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A Two-Dimensional Nuclear Overhauser Experiment with Pure Absorption Phase in Four Quadrants*

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A method is described for obtaining pure absorption phase spectra in four quadrants in a two-dimensional nuclear magnetic resonance spin exchange experiment. It is shown that phase correction results in a substantial increase in resolution and discrimination while maintaining a signal-to-noise ratio comparable to that of the usual magnitude spectrum. Experimental results are presented for the application of the method to a biological macromolecule, the bovine pancreatic trypsin inhibitor.

The use of two-dimensional NMR methods to observe nuclear Overhauser and chemical exchange phenomena have generated considerable excitement (1-3). These techniques are able to resolve many individual spin exchange effects even in complex systems such as biological macromolecules, and they permit rapid and systematic data collection. The quantitative interpretation of this data has been limited by the application of absolute value and power transforms to obtain suitable peak shapes (4). These nonlinear transforms generate positive definite spectra from data that would otherwise have both positive and negative peaks (5) but also result in differential scaling, peak broadening, and cross terms with overlapping peaks and baseline offsets.

In this communication we describe a technique for phasing two-dimensional spectra based on the use of separate quadrature in the two dimensions. We will show that this method can be applied with only minor modifications in the experimental design of the usual two-dimensional spin exchange experiment, and we will present experimental results obtained with the method. Finally we will discuss the impact of these methods on signal to noise ratio and resolution.

BASIS FOR TWO-DIMENSIONAL PHASING IN FOUR QUADRANTS

A two-dimensional exchange experiment can be described by considering an oscillator with frequency ω_1 during the labeling period and a frequency ω_2 after some mixing period. The observed amplitude of this oscillator will then be a function of both the time t_1 used to label the spins and the time t_2 at which it is observed during the acquisition period. The two-dimensional spectrum is usually obtained by taking Fourier transforms in both dimensions

$$F(\omega_1, \omega_2) = \int\int e^{i\omega_1 t_1} e^{i\omega_2 t_2} f(t_1, t_2) dt_1 dt_2 ,$$

where $f(t_1, t_2)$ is the oscillation function in the time domain and $F(\omega_1, \omega_2,)$ is its Fourier transform. Assuming an

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exponentially decaying oscillation we obtain the expression

$$F(\omega_1, \omega_2) = \frac{1/T_2 + i(\omega_1 - \omega_{01})}{1/T_2^2 + (\omega_1 - \omega_{01})^2} \frac{1/T_2 + i(\omega_2 - \omega_{02})}{1/T_2^2 + (\omega_2 - \omega_{02})^2}.$$

It is easily seen that both the real and imaginary parts of this expression are biphasic. Note that for each oscillator $f(t_1, t_2)$ can be separated into the product $f_1(t_1)^* f_2(t_2)$. This permits the integrals to be separated. By accumulating the real and imaginary parts of each dimension independently it is possible to calculate the product of the real part in one dimension and the real part in the other dimension to obtain

$$G(\omega_1, \omega_2) = \frac{1/T_2}{1/T_2^2 + (\omega_1 - \omega_{10})^2} \frac{1/T_2}{1/T_2^2 + (\omega_2 - \omega_{20})^2}.$$

This latter function is linear in the peak amplitude and is positive definite. It decays rapidly away from ω_{01},, ω_{02}., as an absorption phase Lorentzian in both dimensions.

Pure absorption phase spectra from a single-quadrant two-dimensional spin exchange experiment have been reported (6). In a four-quadrant experiment it is not possible to achieve a pure absorption phase two-dimensional spectrum using analytical transformations of the data (5, 7). The transformation outlined above generates a pure absorption phase spectrum in four quadrants using a nonanalytical transformation. It is based on extracting the real part of a complex function, a transformation that is linear to addition and real multiplication, but is not analytical. Having used this nonanalytical transformation, the projection cross-section theorem (8) does not apply, and there is no theoretical objection to a positive definite four-quadrant spectrum.

