

The Measurement of Heteronuclear Transverse Relaxation Times in AX₃ Spin Systems via Polarization-Transfer Techniques

LEWIS E. KAY,^{*,†} T. E. BULL,[‡] L. K. NICHOLSON,[§] C. GRIESINGER,[¶]
H. SCHWALBE,[¶] A. BAX,^{*} AND D. A. TORCHIA[§]

**Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; ‡Biophysics Laboratory, Division of Biochemistry and Biophysics, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892; §Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892; and ¶Institut für Organische Chemie, Universität Frankfurt, Niederurseler Hang, D-6000 Frankfurt 50, Germany*

Received December 31, 1991; revised March 17, 1992

Pulse schemes for the measurement of ¹³C transverse relaxation times in AX₃ spin systems are described which make use of the sensitive ¹H spin for detection. The experiments are based on reverse-DEPT and reverse-INEPT polarization-transfer sequences. It is shown that relaxation rates obtained from ¹³C-direct-observe and from polarization-transfer experiments are identical only if magnetization from each of the ¹³C transitions is transferred equally to the detected ¹H spins. This requires judicious choices in pulse angles and delays in reverse-DEPT and reverse-INEPT experiments. For application to macromolecules, experimental and theoretical results suggest that polarization-transfer schemes based on reverse INEPT are superior to reverse-DEPT-based sequences and give results which are in good agreement with values measured via ¹³C-observe methods. © 1992 Academic Press, Inc.

NMR is a powerful technique for obtaining information regarding internal dynamics of proteins. The measurement of relaxation properties of nuclei such as ¹⁵N and ¹³C is particularly promising in this regard since the relaxation of these nuclei is governed by dipolar interactions with directly attached protons and to a lesser extent by chemical-shift-anisotropy contributions (1). In general, the interpretation of ¹⁵N or ¹³C relaxation data does not require a knowledge of the overall structure of the molecule in question, unlike the situation for ¹H relaxation studies. Resonance assignments made with the recently developed double- and triple-resonance 3D and 4D NMR techniques (2–4) provide a large number of ¹⁵N and ¹³C probes of dynamics that can be studied throughout the protein. Many of these ¹³C probes are attached to methylene or methyl groups and can provide detailed information concerning sidechain dynamics.

Initial measurements of ¹³C relaxation properties in biomolecules were based on direct observation of ¹³C (referred to as ¹³C-direct-observe experiments in what follows) using one-dimensional NMR techniques. Unfortunately, the low sensitivity of these

[†] Present address: Departments of Medical Genetics, Biochemistry, and Chemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

heteronuclei leads to the requirements of large amounts of sample and substantial measuring times, while the lack of resolution in one-dimensional spectra limits the measurements to the extraction of either bulk relaxation times or relaxation times of only a limited number of well-resolved or selectively labeled resonances. These problems led to the development of one-dimensional (5, 6) and two-dimensional NMR experiments (7–9) that enable indirect measurement of relaxation properties of insensitive nuclei, such as ^{13}C or ^{15}N .

Despite the substantial sensitivity advantages enjoyed by these new experiments over ^{13}C -direct-observe methods, significant errors in measured ^{13}C relaxation rates can result. For example, Sklenar *et al.* (5) reported errors as large as 20% in the measurement of the ^{13}C longitudinal relaxation rate of $^{13}\text{CH}_3$ -acetate from a ^1H - ^{13}C - ^1H double-DEPT transfer scheme with the θ pulses in the DEPT and reverse DEPT (REVDEPT) portions of the sequence set to 36° for optimum sensitivity. These errors are, in part, the result of the creation of multispin terms by the $^1\text{H} \rightarrow ^{13}\text{C}$ transfer which are subsequently transferred to observable magnetization by the REVDEPT sequence.

Recently, Palmer *et al.* have shown that the accurate measurement of ^{13}C T_1 values in AX_3 spin systems by double-DEPT- or INEPT-based polarization-transfer schemes (10) requires a judicious choice of θ (REVDEPT) or τ' (reverse INEPT, REVINEPT) (11). With these improved sequences, identical ^{13}C T_1 values were obtained for $^{13}\text{CH}_3$ -acetate measured using both the double-DEPT polarization-transfer sequence and the ^{13}C -direct-observe experiment (11). The extraction of accurate ^{13}C relaxation rates in macromolecules by polarization-transfer methods is, however, more difficult than that for small molecules. Measurements in both small and large molecules are complicated by interference between (a) different ^1H - ^{13}C dipolar interactions (12, 13) and (b) dipolar and chemical-shift-anisotropy interactions (14). In addition, measurements in large molecules may be further complicated by severely nonexponential relaxation of methyl ^1H magnetization (15). For example, for molecules tumbling with an overall correlation time of 10 ns the transverse relaxation times of the fastest-decaying ^1H methyl components for a rapidly rotating methyl group are on the order of 10 ms, while the relaxation times of the other components are over an order of magnitude longer. As we will describe below, this effect can lead to the erroneous measurement of relaxation times for heteroatoms when using pulse sequences that require delays on the order of the relaxation time of the fast-relaxing component of ^1H magnetization.

In this article we describe the origin of the errors that occur in the application of polarization-transfer schemes in relaxation measurements and we present a pulse sequence which minimizes such effects. While we will focus primarily on transverse relaxation measurements, the conclusions presented also apply to the measurement of T_1 relaxation times.

THEORY

Figure 1 shows the pulse schemes for measuring ^{13}C T_2 relaxation times of AX_3 spin systems that will be analyzed in this paper. These schemes are variants of pulse sequences used previously for the measurement of T_1 and T_2 values of low-gamma nuclei (5–9). After a transverse relaxation delay of duration T , ^{13}C magnetization is

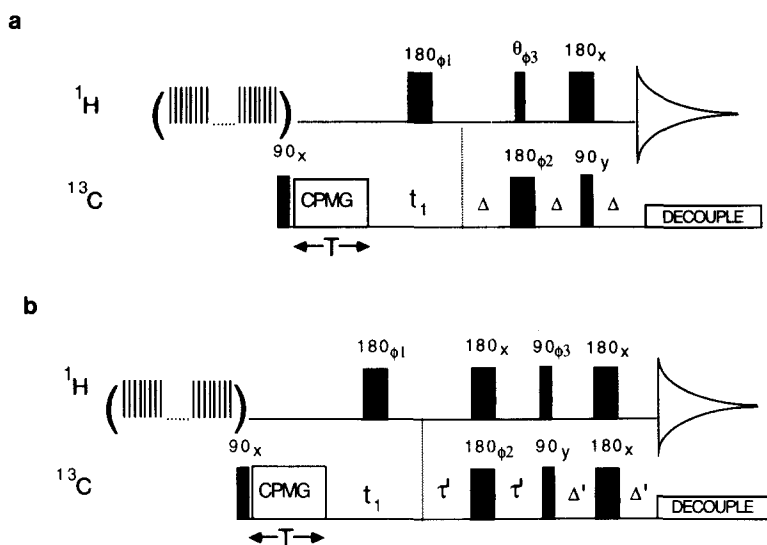


FIG. 1. Pulse sequences used to measure ^{13}C T_2 values via REVDEPT (a) or REVINEPT (b) transfers. Proton saturation is achieved by the application of a train of ^1H 125° pulses, with each pulse separated by 5 ms, prior to the first ^{13}C 90° pulse. A modified CPMG pulse scheme is employed to eliminate the effects of dipolar and CSA cross correlation and minimize the effects of ^1H - ^{13}C dipolar cross correlation (26, 32). This is achieved by application of ^1H 125° pulses, with the center of these pulses at the peak of the spin echo, at a rate fast compared to the rate of decay of transverse magnetization associated with the multiplet components. Typically ^{13}C 180° pulses are applied every 400–500 μs and ^1H 125° pulses every 5 ms. The rapid rate of application of ^{13}C pulses ensures that ^{13}C magnetization remains in-phase during the duration of the CPMG interval. In (a) the value of θ must be set to 54.7° . The results from the present study indicate that Δ must be set $\ll 1/(2J_{\text{CH}})$ if accurate values of ^{13}C transverse relaxation rates are to be obtained. The phase cycling used is $\phi_1 = 8(x), 8(-x)$; $\phi_2 = x, y, -x, -y$; $\phi_3 = 4(x), 4(-x)$; Acq = $2(x, -x), 2(-x, x)$. Quadrature in F_1 is achieved by incrementing the phase of the second ^{13}C 90° pulse via TPPI (39). In (b) a value of τ' must be chosen such that $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad). For a J_{CH} value of 130 Hz, $\tau' = 1.17$ ms. We find that a value of $\Delta' = 1/(8J_{\text{CH}})$ is a sufficiently short delay to allow for the measurement of accurate transverse relaxation times for proteins with $\tau_c < \sim 10$ ns. The phase cycle employed is $\phi_1 = 8(x), 8(-x)$; $\phi_2 = x, y, -x, -y$; $\phi_3 = 4(x), 4(-x)$; Acq = $2(x, -x), 2(x, -x)$. Quadrature in F_1 is achieved by incrementing the phase of the second ^{13}C 90° pulse via TPPI (39).

