

APPLICATIONS OF TWO-DIMENSIONAL NMR SPECTROSCOPY TO ORIENTED MOLECULES

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ABSTRACT

Two-dimensional (2D) NMR spectroscopy has proved exceedingly useful in the study of oriented molecules. In this article the applications of this technique to the study of molecules oriented in liquid crystalline solvent are reviewed. The main applications have been, (a) in simplification of NMR spectra by symmetry filtering, multiple quantum filtering and multiple quantum 2D spectroscopy, (b) in enhancing information content of spectra by spin-echo and correlated 2D spectroscopies, and (c) in the study of multiple quantum relaxation rates by 2D spectroscopy.

INTRODUCTION

TWO-DIMENSIONAL nuclear magnetic resonance (NMR) spectroscopy, in which NMR spectrum is obtained as a function of two-frequency variables, has had a profound effect on the study of biomolecules and other systems which yield complex NMR spectra. One such class is the NMR of molecules partially oriented in liquid crystal matrices. Such molecules have finite intramolecular dipolar interactions, with the intermolecular dipolar interactions averaged to zero. This yields a rich but tractable NMR spectrum which in turn contains detailed information on the conformation of the molecules¹. The spectra are often analysed using iterative computer programs such as LAOCOON and its various modifications¹.

Two-dimensional (2D) NMR methods have been employed, with significant advantage, for the study of spectra of molecules oriented in liquid crystal matrices. This article aims to review the use of the 2D methods in the study of oriented molecules. The main applications are: (a) the use of 2D resolved spectroscopy and 2D correlated spectroscopy, both of which enhance the information content of the spectra, (b) the simplification of the spectra of oriented molecules by symmetry filtering, multiple-quantum filtering and multiple quantum spectroscopy, and (c) study of multiple quantum relaxation rates of oriented molecules.

The methodology of 2D NMR spectroscopy has been very well described in two recent books^{2,3} and several articles⁴⁻⁸. Application of 2D NMR techniques to the study of biomolecules has also been extremely well described in another recent book⁹. The NMR of oriented molecules has been periodically reviewed in several articles and in specialist periodical reports¹⁰. This is a first dedicated review on the application of 2D techniques to oriented molecules and need-based periodic reviews may be planned in future.

Brief outlines of:

2D NMR techniques

In 2D NMR²⁻⁸ one obtains an NMR spectrum as a function of two frequency variables F_1 and F_2 rather than one. The main advantage of the technique is that information transfer takes place between various lines in the spectrum simultaneously in a non-selective manner. This is achieved by allowing the signals to precess in the evolution period t_1 , affecting the information transfer (by either a pulse, a series of pulses or a change of Hamiltonian) and detecting the signal during the detection period t_2 (figure 1). A complete set of time domain data matrix $s(t_1, t_2)$ is collected which on Fourier transformation with respect to t_1 and t_2 yields the two-dimensional spectrum $S(F_1, F_2)$.

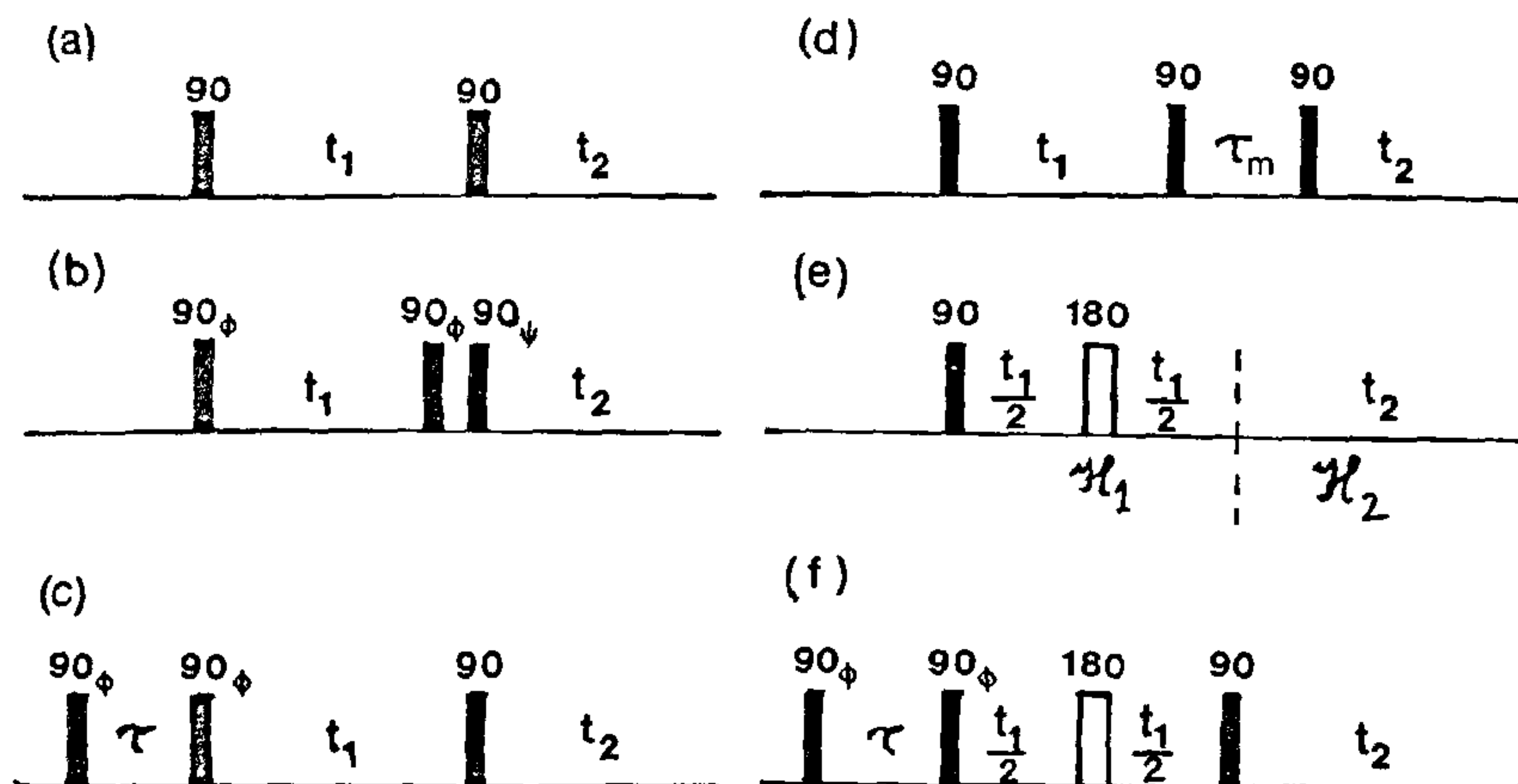


Figure 1. Pulse schemes for various 2D NMR experiments, (a) Correlated 2D spectroscopy (COSY), (b) Multiple quantum filtered COSY (MQFC), (c) Multiple quantum transitions spectroscopy (MQT), (d) Nuclear Overhauser effect spectroscopy (2D NOE or NOESY), (e) spin-echo 2D spectroscopy, (f) Spin-echo multiple quantum transitions spectroscopy (SEMQT). The various orders are separated by time proportional phase incrementation (TPPI).

Schemes (b), (c) and (f) require phase cycling for selection of the desired multiple quantum order, Scheme (d) requires phase cycling for cancellation of transverse and retention of longitudinal magnetization during τ_m . All schemes except (e) require phase cycling for cancellation of axial magnetization. Details of phase cycling can be obtained from ref. 2 and references therein.

The various types of two-dimensional experiments are distinguished by the nature of information transfer taking place in the experiment. If a non-selective coherence transfer is effected by the application of a pulse at the end of t_1 , then this leads to the correlated 2D spectroscopy (COSY), figure 1(a). If the coherence transfer is restricted through an intermediate state of well-defined multiple quantum order (achieved by the application of two pulses with well defined phases) then one obtains what is known as multiple-quantum-filtered COSY (MQFC): of which the most popular is the double-quantum-filtered COSY (DQFC), figure 1(b). On the other hand, if the multiple-quantum orders are allowed to precess during t_1 (generated prior to t_1 by two pulses with a fixed time interval between them) and a coherence transfer to detectable single-quantum transitions is effected by a pulse at the end of t_1 , then one obtains multiple-quantum-transitions (MQT) 2D spectroscopy, figure 1(c). If the information transfer is a

relaxation transfer (achieved by the application of two pulses at the end of t_1 with a fixed interval τ_m between them — the mixing period, and retaining only longitudinal magnetization during τ_m), one obtains nuclear Overhauser effect 2D spectroscopy (2D NOE or NOESY), figure 1(d). If on the other hand no pulse is applied at the end of t_1 and only the effective Hamiltonian of the spins is changed from t_1 to t_2 , one obtains what is known as resolved 2D spectroscopy, figure 1(e).

