

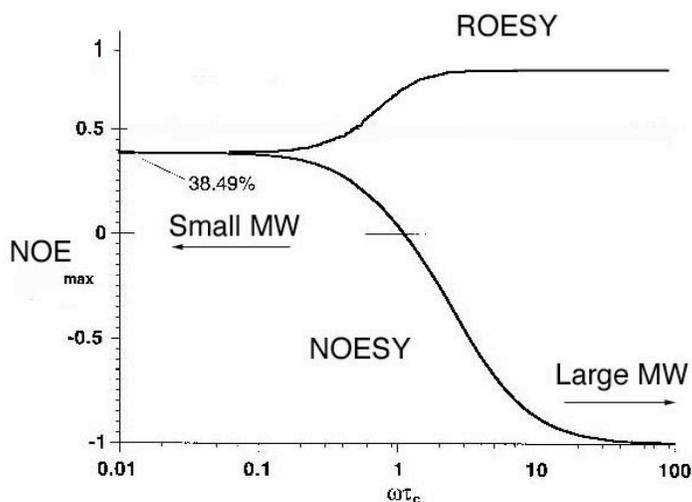
# NOESY on the 400 and 500 Using Topspin

When a proton is saturated or inverted, spatially-close protons may experience an intensity enhancement, which is termed the Nuclear Overhauser Effect (NOE). The NOE is unique among NMR methods because it does not depend upon through-bond J couplings but depends only on the spatial proximity between protons. In other words, the strength of the NOE gives information on how close two protons are. For small molecules, an NOE may be observed between protons that are up to 4Å apart, while the upper limit for large molecules is about 5Å.

There are many different possible NOE experiments (NOE or ROE, steady-state or transient, 1D or 2D, etc). The ones available on the 400 and 500 are 1D selective-NOESY, 2D-NOESY, 2D-NOESY with zero-quantum suppression, and 2D-ROESY. Some understanding of the theory of NOE is necessary for choosing the correct experiment and for interpreting it properly. Some of the important results from the theory are discussed below. For a full understanding, see the excellent text Neuhaus, David, and Williamson, Michael P., *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, 2<sup>nd</sup> ed., WILEY-VCH, New York, 2000.

## Molecular Weight and Maximum NOE

The maximum possible NOE depends on the molecular correlation time (or the inverse of the rate of molecular tumbling), which is in large part determined by the molecular weight and solvent viscosity. Larger molecular weights and higher viscosities lead to larger correlation times. The NOE is positive for small molecules (MW < 600), goes through zero for medium-sized molecules (MW range 700 – 1200), and becomes negative for large molecules (MW > 1200). (These MW ranges are approximate only.) For medium sized molecules, the NOE may be theoretically zero. See the figure below that is adapted from Newhaus and Williamson text. The ROESY experiment (rotating frame NOE) is preferred for medium-sized molecules since the ROE is always positive.

