Two and Three Dimensional Nuclear Quadrupole Resonance in the Investigation of Structure and Bonding

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A variety of two dimensional two- and three-dimensional NQR experiments are reviewed, showing their application to the determination of field gradients for important sites in peptides and proteins, for assigning connected transitions of nuclei with spin $\geq 5/2$, and for determining hexadecapolar coupling constants. The quadrupole coupling tensor for ^{63}Cu in the active site of a protein, bovine erythrocyte (Cu, Zn) superoxide dismutase, has been measured and is compared with the results of *ab initio* calculations.

Introduction

If nuclear quadrupole resonance (NQR) is to approach the same level of usefulness that nuclear magnetic resonance has attained over the last 30 years as a probe of complex systems, several hurdles must be overcome. The most imposing and at the same time most intriguing of these hurdles is the need to increase the information content of NQR spectra. While solid-state NMR is blessed with and sometimes bedeviled by a plethora of nuclear spin interactions — Zeeman, chemical shift, hetero- and homo-nuclear dipolar couplings, and sometimes quadrupolar couplings, the zero-field Hamiltonian most often contains the nuclear quadrupole coupling as the only significant term. This means that for half-integer spin nuclei, whose spectroscopy principally concerns us here, we expect a number of transition frequencies equal to $(I - 1/2)$, where I is the nuclear spin. So for spin 3/2 nuclei, we see only a single transition, and even the principal values of the quadrupolar coupling tensor are unobtainable by simple one-dimensional NQR; spin 5/2 nuclei give two frequencies, sufficient to determine the principal values of the coupling tensor; and spin 7/2 and spin 9/2 nuclei have three and four transitions, respectively, making possible in favorable cases the determination of nuclear hexadecapole coupling parameters.

Two approaches to increasing the information content of NQR spectra will be discussed here. The first, multidimensional NQR spectroscopy [1], has been in use for over a decade. The simplest two-dimensional NQR method, nutation spectroscopy [2, 3], can be used to determine the asymmetry parameter for spin 3/2 nuclei, and can in principle be used to obtain the orientation of the field gradient tensor in samples with a preferential axis system. Two-dimensional correlation spectroscopy [4, 5] links connected spin transitions of higher spin nuclei. It can be used in a similar mode to COSY and its variants [6] in high field NMR to assign NQR spectra of complex materials [7] and to eliminate inhomogeneous distributions of quadrupole couplings [8], so that the ratios of frequencies of connected transitions may be measured more accurately.

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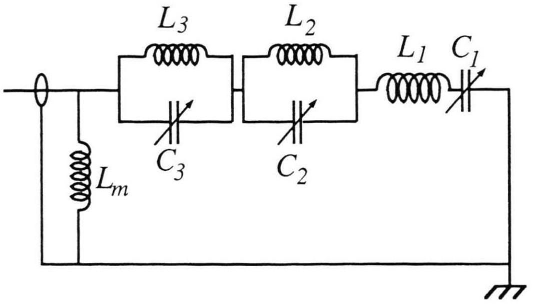

Fig. 1. Triple tuned NQR probe circuit used in the present work.

The second approach to be discussed here is the *ab initio* calculation of electric field gradients which, with the availability of ever-increasing computer power and of high-level electronic structure methods can now be done with a high degree of confidence and accuracy for quite respectably sized molecules, allowing us the luxury of quantitative interpretation of experimentally determined nuclear quadrupole couplings. This two-pronged approach — on the one hand striving through the development of new experimental methods to increase the spectroscopic information available, and on the other hand using high-level theory to make the maximum possible use of the experimental information, is the core theme of the present work.

Methods

All of the experimental data were obtained using a home-built pulsed nuclear quadrupole resonance spectrometer, incorporating three independent radiofrequency channels, all broad banded from 5 MHz to (at least) 300 MHz. For the nutation experiments, a simple inductively matched, parallel tank circuit probe was used, employing a 3 - 30 pF vacuum variable tuning capacitor (FSL Jennings) and an approximately 20 turn, 1 cm diameter, 3 cm long solenoidal coil. Double resonance experiments employed a double resonance probe which has been described in [5].