Spectra of molecules oriented in liquid crystal matrices

Solute molecules partially oriented in liquid crystalline media exhibit a high order of anisotropic reorientations and significant translational diffusion, as a result all intermolecular interactions are averaged, in the first order, to zero, like in liquids, while the intramolecular interactions are anisotropically averaged to residual values dependent on the value of order parameter. The effective Hamiltonian

for this case can be represented as

$$\mathcal{H} = \mathcal{H}_z + \mathcal{H}_J + \mathcal{H}_D + \mathcal{H}_Q, \quad (1)$$

where the first term is the Zeeman term containing the chemical shift $(1 - \sigma_i - \sigma_{ia})$ of the spins, the second is the indirect spin-spin coupling (J_{ij}) term, the third describes the intramolecular dipolar interactions (D_{ij}) and the last is the quadrupolar interaction term. σ_i and J_{ij} represent one third of the traces of corresponding tensors and are identical to the chemical shift and indirect spin-spin couplings observed in high resolution NMR of liquids (isotropic cases). σ_{ia} is the anisotropic part of the chemical shift and for axially symmetric tensor has two components σ_{\parallel} and σ_{\perp} . For spin 1/2 nuclei the quadrupolar term is absent. The intermolecular dipolar interactions are averaged to zero due to large translational diffusion of the molecules. The intramolecular dipolar interactions are reduced to small but finite values governed by the order parameter which defines the degree of orientation of molecules. The high resolution proton spectra of oriented molecules thus exhibit sharp lines with many more lines than the corresponding isotropic spectrum. However, unlike the isotropic case, the spectrum is often not a first order spectrum mainly because the magnitude of the intramolecular dipolar interactions is usually comparable to or larger than the chemical shift differences. This necessitates explicit diagonalization of the Hamiltonian matrix to calculate

the spectra and iterative computer programs to find a fit to the experimental spectra. In the case of spins with $I > 1/2$, the quadrupolar interaction dominates the spectra and the dipolar interactions give additional splittings (1).

2D SPIN-ECHO RESOLVED SPECTROSCOPY

Single-quantum transitions of spin 1/2 systems

The first application of two-dimensional techniques for the study of oriented molecules was a calculation and observation of the 2D spin-echo spectrum of a coupled two-spin system (each spin having $I = 1/2$) (figure 2), showing that due to strong coupling features inherent in the spectra of oriented molecules, one gets additional lines in the 2D spin-echo spectrum some of which appear with negative intensity and that the spin-echo 2D spectrum gives additional information useful in the analysis of the spin-systems^{11,12}. In the above example it was shown that one obtains 8 lines in the 2D spectrum, 4 of which have equal intensities, the difference in the frequencies of which unambiguously determines the coupling parameter $(J + 2D)$, an information which could not be obtained from 1D single resonance spectrum.

A related experimental study, not utilizing two-dimensional algorithm but recording spin-echo spectra of AA'BB' spin systems of 4-cyanopyridine, and 4-acetylpyridine-d₃ (CD₃)

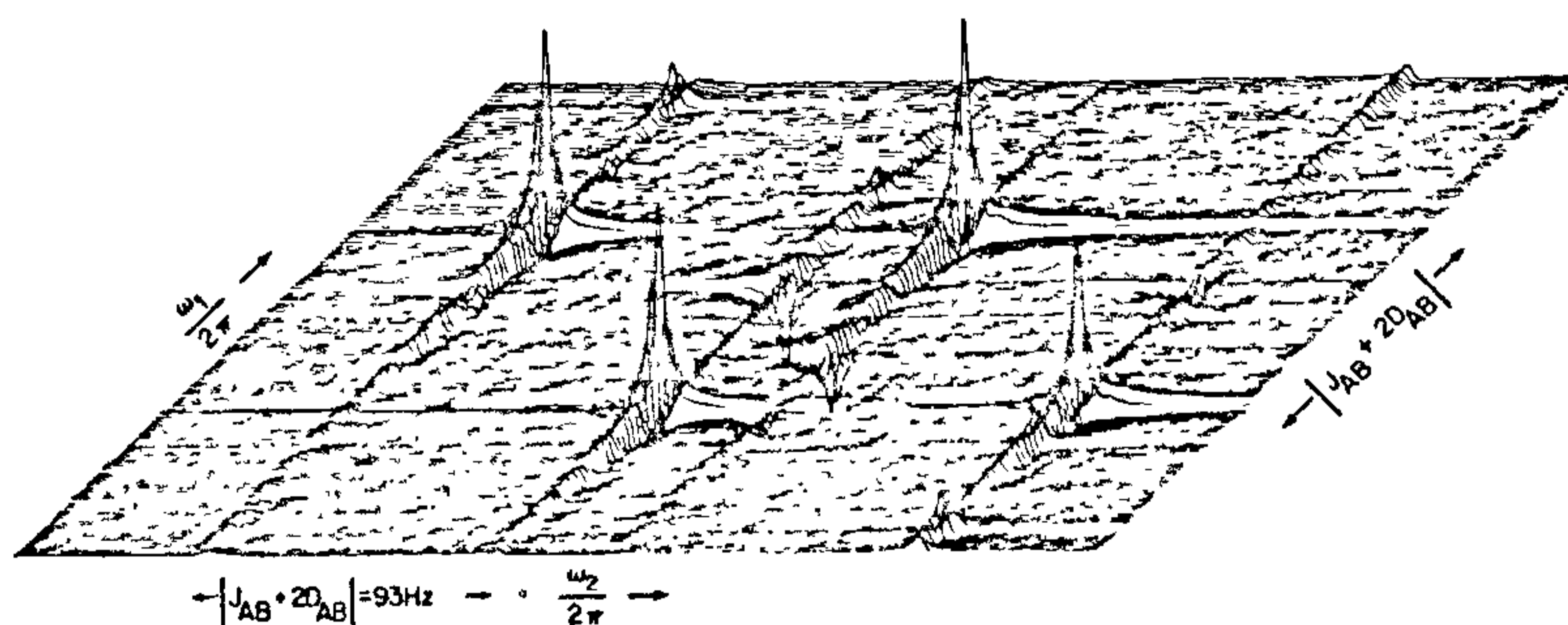


Figure 2. Spin-echo 2D spectrum of two strongly coupled protons of 1-thio-3-selenole-2-thione oriented in MBBA [obtained using scheme (e) of figure 1] recorded at 270 MHz. The phase sensitive spectrum gives 8 lines, 2 of which are of negative intensity, in conformity with the calculations (11) [From ref. 12].

dissolved in phase V*, 4-[$^2\text{H}_1$] pyridine dissolved in E5* and fluorine echo spectra of 2,3,5,6-tetra fluorothioanisole- d_3 dissolved in E5 have been recorded and analysed using modified LAOCOON computer programs¹³. Significant r.f. field inhomogeneity gave rise to artifacts in the spectra which were simulated as error in the 180° refocusing pulse and the spectra were fit to variable angle α of the refocusing pulse in the spin-echo sequence $90-t-\alpha-t$. The angle α varied between 150° and 162° for a good fit. The feasibility of such an analysis was demonstrated as an alternative to spin decoupling for measurements of homonuclear dipolar couplings¹³.