Pure phase spectroscopy can be performed using the usual two-dimensional NMR pulse sequence (Fig. 1). The real part of the t_1 dimension is obtained by taking the difference of the free-induction decays accumulated with the

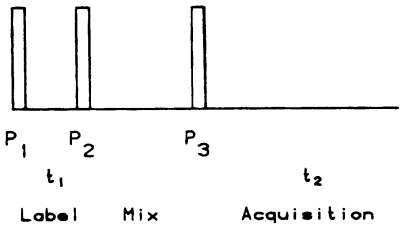

FIG. 1, The pulse sequence used in two-dimensional spin exchange spectroscopy. P_1 and P_2 are two Π/2 pulses used to label spins in the t_1 dimension. The spins are allowed to mix and a final detection pulse P_3, is applied to excite a free-induction decay during t_2.

phase of P_2 at 0 and Π with respect to P_1. The imaginary part of the t_1 dimension is the difference of the free-induction decays accumulated with P_2 at +Π/2 and -Π/2 with respect to P_1. These free-induction decays (a pair for each t_1) are stored and Fourier-transformed separately. They are all phase-corrected using the values appropriate for $t_1 = 0$. After transforming in the t_2 dimension, the complex time domain data in the t_1 dimension is assembled from the extracted real parts of the two spectra for each value of t_1. This complex matrix is transposed and Fourier-transformed to obtain the final phased two-dimensional spectrum. Phase correction can be applied in the second dimension, although in practice only a small linear phase correction is needed to compensate for the finite lengths of P_1 and P_2.

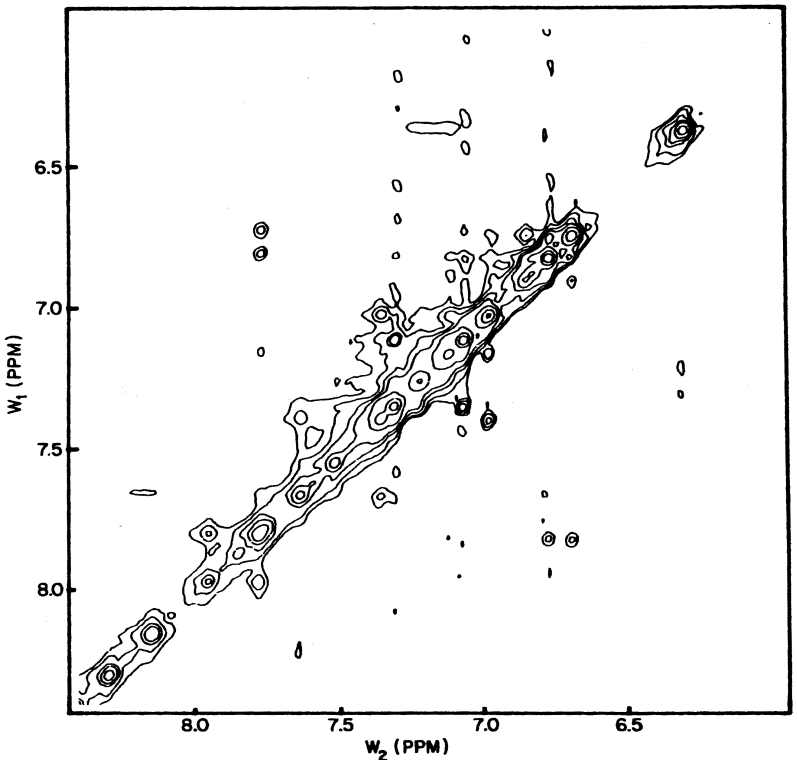

FIG. 2. The pure absorption phase spectrum of the aromatic region of BPT1 as described in the text. The displayed section is 200 points square, extracted from the full 1024 point square spectrum. The spectrum was normalized so that the maximum peak (a dioxane marker) has an amplitude of 10,000. Contours are drawn at 15, 30, 60, 100, 250, 500, and 1000.

Figure 2 illustrates the application of this experiment to a macromolecular system, the bovine pancreatic trypsin inhibitor. Data were collected at 25°C pD at 3.8 using the 500-MHz spectrometer at the Francis Bitter National Magnet Laboratory. A mixing period of 100 msec was used without the application of any homospoil pulse. Cyclic rotation of pulse phases and acquisition modes through the 64 possible phase combinations among the three pulses was used to cancel coherence effects between the labeling and acquisition pulses and to eliminate amplifier imbalance artifacts. The basic sequence of pulse phases is shown in Table 1. This subcycle of 16 phase settings was rotated through a four-phase CYCLOPS sequence (9, 10) to give an overall cycle of 64 acquisitions; 1024 point free-induction decays were collected at 512 t_1 values with a spectral width of 6250 Hz. Gaussian line broadenings of 10 Hz were applied in both dimensions, and the data were zero-filled to 1024 points in the t_1 dimension prior to Fourier transformation.