transferred to proton magnetization via a REVDEPT (16, 17) or REVINEPT (18–20) transfer sequence, thereby increasing the sensitivity of the measurements by a factor of $(\gamma_{\text{H}}/\gamma_{\text{C}})^{1.5} \sim 8$ over direct detection of the heteroatom. The sensitivity of the experiment is improved further by applying proton presaturation so as to develop the full ^1H - ^{13}C NOE. Even for methyl groups attached to macromolecules, the ^1H - ^{13}C NOE can be significant: for $^{13}\text{C}^\delta$ -leucine residues in the protein Staphylococcal nuclease (SNase, M_r 17.5 kDa) an average NOE of 2.5 was measured (21). Furthermore, saturation of the protons prior to the start of the experiment effectively suppresses magnetization originating from protons that are not directly coupled to ^{13}C spins.

In this section we discuss the effects of the parameters θ and τ' in Fig. 1 on relaxation times measured via reverse polarization-transfer schemes and describe how the rapid and multiexponential decay of ^1H magnetization during the fixed delays in the REVDEPT and REVINEPT sequences influences extracted relaxation times. In ad-

dition, we present a strategy for minimizing experimental error due to multiexponential relaxation while eliminating the effects of cross correlation between dipolar and chemical-shift-anisotropy (CSA) interactions on the measurement of relaxation times in macromolecules.

The effects of θ (DEPT) and τ' (INEPT) on measured relaxation times. Figure 2 shows an energy-level diagram and associated wavefunctions for an AX₃ spin system written in an irreducible basis representation. The transitions which give rise to ¹³C single-quantum magnetization are indicated with arrows. The components of the density operator corresponding to ¹³C magnetization can be expressed as linear combinations of single-quantum-transition operators (22) so that we can write, for example,

$$\rho_{8,16} = |8\rangle\langle 16| = |\beta\beta\beta\beta\rangle\langle\alpha\beta\beta\beta| = S_- I_\beta^1 I_\beta^2 I_\beta^3 \quad [1]$$

where $\rho_{8,16}$ is the component of the density operator which connects states $|8\rangle$ and $|16\rangle$, $|k\rangle$ refers to the eigenstate k indicated in Fig. 2, the first spin state in the wavefunction $|k\rangle$ corresponds to the ¹³C spin state with the remaining three states corresponding to the proton spin-states, and S_j and I_j^r ($j = -, +, \alpha, \beta$) are single-spin-transition operators operating on carbon and proton (spin r) spins, respectively. It is possible to express I_j^r (or S_j) in terms of Cartesian components by noting that

$$\begin{aligned} I_+^r &= I_x^r + iI_y^r \\ I_-^r &= I_x^r - iI_y^r \\ I_\alpha^r &= I_z^r + 0.5I^r \\ I_\beta^r &= -I_z^r + 0.5I^r, \end{aligned} \quad [2]$$

where $i = \sqrt{-1}$ and I^r is the identity operator for spin r . Writing the density operators in terms of transition operators, one arrives at

$$\begin{aligned} \rho_{8,16} &= S_- I_\beta^1 I_\beta^2 I_\beta^3 \\ \rho_{7,15} &= (1/3)S_- [I_\beta^1 I_\beta^2 I_\alpha^3 + I_\beta^1 I_\alpha^2 I_\beta^3 + I_\alpha^1 I_\beta^2 I_\beta^3 + I_\beta^1 (2I_x^2 I_x^3 + 2I_y^2 I_y^3) \\ &\quad + I_\beta^2 (2I_x^1 I_x^3 + 2I_y^1 I_y^3) + I_\beta^3 (2I_x^1 I_x^2 + 2I_y^1 I_y^2)] \\ \rho_{6,14} &= (1/2)S_- [I_\beta^1 I_\alpha^2 I_\beta^3 + I_\beta^1 I_\beta^2 I_\alpha^3 - I_\beta^1 (2I_x^2 I_x^3 + 2I_y^2 I_y^3)] \\ \rho_{5,13} &= (1/6)S_- [I_\beta^1 I_\beta^2 I_\alpha^3 + I_\beta^1 I_\alpha^2 I_\beta^3 + 4I_\alpha^1 I_\beta^2 I_\beta^3 + I_\beta^1 (2I_x^2 I_x^3 + 2I_y^2 I_y^3) \\ &\quad - 2I_\beta^2 (2I_x^1 I_x^3 + 2I_y^1 I_y^3) - 2I_\beta^3 (2I_x^1 I_x^2 + 2I_y^1 I_y^2)] \quad [3] \end{aligned}$$

with similar relationships holding for the other density elements. The density elements $\rho_{8,16}$ and $\rho_{1,9}$ give rise to the outer lines of the ¹³C quartet while the other elements, $\rho_{7,15}$, $\rho_{6,14}$, $\rho_{5,13}$, and $\rho_{4,12}$, $\rho_{2,10}$, $\rho_{3,11}$, contribute to the inner two lines of the quartet. The frequencies of each of the lines of the quartet differ by J_{CH} , where J_{CH} is the one-bond ¹H-¹³C coupling. In ¹³C-direct-observe experiments (pulse-acquire experiments, for example) it is easily shown that, in the absence of relaxation, all ¹³C transitions make equal contributions to the observed magnetization so that a 1:3:3:1 quartet results. For the pulse schemes shown in Fig. 1, where magnetization is transferred from ¹³C to ¹H for detection, this is in general not the case.

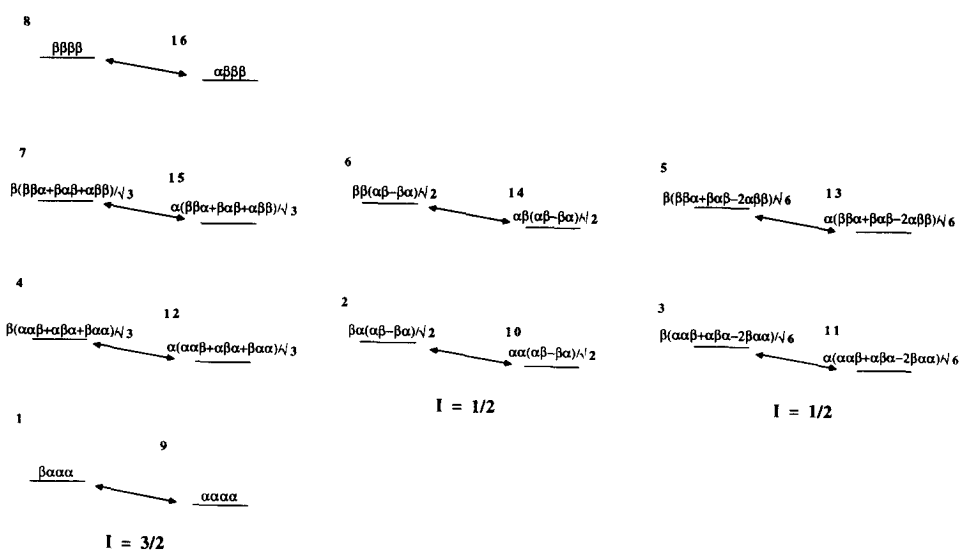


FIG. 2. Energy-level diagram of an isolated AX₃ spin system with the wavefunctions written in an irreducible basis representation. In this representation the wavefunctions can be grouped into three manifolds (a 3/2 manifold and two 1/2 manifolds) and the total spin angular momentum, I , associated with each manifold is indicated. Each of the eigenstates is numbered from 1 to 16. The first spin state in wavefunction $|k\rangle$ corresponds to the ¹³C spin state and the remaining three spin states are associated with the proton spins.

