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Homonuclear Magnetization Transfer Experiments Using Isotropic and Nonisotropic Mixing Schemes

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(Received 30 June 1988)

Abstract. The principles of homonuclear magnetization transfer via isotropic and non-isotropic mixing schemes are discussed. Practical aspects of such experiments concern the minimization of the generation of multiple quantum coherence, minimization of rf power requirements, suppression of NOEs during mixing and minimization of relaxation losses. The analogy between these experiments and heteronuclear decoupling is briefly discussed. The efficiencies of several mixing schemes are compared and it is demonstrated experimentally that the resolution and sensitivity of the homonuclear cross polarization methods can be superior to the conventional COSY experiment.

Jeener's original two-pulse COSY experiment[1,2] formed the basis for the development of a very large number of two-dimensional (2D) NMR techniques. The COSY experiment itself is still one of the most widely used 2D experiments, permitting the unraveling of complicated J coupling networks in solution spectra of many molecules of chemical interest. Powerful and popular as the COSY experiment may be, it also has a number of limitations, all inherent to the fact that no net magnetization transfer takes place in this experiment.[2] Consequently, the cross peaks in a 2D COSY spectrum show antiphase multiplet fine structure, leading to partial cancellation of cross peak intensity (resulting in a loss of sensitivity). Here, a recently developed alternative method for homonuclear magnetization transfer will be discussed which can have significant advantages over the standard COSY experiment. The new method, often referred to as TOCSY[3] or homonuclear Hartmann-Hahn (HOHAHA)[4] spectroscopy, relies on a principle different from the COSY experiment; by using rf irradiation during the mixing period of such a 2D experiment, magnetization can be transferred from one proton to another in a fashion very similar to heteronuclear Hartmann-Hahn cross polarization experiments[5,6] or zero field NMR.[7] The average Hamiltonian during the mixing period ideally contains only the J coupling contribution (isotropic mixing),[3] although in practice this appears difficult to accomplish.[8] Other schemes which are non-isotropic may then have advantages. This paper discusses the criteria for optimizing the mixing sequences and some of the practical problems encountered in this type of experiment.

PRINCIPLES OF ISOTROPIC MIXING

During isotropic mixing, the Zeeman terms of the Hamiltonian are removed, either by suitable rf irradiation or by taking the sample outside the magnetic field. Below, the effect of such isotropic mixing on a system of two isolated J coupled spin-1/2 nuclei, I_1 and I_2, will be considered. Before the isotropic mixing is started, in the weak coupling limit, the stationary eigenfunctions of this two-spin system are the simple basic product functions. During ideal isotropic mixing, only the J coupling terms of the Hamiltonian are present:

$$\mathcal{H}_J = 2\pi J \, \{ (I_1^+ I_2^- + I_1^- I_2^+) / 2 + I_{1_z} I_{2_z} \} \tag{1}$$

During this period, the new stationary eigenfunctions are linear combinations, $(|\alpha\beta\rangle \pm |\beta\alpha\rangle)/\sqrt{2}$, of the basic product functions. The $|\alpha\alpha\rangle$ and $|\beta\beta\rangle$ product functions are unchanged during *ideal* isotropic mixing. The temporary mixing of wave functions of the states that have identical total magnetic quantum numbers, M_r, forms the underlying mechanism for the type of magnetization transfer discussed in this paper. For the simple idealized two-spin case, expressions for magnetization transfer during mixing have been derived by Braunschweiler and Ernst:[3]

$$\begin{aligned} 2I_{1_\alpha} \xrightarrow{\mathcal{H}_J\tau} \; & I_{1_\alpha} (1 + \cos 2\pi J\tau) + I_{2_\alpha} (1 - \cos 2\pi J\tau) \\ & + 2 (I_{1_\beta} I_{2_\gamma} - I_{1_\gamma} I_{2_\beta}) \sin (2\pi J\tau) \end{aligned} \tag{2}$$

Israel Journal of Chemistry Vol. 28 1988 pp. 309–317

with $\alpha,\beta,\gamma = x,y,z$ and cyclic permutations thereof, and τ is the duration of the mixing period. Analogous analytical expressions for more complicated systems (AX_2, AX_3, A_2X_2, etc.) have been derived subsequently.[9] From Eq. (2) it is seen that, for example, in-phase I spin magnetization, I_x, can be transferred into in-phase S spin magnetization, S_x, in a time $1/(2J)$. Complete transfer of magnetization from I to S thus requires a time $1/(2J)$. Note that in the pulse interrupted free precession experiment (for example, COSY) a time $1/J$ would be needed for complete transfer of magnetization: an interval $1/(2J)$ for I spin magnetization to reach antiphase, a pulse, and a time $1/2J$ for S spin magnetization to refocus. For macromolecules, with short T_2 values, the isotropic mixing approach is therefore more effective than pulsed magnetization transfer. Moreover, it can be shown that in the COSY experiment a large fraction of I spin magnetization is converted into multiple quantum coherence by the mixing pulse, especially for more complicated spin systems. During ideal isotropic mixing, no multiple quantum coherence is created, i.e., no magnetization is wasted. Because H_J and I_α ($\alpha = x,y,z$) commute, the vector sum of all magnetizations is a constant of motion, i.e., neglecting relaxation:

$$\sum I_{k_\alpha}(0) = \sum I_{k_\alpha}(\tau) \quad (\alpha = x, y, z) \tag{3}$$

The summation is taken over all coupled spins, k, and the times 0 and τ refer to the beginning and the end of the mixing period. This means that magnetization is merely redistributed throughout the spin system, without any loss of net magnetization.

