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# HOMONUCLEAR HARTMANN-HAHN MAGNETIZATION TRANSFER: NEW ONE- AND TWO-DIMENSIONAL NMR METHODS FOR STRUCTURE DETERMINATION AND SPECTRAL ASSIGNMENT

**A.Bax and D.G.Davies**

Laboratory of Chemical Physics
National Institute of Arthritis, Diabetes,
and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Md. 20205, USA.

## INTRODUCTION

The idea of two-dimensional (2D) Fourier transform NMR, first introduced by Jeener (1) has proven to be very powerful for analyzing the NMR spectra of compounds of high complexity. The general theory of 2D NMR was described by Aue et al. (2) in 1976, and has provided the theoretical basis for the development of a large number of new one- and two-dimensional pulse schemes. Nonetheless, the pulse scheme originally proposed by Jeener, and described in great detail by Aue et al. is currently still one of the most widely used 2D methods. Some minor modifications have been made to this experiment to facilitate data handling and processing (3,4) and to enhance the resolution by recording double quantum filtered absorption mode spectra (5,6). Jeener's experiment is generally referred to as the COSY (Correlated Spectroscopy) method, and has been applied to a variety of problems, varying from small organic molecules to biological macromolecules. The COSY spectrum displays the presence of scalar coupling between coupled spins A and X by the presence of a so-called cross peak centered about the frequency coordinates $(F_1,F_2) = (\nu_A,\nu_X)$ and $(\nu_X,\nu_A)$, where $\nu_A$ and $\nu_X$ are the chemical shift frequencies of spins A and X, respectively. Unfortunately, a more detailed look at such a cross multiplet shows that the individual components of this 2D multiplet are 180° out of phase relative to one another (2,3,7), i.e. no net magnetization is transferred between spins in the COSY experiment. For spin systems with a short transverse relaxation time, $T_2$, or digital resolution that is on the order of $J_{AX}$ or coarser, partial cancellation of the 2D cross multiplet components invariably occurs, resulting in decreased cross peak intensity, and hence, lower sensitivity. For diagonal multiplets, centered around $(\nu_A,\nu_A)$ and $(\nu_X,\nu_X)$, such a mutual cancellation does not occur and the redundant diagonal resonances will often have much stronger intensity than the informative cross peaks, sometimes obscuring nearby cross peaks.

Recently, Braunschweiler and Ernst (8) introduced a new method that provides net magnetization transfer between coupled spins, and that relies on a rather different mechanism than the COSY experiment. With this method, many of the limitations of the COSY experiment can be overcome. They describe their experiment in terms of isotropic mixing, a technique first introduced in solid state NMR by Weitekamp, Garbow and Pines (9) and also used by Caravatti et al. (10). We describe new methods that rely on similar principles but that are experimentally easier to execute since they appear less sensitive to instrument imperfections. Our experiments are most conveniently analysed by considering them as homonuclear Hartmann-Hahn (HOHAHA) coherence transfer methods.

It was first demonstrated by Hartmann and Hahn (11) that net magnetization transfer between heteronuclear coupled spins occurs when one simultaneously switches on two coherent rf fields for spins I and spins S in such a way that

$$H_{1I}\gamma_I = H_{1S}\gamma_S \qquad (1)$$

often referred to as the Hartmann-Hahn match condition. In this expression $H_{1I}$ and $H_{1S}$ denote the effective rf field strengths, experienced by spins I and S, and $\gamma_I$ and $\gamma_S$ are the respective magnetogyric ratios. Under this condition complete magnetization exchange between two coupled spins occurs in a time $1/J_{IS}$. This Hartmann-Hahn magnetization transfer mechanism is the basis of heteronuclear cross polarization in solids (12), and has also been applied in 1D and 2D heteronuclear NMR experiments in liquids (13,14). Unfortunately, the heteronuclear Hartmann Hahn type magnetization transfer in liquids is very sensitive to the match condition (Eq.(1)); so despite its elegance the method has found little use in liquids. As will be shown below, a homonuclear Hartmann Hahn match is much easier to establish, even though the scalar couplings involved are often much smaller than in the heteronuclear case.

## PRINCIPLES OF CROSS POLARIZATION

For the homonuclear case, $\gamma_I$ and $\gamma_S$ are identical and a match occurs when the effective rf field strengths, $H_{1I}$ and $H_{1S}$, experienced by the two coupled spins are identical. In contrast with heteronuclear cross polarization experiments in liquids, this homonuclear Hartmann Hahn match is very insensitive to rf inhomogeneity: since $H_{1I}$ and $H_{1S}$ are generated by the same rf field, originating from a single coil and a single rf amplifier, the ratio $H_{1I}/H_{1S}$ is to a good approximation independent of the location within the sample coil and of the rf power used. Hence, two scalar coupled spins will always experience the same rf field (not necessarily the same effective rf field). For reasons that will become clear later, the magnetization exchange occurs a factor of two faster than in the heteronuclear case, i.e. complete transfer of magnetization from I to S can be achieved in a time, $1/(2J_{IS})$. Hartmann Hahn cross polarization has often been described in the spin temperature formalism, or by using a rather complex mathematical description. Here, we will attempt to present a simpler quasi-vector-picture of this phenomenon. Since Hartmann Hahn transfer will turn out to be closely related to strong coupling in homonuclear spin systems, this latter more familiar case will be addressed first.

Consider two homonuclear coupled spins, I and S, in a static magnetic field of strength, $B_o$, with no rf fields applied. The angular Larmor frequencies are $\Omega_I$ and $\Omega_S$ and the coupling equals $J_{IS}$. The spin operators for x, y and z magnetization of spin I are denoted by $I_x$, $I_y$ and $I_z$, and similar operators are defined for spin S. In the Heisenberg formalism, the transverse spin operators, $I_x$ and $I_y$, precess with frequency $\Omega_I$ about the z axis, and operators $S_x$ and $S_y$ precess with frequency $\Omega_S$. The scalar IS interaction part of the Hamiltonian is given by

$$\mathcal{H}_J = J_{IS}(I_xS_x+I_yS_y+I_zS_z) \qquad (2)$$

If the angular frequencies, $\Omega_I$ and $\Omega_S$ are vastly different, the product terms, $I_xS_x$ and $I_yS_y$, oscillate rapidly (in the Heisenberg formalism) and average to zero. Thus, for the weak coupling limit only the term $J_{IS}I_zS_z$ remains and is reponsible for the multiplet splittings commonly observed in NMR spectra. In the case where the difference between $\Omega_I$ and $\Omega_S$ is small (on the order of $2\pi J_{IS}$ or less), the terms $I_xS_x$ and $I_yS_y$ also have to be considered. As is well known from the strong coupling analysis (15), the stationary wave functions are no longer simple basic product functions but linear combinations thereof. If one suddenly changes the Hamiltonian from the weak coupling to the strong coupling limit or vice versa, this can cause non-equilibrium populations of the energy levels to be converted into off diagonal density matrix elements (and vice versa). As will be shown below, this is essentially the basis of Hartmann Hahn cross polarization.

