NOE Experiments on the Bruker

Choice of experiment

There are several different possible NOE experiments (NOE, ROE, steady-state, transient, 1D, 2D, etc). The ones available are 1D selective-NOESY, 2D-NOESY, and 2D-ROESY. The important factors for choosing a particular experiment are the molecular weight, the amount of material available, and the amount of information required (number of NOEs needed.) The molecular weight most affects the choice of experiment.

Molecular weight

The NOE depends on the rate of molecular tumbling (correlation time), which is in large part determined by the molecular weight (solvent viscosity is also an important factor). The NOE is positive for small molecules, goes through zero for MW range 700 - 1500, and becomes negative for large molecules (MW>1500). For medium sized molecules, where the NOE may be theoretically zero, the ROESY experiment (rotating frame NOE) is often preferred since the ROE is always non-zero and positive. The ROESY experiment has additional shortcomings, however, and should be used only when the NOESY fails.

Amount of material available, information needed

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. The minimum amount of time required for 2D and 1D differ. The minimum time for a 2D NOESY spectrum is relatively long (30 minutes to 1.5 hours) while the minimum time for a single 1D selective NOESY spectrum is about 2 minutes. Thus, if you have a concentrated sample (0.037M), and are interested in less than five or so NOEs, then the 1D version should be chosen. If you have very little material (< 0.005M) or if you are interested in many NOE's, then the 2D version should be chosen. The 1D version has the advantage of easy and fast processing.

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. Find below a prescription for NOE experiments:

Small molecules (MW < 600)

For small molecules, the usual choice is 1D selective NOESY. Exceptions would be if you have very little material (< 0.005M) or peaks to be irradiated are very close to other peaks. In these cases, the 2D NOESY would be chosen.

Medium sized molecules (600 < MW < 1500)

The choice here is more difficult. In general, first try a NOESY. If the desired NOEs are not observed, try a ROESY. If you are only interested in a few well-resolved peaks, the 1D NOESY should be chosen.

Large Molecules (MW > 1500)

The usual choice is NOESY.

Mixing times

The mixing time is the most important parameter for NOE experiments. The choice of value depends on how fast the NOE develops and whether one wants to calculate actual distances (see analysis section). The rate at which the NOE develops depends on molecular correlation time and the distance between spins for the particular NOE. The following are guidelines:

1) small molecules :0.5 -1 or more seconds. Start with 0.5 sec.

2) medium size molecules 0.1 -0.8 sec. Start with 0.25 sec.

3) large molecules 0.05 - 0.2 sec. Start with 0.1 sec.

Sample Considerations:

Preparation: Removing Dissolved Oxygen

Dissolved oxygen 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 CO2/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 N2.

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

Sample size and other tubes

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 500 L without causing serious lineshape problems (shimming problems) and the attendant loss of signal-to-noise. There are special tubes (made by Shigemi) and inserts (made by Wilmad), however, that can be used to restrict the active volume and, hence, reduce the amount of solvent without causing lineshape problems. These work reasonably well, with the Shigemi tubes being superior but more costly.

1D Selective NOESY

1. Take a normal 1D proton spectrum. It is extremely important to tune (**wobb**) the probe for this experiment.

2. You need to obtain the exact frequency positions of the peaks that you want to irradiate. Click on Utilities and click on **o2**, and position the cursor on top of your chosen peak and click the middle mouse button. Type **o2** and record this number. Repeat this process for all peaks that you will wish to irradiate.

3. Change the data set (edc). Type "`rpar selnoegs`". Type **o1** (not **o2**) and now enter one of the values recorded above in step 1. Adjust the value of the mixing time, **d3**. Appropriate values range from 0.2 sec to 1 sec with 0.5 being a good start. The number of scans (**ns**) must be a multiple of 16.

4. 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 your spectrum. Change it if necessary.

5. 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 may want to consider the NOESY experiment).

6. Type **rga**.

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

8. 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 steps 1-6 above if you have not yet done so.

b. Type edc 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 define the peaks 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 FT, 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. To do this click on **dual**. You will get a message that says the second spectrum is not defined. Click on OK and this brings up the edc2 editor. For the second spectrum, enter the exact data set name containing the normal 1D spectrum. To plot two spectra like this, you must use Xwinplot (see Intro handout).