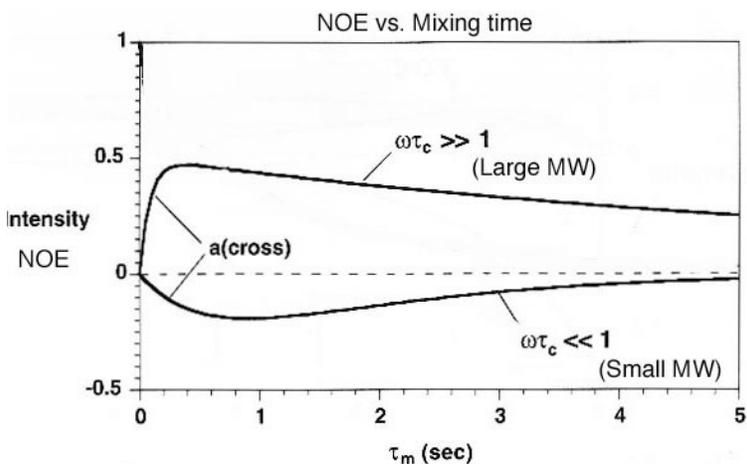


## Time Dependence of NOE - Mixing Times

In transient experiments, such as NOESY and ROESY, the NOE dynamically builds up and then decays due to relaxation during the mixing time, as shown below in the plot of NOE versus mixing time. The NOE, thus, goes through a maximum as function of mixing time. The location of the maximum NOE and rate of build-up depend on the correlation time, or its proxy, the molecular weight, and the distance between protons for a particular NOE. In general, large molecules build-up NOE quickly while small

molecules build-up NOE more slowly. That is, for large molecules the point of maximum NOE is shifted to shorter mixing times. A shorter distance between protons will also lead to faster build-up of NOE and a shift of the maximum to shorter mixing times.

There is only one mixing time specified per NOE experiment, and it is the most important parameter for NOE experiments. For small molecules, a mixing time that maximizes the NOE is desirable, unless you intend to calculate an actual distance (see analysis section). Generally, one is interested in a range of distances so the choice depends on molecular weight rather than a particular distance. For large molecules, the mixing time must be kept small so that the build-up obeys the linear approximation and spin diffusion is avoided (see analysis section). The following are guidelines:



- 1) small molecules 0.5 - 1 sec. Start with 0.5 sec.
- 2) medium size molecules 0.1 - 0.5 sec. Start with 0.25 sec.
- 3) large molecules 0.05 - 0.2 sec. Start with 0.1 sec.

## 1D versus 2D Methods

The choice between 2D (ROESY or NOESY) versus 1D (selective NOESY) depends on the amount of material available and the amount of information needed. A single 2D experiment gives all NOE information simultaneously whereas 1D experiments provide NOEs one at a time. **In general, I recommend the 2D methods.** The minimum amount of time required (which does not depend on sample concentration but on the time necessary for experimental cycling) for 2D and 1D differ. The minimum time for a 2D NOESY spectrum is longer. The standard 2D NOESY often requires a minimum of 1.5 hours but the 2D NOESY with zero-quantum suppression, which uses gradients, has a minimum time of only 25 minutes. The minimum time for a single 1D selective NOESY spectrum is about 2 minutes. Many 1D experiments, however, are usually required. If you have very little material, then signal averaging will be required anyway and the 2D version should be used.

Spectral crowding will affect the choice of experiment. If critical peaks to be irradiated are very close (<30 Hz) to other peaks, then the selectivity of the 1D version will not be sufficient and the 2D version will be needed.

## Artifacts and Their Suppression

Zero-quantum peaks are a common artifact in all NOESY spectra. They occur between peaks that are J-coupled, such as ortho-protons on a ring, as can be identified by their up-down DQF-COSY type of pattern. There is a 2D NOESY sequence that is designed to remove these zero-quantum peaks.

In ROESY spectra, a common occurrence is TOCSY transfer between protons that are J-coupled or symmetric with respect to the center of the spectrum. This latter artifact can be removed by proper positioning of  $\omega_{1p}$ , the center of the spectrum. Finally, the cross-peak intensities have an offset dependence. See analysis section for more detail.

If protons are undergoing chemical exchange, corresponding cross peaks occur in all NOE and ROE experiments. In fact, chemical exchange can be studied with these same NOE methods and are then termed EXESY experiments.

In 1D selective NOESY experiments, there are several types of possible artifacts: zero-quantum (up-down) peaks as well as the unsuppressed residual from very intense singlets. Moreover, the experiment uses selective pulses and their proper calibration is required for optimal results and suppression of artifacts.

## Choice of Experiment – a Prescription

Small molecules (MW < 600)

The usual choice is 2D NOESY with zero-quantum suppression. Exceptions would be if you have a very concentrated sample and you are only interested in one or two NOEs and the peaks to be irradiated are well-separated; then choose the 1D selective NOESY. ROESY has only disadvantages for small molecules.

