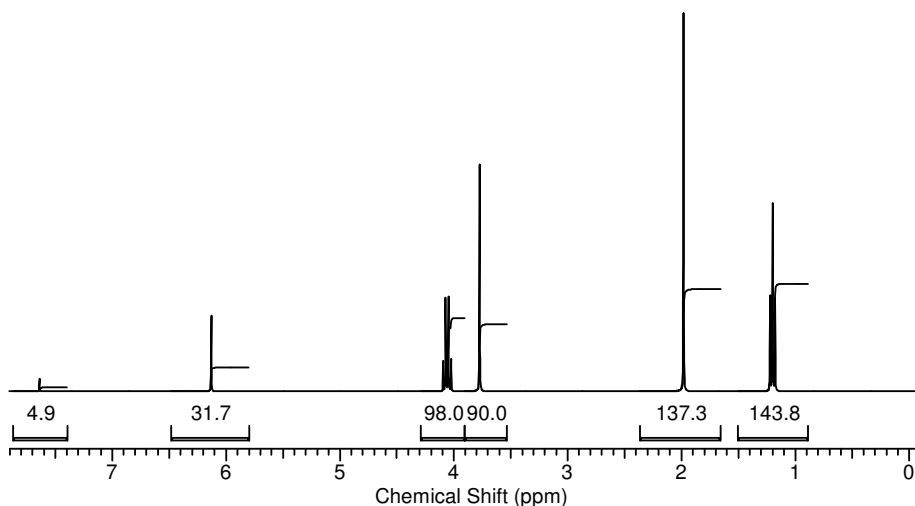
Internal vs. External Standards

Q: How can greater accuracy in proton integrals be obtained?

- $d1 = > 5T_1$
- zero-filling
- appropriate spectral window

Even assuming you do all that, it is still commonplace to add an inert reagent of known concentration to the sample. Here is a sample spectrum of ethyl acetate, to which 1,3,5-trimethoxybenzene has been added:

Q: If the internal standard is present at a concentration of 0.1 M, what is the concentration of ethyl acetate?



A: The internal standard peak shows that at 0.1 M, one proton is worth 10 integral units. (There are evidently some integration errors in this spectrum.) The ethyl acetate methylene is worth 50 integral units. Dividing by two to get one proton is 25 units. Thus, ethyl acetate is at a concentration of $(25/10)0.1 = 0.25$ M.

In the above example, an **internal standard** was added. However, this is not always convenient--the presence of an internal standard can interfere with the chemistry, for example.

Two methods for introducing **external standards** have been developed:

ERETIC: electronic reference to access *in vivo* concentration (Akoka *Anal. Chem.* **1999**, *71*, 2254)

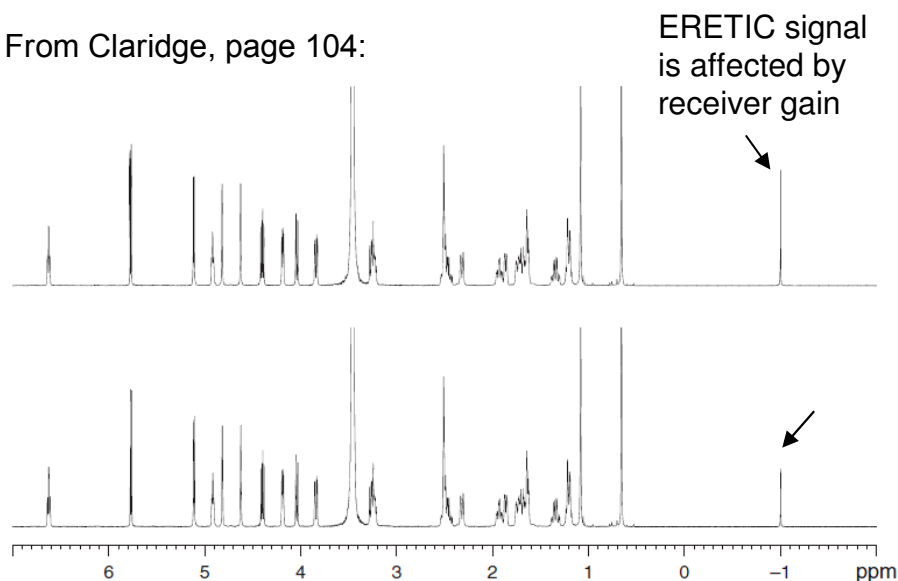
QUANTAS: quantification by an artificial signal (Upton et al. *Mag. Res. Chem.* **2010**, *48*, 753)

Internal vs. External Standards**ERETIC**

- (1) An artificial RF signal is mixed with the detector output. This typically appears at -1 ppm.
- (2) The RF signal intensity and phase are calibrated against a standard sample of known concentration.
- (3) Integrations can be made against this artificial signal.

The accuracy has been shown to be as good as that with an internal standard. The disadvantage is that you have to fiddle with your spectrometer in a complicated way. It is also sensitive to experimental issues like improperly tuned probes.

From Claridge, page 104:

**QUANTAS**

- (1) The reference signal is added using software.
- (2) A scaling factor is used to adjust the size to the peaks in a sample of known concentration.

This has the advantage of not requiring hardware modifications. The signal also does not change with receiver gain or the number of scans. It can also be added to previously recorded spectra, assuming spectrometer stability is good.

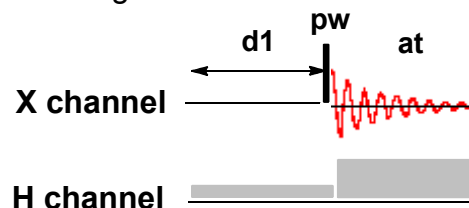
Decoupling in Carbon Spectra

As a reminder:

- (1) We detect signals from ^{13}C -isotopomers only in carbon spectra. But every ^{13}C must be next to a ^1H . Therefore, carbon-proton couplings will be present unless removed by decoupling.
- (2) nOe enhances the intensity of carbons that have protons next to them
- (3) Therefore, ^{13}C integrations are not accurate ***under normal conditions***.

Q: How can coupling or accurate integrals be obtained in carbon spectra?

Ignoring the specifics of just *how* decoupling works for the moment, let's consider the standard pulse sequence. The gray rectangle indicates decoupling. The taller rectangle means the decoupler power is higher.

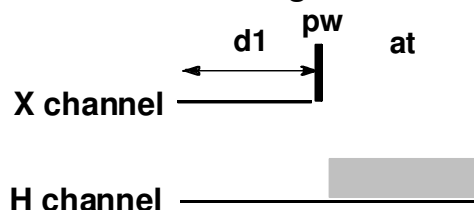


This is called **power gated decoupling**. During the delay time, low-power decoupling allows the nOe to build up. During the acquisition period, stronger decoupling collapses carbon-proton multiplets into single, sharp lines, giving a stronger signal.

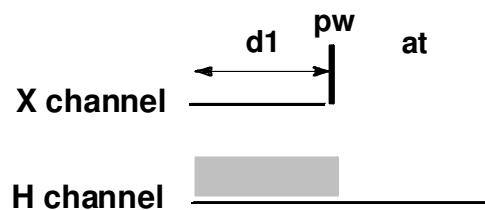
On our Varian instruments, this corresponds to **dm='yyy'**.

Decoupling in Carbon Spectra

What if we **don't want nOe enhancement but do want decoupling**? This means the decoupler has to be off during **d1**, but on during **at**. This is **inverse gated decoupling**. (dm='nny')