In the sequences in Fig. 1, application of the first 90° ¹³C pulse establishes ¹³C transverse magnetization and the density elements $\rho_{8,16}$, $\rho_{1,9}$, $\rho_{7,15}$, $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{4,12}$, $\rho_{2,10}$, and $\rho_{3,11}$ corresponding to the ¹³C single-quantum transitions are all nonzero. A Carr–Purcell–Meiboom–Gill (CPMG) pulse train (23, 24) follows, during which time these density elements relax. Carbon 180° pulses are applied during this interval at a rate much greater than $1/(2J_{\text{CH}})$ so that the magnetization effectively remains locked along the RF field. This ensures that the ¹³C magnetization remains in-phase with respect to the directly coupled ¹H spins. It has been shown previously that, for macromolecules, antiphase heteronuclear magnetization relaxes more rapidly than in-phase magnetization due to very efficient spin flips involving the directly coupled ¹H spins and other ¹H spins in proximity (9, 25–27). As has been long known, the ¹³C transverse relaxation rate measured with a CPMG sequence depends on the spacing between 180° pulses and only when this spacing is much less than $1/(2J_{\text{CH}})$ are “correct” T_2 values obtained from a direct measure of the decay of magnetization (28). The close spacing of the ¹³C 180° pulses also allows cross relaxation among the individual lines of the multiplet. The relaxation of the density elements during the CPMG pulse train can be calculated using Redfield theory (29) according to

$$d\rho/dt = \mathbf{R}\rho, \quad [4]$$

where ρ is a vector containing all density elements corresponding to transverse ¹³C magnetization and \mathbf{R} is a Redfield relaxation operator. For dipolar interactions of various pairs of interacting spins ij , kl the matrix elements of \mathbf{R} are

$$\begin{aligned}
 R_{\alpha\alpha'\alpha''\alpha'''}^{ijkl} = & \sum_{m,n} \{ (-1)^m \delta_{m,-n} 2J_{ijkl}^{00}(\omega_{\alpha\alpha'}) \langle \alpha | T_2^m(ij) | \alpha'' \rangle \langle \alpha''' | T_2^n(kl) | \alpha' \rangle \\
 & - \delta_{\alpha,\alpha''} \sum_{\beta} (-1)^m \delta_{m,-n} 2J_{ijkl}^{00}(\omega_{\alpha''\beta}) \langle \alpha''' | T_2^m(ij) | \beta \rangle \langle \beta | T_2^n(kl) | \alpha' \rangle \\
 & - \delta_{\alpha',\alpha'''} \sum_{\beta} (-1)^m \delta_{m,-n} 2J_{ijkl}^{00}(\omega_{\alpha'\beta}) \langle \alpha | T_2^m(ij) | \beta \rangle \langle \beta | T_2^n(kl) | \alpha'' \rangle \}. \quad [5]
 \end{aligned}$$

In Eq. [5] α , α' , α'' , α''' , and β denote the various eigenstates of an AX_3 spin system, $T_2^m(ij)$ are the components of the second-rank dipolar spin tensor with n or m running from -2 to 2 , and $\delta_{\alpha\alpha'}$ is a Kronecker delta function equal to 0 if $\alpha \neq \alpha'$ and equal to 1 otherwise. The $J_{ijkl}^{00}(\omega_{\alpha\alpha'})$ are the second-rank spherical harmonic spectral densities evaluated at the frequency $\omega_{\alpha\alpha'} = (E_{\alpha'} - E_{\alpha})/\hbar$.

Figure 3 illustrates the time dependence of the density elements that contribute to transverse ^{13}C magnetization in an isolated AX_3 spin system using the Woessner model to describe the internal dynamics (30). These are the density elements that are nonzero after the application of the first 90° ^{13}C pulse in the sequences in Fig. 1 and that will contribute to observed ^1H magnetization after the REVDEPT and REVINEPT transfers. Values of $\tau_c = 10$ ns, $\tau_e = 25$ ps and $\tau_c = 0.5$ ns, $\tau_e = 25$ ps are considered, where τ_c and τ_e are the correlation times for the overall tumbling and for the internal methyl rotation, respectively. Only the effects of ^1H - ^{13}C dipolar interactions are included in the calculation. In general, for methyl groups where $\Delta\delta \sim 25$ ppm (31), the contributions to relaxation due to ^{13}C CSA are much less significant than those from the ^1H - ^{13}C dipolar interaction. Moreover, it has been shown recently that the effects of cross correlation between dipolar and CSA interactions can be eliminated by application of ^1H 180° pulses during the relaxation interval (T in Fig. 1) at a rate which is fast compared to the relaxation of the individual multiplet components (26, 32, 33). Figure 3 indicates that the relaxation of the two outer lines of the ^{13}C quartet (corresponding to $\rho_{8,16}$ and $\rho_{1,9}$) are identical (in the absence of dipolar/CSA cross correlation) and that they relax much more rapidly than the inner lines. Note that for $\tau_c = 10$ ns (Fig. 3A), the density elements $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{2,10}$, and $\rho_{3,11}$ relax at the same rate, which is slightly faster than the rate for $\rho_{4,12}$ and $\rho_{7,15}$. For the case where $\tau_c = 0.5$ ns (Fig. 3B) the relaxation rates of the density elements are more similar and in this case the transverse relaxation of $\rho_{4,12}$ and $\rho_{7,15}$ is more efficient than the relaxation of $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{2,10}$, and $\rho_{3,11}$.

After the CPMG interval, during which transverse relaxation of the density elements occurs, the ^{13}C chemical shift is recorded during t_1 and the ^1H - ^{13}C scalar coupling is refocused by the application of a ^1H 180° pulse at the center of t_1 . Magnetization is subsequently transferred to protons for detection via either a REVDEPT or a REVINEPT sequence. By recording several of these 2D experiments with different CPMG intervals and measuring cross-peak intensities as a function of T , transverse relaxation times can be readily extracted. The transfer of magnetization from the individual ^{13}C transitions back to protons can be calculated by expressing the density elements of Eq. [3] in terms of Cartesian operators and using the expressions given

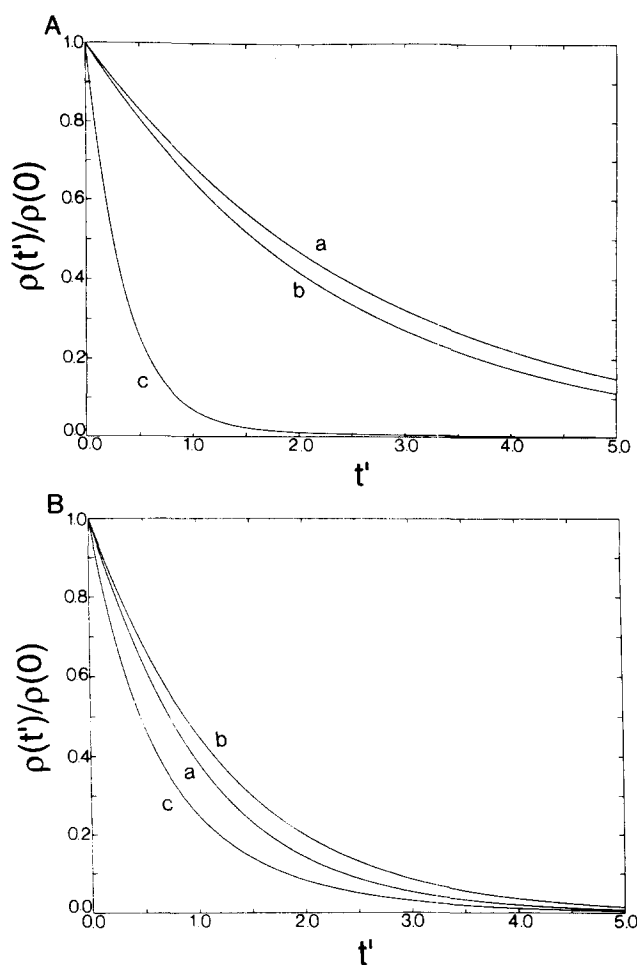


FIG. 3. The decay of the density elements corresponding to ^{13}C single-quantum magnetization in an isolated AX_3 spin system as a function of t' assuming relaxation from ^1H - ^{13}C dipolar interactions only. The reduced variable, t' , is defined as $t' = t/T_2$, where T_2 is the time constant for the decay of the density elements in the absence of cross correlation. Curve (a) shows the decay of $\rho_{4,12}$ and $\rho_{7,15}$, curve (b) the decay of elements $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{2,10}$, and $\rho_{3,11}$, and curve (c) the decay of density elements $\rho_{1,9}$ and $\rho_{8,16}$. In A and B, the Woessner model (30) is used to describe methyl group dynamics with $\tau_c = 10$ ns, $\tau_e = 25$ ps (A) and $\tau_c = 0.5$ ns, $\tau_e = 25$ ps (B).

by Sørensen *et al.* (34) to describe the effects of pulses, chemical shift, and J -coupled evolution on these operators. For example, $\rho_{8,16}$ can be written as

$$\rho_{8,16} = S_- I_\beta^1 I_\beta^2 I_\beta^3 = (1/8) S_- \{1 - 2I_z + 4(I_z^1 I_z^2 + I_z^1 I_z^3 + I_z^2 I_z^3) - 8I_z^1 I_z^2 I_z^3\}, \quad [6]$$

where $I_z = \sum_r I_z^r$.