From the above, it is clear that the isotropic mixing approach has great potential for obtaining J connectivity information with high sensitivity and the high resolution that is inherent to in-phase phase-sensitive experiments. One major impediment to such experiments is, however, that ideal isotropic mixing is difficult to achieve in practice.

HOMONUCLEAR MAGNETIZATION TRANSFER DURING SPIN LOCK

If two coupled spins experience identical Zeeman terms during the mixing period, this causes perfect mixing of the wave functions that have identical M_T values, but it increases the energy difference between states with different M_T. Such a Hamiltonian is present during heteronuclear Hartmann-Hahn cross polarization sequences; the rf parts of the Hamiltonian (which represent Zeeman energy in the rotating frame) of two heteronuclear spins I and S have to be identical for such magnetization transfer to occur. Expressions developed by Mueller and Ernst[10] and Chingas et al.[11] for heteronuclear magnetization transfer in liquids during Hartmann-Hahn cross polarization are, therefore, directly applicable for analyzing homonuclear magnetization transfer during isotropic mixing. In practice, the J coupling during the application of the mixing scheme will be affected too and the simple expression of Eq. (1) no longer applies. This will be analyzed below for the simplest scheme, where mixing is obtained by the application of a continuous rf field (Fig. 1a). This is the direct analog of the conventional heteronuclear Hartmann-Hahn cross polarization experiment, with the only difference that a single rf field is needed to spin lock both spins. Expressions for this simple case have been presented previously.[12,13] To demonstrate the important difference between isotropic and non-isotropic mixing, analysis of this simple case will be repeated here.

The resonance offsets of two protons, I_1 and I_2, are δ_1 and δ_2, and the spin-lock field is of strength ν; the effective fields are labeled ν_1 and ν_2 and make angles α_1 and α_2 with the positive z axis (Fig. 2). The difference $\alpha_1-\alpha_2$, is labeled α. During the spin lock (along the y axis), the Hamiltonian is given by

$$\mathscr{H} = 2\pi\ \{\delta_1 I_{1'_z} + \delta_2 \mathrm{I}_{2'_z} + \nu \{I_{1'_y} + I_{2'_y}) + J I_{1'} I_{2'}\} \tag{4}$$

In this paper, all angular momentum operators with

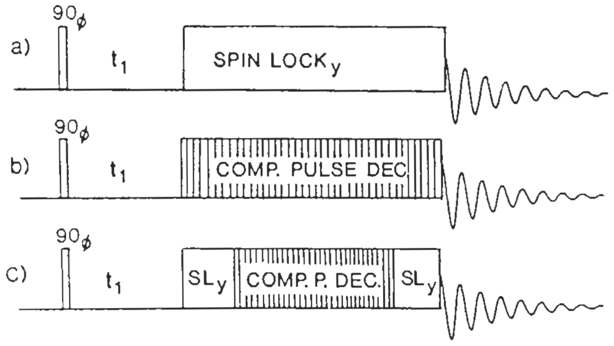


Fig. 1. Pulse schemes for recording 2D homonuclear cross polarization spectra. (a) Using a continuous spin lock field, (b) using a composite pulse mixing sequence and (c) using a composite pulse mixing sequence preceded and followed by trim pulses during which a continuous spin lock field is applied along the y axis. Sequence (a) cross polarizes protons that are within narrowly restricted bandwidths; intense NOE effects may be present. Sequence (b) can cross polarize over a wide bandwidth, depending on the particular composite pulse scheme used; NOE effects can be attenuated; quadrature artifacts may be present in the F_1 dimension of the 2D spectrum. The addition of trim pulses (c) can be used to eliminate the quadrature artifacts. Phase cycling of ϕ_1 can be used in the standard manner to suppress axial peaks and to discriminate between negative and positive F_1 frequencies.[16]

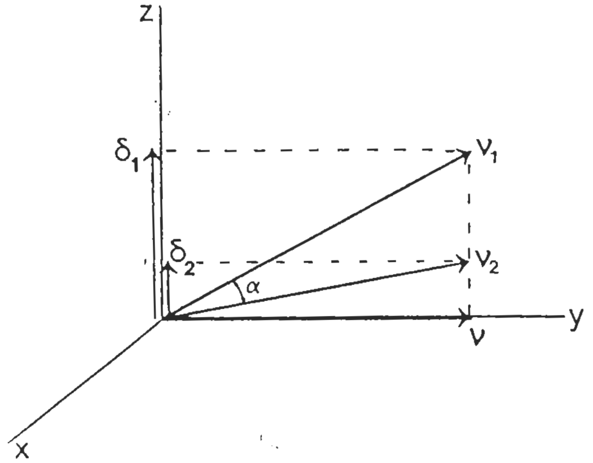


Fig. 2. Orientation of the effective fields, ν_1 and ν_2, for two spins I_1 and I_2, at resonance offset δ_1 and δ_2, during irradiation with an rf field of strength ν along the y axis of the rotating frame.