Single and multiple quantum transitions of spin 1 system of deuterium

The quadrupolar splittings and the chemical shift of deuterium have been separated out by two methods using 2D spectroscopy¹⁴. The first method utilizes the sequence $90^\circ-(\pi/2)-180^\circ-(\pi/2)-90^\circ-t_1-90^\circ-t_2(\text{aq})$. In this method the double quantum coherence is excited in the preparation period by the sequence $90^\circ-(\pi/2)-180^\circ-(\pi/2)-90^\circ$, which is frequency-labelled during t_1 period, at the end of which a coherence transfer is effected by the third 90° pulse into detectable single quantum coherences of deuterium, which are detected during period t_2 . The double quantum frequency during t_1 depends only on twice the chemical shift $[2(\Delta\omega)]$ and is free of quadrupolar coupling while the single quantum frequencies in t_2 depend on both quadrupolar coupling and chemical shift (contains two transitions at frequencies $\Delta\omega \pm \omega_q$). Two-dimensional spectroscopy allows separation of these two interactions in two frequency dimensions. The experiment has been applied to identify the quadrupole split, chemically shifted resonances of seven non-exchangeable deuterons of per-deuterated mannose partially oriented in a liquid crystal matrix. Because of low chemical shift dispersion and larger linewidths of the double quantum coherences, the separation of the chemical shift in these experiments was limited¹⁴. In the second method utilizing the

sequence $90^\circ(\phi_1)-t_1/2-90^\circ(\phi_2)-t_1/2-t_2(\text{aq})$, quadrupolar echoes are formed at the end of the t_1 period. The quadrupolar interaction is focussed at the echo point and the t_1 domain contains only the chemical shifts, while the t_2 domain contains both chemical shifts and quadrupolar interactions. This experiment also yielded only a limited resolution in the F_1 axis¹⁴.

In another study, separation of dipolar and quadrupolar splitting in partially deuterated and oriented systems (CD_2HI oriented in Merck Phase IV* liquid crystal) has been carried out by using 2D NMR techniques for simplification of the spectrum¹⁵. The pulse scheme used is shown in figure 3a. Heteronuclear decoupling during the double quantum preparation period τ facilitates selection of τ for optimal creation of double quantum coherence of interest and during t_2 eliminates $^1\text{H}-^2\text{H}$ dipolar interactions. The evolution of double quantum (DQ) coherences is studied during the period t_1 . Simultaneous application of proton and deuterium π pulses in the middle of the t_1 period refocusses off-set effects, without affecting the evolution of the double quantum coherences. The last 90° pulse converts the DQ coherences into observable deuterium signals. By the use of phase cycling, only DQ coherences are retained during the t_1 period. The F_1 axis contains only dipolar splittings while the F_2 axis contains the quadrupolar splitting and chemical shift. A clear-cut separation of these interactions was obtained in the 2D spectrum (figure 3b), allowing the homo- and heteronuclear dipolar couplings and the quadrupolar coupling to be measured directly from the spectrum. The use of spin-echo and DQ precession allows high resolution to be obtained in F_1 dimension resolving the various dipolar couplings¹⁵.

Study of deuterium relaxation

In order to study the molecular motions the deuterium relaxation has been a subject of several studies¹⁶⁻¹⁹. The deuterium relaxation is studied using spin-echo sequences, for the single and multiple quantum coherences of deuterium. In the first of these studies the

* Eutectic mixtures of Merck liquid crystals.

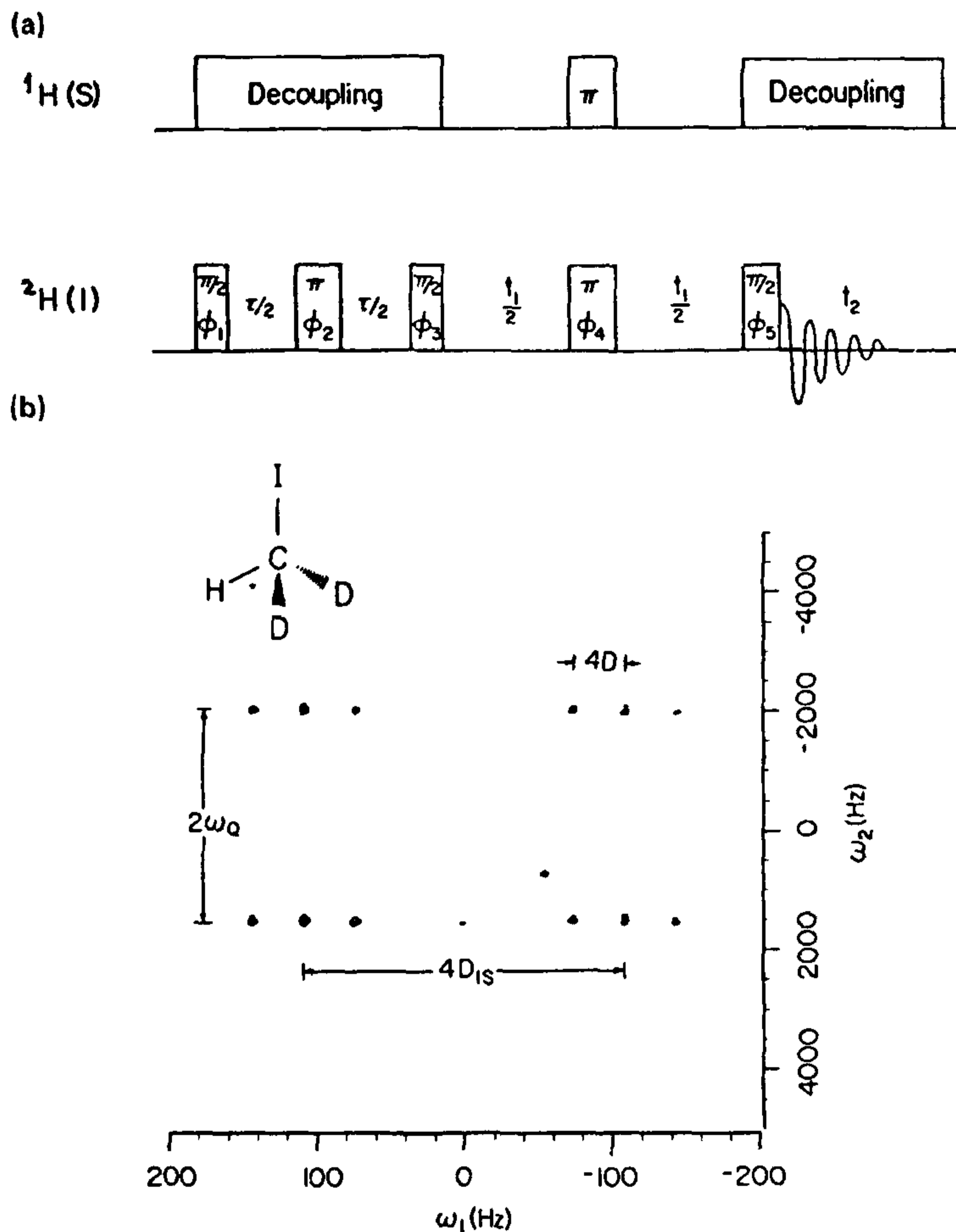


Figure 3a, b. (a) Pulse sequence for obtaining double quantum spectrum of deuterium via two-dimensional MQT spectroscopy which yields a separation of dipolar and quadrupolar splittings along ω_1 and ω_2 axes respectively, of partially deuterated molecules oriented in liquid crystal phase. The phase of pulses is cycled such that all unwanted signals (other than double quantum) are suppressed from ω_1 dimension (2). The resulting spectrum of CD_2HI , oriented in Merck Phase IV, recorded at 11.7 tesla, is shown in (b) [from ref. 15].

relaxation rate of the DQ coherence of deuterium of CDCl_3 oriented in liquid crystal matrix was studied by using multiple-quantum spin-echo spectroscopy¹⁶. DQ coherence was excited by the sequence $90^\circ\text{-}\tau\text{-}90^\circ$. The excited DQ coherence was allowed to evolve during the detection period t_1 , at the end of which another