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. It is suggested the use of categorizing the NOEs into strong, medium, and weak, and only use these comparisons within the same molecule.

Unlike the difference experiment, NOEs are symmetric for the selective NOESY. For a detailed description of the different NOE experiments and the proper interpretation of the NOE, see David Neuhaus and Michael P. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis,* VCH 1989.

ROESY, NOESY

1. Take a 1D spectrum and note the spectral window needed (**sw** and **o1p**). Also note the value of **rg** as found by "`rga`".

2. Change the data set (**edc**) and type "`rpar roesy`" or "`rpar noesy`"**.**

3. Type "`eda`" and 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 fidres (Hz/pt) is about 3 in F2 and 12 in F1. Enter the value of rg found in the 1D spectrum above.

4. 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.

5. Make **ns** a multiple of 8. Type **expt** to determine the experiment time.

6. Type **zg**. (Do not use the **rga** or **start** command.)

Processing

Phasing

NOESY and ROESY spectra are phase-sensitive. They must be phased prior to analysis. For ROESY and small-molecule NOESY, the NOE cross peaks have phase opposite to the main diagonal peaks. 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. Follow this procedure:

1. Type "`rser 1`" to read in a 1D slice. Type "`sinm`" and then "`FT`".

2. Phase this spectrum so that it is absorptive (peaks either completely up or down). It is usually best to phase the diagonal peaks (which will be of greatest intensity) to be negative. Click **return** and then click **save as 2D**. Click on **2D** to return to 2D mode. Type "`xfb`" and wait for it to completely finish.

3. You need to continue phasing. The idea now is to look at and phase individual traces through the 2D spectrum. The above step phased the rows. The columns need to be phased and the rows probably need some fine adjustment. Rows and columns **must** be phased *separately*.

4. You may need to click on the +- button to toggle through the various display modes in order to see both positive and negative peaks.

5. Click on **phase.** Click on **col** and position the cursor on a column that contains peaks. Click the middle mouse button to select the column and then the left button to release the cursor. Click on **mov 1** to move the trace to window 1. Select another column and move it to window 2 by clicking on **mov 2**. Repeat again into window 3, if desired.

6. To phase, click on either **big 1, big 2 or big 3**. This selects the biggest peak from the chosen window as the phasing reference. Use **ph0** and **ph1** to phase the columns. Once satisfied, click on **return** and then **save & return.** To the question "start xf1p", click **OK**, and wait for it to re transform the spectrum.

7. Repeat steps 5 and 6 for the rows.

Linear prediction (optional but recommended) and window functions

Linear prediction is a powerful method of improving the resolution of 2D spectra. 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 that 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 HMQC 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) that was used for the experiment. Type "`eda`" and get this value. The following parameters must be set and are found within the **edp** editor. Type "`edp`" and set the following:

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 create 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.

Linear prediction is normally done only in the F1 dimension. In the F2 dimension, it is better to adjust the digital resolution (**Fidres**) to the appropriate value before acquiring the spectrum.

Window functions affect the signal-to-noise and the resolution and there is a trade-off between the two. The parameter **WDW**, found in the **edp** editor, determines the functional form of the window function; this is normally set to **qsine**. The parameter **SSB** affects the window function greatly. The normal value is 2 but values up to 6 may be useful. You must re-transform with **xfb** to use different window functions.

Noise reduction

Noise reduction should follow linear prediction, if performed. 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.

Click on *utilities*. Towards the bottom, under *f2-axis***,** click on the **calc** button that is adjacent to the **part** button. This displays a horizontal line. Clicking the middle mouse defines one edge of the region. Clicking again defines the other edge. This region should be free from cross peaks all along the F2 axis but should contain the ridges of noise. After defining the region, you are asked for a process number for the calculated projection. Towards the bottom, where it says Type New Name, enter a process number (usually one greater than the current). Now type "`edc2`" and for the 2nd data set, enter this process number and click on **save.** Type "sub2" to perform the noise reduction.

This first step removes positive noise. To remove negative noise, do the following: type "`rep procno`" **(**process number used above) to go to the projection spectrum. Type "`nm`" and click on **OK** to the error message. Return to the 2D spectrum by typing "`rep procno`" (process number of the spectrum, usually 1). Type "`sub2`" again.