It is easily shown that the magnetization transferred from ^{13}C to ^1H by the sequences in Fig. 1a (REVDEPT) or Fig. 1b (REVINEPT) is given by

$$\begin{array}{l}
 \rho_{8,16}, \rho_{1,9} \xrightarrow{\text{REVDEPT}} 1/8 \cos^2\theta \sin \theta M_{\text{TR}} \\
 \rho_{7,15}, \rho_{4,12} \xrightarrow{\text{REVDEPT}} \{(1/8)\cos^2\theta \sin \theta + (1/12)\sin \theta(3 \cos^2\theta - 1)\} M_{\text{TR}} \\
 \rho_{6,14}, \rho_{2,10}, \rho_{5,13}, \rho_{3,11} \xrightarrow{\text{REVDEPT}} \{(1/8)\cos^2\theta \sin \theta - (1/24)\sin \theta(3 \cos^2\theta - 1)\} M_{\text{TR}} \\
 \rho_{8,16}, \rho_{1,9} \xrightarrow{\text{REVINEPT}} (1/8)\sin(6\pi J_{\text{CH}}\tau') M_{\text{TR}} \\
 \rho_{7,15}, \rho_{4,12}, \rho_{6,14}, \rho_{2,10}, \rho_{5,13}, \rho_{3,11} \xrightarrow{\text{REVINEPT}} (1/24)\sin(2\pi J_{\text{CH}}\tau') M_{\text{TR}}, \quad [7]
 \end{array}$$

where M_{TR} is the transverse ^1H magnetization detected during t_2 . The net ^1H magnetization detected during t_2 is given by the sum of the magnetization transferred from the eight density terms indicated in Eq. [7]. In the absence of relaxation (i.e., if the CPMG interval is set to zero and relaxation during the Δ , τ' , and Δ' delays is ignored) the intensity of net observed ^1H magnetization is given by $\cos^2\theta \sin \theta$ and $\cos^2 2\pi J_{\text{CH}}\tau' \sin 2\pi J_{\text{CH}}\tau'$ for the REVDEPT and REVINEPT sequences, respectively. Note that the transfer of magnetization from the density elements corresponding to ^{13}C magnetization to ^1H magnetization differs for the different density elements, even in the absence of relaxation (see Eq. [7]). Since, in general, the relaxation rates of the individual density elements are not the same (Fig. 3), different ^{13}C relaxation times will be measured for different values of θ and τ' in the REVDEPT and REVINEPT experiments, respectively. In theory, only when the transfer of ^{13}C magnetization to ^1H is the same for each of the eight ^{13}C transitions will the ^{13}C relaxation rates measured via the sequences in Fig. 1 be the same as the relaxation rates measured by ^{13}C -direct-observe methods. This is because for ^{13}C -direct-observe experiments, in the absence of relaxation, each ^{13}C transition contributes equally to the observed magnetization. Inspection of Eq. [7] indicates that for $\theta = 54.7^\circ$ ($3 \cos^2\theta - 1 = 0$) in the REVDEPT sequence this condition is met. For the case of the REVINEPT sequence, equal contributions from each ^{13}C transition require that

$$(1/8)\sin 6\pi J_{\text{CH}}\tau' = (1/24)\sin 2\pi J_{\text{CH}}\tau' \quad [8]$$

or that $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad). Identical conclusions were previously reported by Palmer *et al.* for double-DEPT and INEPT sequences for the measurement of ^{13}C T_1 relaxation times in AX_3 spin systems (11).

Figure 4 shows the ratio of T_2 values calculated from the initial rates of decay of transverse ^{13}C magnetization obtained from REVDEPT (Fig. 4A) or REVINEPT (Fig. 4B) and the ^{13}C -direct-observe experiments as a function of θ ($\theta' = 2\pi J_{\text{CH}}\tau'$) for an isolated AX_3 spin system. In the example chosen, the methyl internal dynamics are described by the Woessner model (30) with $\tau_c = 10$ ns, $\tau_e = 25$ ps and $\tau_c = 0.5$ ns, $\tau_e = 25$ ps. Moreover, we have assumed that the delays Δ , τ' , and Δ' in the REVDEPT and REVINEPT sequences indicated in Fig. 1 are short compared to the operative transverse relaxation times during these intervals. As we will discuss later, if transverse relaxation cannot be neglected during these delays, as is most often the case for ap-

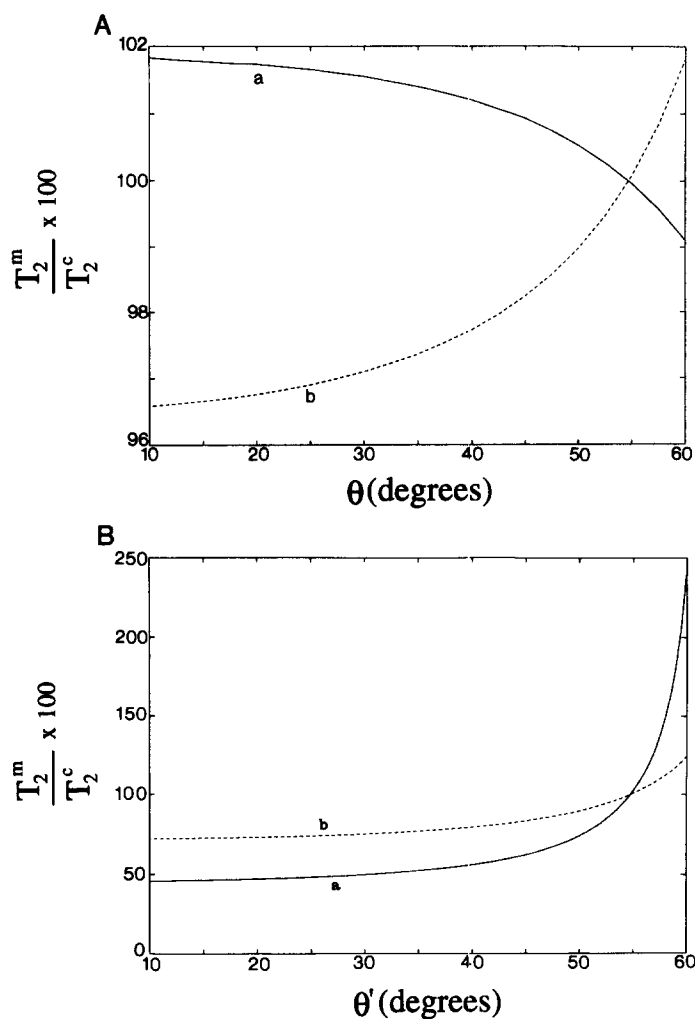


FIG. 4. Theoretical ratios of T_2 values calculated from either the REVDEPT (A) or the REVINEPT (B) experiment (T_2^m) versus T_2 values calculated from ^{13}C -direct-observe methods (T_2^c) as a function of θ (A) or $\theta' = 2\pi J_{\text{CH}}\tau'$ (B). The T_2^m/T_2^c ratio is measured from a sampling of the initial rate of decay of transverse magnetization. Dipolar/CSA cross correlation is not included in this calculation. The Woessner model is used to describe the methyl group dynamics with $\tau_c = 10$ ns, $\tau_e = 25$ ps (a) or $\tau_c = 0.5$ ns, $\tau_e = 25$ ps (b).