Medium sized molecules (700 < MW < 1200)

ROESY is preferred.

Large Molecules (MW > 1200)

The choice here is more complicated. The usual choice is 2D NOESY but 2D ROESY has advantages. ROESY suffers less from spin diffusion and the resulting interpretation errors. However, ROESY is less sensitive for large molecules and has other disadvantages such as TOCSY artifacts. See analysis section.

## Sample Considerations:

Preparation: Removing Dissolved Oxygen

**Dissolved oxygen or other paramagnetic species such as Cu<sup>2+</sup> can reduce or completely quench the NOE.** For small molecules, it is extremely important to remove dissolved oxygen. For large molecules, the removal of oxygen is not critical. Removal of oxygen must be done by the freeze-pump-thaw method. Simply bubbling argon through the sample is not sufficient. The following describes the freeze-pump-thaw procedure:

- 1) freeze the sample in liquid nitrogen or CO<sub>2</sub>/acetone.
- 2) evacuate the space above the solution.
- 3) turn off vacuum but keep sample isolated and allow to thaw. As it thaws, bubbling should be noticed.
- 4) repeat several times (3-4 times).
- 5) backfill with N<sub>2</sub>.

When finished, the sample should, of course, be sealed in some manner. Tubes with attached stopcocks are available.

Sample size and tube options

When sample quantity is very limited, it is advantageous to limit the amount of solvent in which it is dissolved. If a normal 5mm tube is used, however, this cannot be less than about 500µL without causing serious lineshape problems (shimming problems) and the attendant loss of signal-to-noise. There are special tubes made by Shigemi, however, that can be used to restrict the active volume and, hence, reduce the amount of solvent without causing lineshape problems. Shigemi tubes are available from Aldrich.

## Procedure for 1D Selective NOESY (see below for 2D Methods)

1. Take a normal 1D proton spectrum. It is extremely important to tune (**wobb**) the probe for this experiment. Do NOT spin the sample.

You need to obtain the exact frequency positions of the peaks that you want to irradiate. (Make sure the display info is turned. Right click over the spectrum and select Display properties. Make sure the cursor info box is checked.) Position the cursor on the peak and note the frequency in Hz. Repeat for each peak that you will wish to irradiate.

2. Change the data set by typing **new**. Type **rpar selnoegs.top**. Type **getprosol** to update the pulse parameters. Type **o1** and now enter one of the values recorded above in step 1. Adjust the value of the mixing time, **d8**. See discussion above on mixing times. The number of scans (**ns**) must be a multiple of 2.

When you entered a value for **o1** above, you set the center of the spectrum to be the same as your chosen peak. (This is correct!) What you must now consider is whether the **SW** value is large enough to include all of your spectrum. Change it if necessary.

Type **expt** to see how much time the experiment will take. If you have a concentrated sample, (>15mg/ml @ 400g/mol), 32 or 64 scans will be sufficient. If you have <1mg/ml, then 1 or more hours may be necessary (and you should consider the 2D NOESY experiment).

3. Type **rga**.

4. When it is finished, type **zg**.

5. Repeat steps 2-4 for each peak you want to irradiate.

### Automating the acquisition

If you follow the above procedure, you need to be present at the spectrometer when it finishes NS scans in order to start the next acquisition with a different o1 value. This is inconvenient. The command **xau multizg** solves this problem. It executes multiple acquisitions on sequential experiment numbers. For example, assume your first NOE experiment is <noedata 2 1 x jimmy > while the second is <noedata 3 1 x jimmy > and the third is <noedata 4 1 x jimmy >. When you type **xau multizg** it will ask you how many experiments you wish to perform. If your current data set is <noedata 2 1 x jimmy> and you enter 3, it will collect data for <noedata 2 1 x jimmy>, <noedata 3 1 x jimmy>, and <noedata 4 1 x jimmy> in this order. Note that the data sets must already exist and have the correct parameters set. Thus, to use this method, do the following:

- a. Do step 1 and 2 and 3 above if you have not yet done so.
- b. Type **new** and increment the experiment number (expno) by one. This creates a new data set and carries over the current parameters (those for the noe experiment). Type **o1** and enter the next value for the next peak to irradiate. You may change other parameters, if necessary.
- c. Repeat step b for all peaks to be irradiated. Now all these data sets have identical parameters except for o1 values, which defines the peak to be irradiated.
- d. Return to the first experiment number (lowest numerically) in this series of noe experiments, by typing **re #**, where # is the number. Alternatively, you may use the search command.
- e. Type **xau multizg** and enter the number of experiments to be performed.

### Processing the spectra

After **ef**, phase the LARGE, selected peak down. The NOE peaks will be up (positive) for small molecules and down (negative) for large molecules. Artifacts are sometimes present and these will be anti-phase or dispersive (up and down character) but these will occur only for spins J coupled to the selected peak.

It is very helpful to display a normal 1D spectrum along with the NOE spectrum. Click  and drag spectra into the data window. To space them uniformly, click . To give them a horizontal offset, scroll . One can print directly from this window using the Ctrl P print method but one cannot start the Plot Editor from multispectra mode and have more than one spectrum displayed.

## Quantitation

The 1D selective NOESY experiment is a transient NOE experiment which is different from the traditional steady-state NOE difference experiment. The numbers do not directly correspond with the difference experiment. I suggest the use of categorizing the NOEs into strong, medium, and weak, and only use these comparisons within the same molecule. For determining a distance, please see the Analysis section below for more detail.

## Procedure for 2D ROESY and NOESY

1. For long experiments, the temperature should be actively controlled.
2. Do NOT spin the sample.
3. Tune the probe (**wobb**).
4. Take a normal 1D spectrum and note the spectral window needed (**SW** and **o1p**). Also note the value of **rg** as found by **rga**.
5. Change the data set (type **new**) and then choose the experiment you want by typing either **rpar noesyq.top** or **noesy.top** or **roesy.top**. The former is for the zero-quantum suppressed version and is recommended.
6. Type **getprosol** to update all pulse widths.
7. Type **new** and in AcqPars adjust **sw**, and **o1p**. Since these experiments are symmetric, make **sw** in F1 and F2 equal. Adjust the number of points, TD so that the fides (Hz/pt) is about 3 in F2 and 12 in F1. Enter the value of **rg** found in the 1D spectrum above.
8. Select a mixing time. See above discussion on mixing times. For NOESY, type **d8** and enter a value. (For ROESY, type **p15**, and enter a value - start with 200 msec. Values are entered in msec by typing a m after the number.)
9. For NOESY and ROESY, make **ns** a multiple of 8. For the zero-quantum suppressed NOESY, make **ns** a multiple of 2. Click save to exit eda.
10. Type **expt** to determine the experiment time and adjust **ns** or TD in F1 as necessary.
11. Type **zg**. (Do not use the **rga** or **start** command.)
12. When finished type **xfb** to transform. You may transform a 2D spectrum prior to its completion but always transform again upon completion to get the full digital resolution.

## PROCESSING

### Phasing

NOESY and ROESY spectra are phase-sensitive and must be phased prior to analysis. For ROESY and small-molecule NOESY, the NOE cross peaks have phase opposite to the main diagonal peaks (see analysis section for a graphic of this). Thus, if the main peaks are phased down, then NOE cross peaks will be up. For large-molecule NOESY, NOE cross peaks have the same phase as the diagonal. Chemical exchange peaks always have the same phase as the diagonal. Follow this procedure:

Click  to enter phasing. The idea is to look at and phase individual traces through the 2D spectrum. **Phase the rows first.** Position the cursor on a row that contains peaks. Click the right mouse button and select add. Then position the cursor on a row with a peak that has a different shift than the first, and right click and select add. The idea is to choose rows such that the peaks cover a large shift range. You may add more rows but usually two is sufficient. To phase rows, click . There will now be a red line on the

largest peak. Adjust its phase using the zero-order button, **0**. Then adjust the phase of other peaks in the other row(s) using the first-order button, **1**. Click  to save and return.