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

Projections

To use the 1D proton spectrum, taken above, as the projection for the 2D plot, type "edg", click on **edit parameters of f2 projection**. A sub-editor is invoked. Click on **type of projection,** and select **external**. You must then specify the exact filename (path) of the spectrum you want to be plotted as your projection. Click on **save.** Repeat for f1 projection, if desired.

Sometimes, the **y-scaling** of the projection is not as desired. To change the **y-scaling** of the projection, within **edg** and within **edit parameters of f2 projection** (or f1), there is an entry for spectrum y scaling, **PF1CY**. Change this to some large number, e.g., 20 cm.

Analysis — peak identification

NOESY

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 (negative NOE), 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 quantification, as discussed below.

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

ROESY

For all molecules, the diagonal peaks and ROE cross peaks have opposite phase. Chemical exchange cross peaks have the same phase as the diagonal.

TOCSY cross peaks are the major artifact in ROESY spectra. TOCSY peaks have the same phase as the diagonal. 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 **o1p**. A possible complication is the relay of ROE through TOCSY resulting in false ROESY cross peaks. See John Decatur for details.

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, one hopes that cross peak volumes reflect only the distance between the spins giving rise to the cross peak. If spin diffusion is occurring, this is not true. For example, assume there are three protons A, B, and C and B is spatially between A and C. An NOE between A and C will likely have contributions from spin diffusion — the magnetization follows a path from A to B and then from B to C but appears to be directly from A to C. The phase of spin diffusion

peaks for large molecules is the same as for direct contributions and the resulting peaks are impossible to distinguish at a single mixing time.

ROESY spectra may also suffer from spin diffusion but the phase of indirect contributions may be different from direct contributions that 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. (see Bax, *J. Magn. Res*. **70**, 327-331 (1986))

Analysis — distance determination

NOESY

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 6th power, i.e.

$$r_{ij} = r_{ref}\left(\frac{a_{ref}}{a_{ij}}\right)^{1/6}$$

where a_{ij} is the NOE cross-peak volume and r_{ij} is the inter-proton 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 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. For large molecules, the mixing time must be less than 100 msec and for small molecules, it must be less than several hundred milliseconds. In biological work a build-up curve is performed to ensure that one is in the linear region. A build-up curve is a series of NOE spectra taken at different mixing times. If one is within the linear region, 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.

ROESY

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 that between protons at 4 and 5 ppm, even if they have the same inter-proton distance. This dependence is well characterized and can be corrected in the following way (see Ammalahti, *et. al*. *J. Magn. Res. A*, **122**, 230-232 (1996)). Distances are calculated from corrected intensities:

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

where

$$c_{ij} = \frac{1}{\sin^2\theta_i \sin^2\theta_j}$$

and

$$\tan\theta_\alpha = \frac{\gamma B_1}{\omega_\alpha - \omega_0},\ \alpha = i \text{ or } j$$

where (ω_α -ω_0) is the difference between the chemical shift of the peak (in Hz) and **o1p** (in Hz) and γB_1 is the spin lock power (which is about 2500 Hz in this case). Volume corrections of up to a factor of 4, in far off-resonance cases, may be required.

An additional complication with quantification 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.

Volume integration

From the 2D mode, select **integrate**. You are asked for the name of a region file. Enter a name related to the dataset name so that you may reference it in the future. Use the cursor to define the upper left and lower right part of each volume element: click the left mouse button to get cross hairs. Place them on the upper left corner of the volume element and click the middle mouse button to fix it. Then move the cross hairs to the lower right corner and click the middle button again. Now, click the left button and a menu appears which controls how the integration is performed. For red/yellow cross peaks, pick +, for blue/violet cross peaks pick -. Repeat the above procedure for each cross peak to be integrated. If peaks are not sufficiently resolved for proper integration, try re-processing with (increased) linear prediction.

Type "`edo`" and set the name of the current output device to **$screen** and save. This sends the result of the integral calculation to the screen. Type "`int2d`" and you are prompted for a region (ROI) file. The name you gave above should be in the list. Select it. Type "`li`" to generate the integral output. See Page 50-54 of the Bruker processing manual for details.