Figure 1 shows the triple resonance circuit used for the three dimensional experiments. It employs two parallel and one series tank circuit, all in series; inductor L_1 is the sample coil. The three tank circuits give rise (with appropriate impedance values) to three resonances, which are each associated to some extent with one of the tanks, but which are sufficiently coupled to give an adequate radiofrequency field strength in each of the three frequencies in the sample coil. The three resonances can each be matched to a 50 Ohms impedance using a single matching inductor, by adjusting the (through space) inductive coupling between it and the three tank coils. The three resonances can be tuned to a fairly broad range of frequencies.

Glycylglycine hydrobromide monohydrate was obtained by dissolving glycylglycine (Sigma) in a moderate excess of hydrobromic acid and allowing the solution to crystallize at 4 °C. Antimony trichloride and bismuth trichloride were purchased from Fluka (Ronkonkoma, NY) and used without further purification. Methylammonium iodide was prepared following the method of Wagner [10] by slowly cooling a saturated solution of methylamine in hydriodic acid, and was recrystallized from a 1 : 1 mixture of chloroform : methanol. Partially deuterated methylammonium iodides were obtained by dissolving methylammonium iodide in an H_2O/D_2O mixture of appropriate composition, and then recrystallizing from a chloroform and methanol mixture containing O-deuterated methanol and methanol in an appropriate ratio, to yield flat crystalline plates.

Cu(I) superoxide dismutase (SOD) was prepared in a nitrogen atmosphere as follows: approximately 600 mg of SOD were dissolved in 150 mL of 50 mM, pH 7.06 phosphate buffer solution; reduced by adding 280 mg of sodium dithionite, and slowly crystallized by adding MPD up to 64% (v/v). Cu(I)-SOD, a bleached green-yellow precipitate, was packed into double interlocking glass vials and sealed with epoxy glue. *Bis*(thiourea) copper(I) chloride, one of whose ^{63}Cu resonance frequencies falls at 22.177 MHz, was used to calibrate the spectral acquisition parameters.

Ab initio calculations employed the program Gaussian-98 running on one of two 450 MHz Pentium-II workstations under Windows NT 4.0. Hydrogen atom positions were optimized from initial coordinates based on standard hydrogen–heavy-atom bond lengths and angles using the Berke-3 functional and a 6-311+G(2d,p) basis set; this combination generally gives excellent results for such positions. Electric field gradients were calculated using conventional Hartree-Fock with a 6-311+G(d) basis; to convert these to coupling constants and frequencies, an electric quadrupole moment $eQ = 22.0$ fm^2 was adopted for ^{63}Cu.

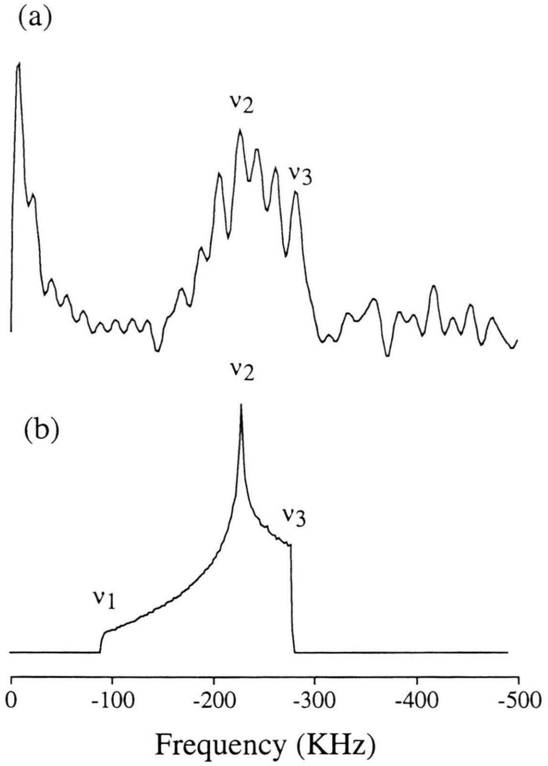

Fig. 2. (a) Experimental nutation NQR spectrum of the peptide glycylglycine hydrobromide monohydrate, compared with (b) a simulation based on an asymmetry parameter of 0.356.