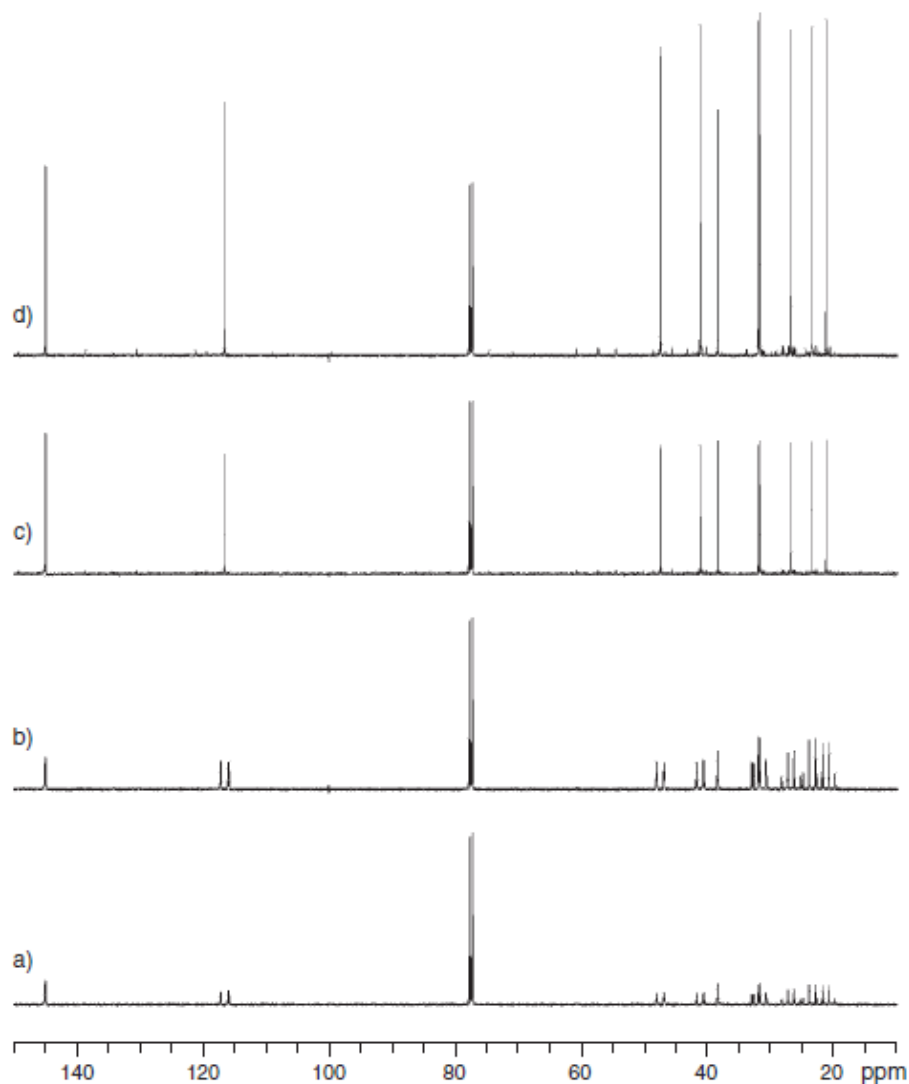
Because the nOe buildup during acquisition is not very significant, these spectra can be integrated. All the other combinations are possible, too. What happens in this pulse sequence? (dm='yyn')



This is **gated decoupling**. It gives CH multiplets *with* nOe enhancement. Finally, if we just leave the decoupler off the whole time (dm='nnn'), we get a coupled spectrum that has no nOe enhancement.

On the facing panel, spectra are plotted to the same scale and were taken under identical conditions (Claridge, page 109).

- (d) power gated decoupled (with nOe, singlets)
- (c) inverse gated decoupling (no nOe, singlets)
- (b) gated decoupling (with nOe, multiplets)
- (a) no decoupling (no nOe, multiplets)



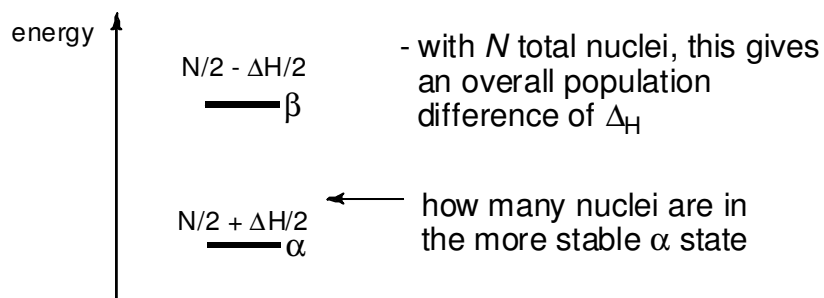
Polarization Transfer in ^{13}C Spectra

Problem: carbon-13 is not as sensitive as hydrogen-1 (receptivity relative to proton, 1.7×10^{-4})

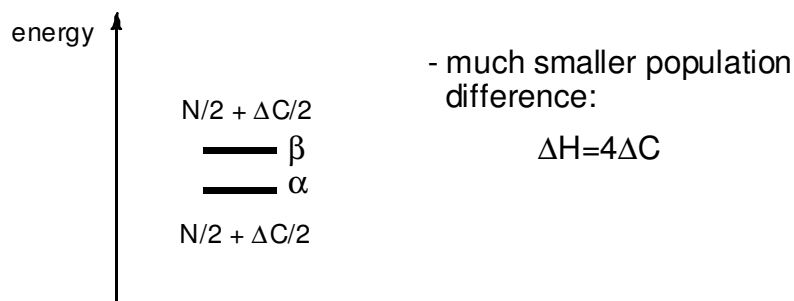
Solution: transfer magnetization from proton to carbon

- through space (via NOE in regular decoupled carbon-13 spectra)
- through bond (via *polarization transfer*)

The rest of this lecture focuses on how polarization transfer works. Consider an isolated proton in a magnetic field:

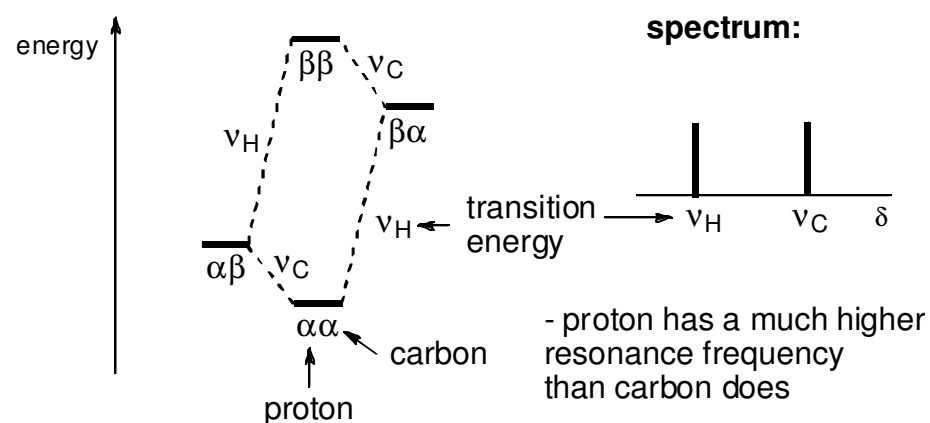


An isolated carbon-13 nucleus looks similar, but because $\gamma_{\text{C}}/\gamma_{\text{H}}$ is 1/4 (ratio of gyromagnetic ratios), the population difference is a lot smaller:

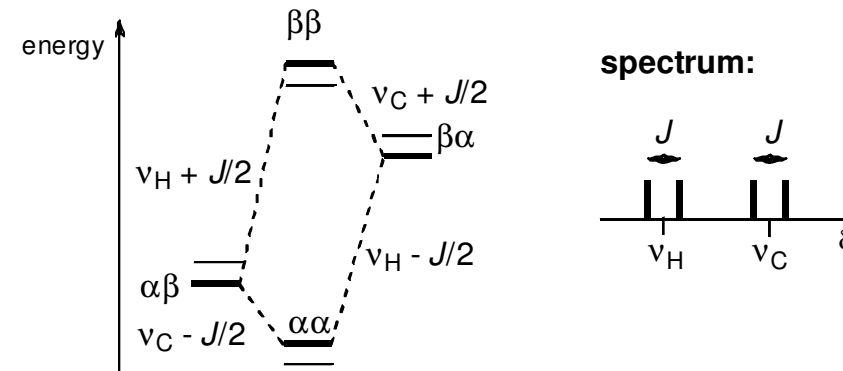


Now, consider an $\text{R}_3^{13}\text{C}-^1\text{H}$ methine (R=a non-magnetic group):

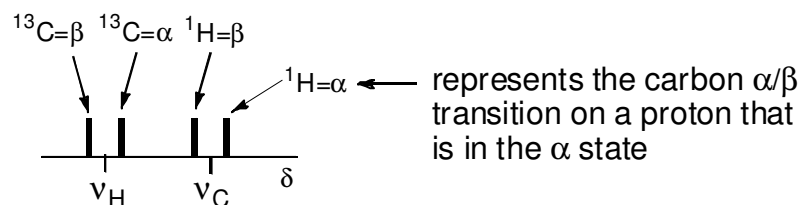
when $^1J_{\text{CH}} = 0$



when $^1J_{\text{CH}} > 0$

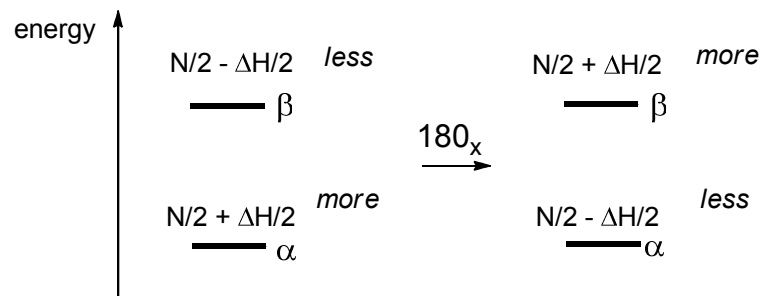


Note that each line in each doublet represents a specific spin state:

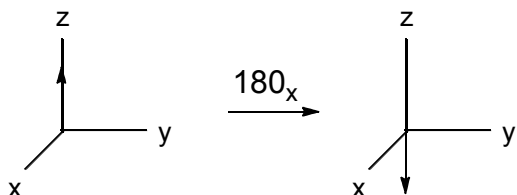


If $^1J_{CH}$ were zero, then the proton and carbon would be effectively independent systems; therefore, irradiating the proton transition would not be expected to perturb the intensity of the carbon transition.