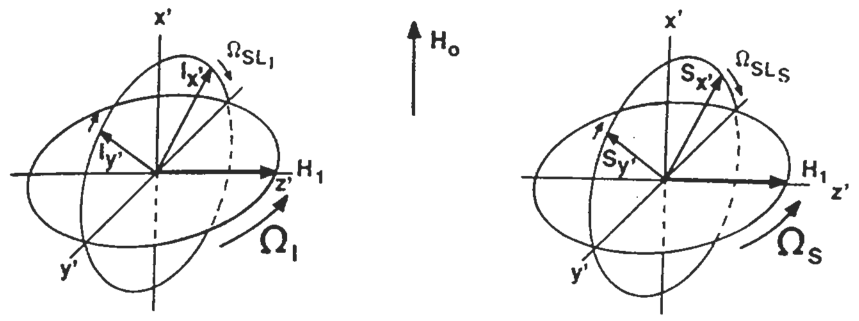


Figure 1 - Rotating frames for two coupled spins, I (left) and S (right). The z' axes are chosen parallel to the respective effective rf fields. The x' axes are parallel to the static magnetic field. $I_{x'}$, $I_{y'}$, $S_{x'}$ and $S_{y'}$ are the Heisenberg spin operators of angular momentum, as discussed in the text.

Consider first a heteronuclear two-spin-1/2 system, IS. If a $90°_x$ pulse is applied to the I spins, followed by a spin lock rf field of strength $\Omega_{SLI}$ along the y axis, the I spin magnetization will be aligned along the I spin rf field, which is the new z axis for this system, and will be labelled z' (Figure 1). The spin operators $I_{x'}$ and $I_{y'}$ in the Heisenberg formalism, rotate with angular frequency $\Omega_{SLI}$ about the z' axis. If simultaneously to the I spin spin-lock field a S spin rf field of strength $\Omega_{SLS}$ is applied, the difference in Zeeman energy for the two spins will be zero if $\Omega_{SLI} = \Omega_{SLS}$. However, the scalar coupling is still present (vide infra). Because the rotating frames of spins I and S rotate at different frequencies, the product $I_{z'}S_{z'}$ averages to zero;

however, the $I_{x'}$, $I_{y'}$, $S_{x'}$ and $S_{y'}$ operators rotate about their respective z' axis, at angular frequency $\Omega_{SLI}$ and $\Omega_{SLS}$ (Fig. 1). Since the components that are perpendicular to the static magnetic field precess at different Larmor frequencies ($\Omega_I$ and $\Omega_S$) about the static magnetic field, only the components of $I_{x'}$ and $S_{x'}$ (and of $I_{y'}$ and $S_{y'}$) that are parallel to the static magnetic field can contribute to the average scalar product. For simplicity we assume that at the beginning of the spin lock period (t=0) both the $I_{x'}$ and the $S_{x'}$ operators are aligned parallel to the static magnetic field. Since these operators rotate at angular frequency $\Omega_{SLI}$ and $\Omega_{SLS}$ about their respective z' axis, the z components of $I_x'$ and $S_{x'}$ are proportional to $\cos(\Omega_{SLI}t)$ and $\cos(\Omega_{SLS}t)$, and hence their product is proportional to $\cos(\Omega_{SLI}t)$ $\cos(\Omega_{SLS}t)$. Similarly, the product of $I_{y'}S_{y'}$ is proportional to $\sin(\Omega_{SLI}t)\sin(\Omega_{SLS}t)$. For calculational purposes it is simpler to work in the Schroedinger formalism, chosen with the z' axis parallel to the spin lock field and the x' axis parallel to the static magnetic field. In this case one is dealing with two rotating frames that precess with different Larmor frequencies about the static magnetic field axis. The scalar interaction, I.S, then reduces to $I_xS_{x'}$ because the y and z components rotate at different Larmor frequencies. Writing $I_x=(I_+ + I_-)/2$, etc., and $\nu_I=\Omega_{SLI}/2\pi$, $\nu_S=\Omega_{SLS}/2\pi$, one obtains for the Hamiltonian under spin-locked conditions:

$$\mathcal{H}' = \nu_I I_{z'} + \nu_S S_z + J_{IS}(I_+S_- + I_-S_+ + I_+S_+ + I_-S_-)/4 \qquad (3)$$

In matrix form, this Hamiltonian is given by:

$$\mathcal{H}' = \begin{bmatrix} \nu_I + \nu_S & 0 & 0 & J/4 \\ 0 & \nu_I - \nu_S & J/4 & 0 \\ 0 & J/4 & \nu_S - \nu_I & 0 \\ J/4 & 0 & 0 & -\nu_I - \nu_S \end{bmatrix} \qquad (4)$$

and this matrix operates in the basic operator product function basis. Therefore, if spins I have unity magnetization at the beginning of the spin lock period and spins S are saturated, the deviations of the average populations of the energy levels $|\alpha\alpha\rangle, |\alpha\beta\rangle, |\beta\alpha\rangle$ and $|\beta\beta\rangle$ are 1,1,-1 and -1, respectively, in units $h\,\Omega_I/4\pi kT$ (h is Planck's constant and k is Boltzmann's constant). It is then easily found that the terms $H_{23'}$ and $H_{32'}$ induce oscillatory changes in the populations of levels $|2\rangle$ and $|3\rangle$. For exactly matched rf fields, i.e. $\nu_I=\nu_S$, after a mixing period of duration 1/J, the populations of levels $|2\rangle$ and $|3\rangle$ are interchanged, and all I spin magnetization has been transferred to the S spins. Note that matrix elements $H_{14'}$ and $H_{41'}$ have very little effect because the energies of levels $|1\rangle$ and $|4\rangle$ differ by $2\nu_I+2\nu_S$, and these elements are therefore nonsecular.

The analysis of heteronuclear cross polarization given above is not new and a number of rather similar descriptions can be found in the literature. The main point we wish to make is that in order to induce magnetization transfer between the two coupled spins, one has to reduce the difference in apparent Zeeman energies ($\nu_I$ and $\nu_S$) for the two spins to less than the size of the interaction between them, i.e. one has to modify a weakly coupled spin system into a strongly coupled one.