Results and Discussion

Nutation NQR

We introduced nutation NQR in 1989 [2] based on the long-recognized principle [9] that the precession frequency of a spin 3/2 under the influence of a radio frequency field depends on the orientation of the quadrupolar coupling tensor with respect to the electric field axis. If the electric field gradient tensor is axially asymmetric, then this frequency depends on two polar angles, θ and ϕ. In a polycrystalline sample, the precession of the ensemble is a powder average over these angles; this precession powder average, Fourier transformed, has three singularities, ν_1, ν_2 and ν_3, which can be used to determine the asymmetry parameter, otherwise unavailable from the one dimensional spectrum of a spin 3/2 nucleus at zero field.

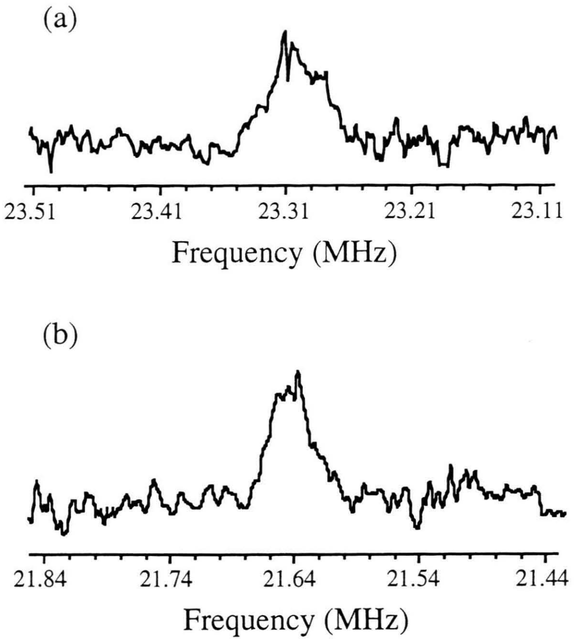

Fig. 3. (a) ^{63}Cu NQR resonance and (b) ^{65}Cu NQR resonance from reduced bovine superoxide dismutase.

$$\eta = \frac{3(\nu_3 - \nu_2)}{\nu_3 + \nu_2} \tag{1}$$

As a typical example of the application of nutation NQR to a peptide hydrohalide, Fig. 2 shows the ^{79}Br nutation spectrum of glycylglycine hydrobromide monohydrate, obtained at 12.2 MHz. The spectrum was actually obtained with a nutation echo sequence [3], to forestall the effect of inhomogeneous broadening. Glycylglycine hydrobromide monohydrate itself is isomorphous with the corresponding hydrochloride, for which a neutron diffraction structure is available. The ν_2 and ν_3 singularities are clearly observable, and can be used to obtain an asymmetry parameter of 0.36, which in turn yields a quadrupole coupling constant of 23.9 MHz, in excellent agreement with the results of single crystal studies. The apparent noise in the nutation spectrum is likely a reflection of the granular nature of the powder. The spectrum is a fair representation of the quality of data attainable for a modestly sized sample of a small molecule at fairly low NQR frequencies.

A more challenging system is the active site of a metalloenzyme. Figure 3 shows the ^{63}Cu and ^{65}Cu NQR spectra of the copper site in bovine erythrocyte (Cu, Zn) superoxide dismutase. These spectra were

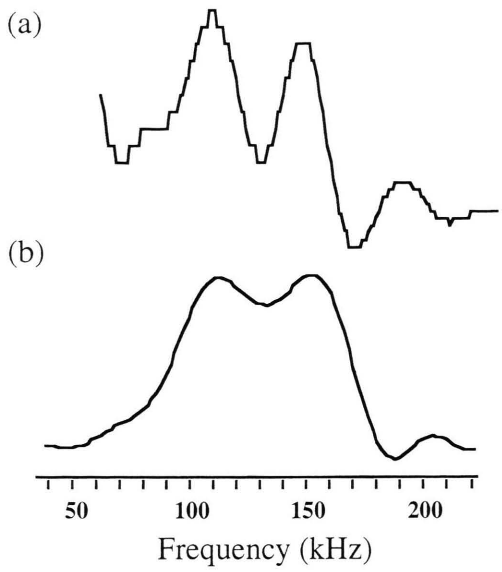

Fig. 4. (a) Experimental and (b) simulated ^{63}Cu nutation spectra of reduced superoxide dismutase.