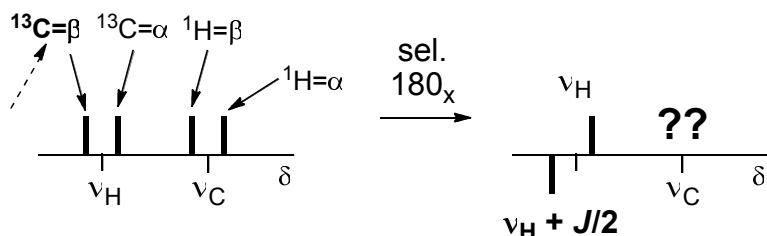
In terms of a single isolated proton, a 180_x pulse corresponds to **population inversion**:



This can be understood via a vector diagram:

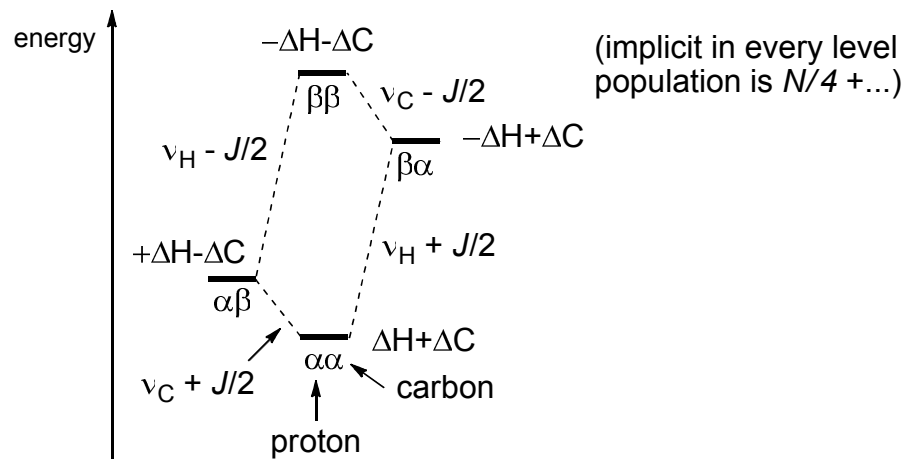


Now, consider applying a selective 180_x pulse to the β half of the 1H doublet:

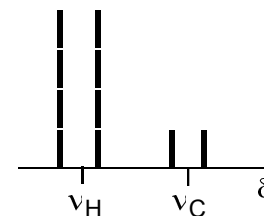


What happens to the intensity of the carbon peaks? Since $J \ll \delta$, let us ignore the effect of J in the energy diagram (even though its presence is required for this to work):

For convenience, let us define the equilibrium population difference across a nucleus as 2Δ , rather than Δ as before. At equilibrium, we have these populations:



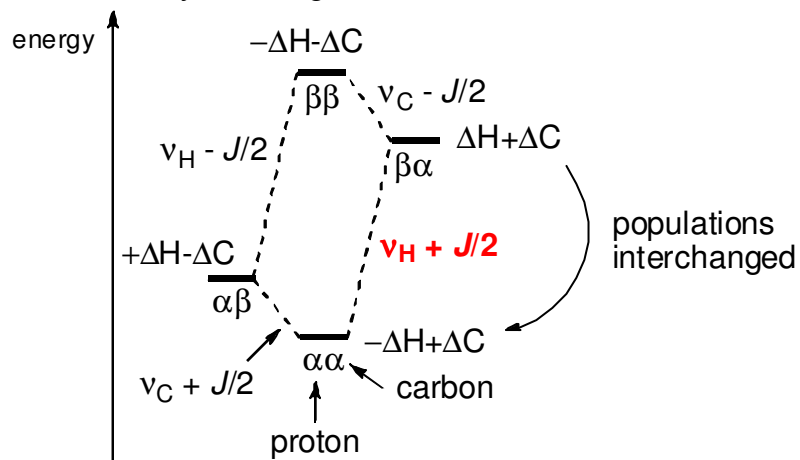
We must now correct the diagram from before, since we know that $\Delta_H = 4\Delta_C$. The proton lines must be four times larger than the carbon lines (see above)! The intensities are actually:



We can check this directly from the energy diagram. For example, the most downfield resonance at $\nu_H + J/2$ has a population difference of bottom population - top population = $(-\Delta H + \Delta C) - (-\Delta H + \Delta C) = 2\Delta H$. Defining $2\Delta C$ as one unit, we have an intensity of +4 units.

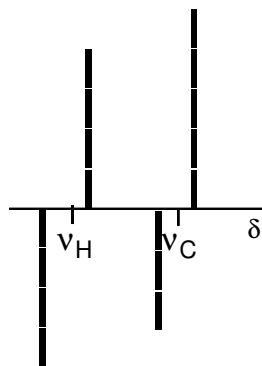
Similarly, the $\nu_C - J/2$ transition as an intensity of $(-\Delta H + \Delta C) - (-\Delta H - \Delta C) = 2\Delta C = +1$ units.

Now, selectively inverting the $\nu_H + J/2$ transition results in:



The resulting population difference across the $\nu_C + J/2$ transition is now $(-\Delta H + \Delta C) - (\Delta H - \Delta C) = -2\Delta H + 2\Delta C = -4 + 1 = -3$ units.

Similarly, the resulting population difference across the $\nu_C - J/2$ transition is now +5 units, and so forth. This means:



Effectively, the carbon lines are now intense because they now incorporate some of the population difference from proton as well as carbon. Now, obviously this is not a very practical approach. Why is that?

(1) I am too lazy to invert the resonances one by one.

(2) The intensities are not perturbed symmetrically.

(3) I would have to wait too long for full relaxation between scans.

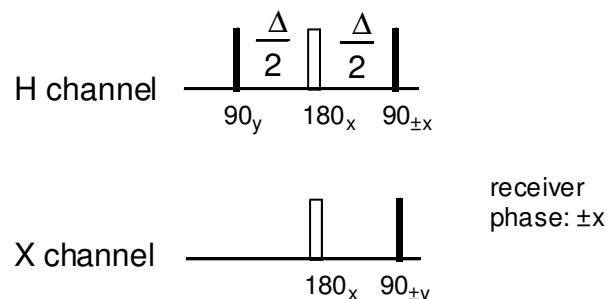
(4) Selective pulses are technically challenging to perform.

What we would like is something that will invert half of the proton resonances *simultaneously*. This sequence is called:

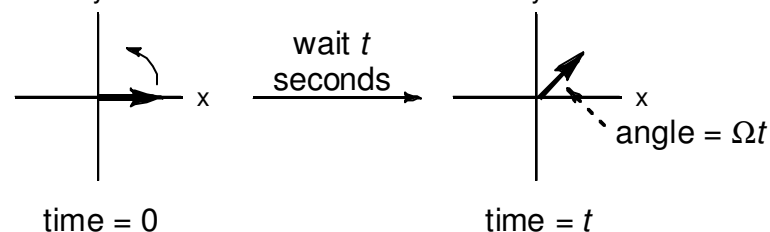
INEPT: Insensitive Nuclei Enhanced by Polarization Transfer

Freeman, *JACS* **1979** 101, 760-762.