So far, we have discussed cross polarization for the heteronuclear case. However, the treatment for the homonuclear case is completely analogous and even slightly simpler since all discussions relate to a single rotating frame. Consider two weakly coupled ($J_{IS} \ll |\Omega_I - \Omega_S|/2\pi$) protons, I and S, and the longitudinal magnetization of spin S has ben destroyed by presaturation. Again a $90°_x$ pulse followed by a spin lock along the y axis is applied. Assuming that the nominal strength of the spin lock rf field, $\nu$, is much larger than the offset frequencies, $\Omega_I/2\pi$ and $\Omega_S/2\pi$, the effective rf fields for the two spins are approximately parallel and their magnitudes, $\nu_I$ and $\nu_S$, are nearly identical. Since the spin lock frames for the two homonuclear spins almost coincide (in contrast with the heteronuclear case), the size the scalar interaction between I and S is unaffected by the spin lock field. In the Schroedinger representation, we find for the Hamiltonian under spin locked conditions:

$$\mathcal{H}' = \nu_I I_{z'} + \nu_S S_{z'} + J_{IS}(I_{x'}S_{x'} + I_{y'}S_{y'} + I_{z'}S_{z'}) \qquad (5)$$

where $\nu_I$ and $\nu_S$ are the effective spin lock field strengths for spins I and S, respectively. In matrix form (in the product basis) this yields

$$\mathcal{H}' = \begin{bmatrix} \nu_I+\nu_S+J/2 & 0 & 0 & 0 \\ 0 & \nu_I-\nu_S-J/2 & J/2 & 0 \\ 0 & J/2 & \nu_S-\nu_I-J/2 & 0 \\ 0 & 0 & 0 & -\nu_I-\nu_S+J/2 \end{bmatrix} \qquad (6)$$

Because the product $I_{y'}S_{y'}$ does not average to zero (in contrast with the heteronulear case), matrix elements $H'_{23}$ and $H'_{32}$ are a factor of two larger than in Eq.(4), and magnetization transfer between spins occurs twice as fast, i.e. for $\nu_I=\nu_S$, complete transfer from I to S is obtained in a time $1/(2J_{IS})$. Again, the form of the Hamiltonian is identical to that of a strongly coupled homonuclear AB spin system.

## THE EFFECT OF RF OFFSET

If the two spins, I and S, have different offsets from the carrier frequency, the magnitude of the effective spin lock fields will differ for the two spins. For offsets, $\Delta$ ,small relative to the nominal rf field strength, $\nu$ , the effective rf field strength is to a good approximation given by

$$\nu_{eff} = \nu + \Delta^2/2\nu \qquad (7)$$

Therefore, if the two offsets $|\Delta_I|$ and $|\Delta_S|$ differ, this causes a mismatch of the Hartmann Hahn condition, which results in an incomplete magnetization transfer between the two spins. Simple matrix calculation, based on Eq.(6), shows that for $|\nu_I-\nu_S|=|J|$ only half the I spin magnetization can be transferred to S and vice versa.

For a system of three coupled spins, AMX, the situation is somewhat more complicated. In this case, an 8x8 Hamiltonian and an 8x8 density matrix have to be considered. However, as is the case for the two spin system, magnetizazion transfer occurs only between levels of equal total spin angular momentum, $\underline{M}$($\alpha\beta$ and $\beta\alpha$for the 2-spin case). For the 3-spin case, one has to consider two sets of energy levels: $\underline{M}=\frac{1}{2}(\alpha\alpha\beta, \alpha\beta\alpha, \beta\alpha\alpha)$ and $\underline{M}=-\frac{1}{2}(\alpha\beta\beta, \beta\alpha\beta, \beta\beta\alpha)$. We therefore can consider separately the reduced Hamiltonians, $\mathcal{H}_{r'}$, for $\underline{M}=\pm\frac{1}{2}$. For $\underline{M}=\frac{1}{2}$ and $J_{AX}=0$, one obtains:

$$\mathcal{H}_{r'} = \begin{bmatrix} \nu_A+\nu_M-\nu_X+(J_{AM}-J_{MX})/2 & J_{MX}/2 & 0 \\ J_{MX}/2 & \nu_A-\nu_M+\nu_X-(J_{AM}+J_{MX})/2 & J_{AM}/2 \\ 0 & J_{AM}/2 & -\nu_A+\nu_M+\nu_X-(J_{AM}-J_{MX})/2 \end{bmatrix} \qquad (8)$$

In this 3-level system, the energies of levels $\alpha\alpha\beta$ and $\alpha\beta\alpha$ differ by $J_{AM}+2\nu_M-2\nu_X$. In other words, the coupling between spins A and M induces a mismatch between spin M and X if $\nu_M=\nu_X$. This mismatch can be reduced by choosing the carrier position such that $\nu_X-\nu_M=J_{AM}/2$ (Eq.(7)), but in this case the magnetization transfer in the second subset ($\underline{M}=-\frac{1}{2}$) between the levels $\beta\alpha\beta$ and $\beta\beta\alpha$ is even further mismatched, i.e. significant transfer will only involve one of the two X-spin doublet components under spin locked conditions. Upon the switchoff of the rf field, the total X spin-locked magnetization will be symmetrically distributed over the two doublet components and this multiplet effect becomes invisible.

The qualitative analysis presented above suggests that the presence of other spins broadens the match condition between M and X (provided that A and M are mismatched) and simultaneously decreases the total amount of transfer that is obtainable between two coupled spins. Nevertheless, significant magnetization transfer between coupled spins I and S remains limited to $|\nu_I - \nu_S| < \Sigma J/2$ where $\Sigma J$ denotes the sum of the multiplet widths of spins I and S (in the absence of heteronuclear coupling).

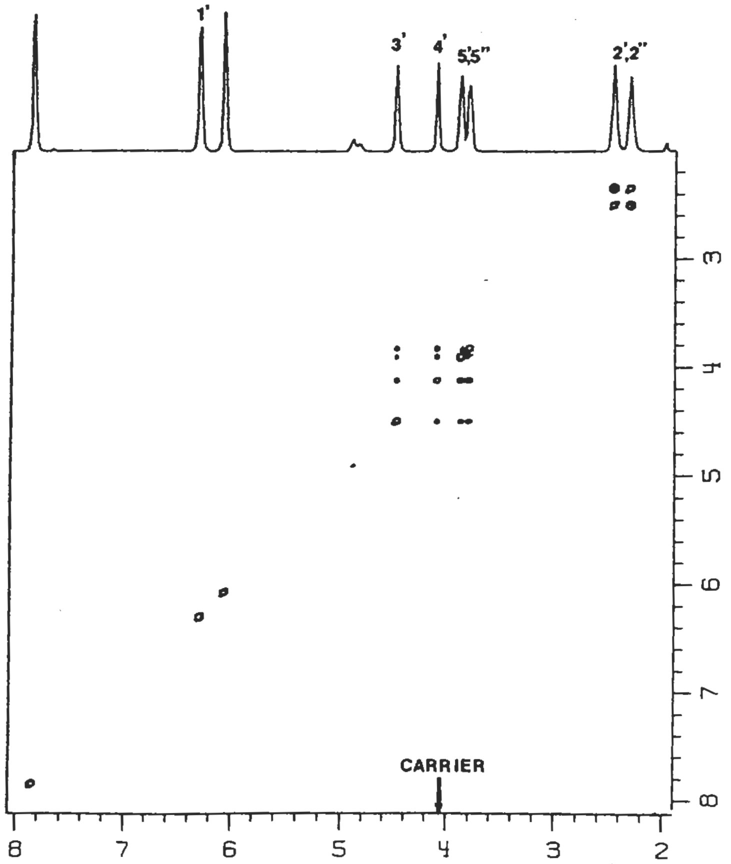


Figure 2 – Part of the two-dimensional spectrum of 2'-deoxycytidine, obtained with a mixing time of 100 ms. The position of the carrier frequency has been indicated on the $F_1$ axis. Magnetization transfer is restricted to protons that experience similar magnitudes of the effective spin lock field