Click  to repeat the above process on columns. The columns will often not need much phase correction. When finished, click  to exit phasing.

## Linear Prediction (optional)

Linear prediction is a powerful method of improving the resolution of 2D spectra. The price, however, is increased noise in the spectrum. Only perform linear prediction on spectra with high signal-to-noise.

Normally the FID in the F1 dimension is not fully sampled; it is cut off. To sample it more completely requires more points (greater TD in F1) and a longer experiment time. Each doubling of TD (in F1) doubles the number of FIDs and thus requires a doubling of experimental time. Linear prediction is a processing method which predicts these cut-off points. It improves resolution in the F1 dimension without any increase in experimental time. It is done after the data is collected and can be optimized by varying the extent of prediction and the NCOEF parameter. It can greatly improve the resolution of HSQC and NOESY spectra (or any phase sensitive spectrum). It is by default turned off and must be turned on.

Crucial parameters are based on the value of TD (in F1), which is found in AcqPars. The following parameters must be set in ProcPars:

ME\_mod : set to LPfr

NCOEF: set to 3 times the number of cross peaks in your spectrum. For NOESY spectra, count symmetric peaks as two. Values that are incorrect by more than a factor of two can degrade the resolution.

LPBIN: set to 2\*TD(in F1)

SI (in F1): set to 2\*TD(in F1) or 4\*TD(in F1) SI is the number of points actually transformed and determines the amount of zero-filling. Caution: Large values of will creat huge data sets. SI should never be smaller than TD (in F1).

To execute linear prediction, you must re-transform the spectrum with **xfb**. Larger values of LPBIN and SI MAY give even higher resolution. It is possible to use 4\*TD for LPBIN and 4\*TD for SI. If you make LPBIN larger, also increase the value of SI. Trial and error is necessary to get the optimum spectrum.

## Baseline Correction

Type **abs2** and then **abs1** for the f2 and f1 dimension, respectively.

Baseline correction is necessary for NOESY and ROESY spectra, It removes the horizontal lines of what looks like noise, and it improves the noise reduction done in the next step. Both baseline correction and noise reduction need to be done after each **xfb** command; they operate only on processed data and each Fourier transformation "erases" their effects. Baseline correction should always precede noise reduction.

## Noise reduction

Noise reduction is done last, after linear prediction and baseline correction. Often in 2D spectra, ridges of noise are present along the F1 axis (vertically) and are especially noticeable for large peaks. This is called t1 noise and its origin is in unavoidable instabilities in the spectrometer. The procedure outlined below reduces this noise. The idea is to select a region of the spectrum that contains the ridges of noise but no cross peaks. A projection of this selected region is then calculated and then subtracted from the entire spectrum.

Display the region to be used for calculating the projection. This region should be free from cross peaks all along the F2 axis but should contain the ridges of noise. Under Processing, select Calculate Projections (proj). Make sure Projection (sum) is set to "rows". Check Update rows/columns from display and click OK. (This sets the row numbers to correspond with the current display) Then, check "Calculate Positive projection". Enter a process number such as 999 and click OK.

Under Processing, select Add/Subtract/Mult. [adsu], and check “Subtract a 1D spectrum from each row, retain sign”. Set the process number (and filename) to the projection you just created above, and click OK. This first step removes positive noise. To remove negative noise, do the following: Type **rep** (process number used above) to go to the projection spectrum. Type **nm** to invert it. Return to the 2D spectrum by typing **rep** (process number of the spectrum, usually 1). Type **sub2** to repeat the subtraction.