primes refer to the eigenbasis in the absence of the rf field; operators without primes refer to the basis where the rf field is present, with the z axis aligned along the effective field direction, and $I_x = I_x'$. The coherence transfer between I and S is computed most conveniently in the tilted frame of the magnetic fields. In this frame, the Hamiltonian is given by

$$\mathscr{H} = 2\pi\,[\nu_1 I_{1_z} + \nu_2 I_{2_z} + J\{I_{1_x} I_{2_x} + (I_{1_y} I_{2_y} + I_{1_z} I_{2_z})\cos\alpha + (I_{1_z} I_{2_y} - I_{1_y} I_{2_z})\sin\alpha\} \tag{5}$$

The J coupling part of this Hamiltonian can be rewritten as

$$\mathscr{H}_J = (\pi J/2)\begin{bmatrix} c & -is & is & (1-c) \\ is & -c & (1+c) & -is \\ -is & (1+c) & -c & -is \\ (1-c) & is & -is & c \end{bmatrix} \tag{6}$$

with $s = \sin\alpha$ and $c = \cos\alpha$. Because $\nu_1,\nu_2 >> J$, all non-diagonal terms apart from $\mathscr{H}_{J_{23}}$ and $\mathscr{H}_{J_{32}}$ can be neglected. This means that the coupling Hamiltonian is reduced to:

$$\mathscr{H}_J = 2\pi J\,\{I_{1_z} I_{2_z}\cos\alpha + (1+\cos\alpha)(I_1^+ I_2^- + I_1^- I_2^+)/4\} \tag{7}$$

The effective size of the J coupling is thus reduced during the rf irradiation.

ISOTROPIC VERSUS NON-ISOTROPIC MIXING

The mixing in the simple example discussed above is clearly non-istropic; the residual Zeeman Hamiltonian in Eq. (5) is much larger than the size of the J coupling ($\nu >> J$). An off-diagonal term $\mathscr{H}_{mn}$ in the Hamiltonian is called non-secular when the difference in energies of the corresponding eigenstates is much larger than the interaction, i.e., $|\mathscr{H}_{mm} - \mathscr{H}_{nn}| >> |\mathscr{H}_{mn}|$. Because the elements $\mathscr{H}_{J_{23}}$ and $\mathscr{H}_{J_{32}}$ are not necessarily much smaller than $E_2 - E_3$ ($= 2\pi\nu_1 - 2\pi\nu_2$), these terms cannot be neglected and they are responsible for transfer of coherence between levels $|2>$ and $|3>$. The condition for efficient magnetization transfer during homonuclear Hartmann-Hahn cross polarization with the scheme of Fig. 1a therefore is

$$|(1+\cos\alpha)J| \gg |\nu_1 - \nu_2| \tag{8}$$

On a high field spectrometer, it would require an extremely strong rf field (>1MHz) to satisfy condition (8) over a typical 10 ppm spectral width. Because the mixing must have a duration comparable to $1/(2J)$ for substantial mixing, this would induce severe rf heating effects if at all possible. Therefore, the sequence of Fig. 1a is not useful for recording 2D J correlation spectra over a wide bandwidth. Different mixing schemes have been developed in recent years that are designed to remove the Zeeman part of the Hamiltonian altogether. As will be commented on later, composite pulse decoupling schemes (originally developed for heteronuclear decoupling experiments[14]) can be very efficient in doing this. For these sequences, an average Hamiltonian can be defined to describe the average evolution during the mixing period.[8,12] For two spins, a general form of this Hamiltonian is

$$\mathscr{H} = \sum_{\gamma=x,y,z} C_{1_\gamma} I_{1_\gamma} + C_{2_\gamma} I_{2_\gamma} + \sum_{\alpha,\beta=x,y,z} C_{\alpha\beta} I_{1_\alpha} I_{2_\beta} \tag{9}$$

In the absence of Zeeman terms ($C_1 = C_2 = 0$), the off-diagonal terms that were non-secular before have to be reconsidered. The fifteen elements of Eq. (9) have been calculated numerically by Waugh[8] for the MLEV16 decoupling cycle.[15] His calculations demonstrate that the size of the Zeeman interaction is reduced to a few Hz over wide bandwidths and that significant off-diagonal terms other than $\mathscr{H}_{J_{23}}$ and $\mathscr{H}_{J_{32}}$ are present. These terms are no longer non-secular and have to be incorporated in the analysis of such a mixing scheme. This means that the sequence loses one of its desirable properties: transverse magnetization is converted into multiple quantum coherence, resulting in a loss of sensitivity. Second, it can be very difficult to eliminate the Zeeman terms from the Hamiltonian completely. This is illustrated by Fig. 3, which shows the residual component of the Zeeman Hamiltonian as a function of resonance offset for a single