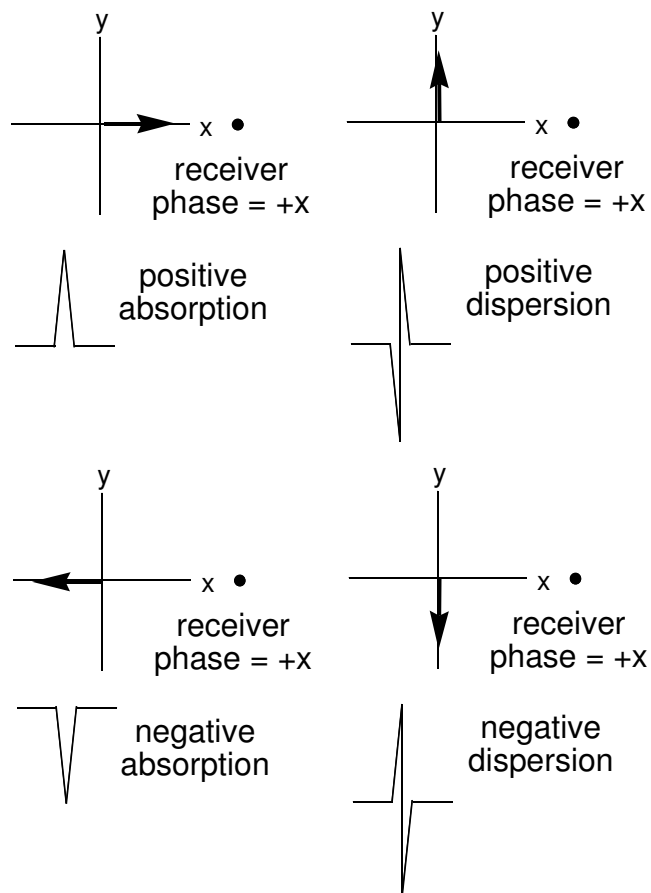
The INEPT sequence is as follows:



Let us analyze this pulse sequence in detail using the vector model. Recall that a time delay of t corresponds to a phase shift of Ωt in the rotating frame, where the offset Ω corresponds to the frequency difference between the resonance ν and the reference frequency: ν

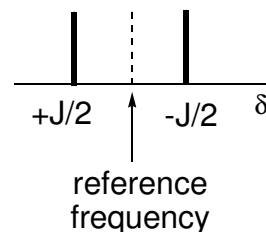


The relationship between the initial phase of the vector and the phase of the receiver at the beginning of the acquisition period determines the lineshape that is observed:



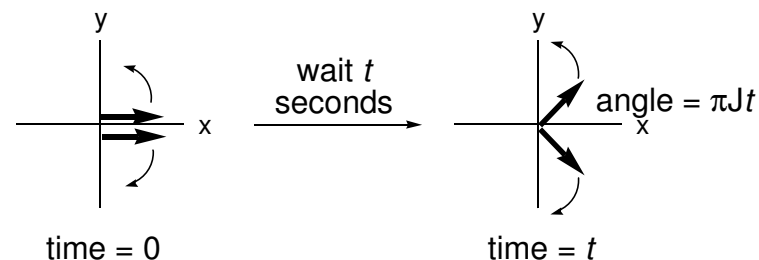
This means that if the phase of the peak ends up at an axis that isn't $+x$, the phase of the receiver will have to be adjusted accordingly to get a positive absorption signal.

What is the effect of a time delay on a J coupling? Consider a doublet to be two resonances, one at $+J/2$ Hz from the reference frequency (defined as zero) and one at $-J/2$ Hz:



Because the $+J/2$ peak has a positive frequency, the peak is rotating *faster* than the reference frequency in the laboratory frame. By convention, the reference frequency rotates CCW. Therefore, the $+J/2$ peak will move CCW in the rotating frame as well.

The same logic says that the $-J/2$ peak will *lag behind* the reference frequency. The overall effect in the rotating frame will be two counter-rotating vectors with the same angular velocity:



To compute the angle, note that: angle (rad) = angle/time (rad/s) \times time (seconds). Each vector is moving at an angular velocity of $J/2$ revolutions per second, or $2\pi \times J/2 = \pi J$ radians per second.

(How long will it take for the angles to be 90 degrees apart?
Answer: $1/4J$ seconds.)

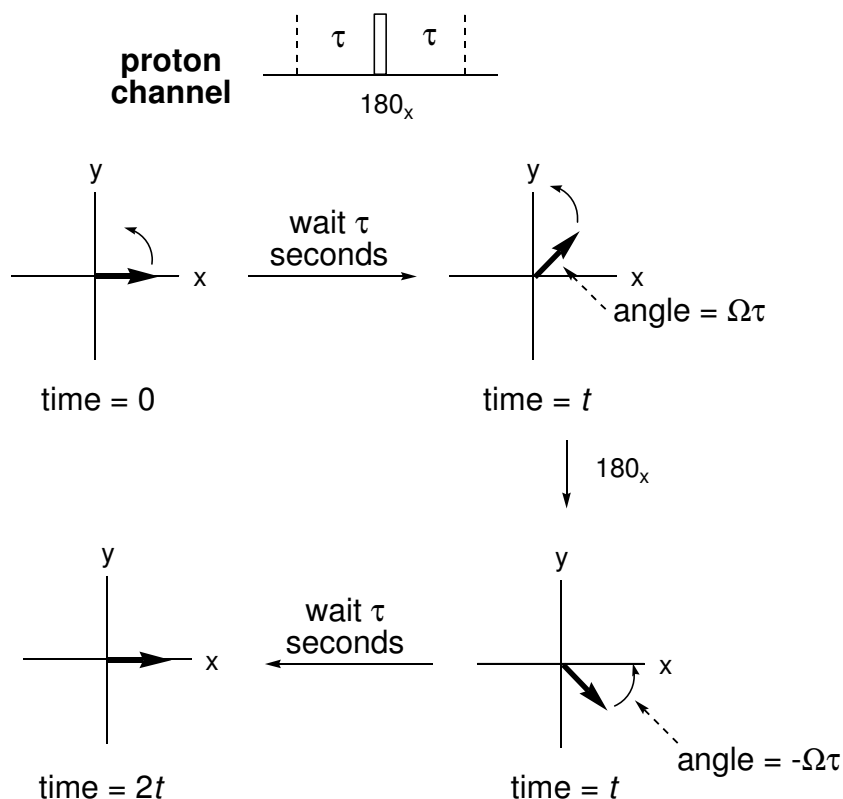
We are now in a position to understand a very important concept in virtually every pulse sequence, the **spin echo**. Consider this pulse sequence, which is part of the INEPT approach:



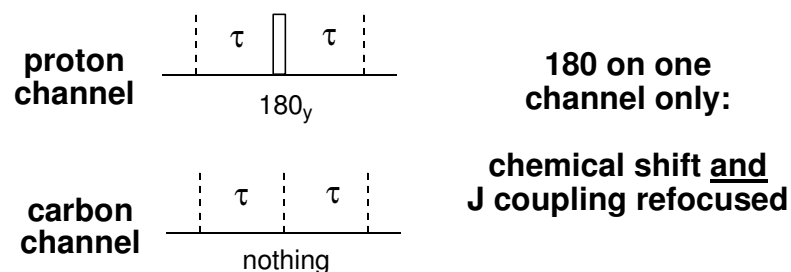
For a $R_3^{13}C-^1H$ pair, what will the effect be? (The dashed lines merely denote time markers; they don't imply a pulse or any other operation.)

Spin Echoes

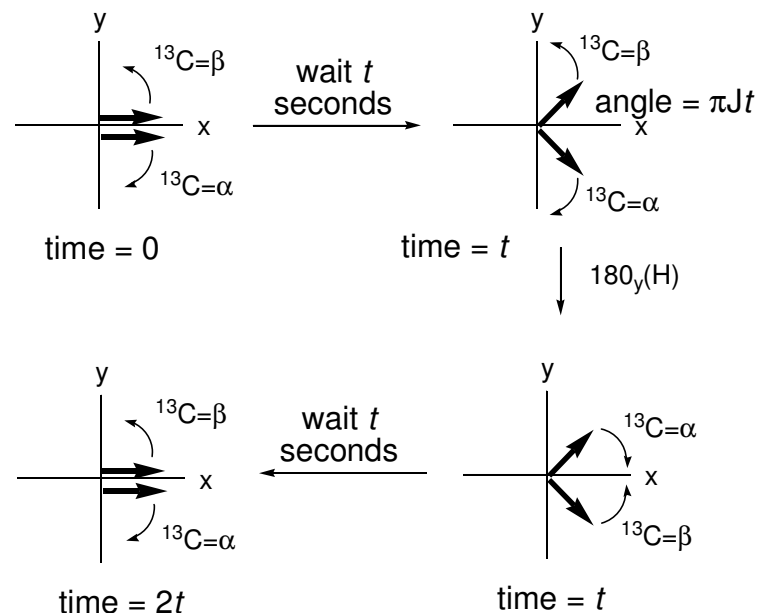
First, consider an isolated proton, which has no couplings to anything else. Only chemical shift evolution need be considered:



Thus, chemical shift evolution has been refocused. As it turns out, the chemical shift and coupling Hamiltonians commute, so it is valid to treat them separately. Such an analysis will show that **spin echoes always refocus chemical shift evolution**. However, they do not always refocus J-coupling evolution. Consider this spin echo for the methine case where 180 degree pulses are applied simultaneously on both nuclei.