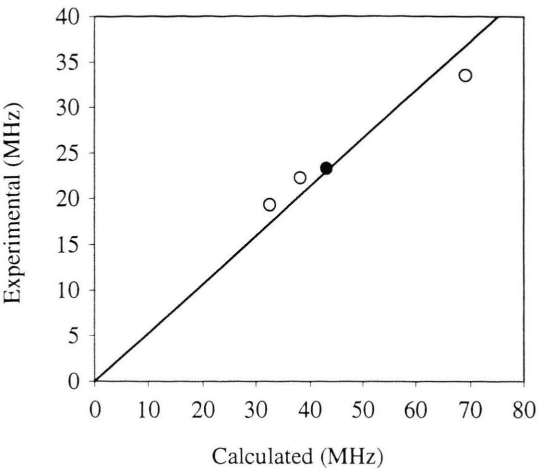

Fig. 5. Comparison of experimental and calculated ^{63}Cu pure NQR frequencies of the (open circles) model compounds listed in the text and (filled circle) reduced superoxide dismutase.

found by sweeping the region from 21 to 24 MHz, where, by extrapolation from earlier ^{111}Cd perturbed angular correlation work [11], the copper resonances were expected, using a simple ($\pi/2$ - τ - π - τ) Hahn echo sequence with a 1.8 μs $\pi/2$ pulse length and a 36 μs echo delay. Frequency sweeps were performed from 21.0 MHz to 24.0 MHz in intervals of 200 kHz, by averaging 200,000 scans. Two resonances were located at 23.320 and 21.650 MHz. Final ^{63}Cu and ^{65}Cu one-dimensional spectra were acquired by averaging 2 million scans with 512 points at 2μs per point and at a recycle delay of 50 ms. Control experiments were performed under the same conditions where acquisitions were taken with an empty coil and a coil with an empty sample tube, both yielding null results. The higher frequency ^{63}Cu resonance of *bis*thiourea copper(I) chloride at 22.17 MHz was used to calibrate the spectrometer and measure the r. f. field strength (113.6 kHz).

The lines are broad and of comparable width and intensity, as expected; their NQR frequencies are in the ratio 1.08:1, which agrees with the established ratio of quadrupole moments of the two copper isotopes. They are, to our knowledge, the first NQR spectra of a single site in a protein. Since both isotopes have spin 3/2, in themselves they are insufficient to define the magnitude of the quadrupolar interaction in Cu(I) SOD. While the signal to noise ratio and the width of the protein signals make two-dimensional NQR difficult, it can be conducted using a very limited ω_1 dimension. Figure 4(a) shows the ^{63}Cu nutation spectrum of Cu(I)-SOD, at a frequency of 23.3 MHz. The spectrum is poorly resolved and highly truncated, but is sufficient to confirm the gyromagnetic ratio of the nucleus to within 10%, verifying that the signal is indeed ^{63}Cu, and to give an asymmetry parameter of 0.7 ± 0.1. This value is close to that measured for ^{111}Cd^{2+} in the copper site. A simulated spectrum for this asymmetry parameter is shown in Figure 4(b). Together with the NQR frequency, this gives a quadrupole coupling constant of 43.2 ± 1 MHz.

Since a crystal structure of superoxide dismutase (albeit in the copper (II) form) is available [12], we can use modern *ab initio* methods to compare the measured quadrupolar Hamiltonian with field gradients calculated for the active site. The feasibility of such calculations is demonstrated in Fig. 5, which compares the NQR frequencies of three copper sites in two compounds, (the two coppers of *bis*thiourea copper(I) chloride, and potassium dicyanocuprate). Both the NQR frequency and the asymmetry parameter of the latter have been previously measured; the NQR frequencies of both copper sites of the former are known, while the asymmetry parameter of the high frequency site for the latter has been measured by us by nutation spectroscopy. Electric field gradients for the copper sites were calculated using Gaussian-98 as already described. There is a good linear relationship between calculated field gradients and experimental quadrupole frequencies; however, the slope of the fit implies an electric quadrupole moment for ^{63}Cu of $eQ = 12.0$ fm^2, considerably lower than the currently accepted value of 22.0 fm^2 [13]. It is clear that the sources of this rather considerable discrepancy remain to be explained.