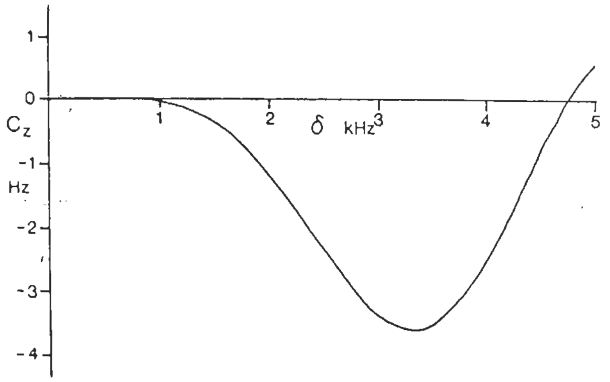


Fig. 3. The z component, C_z, of the Hamiltonian of an isolated spin during an MLEV-16 sequence as a function of resonance offset. An 8 kHz rf field is used for the MLEV16 sequence.

isolated spin during the application of the MLEV16 scheme. Although the size of this residual Zeeman energy is relatively small and does not interfere much with the mixing of magnetization (provided the coupling is much larger than this Zeeman interaction), it can create significant phase distortions in the 2D spectrum. For example, for the conditions of Fig. 3, a spin, at 2.8 kHz offset, experiences, during the MLEV16 irradiation, a 3 Hz rotation about the z axis. In the absence of J coupling, this means that magnetization that started, for example, along the x axis at the beginning of the mixing period ends up along the y axis at the end of an 85 ms mixing period. Computer simulations show that inaccurate phase shifts or imbalances in the rf amplitudes of the phase shifted channels also can contribute to significant residual Zeeman terms.

The two problems mentioned above, magnetization loss due to multiple quantum generation and phase distortions in the final 2D spectrum, can be avoided by making the composite pulse mixing slightly non-isotropic. This is accomplished by the application of a pulse of flip angle, α, at the end of every MLEV-16 cycle. The mixing then consists of an integral multiple of so-called MLEV-17 cycles,[16] with each cycle consisting of [*ABBA BBAA BAAB AABB* α_y], with $A = 90_x 180_y 90_x$ and $B = 90_{-x} 180_{-y} 90_{-x}$. If the duration of each MLEV-17 cycle is τ, the main effect of the seventeenth pulse (α) is to add a component $\alpha I_y/\tau$ to the average Hamiltonian of the mixing period. Typical values are $\tau = 2$ ms and $\alpha = 60°$, causing an average rf field of 83 Hz along the y axis during application of this MLEV-17 sequence, eliminating the phase distortions discussed above. The Zeeman part of the average Hamiltonian will be dominated by this relatively large value, and off-diagonal elements that connect spin states with different M_r will again become non-secular, reducing loss of magnetization. The seventeenth pulse does not have any compensation for offset effects, and, therefore, the bandwidth over which efficient transfer of magnetization is obtained is reduced by the addition of this pulse.

Comparison of the efficiency of magnetization transfer for different mixing sequences is not straightforward; a complete comparison, at least, would have to include the offsets of two coupled spins, the size of the J coupling and the duration of the mixing period, requiring a 4D representation. A simpler alternative chosen here for the comparison of sequences is to use a fixed, but relatively small, value of the J coupling (4Hz), a fixed mixing time of 50 ms, one of the two spins on resonance and the offset of the second spin variable. These parameters reflect the important application in proteins where the carrier is positioned near the center of the spectrum (in the $C_\alpha H$ region) and transfer is observed to an amide proton. Figure 4a compares the magnetization transfer efficiencies of the MLEV-16 sequence with three different flip angles of the seventeenth pulse, 0°, 60° and 180°. For small offsets, δ, of the second spin all three schemes work equally well. However, for $\delta > 1.5$ kHz significant differences are seen. As expected, with the seventeenth pulse being zero, the mixing is most effective, but as discussed earlier, the 2D spectrum cannot easily be phased to the absorption mode. In practice, a seventeenth pulse of flip angle 60° appears to be a good compromise.

Analysis of the WALTZ-16 decoupling scheme[17] for isotropic mixing shows that the residual Zeeman terms are much smaller than for the MLEV sequence; however, the part of the J coupling responsible for the magnetization transfer (containing the I^+S^- terms) decreases more rapidly with offset. In principle, a seventeenth pulse would not be needed with this sequence. In practice small phase errors appear in the spectrum if a pure WALTZ16 is used for mixing, probably caused by transients at the time of rf phase shifting. Upon addition of a seventeenth pulse, the phase distortions disappear, at a minor cost in the efficiency of magnetization transfer (Fig. 4b). Comparison of Figs. 4a and b suggests that the WALTZ mixing should be preferable over the MLEV16 sequence. In practice we find this only to be the case if the phase shifting hardware is perfect, i.e., phase shifts are exact and no imbalance in rf power exists for the phase-shifted channels. Such phase shifting can be obtained by using synthesizer based phase shifting hardware, as incorporated on the newer Bruker and Varian instruments. If phase shift imperfections are present, we and others have found the MLEV17 sequence to be superior over the WALTZ sequence.