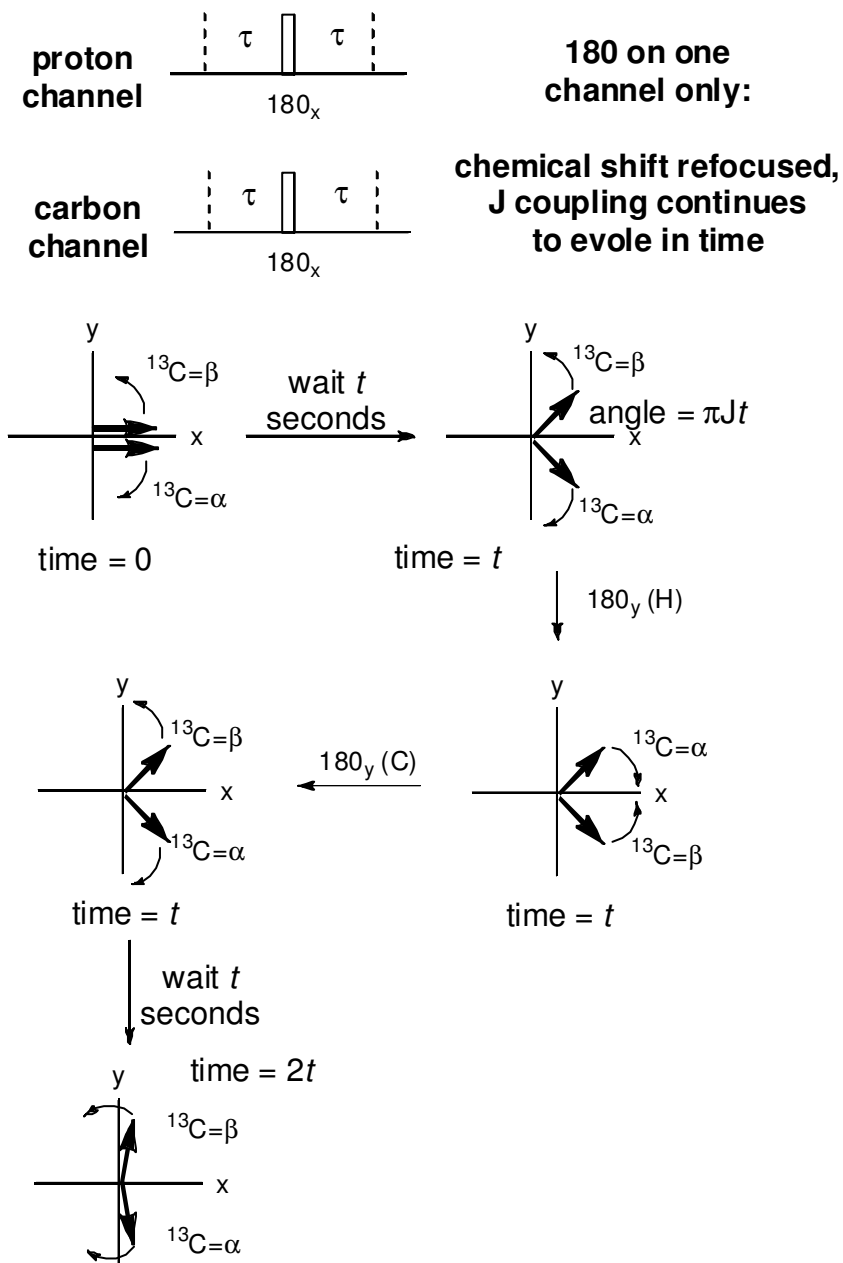


Considering the fate of an initial x-magnetization on the proton (as explained earlier, the faster rotating vector corresponds to the downfield half of the proton doublet, which represents proton transitions on a carbon in the β state):



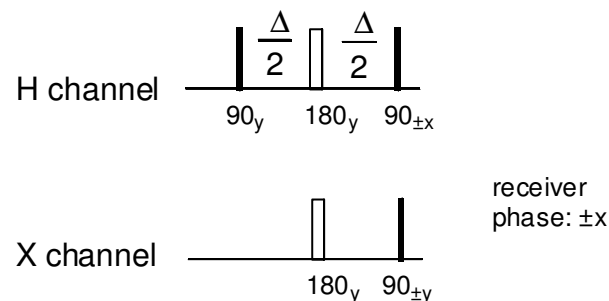
- The α vector is always lagging behind (going CW) and the β vector is always speeding ahead (going CCW).
- This refocused both chemical shift and J coupling. One could repeat this analysis for a non-zero reference frequency to see this; the order of chemical shift and J-coupling evolution would not matter.

The same is *not* true if pulses are applied on both channels:

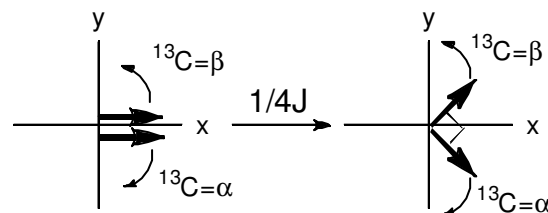


- The α vector is *still* always lagging behind (going CW) and the β vector is always speeding ahead (going CCW).
- However, the 180_y (C) pulse inverts populations, meaning that the protons that were once on carbons in the α state are now on carbons in the β state; hence, the labels interchange.
- Chemical shifts are still refocused. The lack of J-coupling refocusing can be good or bad, depending on what you want to happen. In fact, it is crucial that J-coupling *not* be refocused in the INEPT experiment.

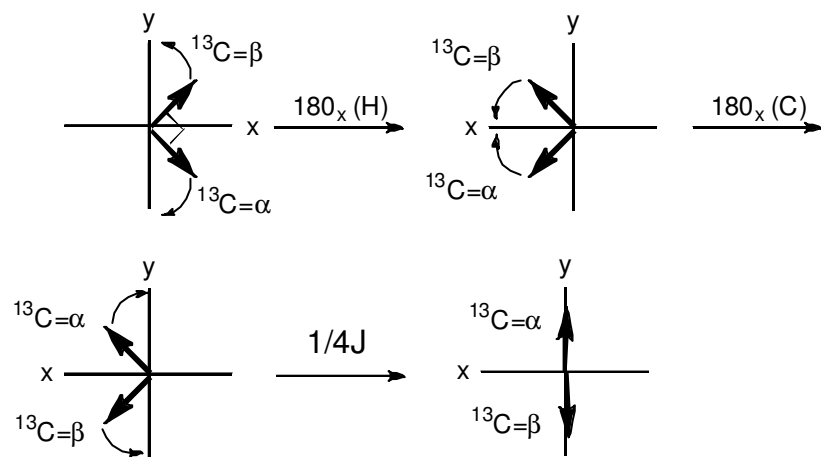
Now, let us return to INEPT. Let us set the delay $\Delta/2$ to $1/4J$:



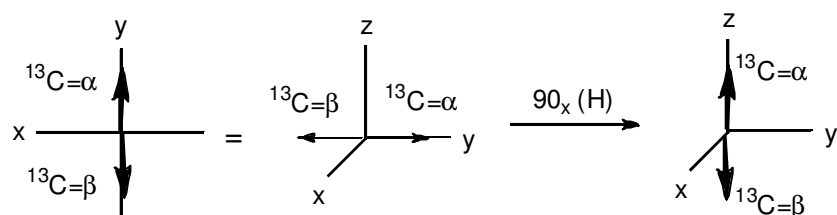
The first 90_y pulse on the proton channel initiates a CCW rotation about the y-axis, placing the vector on the +x axis:



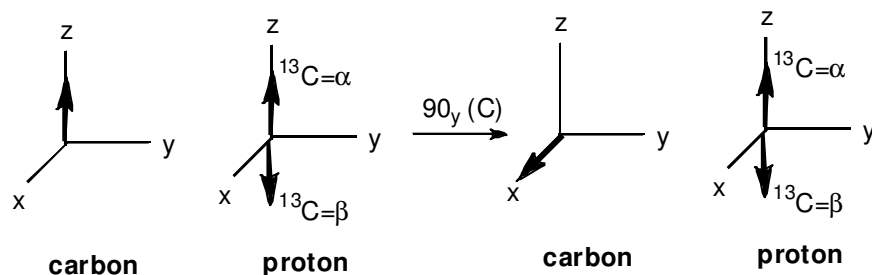
A $1/4J$ time delay corresponds to a 90 degree angle between the vectors since: $1/4J$ (s) \times $2\pi J$ (rad / s) = $\pi/2$ rad. As before, couplings are not refocused:



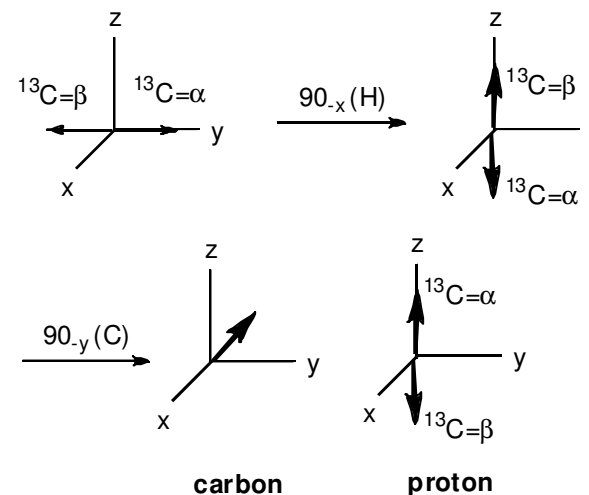
By the end of the echo, the vectors are a full 180 degrees apart. This corresponds to:



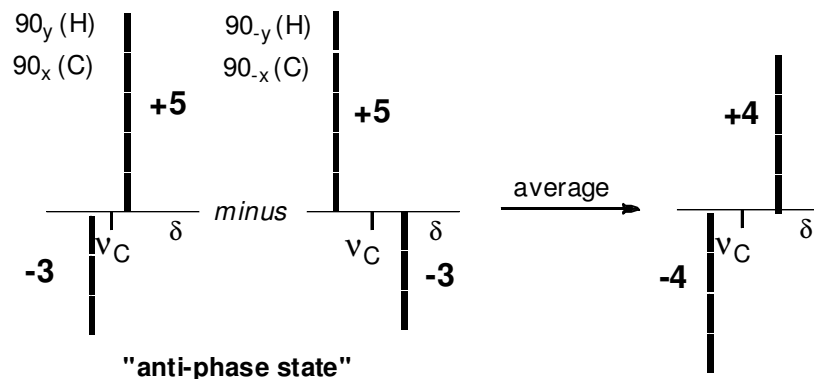
This corresponds to exactly what we were looking for: inverting half of every doublet. The last 90_y on the carbon axis rotates the *carbon* vector so that it can be observed:



Reversing the phases of the last two pulses would invert the other half of the doublet:



Note that the reversal on the carbon nucleus also inverts the sign of the resulting signal. What do the spectra look like? As already argued, the resulting intensities will be +3/-5:



Subtracting the two signals by inverting the receiver phase results in the signals adding to two positive numbers on every pair of successive scans. Note that **decoupling cannot be applied**, since these "anti-phase" doublets would collapse to nothing. But the *effect* of decoupling, i.e., a doubling in signal height *is* desirable.

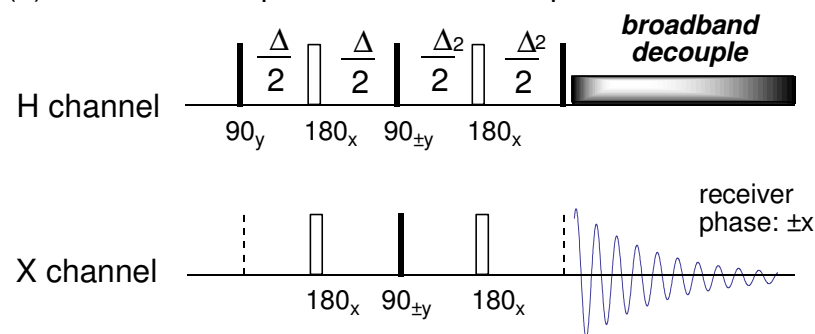
Refocused INEPT

INEPT accomplishes two steps:

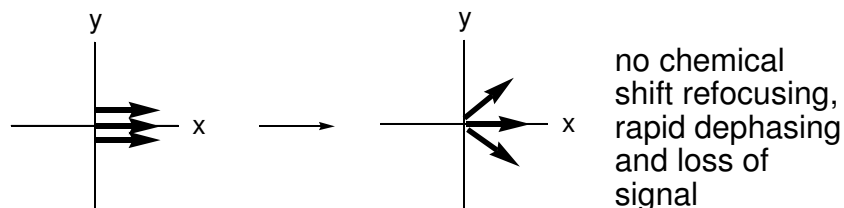
- (1) generate an anti-phase state
- (2) transfer the magnetization to the less sensitive nucleus

With refocused INEPT, we use a second spin echo to turn the transferred anti-phase state back into an in-phase state:

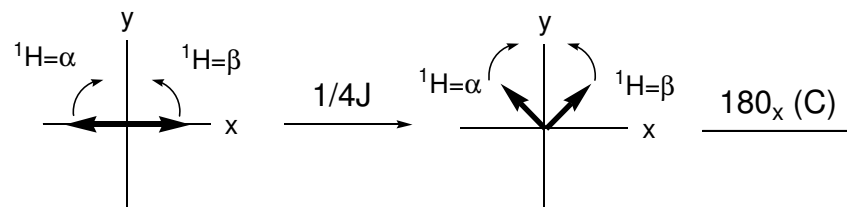
- (3) return the anti-phase state to an in-phase one



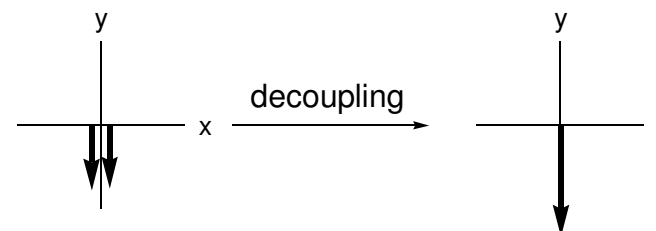
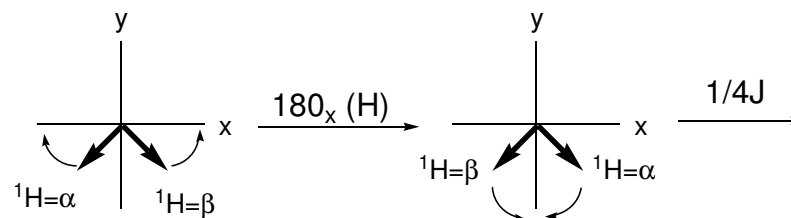
Note that spin echoes are continually required, so that the chemical shifts do not dephase while the couplings are evolving. If they did dephase, the vectors would soon be distributed in a complicated way in the xy plane. On average, their sum would add up to zero--no net signal:



So, at the end of the INEPT sequence, we have an anti-phase state on the carbon-13 nucleus. A further spin-echo of the same $J/4$ duration gives an **in-phase state**, effectively doubling the signal to noise. Setting the second delay Δ_2 to $J/4$ gives:



carbon nucleus at end of INEPT



The actual gain in S/N isn't actually as good as 2, since some relaxation will occur during the second spin echo, but this is still better than doing nothing.

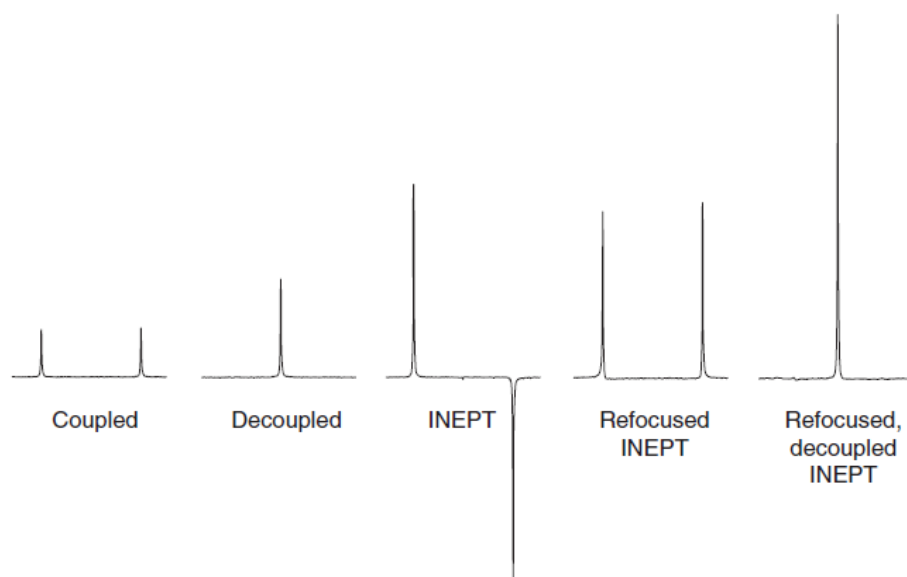
Note that different delays will be optimal for methylenes (CH_2) and methyl (CH_3) groups. The optimal second delay is:

$$\Delta_2 = \frac{1}{\pi J} \sin^{-1} \left(\frac{1}{\sqrt{n}} \right)$$

No delay will be optimal for all systems; every peak will show some phase errors, which will be corrected by decoupling.