This process places the calculated projection into the spectrum’s displayed projection. (you likely will want to replace it with a high-resolution spectrum.)

This process can be repeated using different regions to remove additional noise.

## Projections

One simply right clicks over the projection area and selects “External Projection” and enters the data set information of the 1D spectrum. To alter the intensity of the projection, click the upper right box in the projection area. Then the intensity buttons operate on the projection.

## Plotting

To print using the plot editor, select File, Print. Select “Print with layout – start Plot Editor (plot)”. You can print on either 11x17 or 8.5x11 with this method. The paper size, printer location, and whether the data is 1D or 2D depends on the LAYOUT chosen. See the 1D handout for possible layouts.

## Volume Integration

Peak peaking must be done first and is easiest using manual mode. Under Analysis, select peak picking. At the bottom of the menu, select start manual picker. Place the cross-hairs directly over a peak and right-click the mouse to add or annotate a peak. One may delete an already selected peak. When finished, select save, . To integrate the picked peaks, under Analysis, select integration. Results are found under Integrals tab.

# ANALYSIS – peak identification

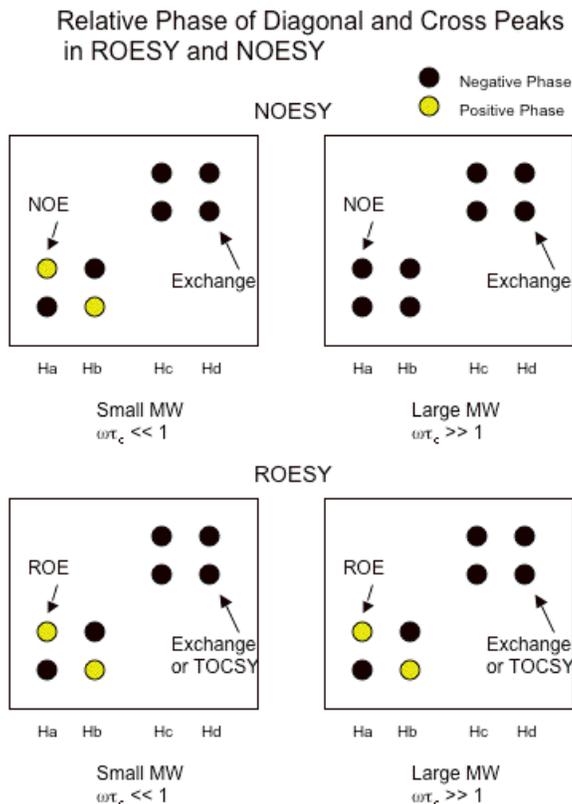
## Relative Phase of Cross Peaks

The phase of ROE, NOE and chemical exchange cross peaks can be different and are summarized in the figure below. In this figure, it is assumed that protons A and B have an NOE or ROE while protons C and D are undergoing chemical exchange. Note that the phase behavior differs for large and small molecules. For small molecules, the diagonal peaks and NOE cross peaks have opposite phase. If the diagonal is negative, then NOE cross peaks will be positive. For large molecules, the diagonal and the NOE cross peaks have the same phase. The phase of the cross peaks, then, indicates whether the molecule is in the large or small molecule region, which has important implications for quantitation, as discussed below. Note that this phase behavior is due to the positive/negative nature of NOE described at the beginning of this handout.

Cross peaks due to chemical exchange, if it is occurring, have the same phase as the diagonal for both small and large molecules in both ROESY and NOESY.

In ROESY, the diagonal peaks and ROE cross peaks have opposite phase for all molecules since the ROE is always positive. TOCSY cross peaks are the major artifact in ROESY spectra. TOCSY peaks have the same phase as the diagonal, and are thus similar to exchange peaks. TOCSY occurs between spins that are J coupled and that are relatively close in chemical shift. It also occurs for peaks that are symmetric about  $\omega_{1p}$ . A possible complication is the relay of ROE through TOCSY resulting in false ROESY cross peaks. For example, geminal methylene peaks often show TOCSY cross peaks. Assume there is a third proton

that should have an ROE to only one of the geminal protons but not its partner. TOCSY can transfer the ROE to its partner and it may appear as if the third proton has an ROE to both geminal protons.