### TABLE I

Phase, $\varphi$, of the initial 90° pulse, and the way data are added to and subtracted from memory in successive scans of the experiments sketched in Figure 3

| scan | $\varphi$ | Acq[a] | |
|---|---|---|---|
| 1 | x | + | |
| 2 | y | | + |
| 3 | -x | – | |
| 4 | -y | | – |

[a] Data in odd- and even-numbered scans are stored in separate memory locations

This limits the straightforward application of homonuclear Hartmann Hahn spectroscopy to spins that have a relatively large coupling and similar magnitudes for the effective rf field strength, i.e. similar magnitudes for the rf offset from the carrier frequency. An example of such a case in Figure 2, for the nucleotide 2' deoxycytidine. The pulse scheme $90^\circ_\varphi - t_1 - SL_y -$ acquire($t_2$) has been employed, with standard phase cycling of $\varphi$ (Table 1) to allow a hypercomplex Fourier transformation (13,16,17) for obtaining an absorption mode 2D spectrum. In this experiment the carrier frequency has been positioned in the center of the 3', 4' and 5', 5" region and a 100 ms spin lock pulse with a rf field strength of approximately 6 kHz has been used. Despite the fact that the coupling between the 3' and 4' protons is rather small (several Hertz), an intense cross peak between the 3' and 4' protons is observed. Cross peaks between the 3' and the 5', 5" protons are also observed, and are due to relay effects: magnetization that has been transferred from the 3' to the 4' proton during the first part of the mixing period is subsequently transferred to the 5', 5" protons during the second part of the mixing period. For very short durations of the mixing period ($\ll 1/J$), these relay effects are absent. Cross peaks between the 2', 2" protons are also observed (large J and small difference in offset frequency), but no cross peak is observed between the 3' and the 2', 2" protons. This is caused by the Hartmann Hahn mismatch, induced by the difference in offset frequencies of the 3' and the 2', 2" protons. The selectivity of this type of Hartmann Hahn experiment can be beneficial: a mismatch between the 2',2" protons enhances the cross peak intensity between the 3' and 4' protons because no 3' magnetization is drawn away by the 2', 2" protons. A major advantage of the homonuclear Hartmann Hahn experiment over the COSY experiment is that net magnetization transfer is obtained, allowing phase sensitive spectra to be recorded, and yielding improved sensitivity in the case of poorly resolved resonances. Several points should be mentioned here:

1. The individual components of a cross multiplet are generally not pure absorptive, but consist of a mixture of absorptive and dispersive components (8,13,14). The dispersive components are in antiphase relative to one another and these unwanted components therefore largely cancel. However, at very high digital resolution and for well resolved couplings this phase distortion is clearly visible. For simple spin systems ($AX, AX_2, AX_3, AMX$) explicit expressions can be derived from density matrix calculations. For a two-spin system these results have been presented by Braunschweiler and Ernst (8). The $AX_2$ case has recently been discussed by Chandrakumar and Subramanian (18).
2. Because the total amount of spin locked magnetization is a constant of motion, magnetization transfer between spins is an exchange process, and the absorptive part of the cross multiplet will be in

phase with the diagonal resonances. As has been pointed out by Bothner-By and co-workers (19), one can use the same pulse sequence as discussed above for measuring transverse NOE effects. Transverse NOE effects are positive for all values of the motional correlation time, $\tau_c$, and therefore give rise to negative cross peaks. Hartmann Hahn and spin locked NOE cross peaks are thus easily distinguished (20,21). In practice, we have always found that for the mixing times most commonly used in the Hartmann Hahn experiment (15-100 ms), the size of NOE cross peaks is usually very small and does not present any serious problems. Cross peaks due to chemical exchange during the spin lock period (22,23) also give rise to positive cross peaks that should not be confused with Hartmann Hahn cross peaks. Note that NOE peaks have opposite phase relative to exchange peaks, independent of the motional correlation time, $\tau_c$. This feature immediately distinguishes these two types of magnetization transfer mechanisms (24).

## REMOVAL OF THE HARTMANN HAHN MISMATCH

Above, it was pointed out that the effectiveness of homonuclear Hartmann Hahn type coherence transfer rapidly decreases if the difference in effective rf field strengths for two coupled spins becomes larger than the scalar coupling. Consequently, only relatively narrow spectral bandwidths can be covered. A solution to this problems is to alternate the rf phase of the spin lock field along the $\pm y$ axis (Figure 3b) (21). We will discuse the reasoning behind this phase alternation in a qualitative manner, based on the Heisenberg vector picture.

As pointed out before, strong homonuclear coupling occurs if the Heisenberg spin operators $I_x$ and $S_x$ precess at similar rates about the static magnetic field (or the spin lock field) and diverge slowly relative to 1/J. As was found several decades ago, one can induce strong coupling effects by the application of a series of 180° pulses that are spaced closely relative to the difference in resonance frequency, $|\Delta_I - \Delta_S|$ (25,26). This type of mixing scheme is also used in the TOCSY scheme, proposed by Branschweiler and Ernst (8). In the Heisenberg vector picture, this strong coupling effect is easily understood: the 180° pulses prevent the $I_x$ and $S_x$ (and the $I_y$ and $S_y$) components from dephasing, and hence the $I_xS_x$ (and the $I_yS_y$) term does not disappear, i.e. strong coupling is induced.