plication to macromolecules, additional errors in the measurement of transverse relaxation rates occur. For $\theta < 54.7^\circ$ and $\tau_c = 10$ ns, the calculations predict that underestimates of the initial decay rate of ^{13}C transverse magnetization are obtained via the REVDEPT sequence. This is because for $\theta < 54.7^\circ$, ^{13}C magnetization associated with the density elements $\rho_{7,15}$ and $\rho_{4,12}$ is transferred more efficiently into ^1H magnetization than magnetization associated with the other six density elements (i.e., $\{(1/8)\cos^2\theta \sin\theta + (1/12)\sin\theta(3\cos^2\theta - 1)\} > (1/8)\cos^2\theta \sin\theta > \{(1/8)\cos^2\theta \times \sin\theta - (1/24)\sin\theta(3\cos^2\theta - 1)\}$; see Eq. [7]). This weights the apparent ^{13}C T_2

measured from the initial decay of magnetization more toward the rate of decay of $\rho_{7,15}$ and $\rho_{4,12}$ than is the case for the ^{13}C -direct-observe experiment. Since for $\tau_c = 10$ ns the decay rates of $\rho_{7,15}$ and $\rho_{4,12}$ are smaller than the rates of decay of the other six density elements, the REVDEPT experiment gives initial decay rates lower than rates measured from the ^{13}C -observe experiment. For $\theta > 54.7^\circ$, the density elements $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{2,10}$, and $\rho_{3,11}$ make a contribution to the final observed ^1H signal larger than that of the elements $\rho_{7,15}$ and $\rho_{4,12}$ so that the apparent decay rate of ^{13}C magnetization is skewed in the opposite direction. For $\tau_c = 0.5$ ns, Fig. 3 shows that the decay rates of $\rho_{7,15}$ and $\rho_{4,12}$ are larger than the decay rates of $\rho_{6,14}$, $\rho_{5,13}$, $\rho_{2,10}$, and $\rho_{3,11}$. This reverses the θ dependence of the error in measured initial ^{13}C decay rates relative to the case where $\tau_c = 10$ ns.

In the REVINEPT experiment a choice of τ' such that $2\pi J_{\text{CH}}\tau' < 54.7^\circ$ ensures that $(1/8)\sin 6\pi J_{\text{CH}}\tau' > (1/24)\sin 2\pi J_{\text{CH}}\tau'$. In this regime, density elements $\rho_{8,16}$ and $\rho_{1,9}$ contribute more to the observed signal than the other density elements. Since these elements decay more rapidly than the other density elements, values of the initial transverse decay rate of ^{13}C magnetization measured with INEPT based sequences are larger than values measured from ^{13}C -direct-observe experiments. In a similar way, values of τ' for which $(1/8)\sin 6\pi J_{\text{CH}}\tau' < (1/24)\sin 2\pi J_{\text{CH}}\tau'$ will lead to measured ^{13}C decay rates that are smaller than the values measured via ^{13}C observation. Only for the case where θ or $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad) and in the absence of relaxation during the delays τ' , Δ , and Δ' in the sequences in Fig. 1 will each of the eight ^{13}C transitions contribute equally to the observed ^1H magnetization. In principle, for these values of θ and τ' , the same ^{13}C relaxation rates should be obtained via ^{13}C -observe and indirect detection pulses schemes.

Figure 4 shows that in order for accurate transverse relaxation times to be measured from REVINEPT-based sequences, θ' must be set as close to 54.7° as possible. In contrast, relaxation times measured from REVDEPT-based sequences are, in theory, much more tolerant of missettings of θ . Based on this, one might conclude that REVDEPT-based sequences are to be preferred over their REVINEPT-based counterparts for the measurement of ^{13}C relaxation times in AX_3 spin systems. However, as will be shown in the following sections, both theoretical and experimental results suggest that more accurate ^{13}C T_2 values of methyl groups in macromolecules can be obtained with REVINEPT-based sequences.

The effects of multiexponential proton relaxation on measured relaxation times from DEPT- and INEPT-based sequences. The extraction of accurate relaxation times from polarization-transfer-based schemes is complicated further by the fact that (a) REVDEPT and REVINEPT sequences do not transfer magnetization from *individual* ^{13}C transitions to *individual* ^1H transitions in an equal manner even when θ and $2\pi J_{\text{CH}}\tau'$ are set to 54.7° (0.955 rad) and (b) the transverse relaxation rates of the individual methyl ^1H transitions are unequal when $\omega\tau_c > 1$ (15, 35, 36). For example, in the limit of a large molecule and rapid internal rotation of the methyl group, the transverse relaxation rates of the ^1H transitions $\pm 3/2 \leftrightarrow \pm 1/2$ are much larger than the rates of the $\pm 1/2 \leftrightarrow \mp 1/2$ transitions, since the former depend on $J(0)$ of the ^1H - ^1H dipolar interaction, while the latter do not (15, 35, 36). The relaxation of both transitions depends on $J(0)$ of the ^1H - ^{13}C dipolar interaction, but the ^1H - ^1H relaxation rate of the $\pm 3/2 \leftrightarrow \pm 1/2$ transitions $[1/T_2 = 9(0.250)\gamma_{\text{H}}^4\hbar^2\tau_c/(5r_{\text{HH}}^6) = (3/2)J_{\text{HH}}(0)]$

(35, 36)] is ~ 20 times faster than the ^1H - ^{13}C rate [$1/T_2 = 0.2(0.111)\gamma_{\text{H}}^2\gamma_{\text{C}}^2\hbar^2\tau_c/r_{\text{HC}}^6 = (2/27)J_{\text{HC}}(0)$ (37)]. Thus the $\pm 3/2 \leftrightarrow \pm 1/2$ transitions relax ~ 20 times faster than the $\pm 1/2 \leftrightarrow \mp 1/2$ transitions. For a protein the size of SNase (M_r , 17.5 kDa), the T_2 values of the $\pm 3/2 \leftrightarrow \pm 1/2$ components of ^1H magnetization are calculated to be less than 12 ms. Hence, a substantial fraction of the ^{13}C magnetization that is transferred to the $\pm 3/2 \leftrightarrow \pm 1/2$ components of ^1H magnetization will decay prior to acquisition if the delay values Δ and Δ' are set for optimum sensitivity [$\Delta = 1/(2J_{\text{CH}})$ and $\Delta' = 1/(4J_{\text{CH}})$]. As is shown below, for the REVDEPT-based transfer, the largest fraction of ^{13}C magnetization associated with the outer components of the ^{13}C quartet is transferred to the fast-decaying components of ^1H magnetization. In contrast, the bulk of magnetization associated with inner quartet components is transferred to the slowly relaxing ^1H $\pm 1/2 \leftrightarrow \mp 1/2$ transitions. Because it is primarily the transfer of ^{13}C magnetization associated with the outer components of the quartet that is affected by the fast relaxation of the $\pm 3/2 \leftrightarrow \pm 1/2$ ^1H components, the REVDEPT experiment results in an admixture of the ^{13}C outer lines smaller than that in the ^{13}C -direct-observe experiment. Since the ^{13}C outer lines relax more rapidly than the inner lines, ^{13}C relaxation rates obtained from the REVDEPT scheme (with θ set to 54.7°) are smaller than the rates measured via ^{13}C -direct-observe experiments for molecules with $\omega\tau_c > 1$.

The previous discussion can be cast in a more quantitative light by considering the REVDEPT sequence and noting that immediately before acquisition, magnetization associated with the individual ^1H transitions $-3/2 \leftrightarrow -1/2$, $-1/2 \leftrightarrow 1/2$, and $1/2 \leftrightarrow 3/2$ of the $3/2$ manifold arising as a result of transfer from $\rho_{8,16}$ is calculated to be

$$\rho_{8,16} \rightarrow (1/4)\cos^5(\theta/2)\sin(\theta/2)\exp(-2\Delta/T_2) \quad (-3/2 \leftrightarrow -1/2) \quad [9a]$$

$$\rho_{8,16} \rightarrow -(1/2)\cos^3(\theta/2)\sin^3(\theta/2) \quad (-1/2 \leftrightarrow 1/2) \quad [9b]$$

$$\rho_{8,16} \rightarrow (1/4)\cos(\theta/2)\sin^5(\theta/2)\exp(-2\Delta/T_2) \quad (1/2 \leftrightarrow 3/2), \quad [9c]$$

where the factor $\exp(-2\Delta/T_2)$ accounts for the rapid transverse relaxation of the ^1H $\pm 3/2 \leftrightarrow \pm 1/2$ transitions. (That is, T_2 is the transverse relaxation time for the $\pm 3/2 \leftrightarrow \pm 1/2$ transitions due to ^1H - ^1H dipolar interactions and it is assumed to be the same for both Δ periods. It should be pointed out that the T_2 values are not rigorously the same for the two Δ intervals since multiple-quantum magnetization is present during the first interval while ^1H single-quantum magnetization is present during the second Δ period. The difference in relaxation rates is small, however, since in both cases the dominant contribution to relaxation is from ^1H - ^1H dipolar interactions.) The transverse relaxation rate of the $\pm 1/2 \leftrightarrow \mp 1/2$ transitions during the pulse sequence can be safely neglected for application to proteins with correlation times ≤ 15 ns. The transfer from $\rho_{1,9}$ is identical to the above with the exception that the expressions for the transfer to the $-3/2 \leftrightarrow -1/2$ and the $3/2 \leftrightarrow 1/2$ transitions must be interchanged. The transfer of magnetization from $\rho_{7,15}$ to the ^1H transitions $-3/2 \leftrightarrow -1/2$, $-1/2 \leftrightarrow 1/2$, and $1/2 \leftrightarrow 3/2$ of the $3/2$ manifold is calculated to be