90° pulse affects a coherence transfer to single quantum coherences detected in period t_2 . Phase cycling was employed to cancel the axial and single quantum peaks¹⁶ of period t_1 . The spin-echo of deuterium DQ was created by an α pulse in the middle of t_1 period and it was shown that for $\alpha = 180^\circ$, complete refocusing

of the DQ coherence takes place. The decay rate of this spin-echo DQ coherence, free of magnetic field inhomogeneity was shown to be due to quadrupolar relaxation mechanism¹⁶. This was extended to the study of relaxation of two coupled deuterons of CDCl₂ partially oriented in a liquid crystal matrix¹⁷. The relaxation of the coupled deuterons is governed by three auto-correlation and three cross-correlation spectral densities. Two of the auto correlation spectral densities were measured by the use of semiselective and nonselective inversion recovery experiments while the remaining spectral densities were determined by 2D techniques using both single and double quantum spin-echoes. The single quantum echoes were obtained by the sequence $\pi/2-t_1/2-\pi-t_1/2-t_2$ (aq) with 4 step EXORCYCLE to cancel the artifacts arising due to imperfections of pulses². The widths of the lines along the t_1 dimension yield the relaxation rates from which the two additional spectral densities could be extracted. The DQ coherence was selectively excited by a weak DQ pulse applied near the resonance. The spin-echo of the excited DQ coherence was obtained by applying a π pulse in the middle of the t_1 period. The 90° pulse at the end of t_1 as usual affects coherence transfer to single quantum coherences detected during t_2 . The line-width of the DQ spin-echo spectrum in the F_1 dimension was used to obtain the relaxation rates, which were used to extract the remaining spectral densities. Limited information on the motional processes which contribute to the relaxation of CD₂Cl₂ in phase V could be extracted from this study¹⁷.

In a continuing study, the deuterium relaxation of partially oriented methyl group in acetonitrile-d₃ oriented in phase V was calculated¹⁸ and measured¹⁹. Multiple-quantum spin-echo spectra were recorded by the usual sequences. The C_{3v} symmetry of the spins was utilized for obtaining symmetrized basis functions and the total spectrum was divided into A₁, A₂ and E irreducible representations. DQ coherences were correlated to single quantum coherences of the same irreducible representation and this aspect was

utilized for assignment of the DQ spectrum¹⁸. All the zero and multiple quantum frequencies were classified into 3 further classes: class 1 in which the frequencies depend only on ν_A , the chemical shift; class 2 in which the frequencies depend also on J and D; and class 3 in which the frequencies depend in addition on ω_Q . The lines belonging to class 3 usually gave broader lines and all the single quantum transitions belong to this class. The class 1 coherences are focussed in the spin-echo experiment and appear at zero frequency in the F_1 dimension of the 2D spectrum. The most striking feature of the observed DQ spectrum was its enhanced resolution. This was due to refocussing of the static field inhomogeneity in the F_1 dimension, bunching of class 1 peaks at $F_1 = 0$ and non-excitation of class 3 peaks due to the particular value of interval τ chosen in the excitation sequence¹⁸.

For studying the relaxation features, these coherences were further classified into degenerate and nondegenerate types¹⁹. The relaxation behaviour of nondegenerate coherences is straightforward, as they decay as single exponentials whose rates can be calculated from the assumed relaxation model. An ideal 180° pulse reverses the class 1 coherences into their complex conjugates while each class 2 coherence is converted into the complex conjugate of its image under spin inversion. The image decays exponentially at the same rate as the original coherence and the decay rate remains single exponential even in the spin-echo experiment and the corresponding line is a simple Lorentzian whose width in F_1 dimension directly yields a relaxation matrix element. Degenerate coherences show multiexponential relaxation and additional complications due to the refocussing 180° pulse. Even order multiple-quantum spin-echo relaxation rates of classes 1 and 2 transitions have been measured, and their relation to various spectral densities pointed out¹⁹.

2D SINGLE QUANTUM CORRELATION SPECTROSCOPY (COSY)

Two-dimensional single quantum correlation spectroscopy (COSY) has been applied for

studying the relationship of various transitions in the dipolar coupled spectra of molecules oriented in liquid crystal matrices. Conventionally double resonance experiments were used for this purpose in high resolution NMR of liquids, but these have had little success in dipolar coupled cases mainly due to the second order nature of the spectra of the oriented molecules. The first attempt in this direction was the study of an AA'BB spin system formed by the protons of benzoselenodiazole oriented in liquid crystal EBBA*. The 2D COSY spectrum obtained at 270 MHz using the pulse sequence $90-t_1-90-t_2(aq)$, yielded cross-peaks only within the transitions belonging to the symmetric and antisymmetric types²⁰, leading to a complete separation of all transitions into the symmetric and antisymmetric manifolds. These manifolds were then separately analysed²⁰. It was found that identification of the transitions of the antisymmetric manifold allowed analytical analysis of the transitions of this manifold²⁰. From this several parameters governing the spectrum could be extracted. The symmetric manifold peaks were classified into three groups, depending on the intensity of diagonal peaks compared to their intensity in one-dimensional spectrum. In strongly coupled spins the diagonal peaks of 2D spectrum have intensities different from the square of their 1D intensities due to contribution of 3-spin-1-quantum type of matrix elements in the 90° pulse rotation operator. Such an element is not possible if a pure state is involved in the transition. The class 1 peaks were thus identified to be those involving a pure state. This identification leads to information useful in the analysis of the spectrum²⁰.

It was further shown that additional information from the COSY spectrum could be obtained by changing the flip angle of the coherence transfer pulse. By decreasing the flip angle below 90° , the relative intensity of the connected to unconnected cross-peaks in a COSY spectrum is increased²⁰. This was

explained by using a pathway description. The intensity of a cross-peak between connected transitions is dependent on matrix elements in the pulse operator which take a total path of lower order than the unconnected transitions. The higher order pathways contribute to the cross-peaks between unconnected transitions, and can be enhanced by increasing the flip angle above 90° . The study demonstrated the utility of COSY in such studies²⁰.

The absence of cross-peaks between symmetric and antisymmetric transitions was further utilized in systems with higher symmetry, leading to a dramatic symmetry, filtering off the spectra of oriented molecules by COSY²¹. In the 2D COSY experiment coherence transfer is affected by the second 90° pulse. The operator representing the 90°_x (or α_x) pulse is given by

$$P_x(\alpha) = \exp(i\alpha I_x) = \mathbf{1} + i\alpha I_x + [(i\alpha)^2/2!] I_x^2 + \dots \quad (2)$$

Each term I_x^n has matrix elements only between states of the same irreducible representation. The coherence transfer operator, therefore, does not effect transfer of coherence between transitions belonging to different irreducible representations of the symmetry group. Spectra of two symmetric molecules, having spin systems denoted by A_3A_3' and $AA'A''A'''A''''A'''''$ arising respectively from protons of acetone and benzene oriented in a nematic phase have been studied²¹. In the case of acetone (A_3A_3'), there are two rotating methyl groups each having C_{3v} symmetry and the spin system is characterized by an intermethyl and an intramethyl H-H dipolar coupling and an intermethyl spin-spin coupling. The eigenstates of the Hamiltonian can be grouped into four irreducible representations A_g, A_u, G_1 and G_{2g} , and analytical expressions are available for the frequencies of all the transitions. The 2D COSY spectrum along with the 1D spectrum is shown in figure 4. The cross-peak linkages in the 2D spectrum separate the spectrum into four sets which are uniquely identified, in a straightforward manner, to the respective irreducible representations. The G_{2g}

* EBBA is *p*-ethoxybenzylidene *p*-*n*-butylaniline and MBBA is *p*-methoxybenzylidene *p*-*n*-butylaniline.

representation has only two transitions. These are immediately identified in the COSY spectrum and yield the value of one of the dipolar couplings. Similarly other representations are also uniquely identified yielding the spectral parameters without the need of complicated calculations²¹.