With the reservation of this unaccountably low value of the quadrupole moment, we can use *ab initio* methods to attempt to reproduce the experimental quadrupole coupling constant of Cu(I) SOD. We model the binding site by using the heavy atom positions from the crystal structure for the four coordinating imidazoles, and hydrogen atom positions from density functional calculations. We assume the fifth ligand is an oxygen, either a hydroxyl anion (which yields a charge-neutral site), or a water. In both cases, as before, the oxygen is placed in its crystallographic position and the position of the proton(s) is optimized by density functional methods, now done for the whole site. Figure 5 shows that there is excellent agreement between the calculated ^{63}Cu frequency of Cu-(1) SOD and the results of *ab initio* calculations, the calculated NQR frequency lying almost exactly on the best fit line between calculated and experimental frequencies obtained for model compounds. More than this, the calculated η for the ^{63}Cu (0.57) is in fair agreement with experimental measurements (0.7 ± 0.1), as are those from model compounds (not listed).

Two Dimensional Correlation Spectroscopy

For nuclei of spin higher than 3/2, measurement of the asymmetry parameter is less difficult, since it can be obtained from the ratios of the several NQR transitions. Following up on our original nuclear quadrupole COSY experiment, we have recently (5) published a detailed treatment of more sophisticated methods for transferring coherence between spin transitions at zero-field, without loss of signal or generation of artifacts.

Briefly; in a system of three or more levels, there are two routes by which coherence can be transferred between connected transitions. The most familiar is via the longitudinal magnetization. If a coherence between states which we shall label $|1\rangle$ and $|2\rangle$ is allowed to evolve, it will acquire a phase ϕ equal to $\omega_{12}t$, where t is the evolution time and ω_{12} the transition frequency. The single quantum coherence itself has an amplitude which must be represented as a complex number $\exp(i\phi)$. If a pulse is now applied to the transition, depending on the phase of the pulse, either the real or the imaginary part of the coherence (but not both) may be converted to longitudinal polarization of the 1-2 transition. Since this perturbed polarization or the 1-2 transition affects the population of the $|2\rangle$ state, it also perturbs the polarization of the 2-3 transition, which, if sampled, will be modulated either by $\cos\phi$ or $\sin\phi$.

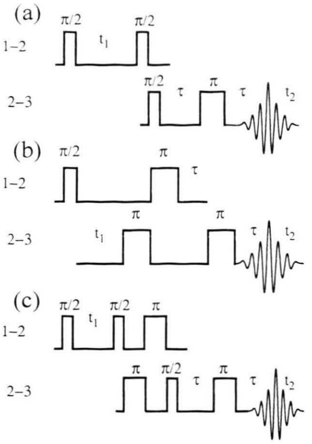

Fig. 6. Pulse sequences for two dimensional NMR correlation spectroscopy at zero field. (a) NQ-COSY. (b) NQ-SCOSY. (c) NQ-MSCOSY.

This can now be made the basis of a 2D NMR experiment. As shown in Fig. 6(a), single quantum coherence of the first transition is excited by a 90° pulse, allowed to evolve and acquire a phase ωt_1, then returned to longitudinal polarization with a second 90° pulse. The second transition is then sampled via a $90° - \tau - 180° - \tau$ sequence, and after two dimensional Fourier transformation using quadrature detection via the method of States *et al.* [14], gives a NQ-COSY spectrum as shown in Fig. 7(a) for the 1/2 – 3/2 and 3/2 – 5/2 transitions of ^{123}Sb in SbCl$_3$.