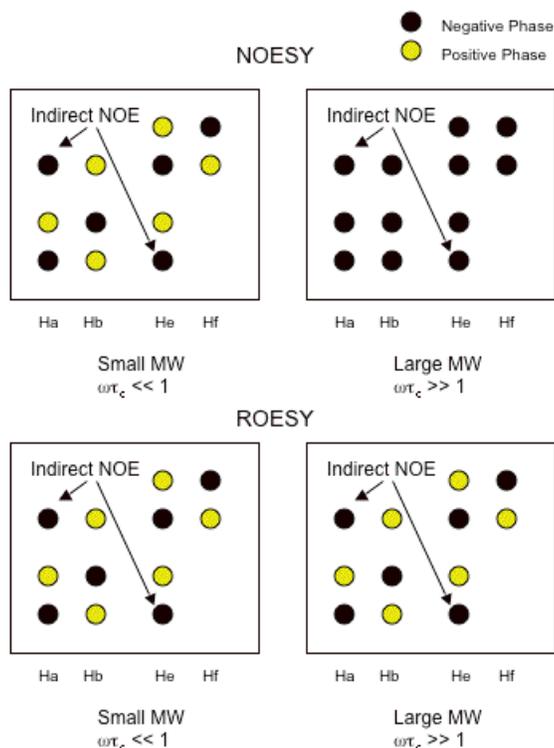


## Spin Diffusion

When analyzing NOESY spectra, one must understand the consequences of spin diffusion. Spin diffusion occurs primarily for large molecules and for long mixing times outside the “linear approximation”. In NOESY spectra, spin diffusion can lead to misleading cross peaks and incorrect distances. In this section, I describe the presence of extra, misleading cross peaks and in the quantitation section, I discuss incorrect distances. Assume there are four protons A, B, and E and F and that A and B, B and E, and E and F are close. That is, you expect NOEs between those three pairs, as shown in the figure below. These expected cross peaks between protons that are close are termed direct contributions. When spin diffusion is present, indirect contributions will also be present and a cross peak between A and E (and B and F) will likely be present. In spin diffusion, the magnetization follows a path from A to B and then from B to E but appears to be directly from A to E. In NOESY spectra of large molecules, the phase of these indirect peaks is the same as for direct contributions and the resulting cross peaks are impossible to distinguish at a single mixing time. The appearance of the A to C cross peak could lead you to erroneously conclude that protons A and C are close.

ROESY spectra suffer much less from spin diffusion; the phase of indirect contributions may be different from direct contributions and allows their easy identification. The phase of indirect contributions alternates with number of steps of transfer. That is, the phase of 2-step indirect contributions is opposite to direct contributions, while that of 3-step indirect contributions is the same as direct contributions. 3-step contributions are rare, however. (see Bax, J. Magn. Res. 70, 327-331 (1986))

## Effects of Spin Diffusion in ROESY and NOESY

Analysis – Quantitative Distance Determination

For organic molecules, it is generally sufficient to classify NOE peak intensities as strong, medium, and weak and make qualitative deductions about relative distances. If an actual distance is needed, one may use the well-known approach in which the NOE is inversely proportional to the distance to the 6<sup>th</sup> power, i.e.,

$$r_{ij} = r_{ref} (a_{ref}/a_{ij})^{1/6}$$

where  $a_{ij}$  is the NOE cross-peak volume and  $r_{ij}$  is the interproton distance of the two protons  $i$  and  $j$ . Given a known distance between two protons ( $r_{ref}$ ) and its NOE volume ( $a_{ref}$ ), a distance can be calculated from another NOE volume.