Benzene has D_{6h} symmetry and its proton NMR spectrum in oriented phase is characterized by six parameters namely (D_o, J_o) , (D_m, J_m) , (D_p, J_p) respectively the ortho, meta and para dipolar and spin-spin couplings. However, for regular hexagon the dipolar couplings are interrelated reducing the independent para-

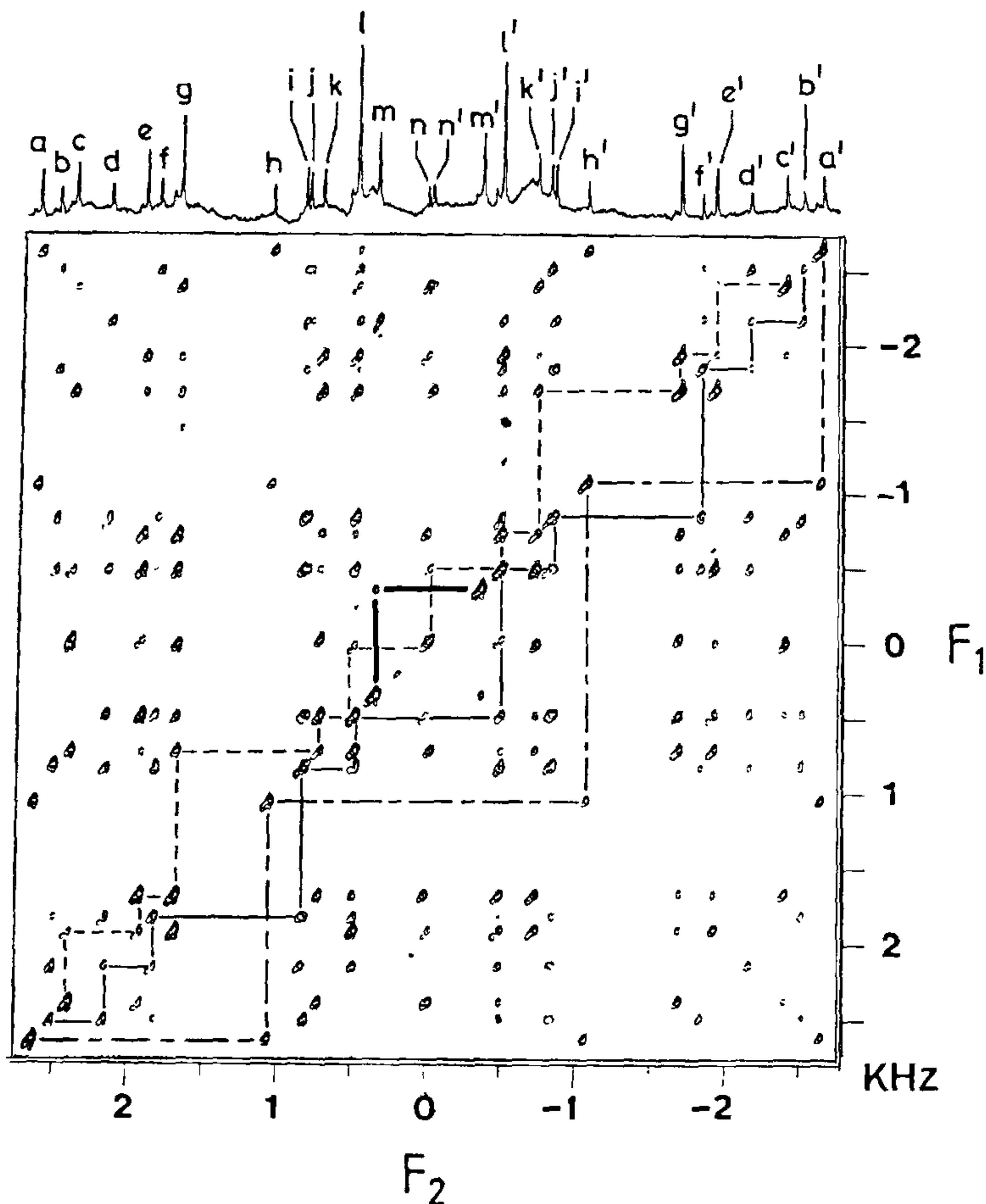


Figure 4. 270 MHz proton NMR spectra of acetone oriented in MBBA recorded at room temperature. (Top) one-dimensional spectrum. (Bottom) two-dimensional COSY spectrum (with 512×1024 data points), showing cross-peak linkages and separation of the lines into four irreducible representations. G_{2g} (lines m and m') is shown by thick continuous lines, A_g (lines b, d, f, i, j, l, l', j', i', f', d', b') by thin continuous lines, G_1 (lines c, e, g, k, l, n, n', l', k', g', e', c') by dashed lines and A_u (lines a, h, h', a') by alternate long and short dashes [from ref. 21].

meters to only 4. The spin states are divided into six irreducible representations (A_1 , A_2 , B_1 , B_2 , E_1 , E_2). The A_2 representation has only one spin state and no transition. Transitions belonging to the remaining 5 irreducible representations have been identified with the help of the COSY spectrum. Separate iterations on each irreducible representation using a modified LAOCOONOR programme yielded all the parameters in an efficient manner²¹.

2D MULTIPLE QUANTUM TRANSITIONS SPECTROSCOPY

In the previous section, utility of the experiments which correlate single quantum transitions with each other (COSY) was described. In this section the utility of correlation of multiple quantum transitions to single quantum transitions and the use of multiple quantum filtering in the study of oriented systems are described.

MQT spectroscopy

2D multiple quantum spectra are obtained by the use of sequence shown in figure 1(c). The multiple quantum transitions are excited at the end of second 90° pulse; they are frequency-labelled during t_1 and converted into observable single quantum magnetization by the last 90° pulse. This observable magnetization is detected as a function of t_2 , yielding a 2D spectrum in which the F_1 axis contains the various multiple quantum transitions and the F_2 axis contains the single quantum transitions (SQT). The 2D spectrum indicates the correlation of the MQT with the SQT. The magnetization in t_2 can also be detected at a single fixed point τ , resulting in a one-dimensional spectrum as a function of F_1 . This one-dimensional spectrum is equivalent to a projection of the 2D absolute mode spectrum on the F_1 axis. While most of the applications have been demonstrated using such one dimensional spectra²²⁻³⁰, 2D spectra have also been recorded³¹⁻³⁴. Figure 5 shows the 2D multiple quantum NMR spectrum of oriented benzene³⁴. The spectrum becomes