The clearest disadvantage of the NQ-COSY method is that it wastes half of the initial polarization. This is inevitable for any method which relies on transfer via longitudinal polarization; because the density matrix is Hermitian, diagonal elements must be purely real, while off-diagonal elements may be complex. An alternative route of transfer is via the double quantum coherence which spans the two single-quantum transitions. Single quantum coherence of the 1-2 transition can be converted to double quantum 1-3 coherence by means of a pulse on the 2-3

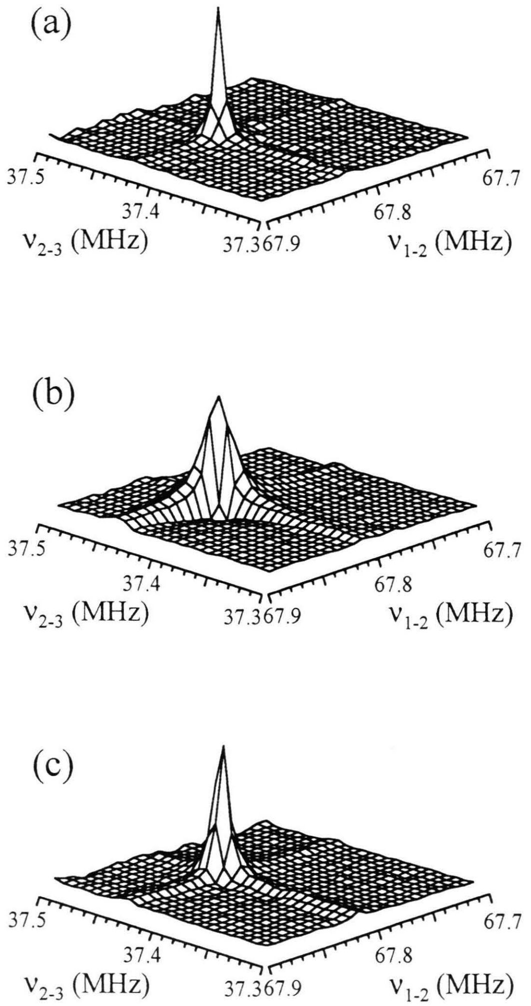

Fig. 7. The two dimensional zero-field NMR correlation spectrum of the ^{123}Sb nucleus in antimony(III) chloride recorded by (a) the NQ-COSY pulse sequence. (b) the NQ-SCOSY pulse sequence. (c) the NQ-MSCOSY pulse sequence.

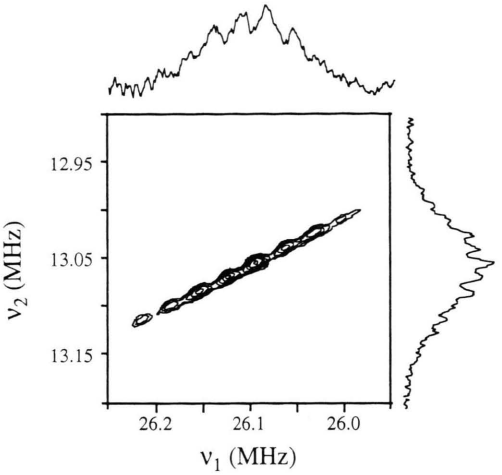

Fig. 8. The two dimensional zero-field ^{127}I correlation spectrum of 95%-deuterated methylammonium iodide, with observation frequencies set at 13.050 MHz for the (3/2-1/2) transition and 26.100 MHz for the (5/2-3/2) transition.

transition; the phase of the resulting coherence will be the sum of the initial phase and the phase of the pulse, so that full phase information is retained. The double quantum coherence is converted to 2-3 single quantum coherence by means of a pulse on the 1-2 transition, again with full retention of phase information. This 2-3 coherence can then be sampled directly or after a spin-echo to minimize dead-time problems (Fig. 6(b)). This we call the Spinor-COSY or S-COSY experiment. (The use of 180° pulses instead of 90° pulses is a consequence of the spinor property of half-integer spins [15]). The principal drawback of the experiment is an artifact of two-dimensional Fourier transformation called phase twist, which can only be eliminated by collecting data with a reversed sign of precession in the first dimension, and in this experiment such reversed precession is not easily manufactured.