This technique offers several practical advantages when compared to magnitude or power spectrum methods. Primary among these is the improved discrimination of the method. Figure 3 shows an absolute value spectrum calculated from the data presented in Fig. 2 with the same line broadenings and the same contour levels. It is much easier to recognize peaks in the phased spectrum and to distinguish them from the noise present in the ω_1 dimension and from overlapping tails of larger peaks. In the pure phase spectrum the noise is seen to be randomly phased, but in the magnitude spectrum it is positive definite like the signal of interest. Line-widths in the phased spectrum are narrower than those in the magnitude spectrum minimizing the need to apply resolution enhancements with their associated artifacts, and resulting in an effective increase in signal-to-noise ratio. For Lorentzian lines the peak widths at half-height are $1/T_1$ an improvement of the square root of three over the spectrum width at half-height. Even more dramatic resolution improvements occur when one considers widths at lower heights because the limiting behavior of Lorentzian peaks is a decay with $(\omega - \omega_0)^{-2}$ while magnitude peaks decay as $(\omega - \omega_0)^{-1}$. The long tails present in magnitude spectra are particularly bothersome in samples with large

TABLE 1

PULSE PHASES USED TO ACCUMULATE THE REAL AND IMAGINARY FREE-INDUCTION DECAYS FOR EACH t_1 VALUE

Pulse phases			
P_1	P_2	P_3	Accumulation
X	X	X	+ real
Y	Y	X	+ real
$\bar{X}$	$\bar{X}$	X	+ real
$\bar{Y}$	$\bar{Y}$	X	+ real
$\bar{X}$	X	X	− real
$\bar{Y}$	Y	X	− real
X	$\bar{X}$	X	− real
Y	$\bar{Y}$	X	− real
X	Y	X	+ imaginary
Y	$\bar{X}$	X	+ imaginary
$\bar{X}$	$\bar{Y}$	X	+ imaginary
$\bar{Y}$	X	X	+ imaginary
$\bar{X}$	Y	X	− imaginary
$\bar{Y}$	$\bar{X}$	X	− imaginary
X	$\bar{Y}$	X	− imaginary
Y	X	X	− imaginary

Note. Four-phase CYCLOPS rotation is applied to this set of pulse sequences resulting in a cycle of 64 accumulations.

dynamic ranges where they obscure significant regions of the spectrum. Finally, in crowded regions of the spectrum prominent overlap effects are seen in the magnitude spectrum which are absent in the phased spectrum. The amplitude in the pure phase spectrum is a linear function of the resonance amplitude without cross effects between

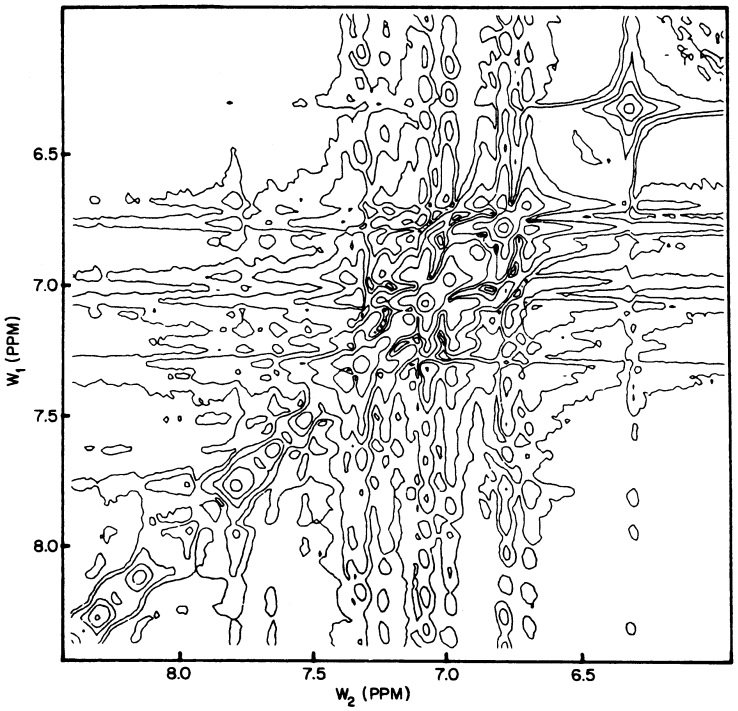

FIG. 3. An absolute value spectrum calculated from the same data shown in Fig. 2. The same normalization was applied, and the same contour levels are drawn.

overlapping peaks. For these reasons, pure phase spectra should greatly simplify quantitative data analysis.