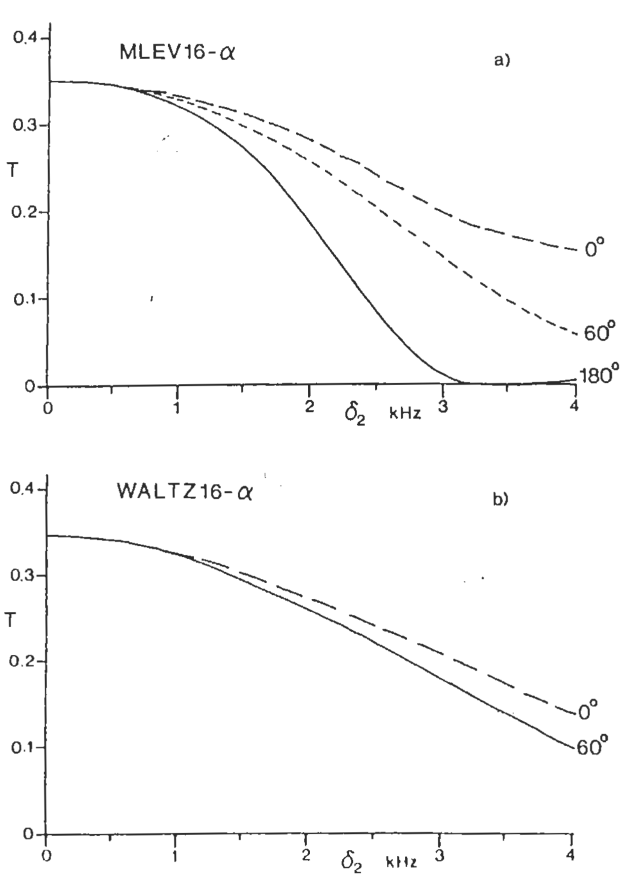


Fig. 4. The fraction, T, of net magnetization transferred from spin I_1 to spin I_2 during (a) an MLEV-17 sequence and (b) a WALTZ17 sequence as a function of the resonance offset of I_2, for various values of the flip angle α of the seventeenth pulse. Spin I_1 is on resonance, an 8 kHz rf field is used, the J coupling is 4 Hz and the duration of the mixing period is 50 ms. No relaxation effects are taken into account.

MINIMIZATION OF RELAXATION LOSS AND SUPPRESSION OF NOE

During the scheme of Fig. 1a, spin-locked magnetization during the mixing period decays with a time constant, $T_{1\rho}$. For macromolecules, $T_{1\rho}$ is equal to T_2; this decay of magnetization is the main limitation for obtaining a large amount of magnetization transfer between J coupled spins. As shown previously, the $T_{1\rho}$ decay of spin-locked magnetization can be prolonged substantially by application of a composite pulse mixing scheme, such as the MLEV decoupling sequence. During the application of such a scheme, transverse magnetization that starts, for example, along the y axis is rotated by one of the $90_x180_y90_x$ elements of the MLEV cycle from the y axis to the $-z$ axis to the $+z$ axis to the y axis. During this trajectory, magnetization decays at a rate $1/(2T_1)+1/(2T_2)$ which can be slower by up to a factor of 2 relative to $T_{1\rho}$. Note that the relaxation rate for magnetization that starts along the x axis differs from y magnetization. If one attempts to mix simultaneously both the x and the y components of transverse magnetization, this differential relaxation can result in significant quadrature artifacts in the F_1 domain of the 2D spectrum. To make sure that only the y component of magnetization is present at the beginning of the mixing period, a so-called trim pulse (Fig. 1c) can be applied prior to the composite pulse mixing. This trim pulse consists of a short spin lock, using rf inhomogeneity for defocusing any magnetization perpendicular to the spin lock axis.

During the mixing period, NOE effects can also contribute significantly to magnetization transfer. In fact, the sequence of Fig. 1a was proposed by Bothner-By and coworkers for measuring spin-locked NOEs.[18] For macromolecules the buildup rate of the spin-locked NOE (ROE for *r*otating frame *O*verhauser *e*ffect) is twice as large and of opposite sign compared to the regular NOE.[19,20] This means that in a typical 50 ms mixing period significant ROE effects can be observed. By using a mixing sequence such as the MLEV or WALTZ scheme where the magnetization is rotated back and forth between the z axis and the transverse plane, the NOE and the opposite signed ROE will largely cancel.[21] The size of the residual NOE effect will also depend on the orientation at the beginning of the mixing period. For example, the WALTZ scheme consists of 90, 180 and 270° pulses applied along the $+x$ axis. Magnetization that starts along the x axis will remain there during the entire WALTZ sequence, displaying the full ROE. Magnetization starting along the y axis rotates back and forth in the yz plane and will display the reduced NOE. Again, trim pulses assure that only the component that has the smallest average NOE will be conserved.