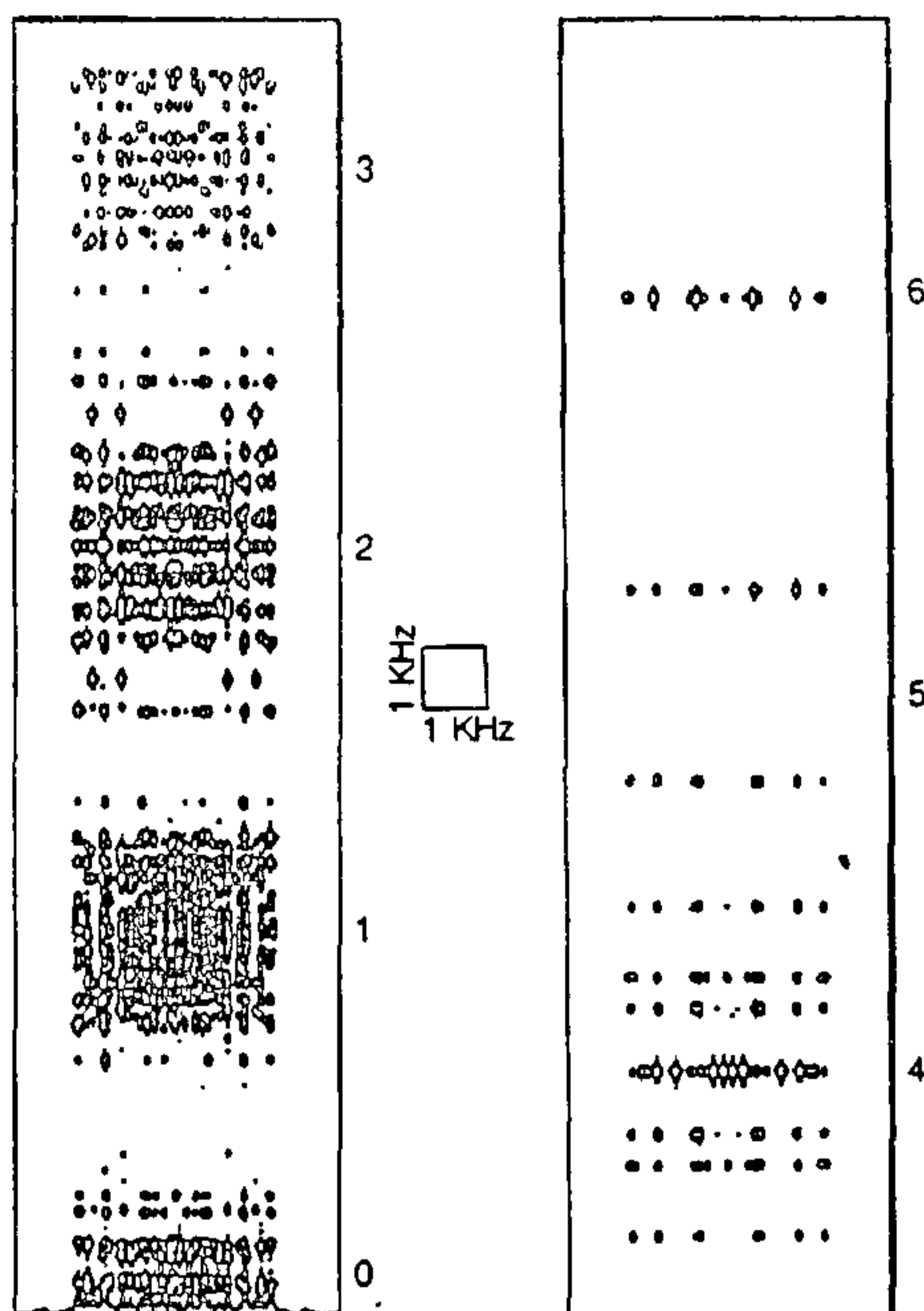


Figure 5. Contour plot of multiple quantum (vertical axis) versus single quantum (horizontal axis) two-dimensional correlation proton spectrum of oriented benzene [from ref. 34].

progressively simple as one goes to higher multiple quantum orders. For example the 6th quantum has only one transition which correlates to many single quantum transitions. There are two 5 quantum transitions and seven 4 quantum transitions.

The various multiple quantum orders are separated out by offsetting the carrier from exact resonance²². If $\Delta\omega$ is the offset of carrier from the centre of a single quantum spectrum, then the centre of the n th order appears at $n(\Delta\omega)$. The linewidths due to field inhomogeneity along F_1 axis are increased proportionately for various orders. To refocus the field inhomogeneity, a 180° pulse has been introduced in the middle of t_1 period [figure 1(f)]. This focuses the field inhomogeneity as well as offset dependence of various orders. The orders in such cases are separated out by time proportional phase incrementation

(TPPI)^{22,34}. In this method the phase of the first two 90° pulses is incremented in sequential t_1 experiments as $\phi = (\Delta\omega)t_1$. This results in a separation of various multiple quantum orders by an offset of $n(\Delta\omega)$. In case the chemical shifts are present along with dipolar couplings, the 180° pulse in scheme (f) of figure 1 causes coherence transfer within each order, giving rise to new transitions and additional frequencies in a manner similar to the strong coupling isotropic cases discussed earlier³⁵.

It is possible, by additional phase cycling, to retain only one of the orders along F_1 axis resulting in a much simpler spectrum; say a DQ spectrum^{23,31}. Figure 6 shows a part of the two quantum proton spectrum of 81% randomly deuterated *n*-hexane³¹. The deuterium spins

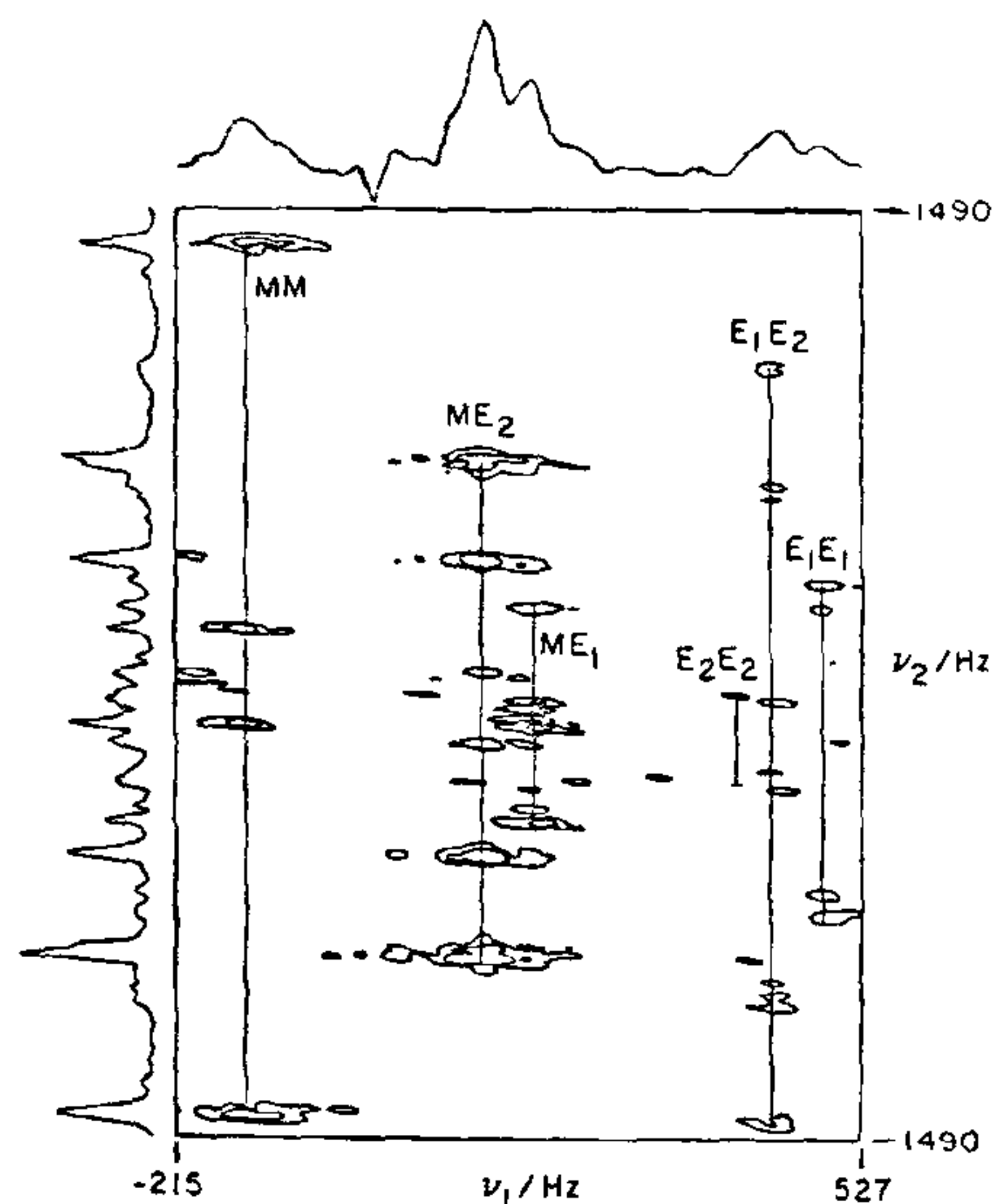


Figure 6. Part of double quantum (ω_1 axis) versus single quantum (ω_2 axis) proton spectrum of 81% randomly deuterated *n*-hexane, 23% mole in liquid crystal EK 11650. The deuterium spins have been decoupled during the experiment. Vertical lines parallel to ω_2 axis indicate the six possible double-quantum frequencies of molecules with two protons. Identified are the molecules having the two protons in the configurations *MM*, *ME*₂, *ME*₁, *E*₂*E*₂, *E*₁*E*₁ and *E*₁*E*₂, identifying the dipolar couplings in each case [from refs 31 and 34].

have been decoupled during the experiment. Deuteration by large amount reduces the number of coupled protons. Molecules having only one proton give no DQ spectrum and the spectrum essentially consists of molecules having two protons. The number of molecules having more than two protons is negligibly small. The two protons can be in any of the two methyl (*M*) or four methylene (*E*₁ and *E*₂) positions yielding a possible eight *AB* and eight *A*₂ type dipolar coupling patterns. The *A*₂ type are formed by the molecules having the two protons in the configuration *MM*, *MM'*, *E*₁*E*₁, *E*₁*E*_{1'} (*cis*), *E*₁*E*_{1'} (*trans*), *E*₂*E*₂, *E*₂*E*_{2'} (*cis*), *E*₂*E*_{2'} (*trans*) and the *AB* type by *ME*₁, *ME*_{1'}, *ME*₂, *ME*_{2'}, *E*₁*E*₂ (*cis*), *E*₁*E*₂ (*trans*), *E*₁*E*_{2'} (*cis*), *E*₁*E*_{2'} (*trans*). In figure 6, vertical lines parallel to the single quantum axis identify six of the DQ frequencies³¹.