$$\rho_{7,15} \rightarrow (1/4)[\cos^5(\theta/2)\sin(\theta/2) - 2\cos^3(\theta/2)\sin^3(\theta/2)]\exp(-2\Delta/T_2) \quad (-3/2 \leftrightarrow -1/2) \quad [10a]$$

$$\rho_{7,15} \rightarrow (1/6)[2\cos^5(\theta/2)\sin(\theta/2) - 5\cos^3(\theta/2)\sin^3(\theta/2) + 2\cos(\theta/2)\sin^5(\theta/2)] \quad (-1/2 \leftrightarrow 1/2) \quad [10b]$$

$$\rho_{7,15} \rightarrow (1/4)[\cos(\theta/2)\sin^5(\theta/2) - 2\cos^3(\theta/2)\sin^3(\theta/2)]\exp(-2\Delta/T_2) \quad (1/2 \leftrightarrow 3/2). \quad [10c]$$

The transfer from $\rho_{4,12}$ is identical to the transfer from $\rho_{7,15}$ with the exception that the expressions for the transfer to the $-3/2 \leftrightarrow -1/2$ and $3/2 \leftrightarrow 1/2$ transitions must be interchanged. (It can be verified that in the absence of relaxation during Δ the sum of magnetization transferred from $\rho_{8,16}$ or $\rho_{7,15}$ to all three ^1H coherences is identical to the net transfer of ^{13}C magnetization from $\rho_{8,16}$ or $\rho_{7,15}$ to ^1H magnetization indicated in Eq. [7].) The transfer of magnetization from $\rho_{6,14}$, $\rho_{2,10}$, $\rho_{5,13}$, and $\rho_{3,11}$ to the $\pm 1/2 \leftrightarrow \mp 1/2$ transitions of the $1/2$ manifold follows the dependence in Eq. [7]. [It should be noted that magnetization cannot be transferred between manifolds by the action of rf pulses (34, 37).] If θ is set to 54.7° and $T_2 = 2\Delta$, these expressions indicate that the outer and inner components of the ^{13}C quartet are transferred to ^1H magnetization in the ratio $-1:11.4$ rather than the expected ratio of 1:3. Clearly, such results can lead to relaxation-time measurements that are significantly different from values obtained via ^{13}C -direct-observe experiments. Illustrations of the anomalous results which can be obtained via REVDEPT transfer are shown in Figs. 5 and 6. The spectra in Figs. 5 and 6 were recorded using the REVDEPT pulse sequence in Fig. 1 with $\theta = 54.7^\circ$, the ^1H 180° pulse removed from the center of the t_1 period, and the CPMG period removed from the sequence. In Figure 5 a cross section from a ^1H - ^{13}C correlation spectrum taken at the center of the methyl proton resonance of $^{13}\text{C}^\beta$ -alanine dissolved in perdeuterated glycerol is presented. As will be discussed later, at the temperature used to record the spectrum, alanine tumbles with an overall correlation time of ~ 8 ns, well within the large-molecule limit. In Fig. 6 the 2D ^1H - ^{13}C correlation map of the protein SNase labeled with ^{13}C in the leucine C^δ -methyl positions is presented. Note that the 1:3:3:1 quartet structure normally associated with the ^{13}C spectrum of a methyl group is absent and that only the inner multiplet components are transferred to ^1H magnetization.

For the REVINEPT sequence, a similar calculation shows that the transfer of magnetization from either $\rho_{8,16}$ or $\rho_{1,9}$ to the individual ^1H transitions $-3/2 \leftrightarrow -1/2$, $-1/2 \leftrightarrow 1/2$, and $1/2 \leftrightarrow 3/2$ of the $3/2$ manifold is

$$\rho_{8,16}(\rho_{1,9}) \rightarrow (1/32)\sin 6\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) \} \quad (-3/2 \leftrightarrow -1/2) \quad [11a]$$

$$\rho_{8,16}(\rho_{1,9}) \rightarrow (1/16)\sin 6\pi J_{\text{CH}}\Delta' \quad (-1/2 \leftrightarrow 1/2) \quad [11b]$$

$$\rho_{8,16}(\rho_{1,9}) \rightarrow (1/32)\sin 6\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) \} \quad (1/2 \leftrightarrow 3/2), \quad [11c]$$

while the transfer from $\rho_{4,12}$ and $\rho_{7,15}$ is calculated to be

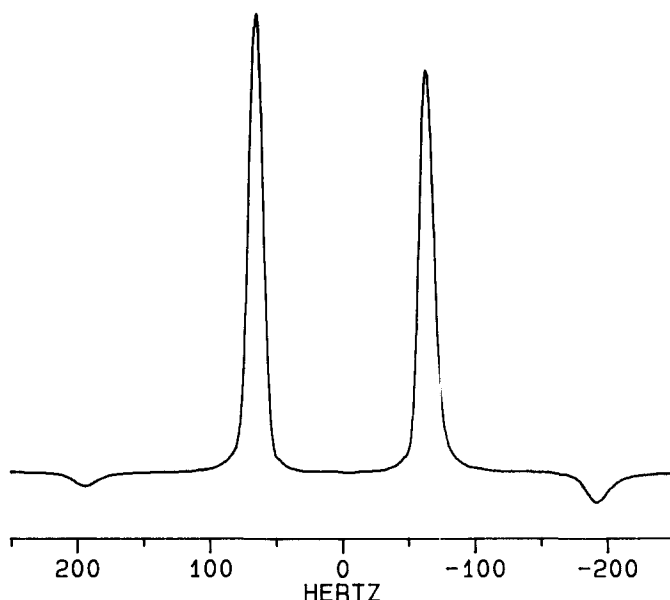


FIG. 5. Cross section at the center of the methyl proton resonance of $^{13}\text{C}^{\beta}$ -alanine dissolved in perdeuterated glycerol from a ^1H - ^{13}C correlation spectrum recorded at 500 MHz using the REVDEPT pulse sequence of Fig. 1 with $\theta = 54.7^\circ$, with the ^1H 180° pulse removed from the center of the t_1 period, and with the CPMG period removed from the sequence. The spectrum was recorded at a temperature of 288 K.

$$\rho_{4,12}(\rho_{7,15}) \rightarrow (1/32)\sin 2\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) \} \quad (-3/2 \leftrightarrow -1/2) \quad [12a]$$

$$\rho_{4,12}(\rho_{7,15}) \rightarrow (-1/48)\sin 2\pi J_{\text{CH}}\Delta' \quad (-1/2 \leftrightarrow 1/2) \quad [12b]$$

$$\rho_{4,12}(\rho_{7,15}) \rightarrow (1/32)\sin 2\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) \} \quad (1/2 \leftrightarrow 3/2). \quad [12c]$$

It should be noted that as with the REVDEPT sequence, ^{13}C magnetization from the $3/2$ manifold cannot be transferred to ^1H transitions in the $1/2$ manifolds and vice versa. Therefore, there is no transfer of magnetization from elements $\rho_{8,16}$, $\rho_{1,9}$, $\rho_{4,12}$, or $\rho_{7,15}$ to the ^1H transitions in the $1/2$ manifold. From Eqs. [7], [11], and [12] it is easy to show that for $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad), so that $(1/3)\sin 2\pi J_{\text{CH}}\tau' = \sin 6\pi J_{\text{CH}}\tau'$, the total transfer from the outer components of the ^{13}C quartet to ^1H magnetization is given by

$$(1/24)\sin 2\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) + 1 \}, \quad [13]$$

while the transfer from the inner components of the quartet to ^1H magnetization is given by

$$(1/8)\sin 2\pi J_{\text{CH}}\Delta' \{ \exp(-2\Delta'/T_2) + 1 \}. \quad [14]$$

These results suggest that, in sharp contrast to the REVDEPT sequence, the ratio of the transfer of ^{13}C magnetization from the inner and outer quartet components to protons via the REVINEPT scheme is insensitive to the fast relaxation of the $\pm 3/2 \leftrightarrow \pm 1/2$ ^1H transitions. As will be described later, experimentally we find that although