HETERONUCLEAR DECOUPLING SEQUENCES FOR ISOTROPIC MIXING

All methods for homonuclear cross polarization proposed so far are variations of heteronuclear decoupling experiments. The continuous spin lock corresponds to coherent decoupling; the phase alternated spin lock[4] corresponds to the square wave decoupling method.[22] The MLEV and WALTZ schemes were developed for obtaining improved decoupling performance. A discussion of heteronuclear decoupling which shows that this similarity is not coincidental has been given by Waugh.[23] In his discussion of decoupling he considers a heteronuclear IS spin system, with coupling, J. If the two I spin magnetization vectors, corresponding to the α and β spin states of the S spin, follow identical trajectories through threedimensional space during the application of a pulse sequence applied to spins, I, this means that spins I are "unaware" of the polarization of spin S. In this case, the S spin cannot "see" the I spin either; i.e., it is decoupled. Hence, a criterion for good heteronuclear decoupling is that magnetization vectors of two spins in the ^{1}H spectrum that are displaced in frequency by an amount J will follow identical trajectories through space. Without decoupling, the I spin experiences the presence of S by an increase or decrease of its resonance frequency by $J/2$, i.e., a change in its "chemical shift". Thus, a good decoupling sequence would also remove small differences in chemical shift (typically 150 Hz for a ^{13}C–^{1}H coupling) between two chemically unequivalent protons. According to the Waugh criterion for good decoupling, the two nonequivalent I spins, I_1 and I_2, would follow near-identical trajectories through space which means that the scalar product $I_1 \cdot I_2$ would remain close to unity. Thus, the ideal mixing of the magnetizations would be accomplished.

The Waugh picture of decoupling illustrates why good decoupling sequences may be suitable for homonuclear cross polarization. It also illustrates why wide band homonuclear cross polarization is more difficult than heteronuclear decoupling; the heteronuclear coupling is at most a few 100 Hz, whereas the difference in chemical shift to be removed during homonuclear cross polarization can be several kHz. Therefore, a good decoupling sequence does not necessarily provide optimal cross polarization. Sequences, such as GARP,[24] that provide very wide band heteronuclear decoupling appear unsuit-

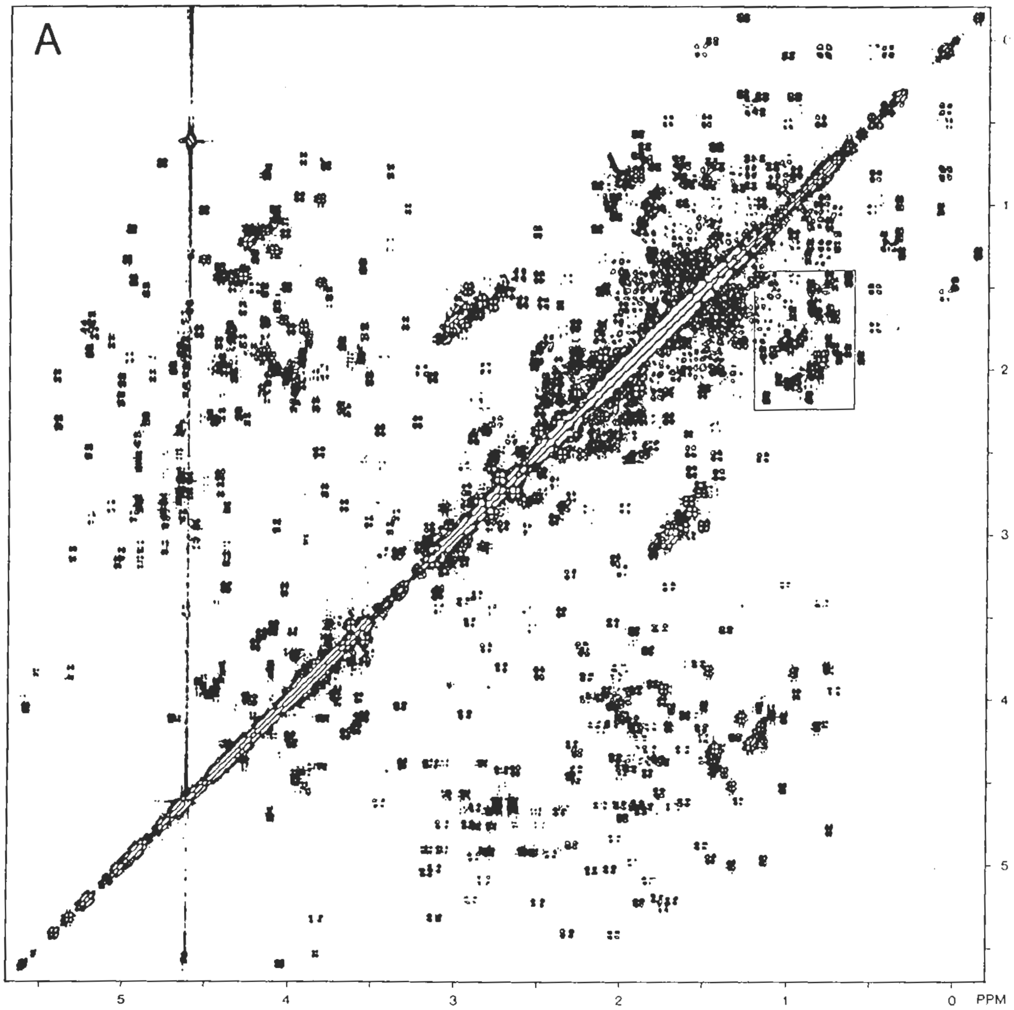
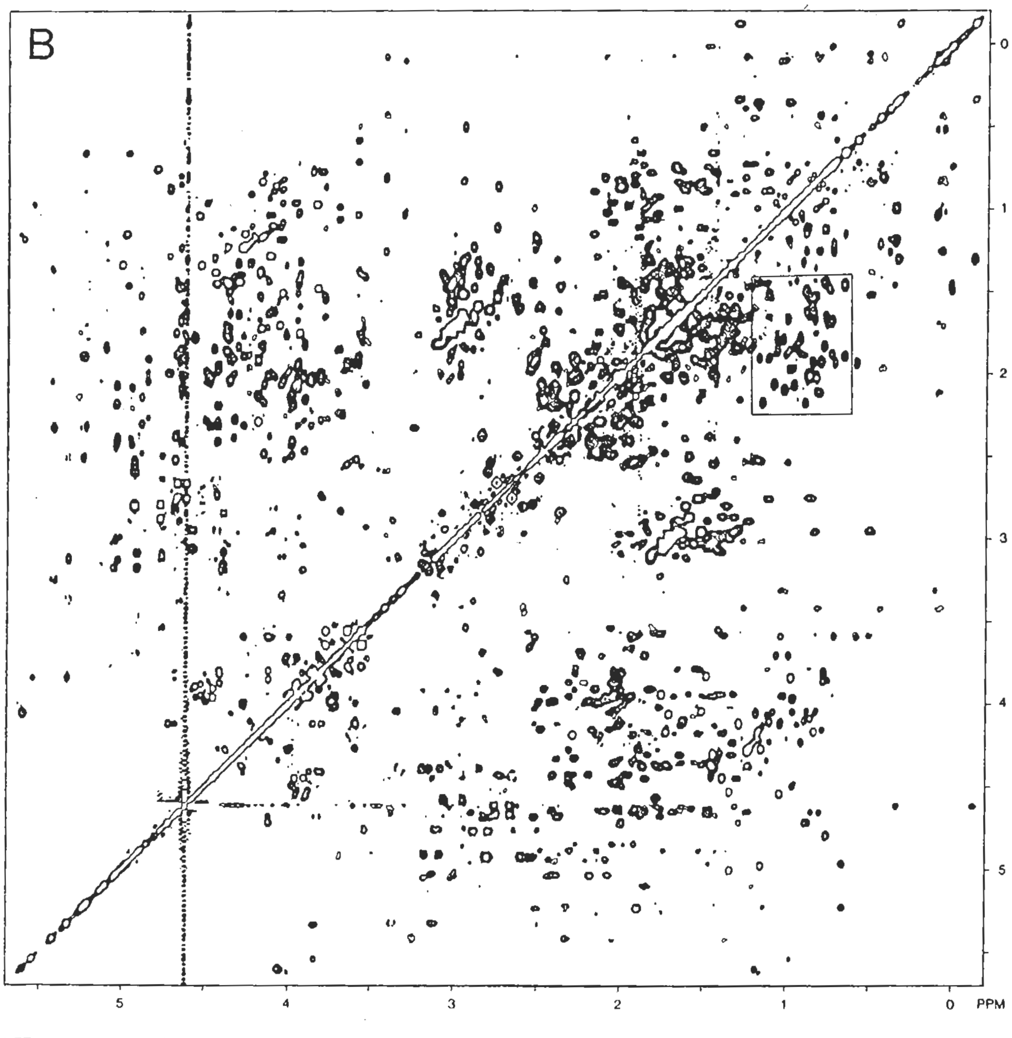