The 6 quantum and 7 quantum spectra of *n*-hexane fully deuterated at both the methyl positions have also been reported recently and the various spectral parameters have been derived³². There are 8 coupled protons in this case and it is shown that the (*n*-1) and (*n*-2) order spectra yield the various dipolar couplings and their signs. From these results an estimate of dynamic molecular structure in the form of conformational probabilities was obtained³².

In another study of 34% randomly deuterated benzene oriented in a nematic liquid crystal, 5-quantum versus 1-quantum proton 2D spectrum was recorded. The 5-quantum spectrum can be obtained either from fully protonated benzene or from singly deuterated species³³. The singly deuterated species has only one 5 quantum transition while the fully protonated species has two 5 quantum transitions. From their correlation to the single quantum transitions, various spectral parameters could be derived³³.

Multiple-quantum filtered COSY

Instead of obtaining multiple quantum 2D spectra which need large computer memory and yield rectangular data matrices, one can obtain a COSY type spectrum, in which the single quantum transitions are correlated to

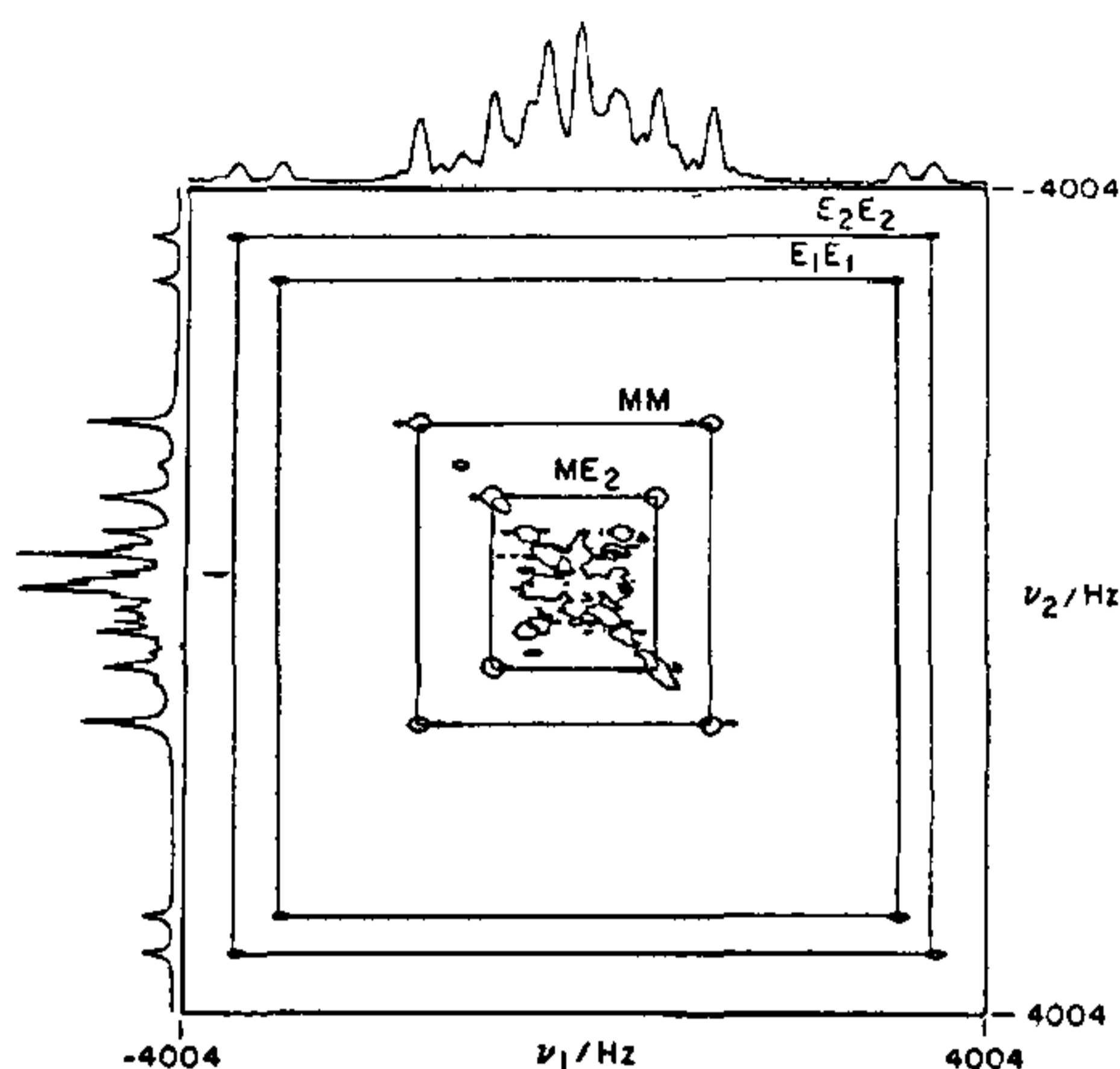


Figure 7. 360 MHz double quantum filtered proton COSY spectrum of the same system as in figure 6. The square patterns, which reveals dipolar couplings in individual isotopomers, are shown for four of the sixteen possible proton pairs (from refs 31 and 34).

each other but the magnetization transfer process is restricted to a well-defined order^{23,31,34}. The scheme of figure 1(b) can be used for this purpose in which the phase cycling determines the multiple quantum order to be selected. The most popular of these is the DQ filtered COSY (DQFC)²³. The DQFC spectrum of 81% deuterated *n*-hexane is shown in figure 7 which has similar information as in DQ spectrum of figure 6³¹. The single quantum transitions on both axes are correlated to each other. Only those transitions appear which have DQ magnetization between second and third 90° pulses of DQFC scheme figure 1(b). The singlets are eliminated from such a spectrum. The DQ filtered spectrum also allows identification of various spin systems in a manner similar to the 2-quantum spectrum of figure 6³¹.

The DQFC spectroscopy has also been utilized for the study of 78.7% randomly deuterated benzene oriented in nematic phase³³. Subspectra of 3 diprotonated and 3 triprotonated species could be identified in the spectrum. The subspectra were compared with the simulated spectra for assignment and for obtaining the various coupling values.

CONCLUSIONS

Two-dimensional NMR spectroscopy has been successfully utilized in the study of spectra of molecules oriented in liquid crystal matrices. The spin-echo experiment has been used for enhancing the information content of the spectra and for studying the relaxation behaviour of the single and the multiple quantum coherences. The COSY experiment has been utilized for finding interrelation between various transitions and for symmetry filtering of the spectra. The MQT and MQFC have proved highly effective for filtering the spin systems and for identification of coupling networks. These have been applied to partially deuterated species leading to information on the spectral parameters and the conformations.

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1. Diehl, P. and Khetrpal, C. L., *NMR-basic principles and progress*, Springer-Verlag, New York, 1969, Vol. 1; Emsley, J. W., and Lindon, J. C., *NMR spectroscopy using liquid crystal solvents*, Pergamon Press, Oxford, 1975.
2. Ernst, R. R., Bodenhausen, G. and Wokaun, A., *Principles of nuclear magnetic resonance in one and two dimensions*, Clarendon, Oxford, 1987.
3. Chandrakumar, N. and Subramanian, S., *Modern techniques in high-resolution FT-NMR*, Springer-Verlag, New York, 1987.
4. Aue, W.P., Bartholdi, E. and Ernst, R. R., *J. Chem. Phys.*, 1976, **64**, 2229.
5. Bodenhausen, G., *Progr. NMR Spectrosc.*, 1981, **14**, 137.
6. Ad Bax, *Two-dimensional nuclear magnetic resonance in liquids*, Delft Univ. Press, Dordrecht, 1982.
7. Benn, R. and Günther, H., *Angew. Chem. Int. Eng. Ed.*, 1983, **22**, 350.
8. Turner, D. L., *Prog. NMR Spectrosc.*, 1985, **17**, 281.