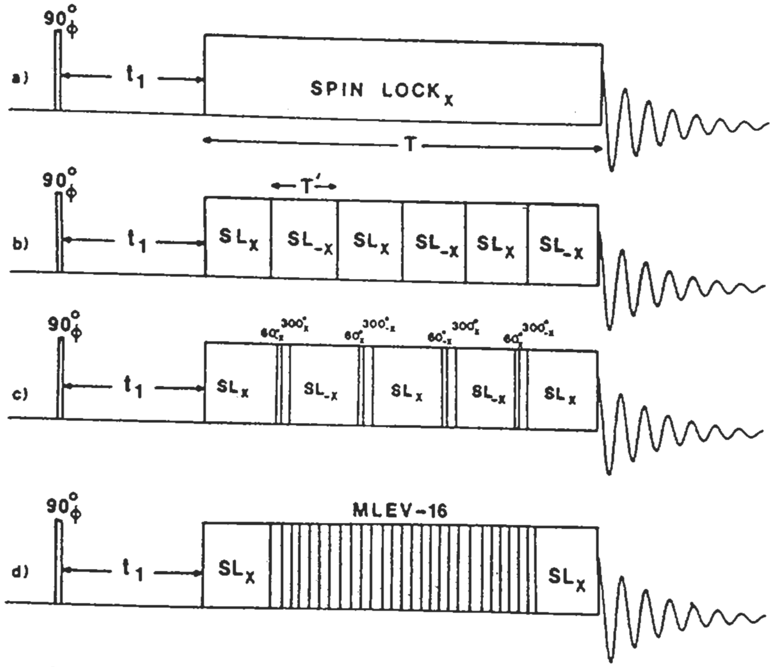


Figure 3 - Various 2D pulse schemes for establishing HOHAHA magnetization transfer.
(a) using a continuous spin-lock field,
(b) a phase alternated spin lock field,
(c) a compensated phase alternated spin lock field,
(d) an MLEV sequence with trim pulses

In the homonuclear Hartmann Hahn experiment, where the spin lock field is applied along the y axis (relabeled for convenience as z'), the $I_{x'}$

and $S_{x'}$ operators precess at different rates about the z' axis if the effective field strengths differ. However, the dephasing of $I_{x'}$ and $S_{x'}$ can be refocused by changing the phase of the spin lock field (Fig. 3). This causes the precession of the $I_{x'}$ and $S_{x'}$ vectors to reverse, and prevents the product $I_{x'}S_{x'}$ from averaging to zero. For effective Hartmann Hahn mixing, the $I_{x'}$ and $S_{x'}$ vectors should not diverge more than about $\pi/2$, and hence, one wants to alternate the phase of the spin lock field at rate, $1/\tau'$, given by

$$1/\tau' > 4|\nu_I - \nu_S| \qquad (9)$$

For $1/\tau' = |\nu_I - \nu_S|$ no Hartmann Hahn type coherence transfer will occur, and for $1/\tau' = 2|\nu_I - \nu_S|$, the rate will be reduced by about 30% relative to the matched Hartmann Hahn transfer rate. Note that in Eq. (8) the size of the coupling does not appear. As mentioned before, however, the transfer rate is directly proportional to the size of the scalar interaction.

In order to obtain 2D absorption mode spectra, it is important that only the magnetization components parallel to the spin lock axis remain, i.e. transverse components have to be defocused. Phase alternation of the spin lock field causes the introduction of rotary echoes (27) for magnetization that is transverse to the spin lock field. In principle, these echoes contain useful signal (10) since they provide the information about magnetization that is perpendicular to the spin lock field, i.e. this signal provides the complementary half needed for quadrature detection in the $t_1$ dimension. In practice, however, we find that the decay of these echoes occurs much faster than $T_{1\varrho}$ (caused by experimental imperfections) and we therefore try to eliminate them in order to allow the recording of absorption mode spectra. These rotary echoes can be effectively removed by applying a "trim pulse" of approximately 3 ms at the end of the mixing period, with the same rf phase as the spin lock pulses. The inhomogeneity of the rf field will defocus magnetization that is perpendicular to the axis along which the trim pulse is applied (with the exception of certain antiphase multiplet components) and spectra are therefore modulated in amplitude as a function of $t_1$, and have the required format for obtaining 2D absorption mode spectra (13,16,17).

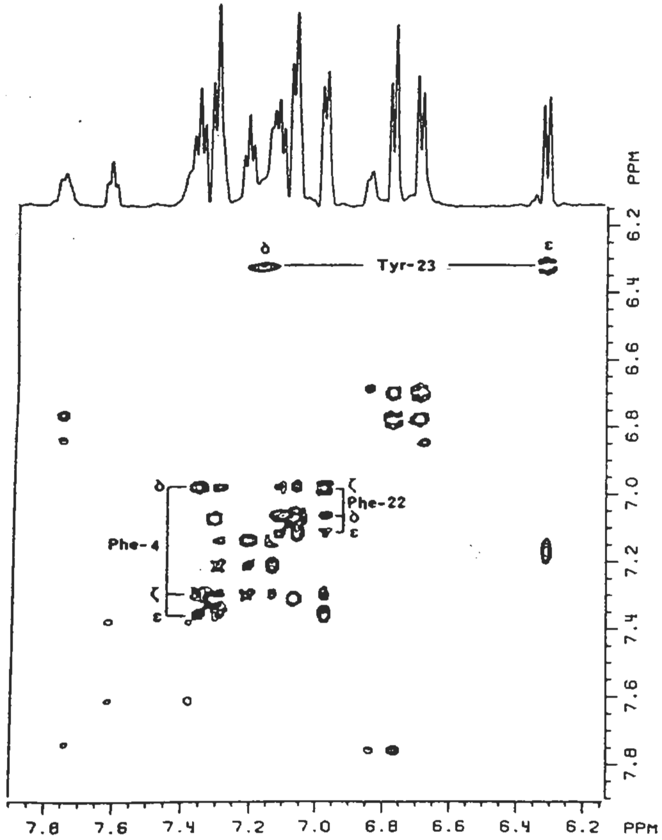
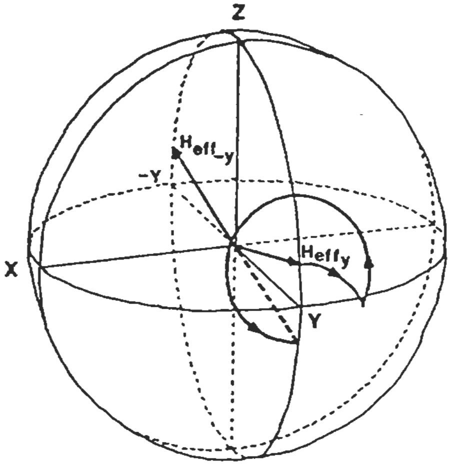


Figure 4 - 2D HOHAHA spectrum of the aromatic region of a 5 mM solution of bovine trypsin inhibitor in $D_2O$, recorded at 500 MHz, 42°C, pH 4.6, using a 35 ms spin lock period. The spectrum confirms the reassignment of the Phe-4 protons by Rance et al.(6), and additionally suggests that resonances of Phe-22 should be reassigned as indicated