DEPT - Distortionless Enhancement by Polarization Transfer

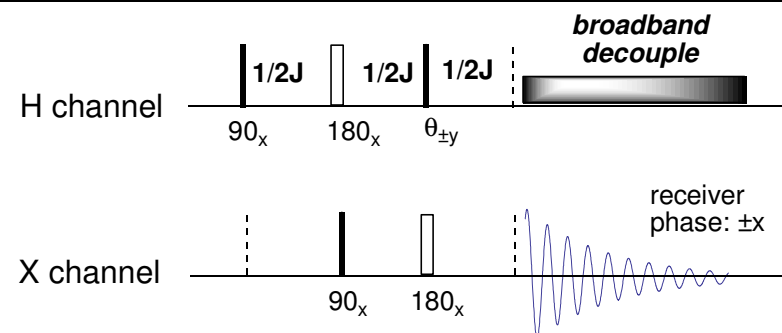
Here is a graphical summary of the sequences we've talked about (Claridge, page 118):



Even refocused, decoupled INEPT is no panacea. For one thing, the intensities relative to normal multiplets is distorted:

	normal intensities				INEPT			
CH	1	1			-3		+5	
CH₂	1	3	1		-7	+2	+9	
CH₃	1	3	3	1	-11	-9	15	13
INEPT with suppression of natural magnetization					-1	1		
					-1	0	1	
					-1	-1	1	1

(The natural ¹²C-¹H magnetization will not be affected by phase cycling of the last pulse, so will be removed on successive pairs of scans.) The DEPT sequence seeks to avoid any distortion, while providing a way to distinguish between methine, methylene, and methyl carbons. The sequence is:



(Doddrell *et al.* *J. Magn. Reson.* **1982**, *48*, 323-327.) Though this looks similar to the INEPT sequence and is based on similar ideas, "multiple quantum coherence" is developed during this sequence. MQC cannot be described by the vector model, and we will have to wait until we understand the product operator formalism to have any idea of what's going on.

The variable angle θ is what gives rise to the various flavors of DEPT. In each, peaks of different multiplicity give DEPT peaks of different sign:

	DEPT-45	DEPT-90	DEPT-135
C	0	0	0
CH	+	+	+
CH₂	+	0	-
CH₃	+	0	+

Although these spectra can be interpreted separately, it is generally a good idea to use a program to process them into DEPT "sub-spectra," just in case there is any spurious overlap of positive and negative signals in the same spectrum.

Note that quaternary carbons do not appear. Since the sequence depends on the value of ¹J_{CH}, which varies throughout the molecule, the actual phases can deviate from the values given in the table above. Additionally, large two-

bond couplings, such as those in alkynes, can give rise to spurious phases (DEPT assumes that magnetization is only transferred through one-bond carbon-proton couplings). Usually a compromise value of $^1J_{CH} = 140 \text{ Hz} / 3.6 \text{ ms}$ is chosen.

It is useful to compare the signal enhancement obtained from INEPT vs. the NOE (Claridge, page 119):

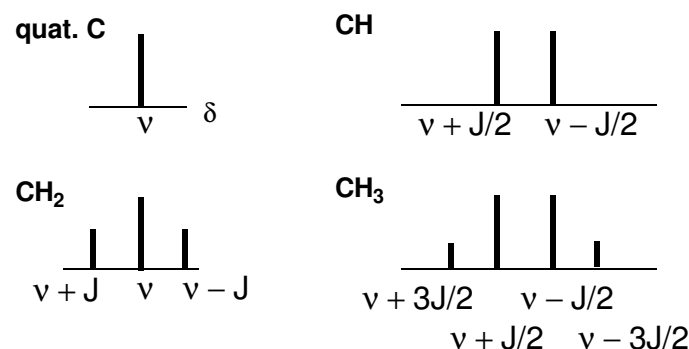
$$I_{\text{INEPT}} = I_0 \left| \frac{\gamma_H}{\gamma_X} \right| \quad I_{\text{NOE}} = I_0 \left(1 + \frac{\gamma_H}{2\gamma_X} \right)$$

Because some gyromagnetic ratios, NOEs can actually give negative enhancements. Additionally, the benefits for groups of different multiplicities are *not* additive, such that CH_3 groups are not enhanced three times more than CH groups. However, there is an advantage to INEPT-type transfer: because the magnetization originates on the protons, it is the T_1 relaxation times of the protons (fast) rather than the relaxation times of the carbons (slow) that matters. The disadvantage is that several DEPT sub-spectra need to be run, so that regular decoupled (NOE) spectra are probably the best for routine work. If multiplicity editing is desired, DEPT is the best choice.

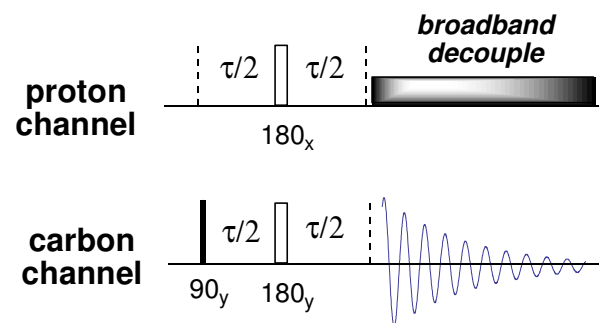
X	^{13}C	^{15}N	^{29}Si	^{31}P
I_{INEPT}	3.98	9.87	5.03	2.47
I_{NOE}	2.99	-3.94	-1.52	2.24

Spectral Editing

Although I can't yet tell you how DEPT works, I *can* talk about how spectral editing works in general. The goal is to sort C, CH, CH_2 , and CH_3 resonances by taking advantage of the differences in their multiplet structure. This diagram will make it more clear:

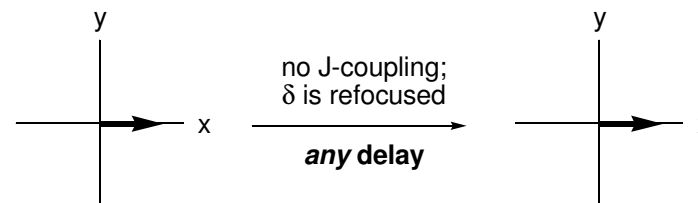


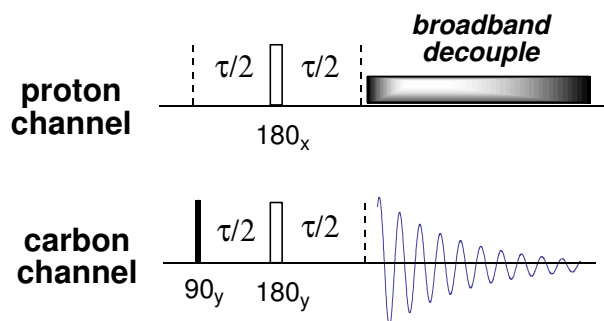
The basic sequence that sorts these out is the **J-modulated spin echo**. This is really the same as the spin echo we already dissected earlier, except now we'll pay explicit attention to what delay is optimal for the refocusing of the various possible multiplicity groups:



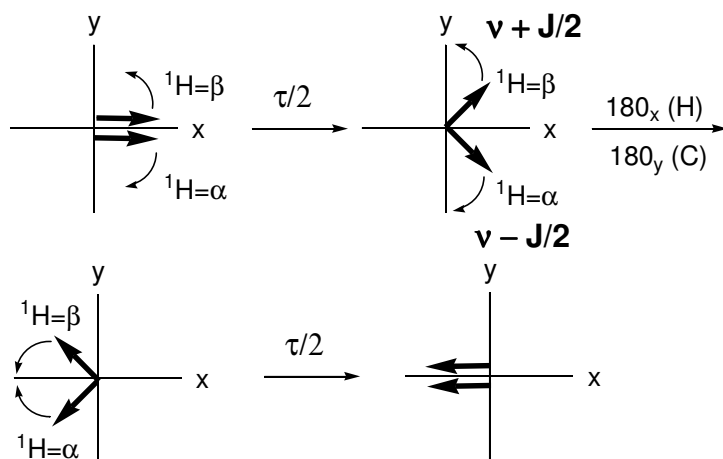
We recognize this as a heteronuclear spin echo that refocuses chemical shifts without refocusing couplings, which evolve for τ seconds total. What delay is optimal for refocusing?