Fig. 5. Aliphatic regions of (a) the double quantum filtered COSY and (b) the HOHAHA spectrum of a 1.5 mM solution of the staphylococcal nuclease/pdTp/Ca^{2+} complex (18 kD) in D$_2$O, p^{2}H 7.6, 100 mM NaCl, recorded at 600 MHz on a Bruker AM600 spectrometer. Both spectra were recorded at 39°C and they result from 800*2048 ανδ 1024*2048 data matrices, with acquisition times of 56 and 144 ms (HOHAHA) and 72 and 144 ms (COSY) in the t_1 and t_2 dimensions, respectively. The number of scans per t_1 value

able for cross polarization purposes. Decoupling sequences, such as the DIPSI sequences developed by Shaka et al.,[25] that provide excellent decoupling over a relatively narrow bandwidth are much more promising in this respect.

DISCUSSION AND CONCLUSIONS

The homonuclear cross polarization schemes provide a powerful approach for the analysis of scalar coupling networks. In practice, sensitivity and resolution can be significantly higher than for the alternative COSY or relayed COSY experiment. Figure 5 compares identical regions of the double-quantum filtered COSY and HOHAHA spectra, recorded for the protein staphylococcal nuclease, complexed with pdTp and calcium. To obtain acceptable sensitivity, the COSY spectrum was recorded with four times the number of scans per t_1 increment compared to the HOHAHA spectrum. However, inspection of slices through these 2D spectra shows that the signal-to-noise ratio of many COSY cross peaks is lower than for the corresponding HOHAHA cross peaks. Moreover, the COSY cross multiplets appear broader; this is caused by the antiphase nature of the COSY cross multiplet, which accentuates the edges of the cross peak with relatively little intensity at its center. In contrast, the absorptive components of the HOHAHA cross peaks are in phase, and do not exhibit this broader multiplet appearance. This is seen most clearly in the expanded region of the spectra of Fig. 5 as shown in Fig. 6.



was 4 (HOHAHA) and 16 (COSY), preceded by one dummy scan. Total measuring times were 3.5 h (HOHAHA) and 14h (COSY). The HOHAHA spectrum was recorded with a WALTZ17 sequence ($\alpha = 60°$), using a 9 kHz rf field, 1.5 ms trim pulses and a 40 ms duration of the 15 WALTZ17 cycles. Identical digital filtering (negative Lorentzian and positive Gaussian) was used for the two spectra.