The phase alternated spin lock version of the Hartmann Hahn experiment makes it possible to cover fairly large spectral widths. On our 500 MHz spectrometer, and 8 W linear amplifier was used for generating the spin lock rf field of approximately 7.5 kHz rf field strength. Although the amplifier generated 8 W, 6 dB of this power was lost in the electronic circuitry (tuned diodes, bandpass filter, gating circuitry) and only 2 W rf power was dissipated in the high resolution probehead of our 500 MHz $^1$H probe. With a 7.5 kHz rf field strength we can cover a bandwidth of approximately ±1 kHz, i.e. ± 2 ppm on our instrument, if the spin lock phase is alternated every 5 ms (21). As an example, Fig. 4 shows the Hartmann Hahn spectrum obtained for the aromatic region of a 8 mM solution of bovine trypsin inhibitor, a compound extensively investigated in the past by Wüthrich, Wagner, and co-wokers (28). The spectrum, recorded at 42 °C and using a 35 ms mixing time, shows the connectivities within each of the aromatic rings of the Tyr and Phe residues. The spectrum is displayed in the absorption mode and shows the excellent resolution that is obtained with this method. The net magnetization transfer provides high sensitivity because no mutual cancellation of the absorptive components of the cross multiplet occurs. This allows the observation of a cross peak between the δ and ε protons of Tyr-23, even while the resonances of the δ protons are broadened by slow flipping of the ring. In a COSY spectrum this cross peak could not be observed at this temperature. For the Phe residues, relay peaks are also observed. For example, for Phe-22 the ζ proton (7.00 ppm) shows connectivity to both the ε protons (7.15 ppm) and relay peaks to the δ protons (7.09 ppm). The fact that both the δ protons and the ε protons have identical chemical shifts suggests, contrary to earlier assumptions (29), that this residue is flipping fast relative to the difference in chemical shift frequency.

## COMPENSATED PHASE-ALTERNATED SPIN LOCKING

As mentioned above, with the phase alternated spin lock mixing sequence, a substantial bandwidth can be covered. The typical spectral width for many compounds of interest is about 8-10 ppm. On the basis of Eq.(8), one then calculates that one has to alternate the rf phase as often as every millisecond or faster. Sice the effective rf field vectors for spin locking along the x and -x axes are not 180° out of phase relative to one another in the case of resonance offset (Fig.5), during each phase alternation some magnetization is lost. For resonance offset, $\Delta$ , the effective field vectors are at an angle of approximately $\pi-2\Delta/\nu$ , and during each phase alternation a fraction $1-\cos(2\Delta/\nu)$ is lost. For large offsets and relatively rapid phase alternation this leads to a rapid decay of spin locked magnetization. One way to avoid this problem is to rotate the magnetization that is initially locked along the +x effective rf field to the -x effective rf field axis. A simple way to accomplish this is the insertion of a $60°_{-x}300°_{x}$ pulse pair after the spin lock along the +x axis (Fig. 3c). The rotation by such a pulse pair of magnetization, initially parallel to the effective spin lock field, to the new effective field vector with inverted rf phase, is sketched in Fig. 5. Similarly, a $60°_{x}300°_{-x}$ pulse pair is inserted when the phase is changed from -x to +x. In pratice, we "fine tune" both the 60° and the 300° pulse by maximizing the spin locked signal for an isolated resonance that is approximately 2 kHz off resonance, using for example, 1.5 ms $\tau'$ values and about 40 phase alternations. This approach allows us to cover about $\pm2$ kHz (with a 7.5 kHz rf field), and is sufficient for most applications. For covering narrower spectral widths, no fine tuning of the 60° and 300° pulses appears necessary.



$(SL_Y\,60°_{-Y}300°_Y\,SL_{-Y}60°_Y300°_{-Y})_n$

Figure 5 - The rotation of magnetization, initially parallel to the y effective field, to the effective field direction for a -y rf field (parallel to the boldface broken line). The $60°_{-y}300°_{y}$ pulse pair rotates magnetization from the y effective field to the -y effective field direction. A counterclockwise rotation of magnetization about the magnetic field is assumed

As an example, Fig. 6 shows the entire 2D spectrum of the bovine trypsin inhibitor, recorded in 3 h., and using a mixing time of 34 ms. The sample has been dissolved in $D_2O$, at pH 4.6, and only the non-exchanging amide protons are visible in this spectrum. Connectivity between the amide and $\alpha$ protons can be seen, and significant amounts of relay to the $\beta$ protons is also observed. At lower contour levels (not shown) nearly all amide-$H(C_\beta)$ relay connectivities are observed. The inset shows the connectivity between the $\delta$ and $\varepsilon$ protons of Tyr-23, and shows the broadening of the $\delta$ protons due to slow ring flipping. Similar broadened cross peaks are observed for Phe-45 (at about 8ppm). The low intensity negative resonances in the inset are not due to spin locked NOE but are a common feature in many types of 2D absorption mode spectra. These negative "ditches" running parallel to the $F_2$ axis, are caused by the fact that the first couple of points of the free induction decay (acquired during $t_2$) are too low in intensity, caused by the filter bandwidth of the audio filters. Using a wider filter bandwidth and minimizing the delay between the end of the mixing period and the opening of the receiver gating can be used to minimize these artefacts.