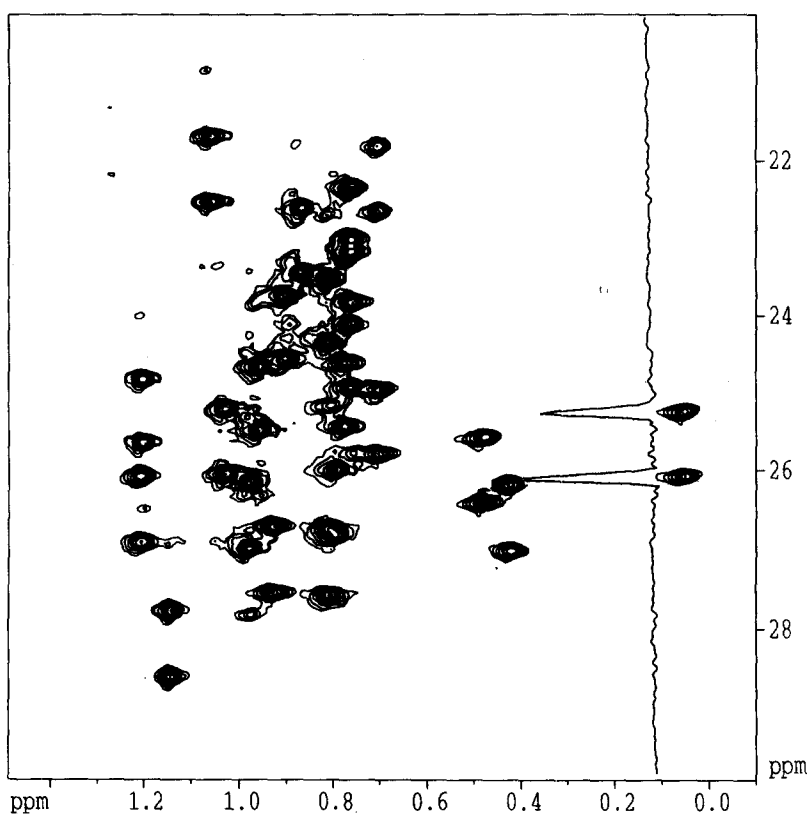


FIG. 6. Two-dimensional ^1H - ^{13}C correlation map of SNase labeled with ^{13}C in the leucine C^δ -methyl positions. The spectrum was recorded at 600 MHz, $T = 308$ K, with $\theta = 54.7^\circ$, with the ^1H 180° pulse removed from the center of the t_1 period, and with the CPMG period removed from the sequence. Acquisition times of 100 and 53 ms were used in t_1 and t_2 , respectively. No digital filtering was used in the processing. A trace through the δ carbon of leucine 25 is indicated.

the REVINEPT scheme is less sensitive to the effects of the differential relaxation of the ^1H transitions than the REVDEPT sequence (in agreement with the equations above), it appears that the inner ^{13}C transitions are transferred slightly more efficiently to ^1H magnetization than the outer transitions.

A strategy for minimizing the effects of cross correlation between ^1H - ^{13}C dipolar interactions and cross correlation between ^1H - ^{13}C dipolar and chemical-shift anisotropy interactions. Recently it has been shown that in the limit where $J(0)$ spectral density terms dominate ^{13}C transverse relaxation ($\omega\tau_c \gg 1$) in an isolated methyl group rotating rapidly around its C_3 axis, a simple expression describing the relaxation of the outer and inner components of the ^{13}C quartet pertains (38). In this limit, $J_{\text{CH}}(0) = K_{\text{HCH}}(0)$, where $J_{\text{CH}}(0)$ is the autocorrelation spectral-density function from ^1H - ^{13}C dipolar interactions evaluated at zero frequency and $K_{\text{HCH}}(0)$ is the three-spin cross-correlation spectral-density function where two distinct ^1H spins share the same ^{13}C spin. In addition, auto- and cross-spectral-density terms at zero frequency due to intramethyl

group ^1H - ^1H dipolar interactions which normally enter into the ^{13}C relaxation equations exactly cancel. The relaxation of ^{13}C magnetization in the 3/2 and 1/2 manifolds becomes uncoupled and it can be shown that for relaxation arising from ^1H - ^{13}C dipolar interactions only, ^{13}C magnetization decays according to

$$A(t) = A(0)\{0.75 \exp[(-2/27)J(0)t] + 0.25 \exp[(-2/3)J(0)t]\}, \quad [15]$$

where $A(t)$ is the intensity of ^{13}C magnetization at time t and $J(0) = J_{\text{CH}}(0) = K_{\text{HCH}}(0) = 0.3 \gamma_{\text{H}}^2 \gamma_{\text{C}}^2 \hbar^2 / r_{\text{HC}}^6 \tau_c$ (38). For an isolated AX_3 spin system attached to a macromolecule in the limit that $\omega \tau_c \gg 1$, therefore, the decay rate of $A(t)$ is biexponential with decay constants that differ by a factor of 9. For the case of a rapidly spinning methyl group in a macromolecule tumbling with a correlation time of 10 ns, these two decay times are calculated to be ~ 23 and ~ 210 ms, respectively. In principal, these decay rates could be extracted accurately by measuring the decay of magnetization, $A(t)$, for a number of different t values and fitting the magnetization vs time profile to a biexponential. In practice, however, it can be difficult to interpret the measured relaxation times in terms of the spectral-density functions given by Eq. [15] because the assumption of an isolated methyl group is invalid and spin flips between the protons directly attached to the ^{13}C and neighboring protons tend to partially average the different relaxation rates of the various ^{13}C multiplet components. A proper account of these effects requires a detailed knowledge of the structure of the molecule, which is often not available. It is therefore important that the initial rate of the decay of ^{13}C magnetization be used (i.e., time points obtained in the regime where the decay can be described by a single exponential) to extract proper values of the spectral densities indicated in Eq. [15]. This is extremely difficult to do with any degree of accuracy for ^{13}C methyl groups attached to macromolecules where the fastest-decaying component makes up only 25% of the net magnetization and its relaxation can be as short as ~ 25 ms for a molecule with $\tau_c = 10$ ns.

Our approach to minimizing this problem is to exchange ^{13}C magnetization among the various transitions so as to average out differences in the relaxation rates of the multiplet components as much as possible. For the measurement of transverse relaxation rates this is achieved by the application of ^1H 125° pulses at the center of the ^{13}C spin echo during the CPMG interval at a rate that is fast compared to the decay rate of the fastest-decaying ^{13}C transition (\sim every 5 ms). For the measurement of T_1 values, ^1H 125° pulses are applied every 5 ms during the inversion-recovery period. In this way the relaxation rates of the four ^{13}C transitions associated with the 3/2 manifold become equal. The decay of the total transverse ^{13}C magnetization (i.e., magnetization from the 3/2 and the 1/2 manifolds), $A(t)$, is now given by

$$A(t) = A(0)\{0.50 \exp[(-10/27)J(0)t] + 0.50 \exp[(-2/27)J(0)t]\}. \quad [16]$$

It is important to recognize that the application of the proton pulses does not mix ^{13}C magnetization from the 3/2 and 1/2 manifolds, so that a complete averaging is not possible. For a molecule with $\tau_c = 10$ ns, the transverse relaxation time of the fastest-decaying component is now ~ 45 ms and composes 50% of the net ^{13}C magnetization, so that it is easier to extract accurate relaxation times from the initial rate of decay of magnetization than in the previous case. In addition, the 125° ^1H pulses also minimize the effects of cross correlation between ^1H - ^{13}C dipolar interactions and CSA in a

manner completely analogous to the situation where ^1H 180° pulses are applied (26, 32, 33).