We can, however, hybridize these two techniques to produce a sequence which eliminates phase twist but uses all of the initial polarization. The sequence itself is complex (Fig. 6(c)) but the concept is simple. We start with a 90° - t - 90° sequence on the 1-2 transition, so that the longitudinal polarization is modulated by the cosine or the sine of the acquired phase. The remaining coherence remains with a phase equal to that of the second 90° pulse. This residual coherence is transferred by a pair of 180° 'spinor' pulses, via double-quantum coherence, to single-quantum coherence of the 2-3 transition. The effect of the 180° pulses on longitudinal magnetization can be accounted for in the phase cycling. Finally, a 90° pulse is applied to the 2-3 transition; this pulse has the same phase as that of the coherence transferred via the double quantum route, and so leaves it unaffected. The result is a single quantum coherence of the 2-3 transition which is a vector sum of two orthogonal components, one transferred via the diagonal elements of the density matrix, the other by the double quantum coherence. After appropriate phase cycling to balance differential transfer via the two routes and to allow phase quadrature in the first dimen-

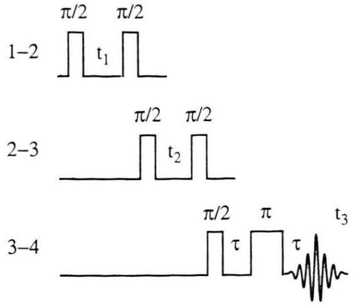

Fig. 9. Three-dimensional NQR correlation pulse sequence.

sion, the final NQ-MSCOSY spectrum is obtained (Fig. 7(c)).

Figure 8 shows the application of this method to a complex ^{127}I spectrum, that of methylamine hydriodide, 95.05% deuterated on the exchangeable positions. Both iodine transitions are broken up into a series of poorly-resolved lines by the multiplicity of numbers of deuterons in the twelve sites nearest the iodines, and it is not easy to determine by inspection which 1/2-3/2 transition is connected to which 3/2-5/2 line. However, in the 2D spectrum, the correlation is obvious, and the multiple ^{127}I lines are clearly resolved.

3D-NQR

An obvious extension of the above methods is three-dimensional NQR spectroscopy. Figure 9 shows a simple 3D-NQR pulse sequence based on the principles of NQ-COSY. Magnetization is transferred sequentially from 1-2 to 2-3 and then 3-4 transitions, at each stage acquiring a phase component modulated by $\omega_{mn} t_m$. Three dimensional hypercomplex Fourier transformation then gives a 3D spectrum which can obviously not be fully represented on a two dimensional surface; however, slices through the 3D spectrum correlating the ν_3 (1/2-3/2), ν_2 (3/2-5/2) and ν_1 (5/2-7/2) transitions of ^{123}Sb in $SbCl_3$ are shown in Figure 10. It is clear that the inhomogeneous broadening of the ν_1 and ν_2 transitions in particular are strongly correlated, leading to an elliptical peak shape.

The simultaneity of the three frequency measurements, combined with the elimination of inhomogeneous broadening for single spin-packets, makes it possible to very accurately measure the ratios of the three frequencies of a spin 1/2 nucleus, and thus search for a possible hexadecapole interaction, which was proposed for $SbCl_3$ in the first NQR study [16], and has been argued about ever since. The present spectra sets the hexadecapolar coupling constant $e^2 m_{16} M_{16}/h$ equal to 18 kHz, close to the original measurement by Wang, and considerably at variance with later measurements.

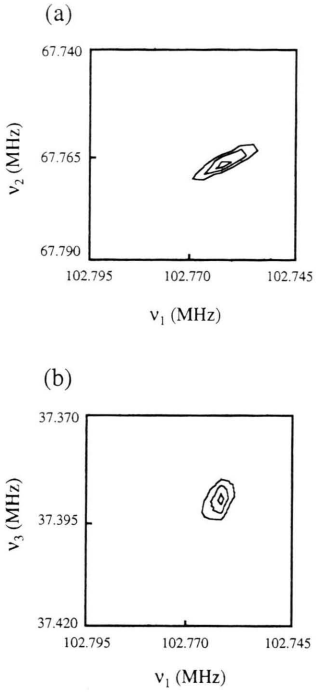

Fig. 10. Projections from the three-dimensional zero-field NMR spectrum of the ^{123}Sb nucleus in antimony trichloride, showing the correlation between (a) the (7/2-5/2) and (5/2-3/2) transition and (b) the (7/2-5/2) and (3/2-1/2) transition.

Conclusions

Multi-dimensional NQR measurements are a valuable extension of the NQR technique, which greatly extend the information available and the accuracy of parameters obtainable. We anticipate that in the future they will be of even more value in extending the value of this now half-century old spectroscopy.

Acknowledgements

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