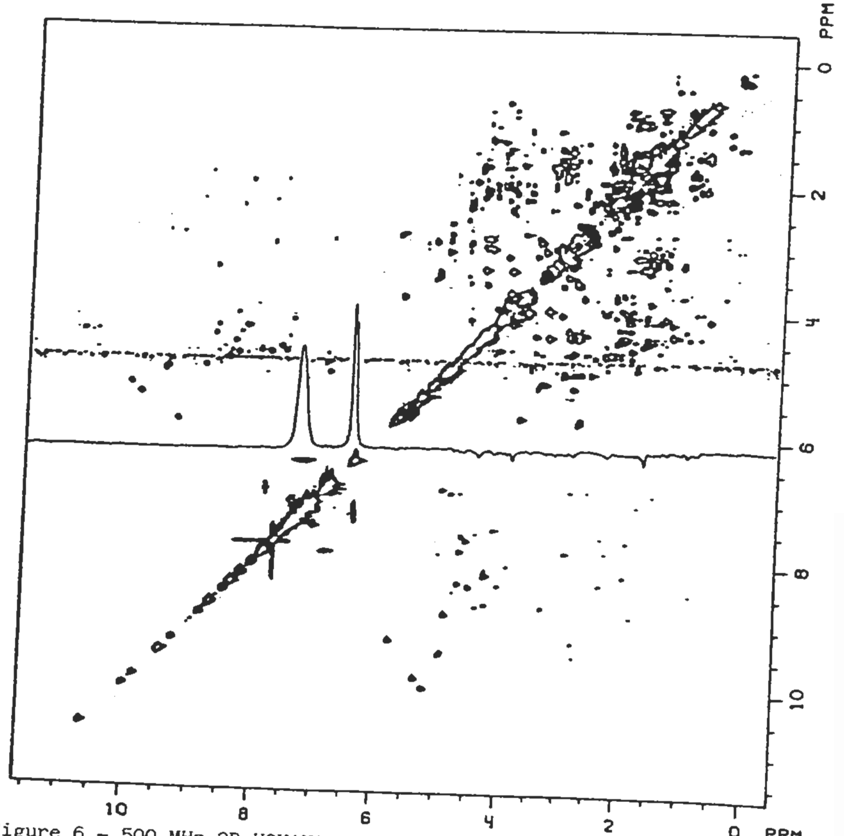


Figure 6 – 500 MHz 2D HOHAHA spectrum of 8 mM bovine trypsin inhibitor in $D_2O$, pH 4.6, at 37°C, using a 34 ms mixing period and $\tau$' durations of 1.5 ms. The total measuring time was 3 h. The inset shows a $F_1$ section taken at the $F_2$ frequency of the Tyr-23 $C_\varepsilon$ protons and displays the connectivity to the $C_\delta$ protons, despite the broadening introduced by intermediately slow ring flipping

## MIXING WITH A MLEV-16 COMPOSITE PULSE CYCLE

The major limitation in the application of homonuclear Hartmann Hahn mixing is the decay of the magnetization during the spin lock, with decay constant $T_{1\rho}$ which is approximately equal to the transverse relaxation time $T_2$. However, mixing schemes can be developed that prolong this apparent decay constant, the theoretical limit being $T_1$. The best we have been able to achieve, so far, is a "spin lock" period using a MLEV-16 composite pulse decoupling cycle (31), instead of the phase alternated spin lock field (Fig. 3d). A single MLEV-16 cycle consists of a repetitive series of 16 composite pulses, A and B, applied in the order: AABB ABBA BBAA BAAB. The composite pulse A consists of $90°_x 180°_y 90°_x$, and B is the inverse of this: $90°_{-x} 180°_{-y} 90°_{-x}$. The net effect of A or B is a rotation of 180° about the +y (A) or -y (B) axis. Thus, the MLEV sequence can be considered as a rapidly phase alternating spin lock sequence along the $\pm y$ axis. However, composite pulses A and B rotate magnetization vectors, initially parallel to the y axis, through space in such a way that effectively one half of the time the magnetization is aligned along the static magnetic field. During this time, the relaxation is determined by $T_1$, which for macromolecules is usually much longer than $T_{1\rho}$ and $T_2$. In the limit, $T_1 \gg T_{1\rho}$ one can therefore expect an apparent lengthening of the decay constant by up to ~100%. Experimentally, we have observed a lengthening of 96% for the non-exchangeable amide protons in ribonuclease. Unfortunately, the working of the MLEV sequence for obtaining efficient magnetization transfer between homonuclear coupled spins is very sensitive to pulse imperfectione (imperfect 90° phase shifts and unbalanced rf amplitudes) and therefore requires properly adjusted hardware. As shown elsewhere (31), these pulse imperfections during MLEV mixing can slow down the transfer rate and can also cause difficulties in obtaining absorptive spectra. However, the

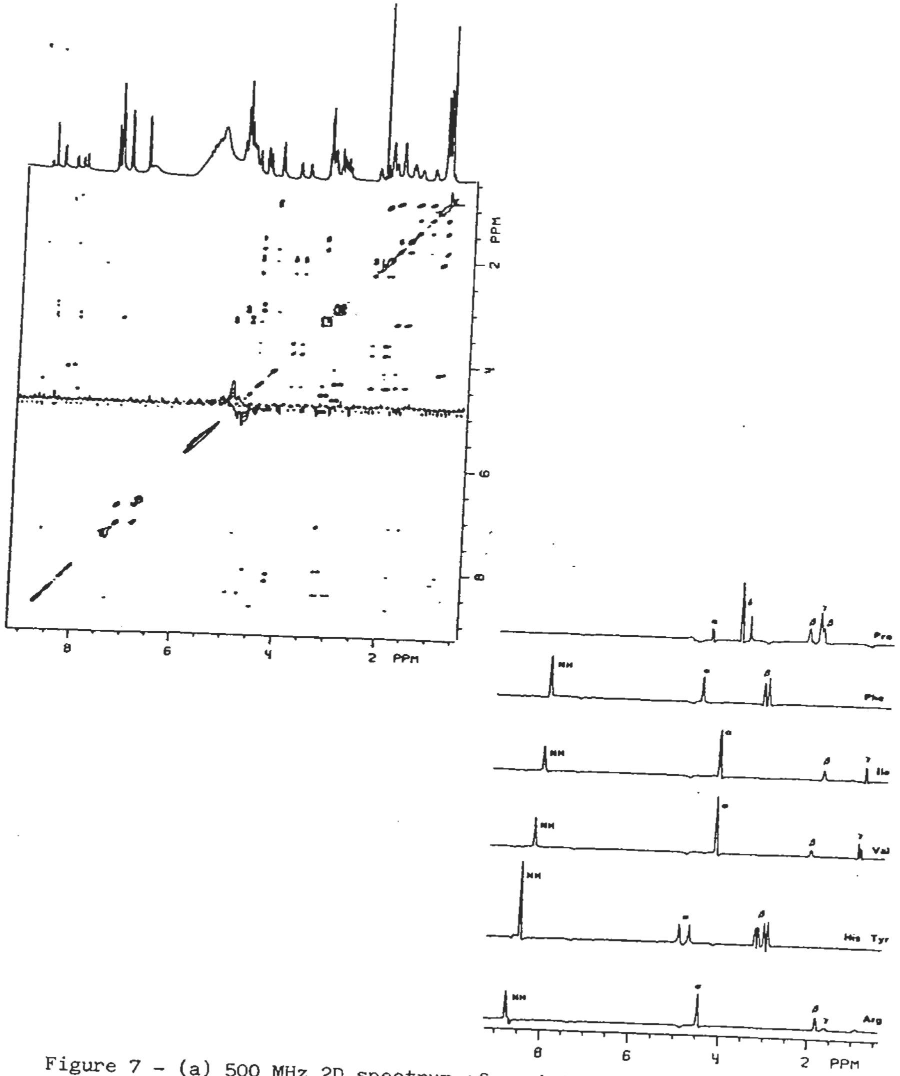


Figure 7 - (a) 500 MHz 2D spectrum of angiotensin-II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) obtained with an MLEV mixing period of 54 ms, preceded and followed by a 3 ms trim pulse. The sample was dissolved in 80%$H_2O$/20%$D_2O$, pH 3, at 30°C. The total data acquisition time was 2h.