9. Wuthrich, K., *NMR of proteins and nucleic acids — The Baker Lecture Series*, Wiley, New York, 1986.
 10. Khetrapal, C. L., Kunwar, A. C., Tracey, A. S. and Diehl, P., *NMR — Basic principles and progress*, Springer-Verlag, New York, 1975, Vol. 9; Khetrapal, C. L. and Kunwar, A. C., *Advances in magnetic resonance*, Academic Press, New York, 1977, Vol. 9; Khetrapal, C. L. and Kunwar, A. C., *Advances in liquid crystals*, Academic Press, New York, 1977, Vol. 6; Khetrapal, C. L. and Kunwar, A. C., *Specialist periodical reports NMR: oriented molecules*, The Royal Society of Chemistry, London, 1979, Vol. 8; 1980, Vol. 9 and 1982, Vol. 11; Khetrapal, C. L., *ibid*, 1984, Vol. 13; Khetrapal, C. L. and Ramanathan, K. V., *ibid*, 1986, Vol. 15; Khetrapal, C. L., Arun Kumar, B. S. and Raghobhama, S., *ibid*, 1988, Vol. 17.
 11. Anil Kumar and Khetrapal, C. L., *J. Magn. Reson.*, 1978, **30**, 137.
 12. Khetrapal, C. L., Anil Kumar, Kunwar, A. C., Mathias, P. C. and Ramanathan, K. V., *J. Magn. Reson.*, 1980, **37**, 349.
 13. Avent, A. G., Emsley, J. W. and Turner, D. L., *J. Magn. Reson.*, 1983, **52**, 57.
 14. Miner, V. W., Tyrell, P. M. and Prestegard, J. H., *J. Magn. Reson.*, 1983, **55**, 438.
 15. Ramachandran, R., Kunwar, A. C., Gutowsky, H. S. and Oldfield, E., *J. Magn. Reson.*, 1984, **60**, 352.
 16. Bodenhausen, G., Vold, R. L. and Vold, R. R., *J. Magn. Reson.*, 1980, **37**, 93.
 17. Vold, R. L., Vold, R. R., Poupko, R. and Bodenhausen, G., *J. Magn. Reson.*, 1980, **38**, 141.
 18. Jaffe, D., Vold, R. L. and Vold, R. R., *J. Magn. Reson.*, 1982, **46**, 496.
 19. Jaffe, D., Vold, R. R. and Vold, J. R., *J. Magn. Reson.*, 1982, **46**, 475.
 20. Albert Thomas, M., Ramanathan, K. V. and Anil Kumar, *J. Magn. Reson.*, 1983, **55**, 386.
 21. Rukmani, K. and Anil Kumar, *Chem. Phys. Lett.*, 1987, **133**, 485.
 22. Drobny, G., Pines, A., Sinton, S., Weitekamp, D. P. and Wemmer, D., *Far. Symp. Chem. Soc.*, 1979, **13**, 49.
 23. Sinton, S. and Pines, A., *Chem. Phys. Lett.*, 1980, **76**, 263.
 24. Tang, J. and Pines, A., *J. Chem. Phys.*, 1980, **73**, 2512.
 25. Warren, W. S., Weitekamp, D. P. and Pines, A., *J. Chem. Phys.*, 1980, **73**, 2084.
 26. Warren, W. S. and Pines, A., *J. Chem. Phys.*, 1981, **74**, 2808.
 27. Weitekamp, D. P., Garbow, J. R. and Pines, A., *J. Magn. Reson.*, 1982, **46**, 529.
 28. Weitekamp, D. P., Garbow, J. R. and Pines, A., *J. Chem. Phys.*, 1982, **77**, 2870.
 29. Murdoch, J. B., Warren, W. S., Weitekamp, D. P. and Pines, A., *J. Magn. Reson.*, 1984, **60**, 205.
 30. Sinton, S., Zax, D. B., Murdoch, J. B. and Pines, A., *Mol. Phys.*, 1984, **53**, 333.
 31. Gochin, M. and Schenker, K. V., Zimmermann, H. and Pines, A., *J. Am. Chem. Soc.*, 1986, **108**, 6813.
 32. Gochin, M., Zimmermann, H. and Pines, A., *Chem. Phys. Lett.*, 1987, **137**, 51.
 33. Gochin, M., Hugi-Cleary, D., Zimmermann, H. and Pines, A., *Mol. Phys.*, 1987, **60**, 205.
 34. Pines, A., *Lectures on pulsed NMR*, presented at the 100th Fermi School of Physics, Varenna, Italy, July 8–18, 1986, (to be published in its proceedings), (ed.) B. Maraviglia, North-Holland, Amsterdam.
 35. Albert Thomas, M. and Anil Kumar, *J. Magn. Reson.*, 1984, **56**, 479.
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HUMAN TUMOUR XENOGRAFTING AND RADIOIMMUNOTARGETTING

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ABSTRACT

The transplantability of human tumours especially breast carcinoma as xenografts has been examined in Swiss albino mice that had been immune-suppressed. Tumour takes have been noticed as early as on the eleventh day. Tumour targetting which has assumed great importance both for radioimmunodetection as well as radioimmunotherapy has been achieved in these models using ^{125}I labelled anti-HMFG-2 monoclonal antibodies. Such an immune-suppressed model is feasible and economical as compared to athymic nude mice.

INTRODUCTION

WE are currently interested in a programme of research on economical techniques for the growth of human tumours in immune-suppressed mice and of creating *in vivo* model as well of infective lesion e.g. tuberculoma¹ to evaluate radiolabelled monoclonal antibodies or engineered cocktail² for radioimmunodetection (RID) with the hope that the work on tumours can be later extended to radioimmunotherapy (RIT).

The idea of human tumour implantation into experimental animal hosts is not new. Such xenografting is, in some sense, an extension of the many ways in which transplantation of animal tissues and organs to the human species was attempted as replacement therapy. Heterografting of human tissues, chiefly pioneered by Handler³ has attracted growing interest and brought an increasing number of human tumours within the scope of *in vivo* experimentation. Such xenografts can be used for diverse investigations such as chemotherapy testing and study of tumour-cell kinetics, hormone production mechanisms, RID and RIT.

There have been many attempts to devise xenograft systems in the past but the results were disappointing and had numerous setbacks. The initial use of immunologically privileged sites for xenografting—e.g. the anterior chamber of the eye, the brain, the hamster cheek pouch and others—did not fulfil early expectations. With a fuller knowledge of the biology of tumour graft rejection mediated through cellular immunity (T-lymphocytes)⁴, it became feasible to immune-suppress the recipient animals usually mice in various ways right from the use of whole body irradiation supplemented with corticosteroids, and other immune-suppressive drugs reported to be extremely toxic for the host animal⁵ or thymectomy at 3–4

weeks of age and treatment by antilymphocytic sera (ALS)/antithymocytic sera (ATS)^{6–9} together with marrow reconstitution to combat high radiation dose and use of marrow rescuers e.g. Ara-C^{10–12}. Several human tumours were successfully established and propagated as xenografts in these systems though certain technical problems were encountered including the disadvantages arising from reconstitution by syngeneic bone marrow which contains T cells or their precursors.

Such problems appeared to have become redundant, however with the introduction of genetically immune-suppressed recipients principally the athymic nude mice. These animals lack the capacity to reject foreign tumour grafts to a degree which compares favourably with the most profoundly immune-suppressed animals by any of the previous methods. However, these did not prove ideal for laboratories in India because their maintenance is not economic; further they soon become diseased altering the drug response¹³; not all tumours grow in them specially those possessing tumour rejection antigen (TRA)¹⁴ and metastases are rare probably due to high natural killer (NK) cells level¹⁵.

Although thymectomy under general anaesthesia sounds essential for better immune-suppression, it is not always easy in new hands and that too on only 3–4 weeks old mice. Secondly, T-deprived mice may recover their immune competence after 6–8 weeks of irradiation. Use of ALS and ATS antisera is limited by availability and cost etc. We therefore thought it necessary to maintain immune-suppression with a titrated dose of corticosteroids which is neither lethal to the graft nor to the host and yet does not carry a significantly enhanced infection rate making the protocol quite economic.

It has been reported that some tumours e.g. malignant melanomas, colorectal adenocarcinoma,