For this relation to be valid, a strict experimental protocol must be followed. First, the mixing time must be relatively short so that the linear approximation is valid and spin diffusion is avoided. For small molecules, the mixing time must be less than several hundred milliseconds. For large molecules, there may be no practical value for a mixing time that completely avoids spin diffusion but, in general, the mixing time must be less than 100 msec. Whether spin diffusion leads to an apparent increase or decrease in distance depends on the details of the molecular geometry. Linear geometries lead to shorter apparent distances while non-linear geometries may lead to longer distances (See Neuhaus and Williamson p117-122). To help ensure that the mixing time is within the linear region, a build-up curve is performed. A build-up curve is a series of NOE spectra taken at different mixing times. If one is within the linear region, then the NOE will linearly increase with mixing time. A second requirement for quantitative work is that the relaxation delay must be long enough to allow reasonable recovery of the magnetization between scans. The normal time of 2 sec for D1 is not sufficient and one must increase this. The proper D1 should be at least 3 times  $T_1$ . A  $T_1$  determination may be necessary.

## Effect of Conformational Mobility

The purpose of running a NOESY is often to determine conformation by establishing a distance between two protons. When more than one conformation is present, however, the NOE may give misleading distances. If the conformations are being averaged over the time scale of the experiment (the mixing time) the NOE will not reflect the average distance between the protons but rather the average of the inverse sixth power of the distance. Since

$$\left\langle \frac{1}{r^6} \right\rangle > \frac{1}{\langle r \rangle^6}$$

the effective distance for NOE is less than the average distance,  $r_{\text{eff}} < r_{\text{ave}}$ . and the effective distance is weighted towards that of the “closest approach”. For example, assume there are two conformations A and B and that the A conformation is 10% populated. If the protons are 0.2 nm apart in conformation A and 0.6 nm apart in conformation B, then the effective distance is 0.293 nm which is much closer than the 0.6 nm separation that is present in the dominant conformer. This is summarized in the table below. Thus, in such cases the NOE will reflect the conformation where the protons are closer.

Conformation	A (10%)	B (90%)
r	0.2 nm	0.6 nm
$r_{\text{ave}}$	0.56 nm	
$\left\{ \left\langle \frac{1}{r^6} \right\rangle_{\text{ave}} \right\}^{-1/6}$	0.293 nm	

## ROESY – Quantitative Distance Determination

In addition to the above considerations for NOESY, the ROESY has additional complications. The cross peak intensities have an offset dependence relative to the transmitter center, o1p. Cross-peaks are less intense the further they are from the center, regardless of spatial distance. For example, assuming o1p is 5 ppm, then a cross-peak between protons at 1 and 2 ppm will have lower intensity than between protons at 4 and 5 ppm, even if they have the same interproton distance. This dependence is well characterized and can be corrected in the following way (see Ammalahiti, et al., J. Magn. Res. A, 122, 230-232 (1996)). Distances are calculated from corrected intensities:

$$r_{ij} = r_{\text{ref}} (a_{\text{ref}} c_{\text{ref}} / a_{ij} c_{ij})^{1/6}$$

where

$$c_{ij} = 1 / (\sin^2 \theta_i \sin^2 \theta_j)$$

and

$$\tan \theta_i = \gamma B_1 / (\omega_i - \omega_0)$$

where  $(\omega_i - \omega_0)$  is the difference between the chemical shift of the peak (in Hz) and o1p (in Hz) and  $\gamma B_1$  is the spin lock power (which is about 2500 Hz in our case). Volume corrections of up to a factor of 4, in far off-resonance cases, may be required.

An additional complication with quantitation of ROESY spectra is that TOCSY transfer may occur and cancel or partially cancel ROESY cross peaks. This obviously has deleterious effects on distance determination. This is a particular problem for the reference ROE for which a J-coupled methylene pair is often chosen. See John Decatur for advice if detailed distance information is needed.