(b) Sections parallel to the $F_1$ axis through the 2D spectrum, taken at the $F_2$ frequencies of the various amide protons and the high-field Pro $C_\delta$ proton

advantage of lengthening the decay constant during the mixing period may be crucial in the study of macromolecules. Improvements to the MLEV sequence that will make it less sensitive to pulse imperfection are currently under investigation.

An example of a 2D spectrum of angiotensin-II, a linear octapeptide, dissolved in 90% $H_2O$/10% $D_2O$, obtained with a MLEV mixing period of 54 ms is shown in Figure 7a. Intense relay peaks between NH and $C_\beta$ and $C_\gamma$ protons are observed. Cross sections parallel to the $F_1$ axis taken at the $F_2$ frequencies of the various amide resonances provide partial subspectra of the various amino acids (Figure 7b).

## GENERATION OF ONE-DIMENSIONAL SUBSPECTRA

The spin propagation idea can easily be used to generate subspectra of parts of a molecule that are not scalar coupled to other parts. For example, it is possible to generate subspectra of individual sugar units in an oligosaccharide, or individual amino acids in a peptide. The idea is very similar to 1D NOE difference spectroscopy: a selective 180° pulse is used to invert the $^1H$ multiplet of one particular hydrogen, and a 90° pulse followed by a propagation period of variable length is employed. This spectrum is then substracted from a spectrum obtained in an identical way, but without the selective 180° pulse (Fig. 8). Only magnetization that originates from the inverted $^1H$ multiplet will appear in the difference spectrum. For short propagation periods ( $< 25$ ms), magnetization that has not propagated much further than to geminal or vicinal protons will appear in the difference spectrum. For longer propagation delays (100-200 ms), one can obtain entire subspectra of individual parts of the molecule. As an example, Fig. 9 shows how the spectrum of sucrose can be separated into a glucose and a fructose subspectrum. Note that in the fructose subspectrum tha C1 protons are absent because coupling to the C3 proton is vanishingly small.

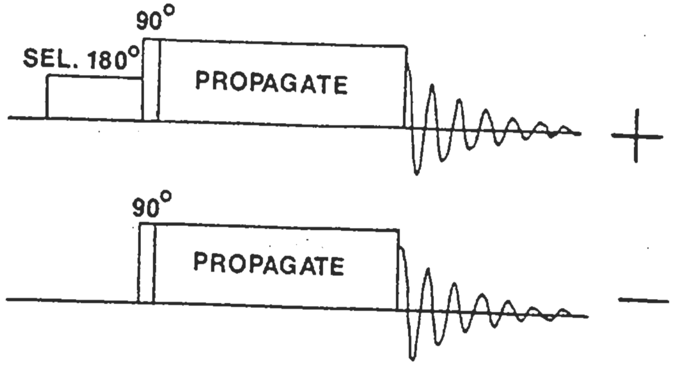


Figure 8 - Pulse scheme for the generation of one-dimensional subspectra. Any of the mixing schemes used in Fig.3 can be used to induce spin propagation. A difference experiment, with and without a selective 180° pulse, generates a subspectrum of all protons directly or indirectly coupled to the selectively inverted proton

We find the one-dimensional propagation method very useful for $^1H$ resonance assignment in complex overlapping spectra of rather small molecules ($M_r < 1500$) that often have a relatively long $T_{1\varrho}$ and permit the use of 1D spin propagation without excessive loss in sensitivity.

## DISCUSSION AND CONCLUSIONS

Above, the idea of spin propagation has been discussed in very qualitative terms and a attempt has been made to convey some of the ideas that are helpful in understanding the propagation mechanism. A more rigorous treatment requires diagonalization of the propagation Hamiltonian and generally requires a substantial amount of computer arithmetic. An advantage of the computer analysis of the problem is that resonance

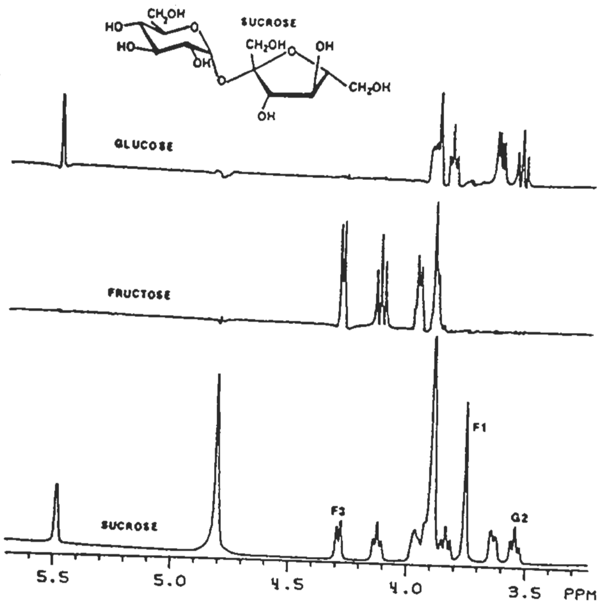


Figure 9 - Spectra of sucrose.(a) Regular $^1H$ spectrum and (b) propagation spectrum of fructose, obtained by selective inversion of H3 and (c) propagation spectrum of glucose, obtained by inverting glucose H2. Propagation periods of 100 ms were used for both spectra (b) and (c), and the compensated alternated spin lock mixing (Fig.3c) was used. Note that fructose protons H1 do not appear in (b) due to the absence of a scalar coupling pathway to the rest of the fructose protons

offset effects do not provide major additional problems. A second simple computer solution to describe the behaviour of the spin system during propagation is to use a numerical evaluation of the Liouville equation:

$$\dot{\sigma} = -i\,[\,\sigma, H\,] \qquad (10)$$

where no diagonalization of H is required. Using such computer calculations one can estimate the size of unresolved scalar couplings from the buildup rate of cross peaks in the 2D spectra recorded for a series of propagation delays. This application is currently under investigation.

We do not expect that the HOHAHA method will replace the COSY technique because the latter has the advantage of showing exclusively direct scalar connectivity, whereas the HOHAHA method can show weak relay peaks for mixing times as short as 15 ms. In this case it may not always be obvious whether such a peak originates from a direct coupling or whether it concerns a relay effect. However, it is expected that HOHAHA spectroscopy will be used as an additional device for cases where the COSY method does not provide an unambiguous answer. In our experience, the HOHAHA method appears more effective, both in sensitivity and resolution, than the homonuclear RELAY experiment (32-34) and we expect this RELAY method to become obsolete for most common cases of interest.

A large number of other, more complicated pulse sequences, based upon the homonuclear Hartmann Hahn effect can be envisioned. For example, all current techniques that rely on COSY type relay of magnetization can be modified in such a way that magnetization relay occurs via the Hartmann Hahn mechanism. For the heteronuclear $^{1}H-^{1}H-^{13}C$ relay experiment (35) such an experimental modification has shown to be very useful, especially when looking at saturated aliphatic ring systems (36).

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