The sensitivity of two-dimensional spectroscopy has been examined in detail previously (11). The effect of phasing on the sensitivity depends on how sensitivity is defined. A pure phase two-dimensional NMR experiment results in four linearly independent spectra; four-quadrant and one-quadrant experiments are equivalent (assuming that quadrature phase data acquisition is used in both). Each component will contain noise with a standard deviation of σ. All four components of the noise would appear in the power or magnitude spectrum

$$A(\omega_1\omega_2) = [(R(\omega_1)R(\omega_2) - I(\omega_1)I(\omega_2))^2 + (R(\omega_1)I(\omega_1) + I(\omega_1)R(\omega_2))^2]^{1/2}.$$

As Aue *et al.* note, there is a systematic shift of the data in a magnitude spectrum resulting from the average noise power

$$\langle N \rangle = \int dN_1 dN_2 (N_1^2 + N_2^2)^{1/2} f_1(N_1) f_2(N_2),$$

where f_1 and f_2 are the noise distribution functions for the two parts of the absolute value spectrum. Because each is composed of the sum of two of the four independent parts of the phased two-dimensional spectrum each will have a standard deviation of $2^{1/2}\sigma$. Assuming a Gaussian distribution for the noise the integral can be evaluated:

$$f(N_i) = \frac{1}{2\sigma(\pi)^{-1/2}} \exp(-N_i^2/4\sigma^2),$$

$$\langle N \rangle = \pi^{1/2}\sigma,$$

$$\langle N^2 \rangle = \langle N_1^2 \rangle + \langle N_2^2 \rangle$$

$$= 4\sigma.$$

The variance of the absolute value spectrum is therefore

$$\sigma_{abs}^2 = \langle N^2 \rangle - \langle N \rangle^2$$

$$= (4 - \pi)\sigma^2.$$

In the two-dimensional experiment the magnitude spectrum has a lower noise variance by a factor of 0.86. In a one-dimensional experiment there are only two components so the noise variance in a magnitude spectrum is lower than the phased spectrum by a factor of 2 - Π/2 or 0.42. Defining the noise level as the square root of the variance, the phased spectrum has a lower signal-to-noise ratio by a factor of 0.93 in two-dimensional spectroscopy (or 0.65 in one-dimensional spectra). It should be noted that the noise distribution in the magnitude spectrum has a significant third moment (skew) while the noise in a pure phase spectrum is symmetric. For practical purposes the magnitude and pure phase spectra appear to be essentially equivalent in signal-to-noise ratio. The rms error in the spectrum resulting from the addition of white noise of amplitude σ to each component of the data during acquisition is improved by a factor of 2 for phased spectra compared to magnitude spectra because there is no systematic offset of the data.

In summary, a method is presented for obtaining absorption phase two-dimensional NMR data. Experimental results are presented and several advantages of the method are discussed. These experiments should provide unique insight into the solution structure and dynamics of biological macromolecules.

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REFERENCES

1. J. JEENER, B. H. MEIER, P. BACHMANN, AND R. R. ERNST, *J. Chem. Phys. 71,* 4546 (1979).

2. R. FREEMAN AND G. A. MORRIS, Bull. *Magn. Reson. 1,* 5 (1979).

3. K. WUTHRICH, K. NAGAYAMA, AND R. R. ERNST, *Trends Biochem. Sci. 4,* N178 (1979).

4. A. KUMAR G. WAGNER, R. R. ERNST, AND K. WUTHRICH, *J. Am. Chem. Soc.* 103, 3651 (198 1).

5. W. P. AUE, E. BARTHOLDI, AND R. R. ERNST, *J. Chem. Phys. 64,* 2229 (1976).

6. S. MACURA AND R. R. ERNST, *Mol. Phys. 41,* 95 (1980).

7. K. NAGAYAMA, P. BACHMANN, K. WUTHRICH, AND R. R. ERNST, *J. Magn. Reson.* 31,75 (1978).

8. R. N. BRACEWELL, *Aust. J. Phys. 9,* 198 (1956).

9. D. I. HOULT AND R. E. RICHARDS, Proc. *Roy. Soc. London Ser.* A 3", 311 (1975).

10. E. 0. STEJSKAL AND J. SCHAEFER, *J. Magn. Reson. 14,* 160 (1974).

11. W. P. AUE, P. BACHMANN, A. WOKAUN, AND R. R. ERNST, J. *Magn. Reson. 29,* 523 (1978).