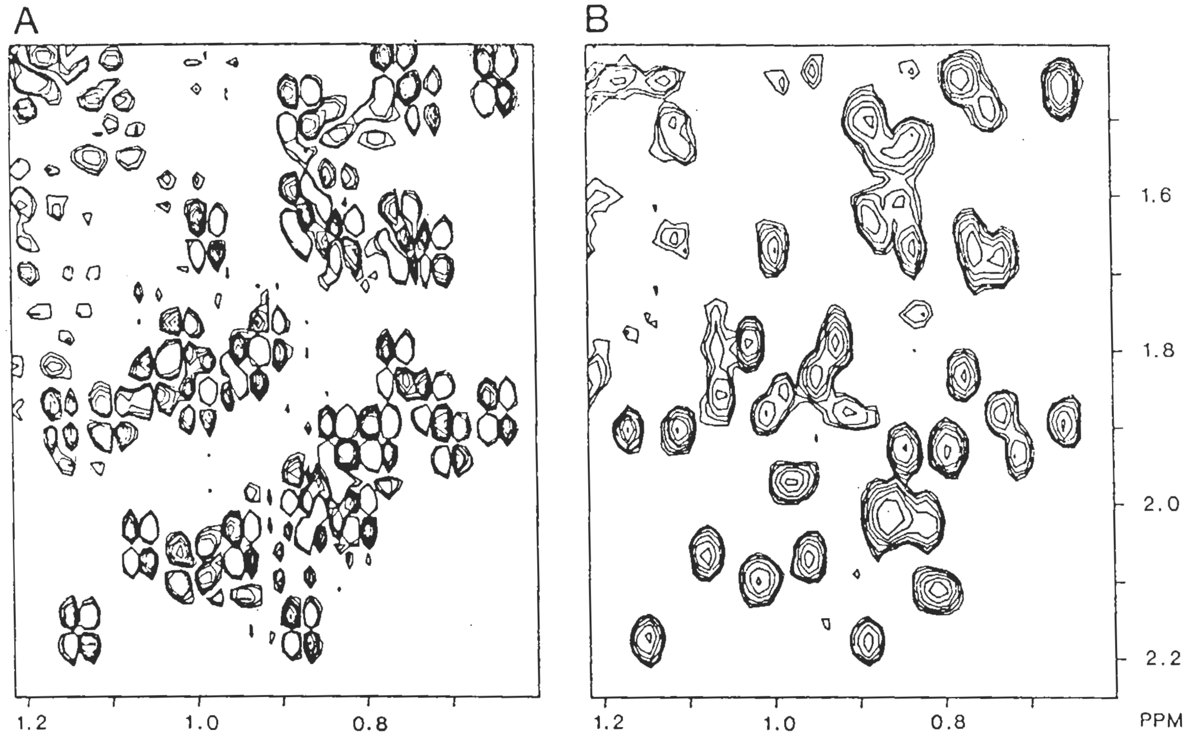


Fig. 6. Expansions of the boxed regions in Fig. 5, comparing the resolution obtainable in the two experiments. The visual appearance of the COSY spectrum improves somewhat if negative and positive levels are displayed with different colors.

Depending on the duration of the mixing period, a variable amount of relayed connectivity can be obtained. For short mixing periods (<20 ms) magnetization transfer is restricted almost exclusively to directly coupled protons; for longer mixing periods an increasing amount of relay can be observed. For example, for a 70 ms mixing period, intense cross peaks could be observed between the NH and $C_\varepsilon H_2$ resonances (7-bond connectivity) of a lysine residue in a 23 amino acid peptide,[26] despite a relatively small NH–C_αH *J* coupling (4 Hz). The presence of relay in HOHAHA spectra can severely attenuate cross peak intensity because intensity of a direct correlation is spread over all the indirectly connected protons. For this reason, at longer mixing times (>30 ms) connectivities in larger spin systems (e.g., lysine, arginine and proline) are significantly weaker than in small systems such as alanine, tyrosine, etc. Successive relays of magnetization can be a blessing because of the extra information available, but also a disadvantage in increasing spectral crowding. Moreover, sometimes it may be difficult to distinguish between direct and relayed connectivities. Therefore, it is recommended to also record a complementary COSY spectrum.

The composite pulse mixing schemes based on the MLEV16 and WALTZ16 decoupling schemes function very well for homonuclear cross polarization purposes. They permit covering a 5 kHz bandwidth with a few watts rf power. For covering larger bandwidths, either higher rf power is needed or a more efficient mixing scheme. Considering that there are significant undesirable terms in the average Hamiltonian of the MLEV and WALTZ sequences, it is expected that improved mixing schemes can be developed.

Acknowledgment. I wish to thank Dennis Torchia and Steven Sparks for providing the sample of Staphylococcal Nuclease. The simulation software used for generating the graphs of Figs. 3 and 4 was kindly provided by Technic de Bouregas. This work was supported by the AIDS Antiviral Program of the Office of the Director of National Institutes of Health.

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