Quaternary carbons represent a trivial case; *any* delay will give refocusing:

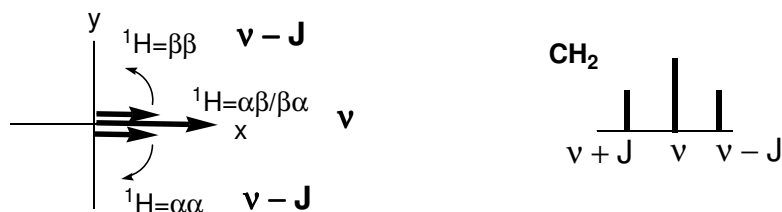




Methines will be refocused by a delay of $\tau=1/J$.

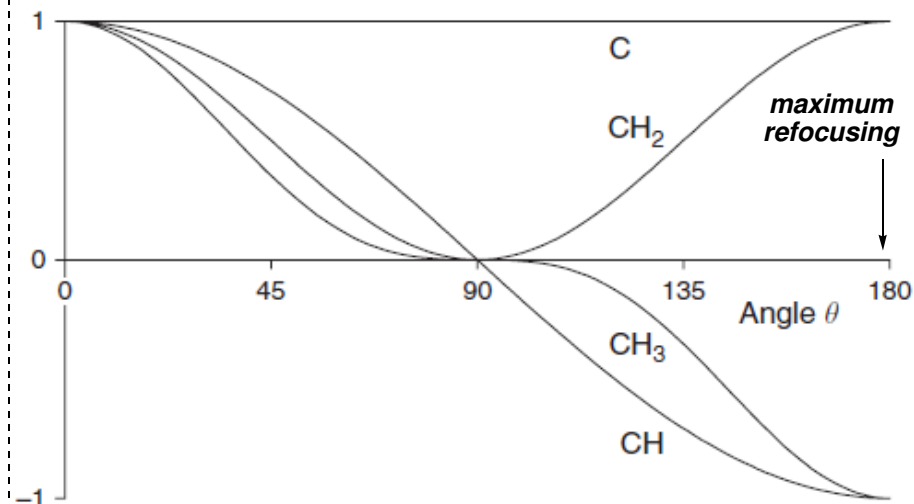


The key calculation is that the angle between the vectors will increment by $\pi(J/2)(\tau/2) = \pi(J/2)(1/2J) = \pi/4$ during every delay $\tau/2$. That means both vectors move by 45 degrees during each delay. **Methylenes** will also be refocused by a delay of $\tau=1/J$, except we now have to use three vectors:

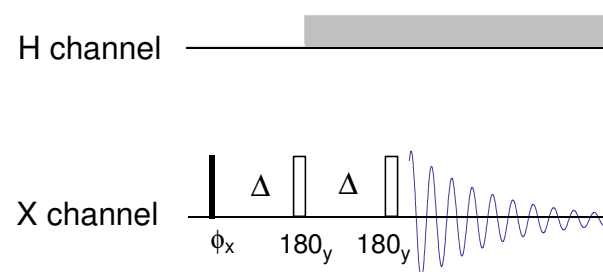


The outer two vectors will be moving at relative angular rates of $+J$ and $-J$, while the inner one will have a relative frequency of 0. The inner one is also twice as large, since it is composed of two degenerate components.

Defining $\theta = 180 J \tau$ degrees, the intensities vary as $\cos^n(\theta)$, where n is the multiplicity of the CH_n group ($n = 0, 1, 2, 3$):

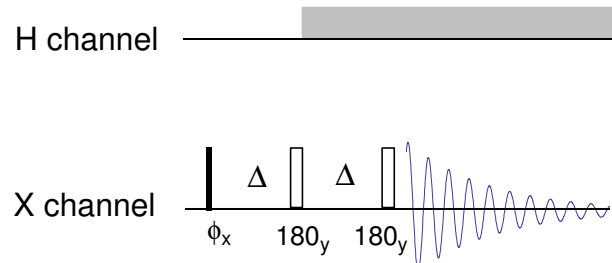


In practice, the J-modulated spin echo is not that practical, since it uses a 90 degree excitation pulse (this is bad for signal averaging). The solution is to use **APT - Attached Proton Test** (Schoolery *et al. J. Magn. Reson.* **1982**, 46, 535-539.):

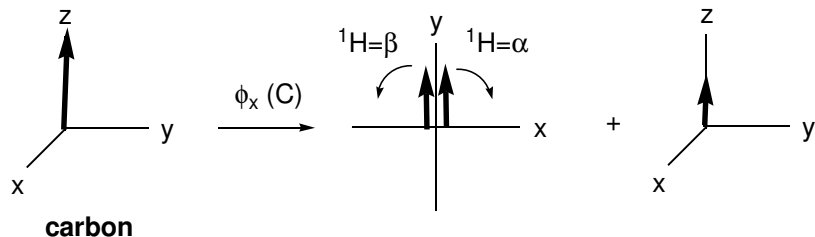


Why is an "extra" 180 pulse needed *during* the decoupling period?

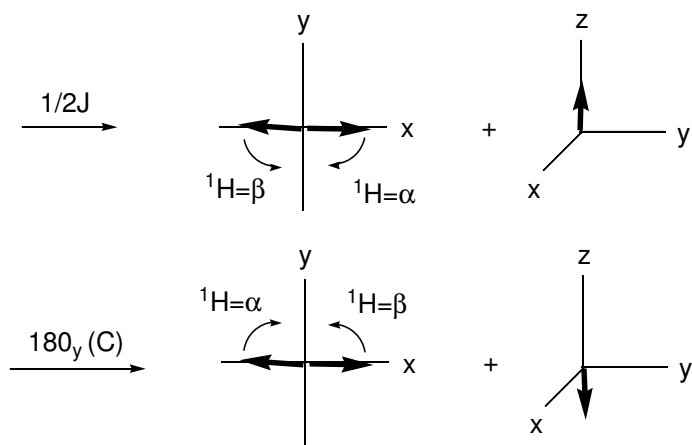
APT



Consider a methine carbon again, with a delay $\Delta=1/2J$. Here, we explicitly examine the natural magnetization (^{12}C - ^1H), which will not be affected by any carbon pulses:

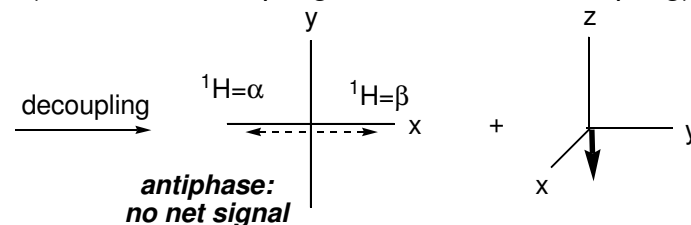


The reduced angle ϕ rotates some of the $^{13}\text{C}/^1\text{H}$ magnetization into the xy -plane, but leaves some at $+z$. The $+z$ component also includes a natural magnetization contribution.

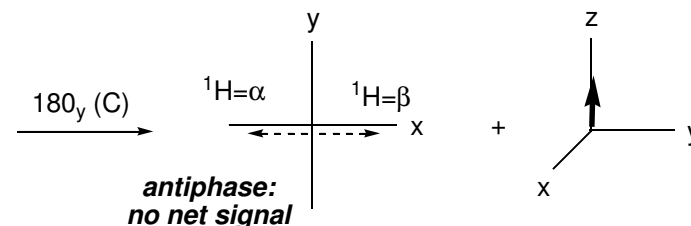


(On subsequent scans, the phase of this 180 pulse can be

alternated so that the sign of the natural magnetization will flip and therefore get cancelled out with signal averaging.) Finally, we apply decoupling, during which coupling evolution remains frozen (since the decoupling "turns off" the J coupling):



As soon as another delay period has passed, the chemical shifts have been refocused and we can start acquiring. However, the natural magnetization and labeled, but unexcited magnetization must be placed back on the $+z$ axis, so proper relaxation can occur:



You might be surprised that there is no signal there. But with a delay of $1/2J$, corresponding to an angle of 90 degrees, the graph on the previous page predicts a null signal for methines. So this is the expected result. We have accomplished the same thing as we expect a J -modulated spin echo to, but we've used less than full 90 degree pulse at the beginning, and optimized the (slow) relaxation process.

In subsequent lectures, you will see that the HSQC experiment is essentially a 2D version of what I described here. This is very important for assigning protons to their directly attached carbons; sensitivity gains and spectral editing are made in the same way: INEPT transfer, followed by J -modulated spin echoes.