It should be stressed that ^1H 180° pulses which eliminate dipolar/CSA cross-correlation effects (26, 32, 33) do not eliminate the effects of cross correlation between ^1H - ^{13}C dipolar interactions. Applications of ^1H 180° pulses merely interconverts ^{13}C magnetization from the outer components ($\rho_{1,9} \leftrightarrow \rho_{8,16}$) and interconverts ^{13}C magnetization from the inner components ($\rho_{7,15} \leftrightarrow \rho_{4,12}$, $\rho_{6,14} \leftrightarrow \rho_{2,10}$, and $\rho_{5,13} \leftrightarrow \rho_{3,11}$) but does not exchange magnetization between inner and outer components. In contrast, the application of ^1H 125° pulses mixes magnetization from the outer and inner components from the 3/2 manifold very efficiently, thereby minimizing the effects of nonexponential recovery of carbon magnetization. Using the equations describing the effects of X pulses of flip angle ϕ on the A lines (from the 3/2 manifold) of an AX_3 spin system given in the Appendix, it is straightforward to show that the sum of the outer and inner lines of the A quartet evolve as

$$\begin{aligned}\Delta_1 &= (1 + 3 \cos^2\phi)\Delta_1^0/4 + 3 \sin^2\phi\Delta_2^0/4 \\ \Delta_2 &= 3 \sin^2\phi\Delta_1^0/4 + (1 + 3 \cos^2\phi)\Delta_2^0/4,\end{aligned}\quad [17]$$

where Δ_1^0 (Δ_1) and Δ_2^0 (Δ_2) correspond to the sum of the outer and inner lines, respectively, of the A quartet before (after) application of the X pulse. Taking the sum and difference of Δ_1 and Δ_2 yields

$$\begin{aligned}\Delta_1 + \Delta_2 &= \Delta_1^0 + \Delta_2^0 \\ \Delta_1 - \Delta_2 &= [(1 + 3 \cos 2\phi)/4](\Delta_1^0 - \Delta_2^0).\end{aligned}\quad [18]$$

Thus, the sum of the four multiplet components from the 3/2 manifold is invariant to rotation, while the difference decreases with each X pulse if $\phi \neq n\pi$, since $(1 + 3 \cos 2\phi)/4 < 1$.

Assuming that the decay of Δ_1 and Δ_2 are single-exponential with time constants T_{2f} and T_{2s} respectively, it is straightforward to show from Eq. [18] that for $\phi = \pm 54.7^\circ$ or $\pm 125.3^\circ$ (i.e., $1 + 3 \cos 2\phi = 0$),

$$\Delta_1(n\tau) = \Delta_2(n\tau) = (1/2)\{\exp[-\tau/T_{2f}] + \exp[-\tau/T_{2s}]\}/2\}^n \quad [19]$$

where $\Delta_1(n\tau)$ and $\Delta_2(n\tau)$ are the values of Δ_1 and Δ_2 immediately after application of the n th ^1H ϕ pulse and τ is the spacing between consecutive ^1H pulses. In the limit that $\tau \ll T_{2f}, T_{2s}$, Eq. [19] reduces to

$$\Delta_1(n\tau) = \Delta_2(n\tau) \approx (1/2)\{\exp(-n\tau/T_{2a})\}, \quad [20]$$

where $1/T_{2a} = (1/2)(1/T_{2f} + 1/T_{2s})$ and efficient mixing of the multiplet components occurs. We have chosen $\phi = 125.3^\circ$ rather than 54.7° so that the net rotation rate of the X spins is sufficiently fast to minimize dipolar/CSA cross-correlation effects in addition to minimizing the effects due to ^1H - ^{13}C dipolar cross correlation. It should be noted that the assumption that the decay of Δ_1 and Δ_2 are single-exponential is valid for the case where the ^1H 125° pulses are applied at a rate fast compared to either the decay of the various ^{13}C multiplet components or the cross-relaxation rates between the components. Moreover, in the limit where $\omega\tau_c \gg 1$ and for a rapidly spinning methyl group, the ^{13}C cross-relaxation terms vanish.

RESULTS AND DISCUSSION

In order to obtain experimental verification of these results we choose to measure ^{13}C T_2 values for $^{13}\text{C}^\beta$ -alanine dissolved in perdeuterated glycerol. Our choice of this "test system" was motivated by the steep viscosity dependence of glycerol with temperature, providing a simple experimental approach for "tuning" the correlation time of the solute over a wide range of values. Table 1 shows the ^{13}C T_2 values measured for $^{13}\text{C}^\beta$ -alanine obtained from the initial decay rates of magnetization using the sequences given in Fig. 1. In addition, ^{13}C T_1 , T_2 and ^1H - ^{13}C NOE values were measured via ^{13}C -direct-observe experiments and values for $\omega_c\tau_c$, where ω_c is the ^{13}C Larmor frequency, were calculated by fitting the T_1 , T_2 , and NOE data using the Woessner model (30). When measurements are made at 318 or 308 K and θ and $2\pi J_{\text{CH}}\tau'$ are set to 54.7° (0.955 rad), T_2 values are obtained that are in good agreement with values measured via direct detection. However, for other θ and τ' values, large discrepancies result. For example, when $2\pi J_{\text{CH}}\tau' = 30^\circ$ (0.524 rad), so that the outer components of the ^{13}C quartet are transferred preferentially to observable ^1H magnetization, REVINEPT results in an overestimate of the initial ^{13}C transverse decay rate by $\sim 20\%$ at a temperature of 308 K.

TABLE I
 ^{13}C Transverse Relaxation Times of $^{13}\text{C}^\beta$ -alanine in Perdeuterated Glycerol^a

Temperature (K)	^{13}C T_2^b	^{13}C T_2^c	^{13}C T_2^d	^{13}C T_2^e	^{13}C T_2^f	^{13}C T_2^g	$\omega_c\tau_c^h$
288	66	70	74	53	77	86	6.4
298	149	148	161	116	165	170	2.7
308	266	259	262	225	258	269	1.2
318	372	370	381	337	363	379	0.4

^a The decay of ^{13}C transverse magnetization in an AX_3 spin system is nonexponential (12, 13). The T_2 values reported are measured from a fit of the initial decay of transverse magnetization to an equation of the form $y = A \exp(-t/T_2)$ using conjugate gradient minimization techniques. Error limits for T_2 values are approximately $\pm 5\%$ based on multiple (three to four) measurements of individual values.

^b Values measured from ^{13}C -observe experiments. A CPMG sequence with ^1H 125° pulses applied every 5 ms at the height of the carbon spin echo was used.

^c Values measured from the REVINEPT sequence in Fig. 1b with $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad) and Δ' set to $1/(8J_{\text{CH}})$. A CPMG sequence with ^1H 125° pulses applied every 5 ms at the height of the carbon spin echo was used.

^d Values measured from the REVINEPT sequence in Fig. 1b with $2\pi J_{\text{CH}}\tau' = 54.7^\circ$ (0.955 rad) and Δ' set to $1/(4J_{\text{CH}})$. A CPMG sequence with ^1H 125° pulses applied every 5 ms at the height of the carbon spin echo was used.

^e Values measured from the REVINEPT sequence in Fig. 1b with $2\pi J_{\text{CH}}\tau' = 30.0^\circ$ (0.524 rad) and Δ' set to $1/(8J_{\text{CH}})$. A CPMG sequence with ^1H 125° pulses applied every 5 ms at the height of the carbon spin echo was used.

^f Values measured from the REVDEPT sequence in Fig. 1a with $\theta = 54.7^\circ$ and Δ set to $1/(2J_{\text{CH}})$. A CPMG sequence with ^1H 125° pulses applied every 5 ms at the height of the carbon spin echo was used.

^g Values measured from the REVDEPT sequence in Fig. 1a with $\theta = 54.7^\circ$ and Δ set to $1/(2J_{\text{CH}})$. A CPMG sequence with ^1H 180° pulses applied every 5 ms at the height of the carbon spin echo was used.

^h Calculated from a best fit of ^{13}C T_1 , T_2 and ^1H - ^{13}C NOE values (measured by ^{13}C -direct-observe experiments) using the Woessner model (30) to describe methyl group dynamics.

At temperatures of 318 or 308 K, the tumbling time of alanine is in the regime $\omega\tau_c \leq \sim 1$ and the decay rates of the individual ^1H transitions of the methyl group are similar. Therefore, similar ^{13}C T_2 values are obtained from REVDEPT and REVINEPT sequences and from REVINEPT-based sequences, where Δ' is varied from $1/(4J_{\text{CH}})$ to $1/(8J_{\text{CH}})$. In contrast, as the temperature is lowered below 308 K, $\omega\tau_c > 1$ and the errors in measured ^{13}C T_2 values increase for some of the polarization-transfer pulse schemes. As expected, in the slow-correlation-time limit, the trend in the data suggests that errors in T_2 values measured from REVINEPT sequences are somewhat smaller than the errors measured from the corresponding DEPT-based experiments.

The errors associated with the measurement of transverse relaxation rates via the REVDEPT transfer scheme can be decreased by minimizing the net delay times during which ^1H magnetization evolves during the sequence. This involves minimizing the delay Δ in the REVDEPT sequence in Fig. 1. As the REVDEPT sequence is written in Fig. 1, the transfer function ($^{13}\text{C} \rightarrow ^1\text{H}$) is given by $\sin^6 \pi J_{\text{CH}} \Delta$. In practice, minimizing the delays involves a trade-off between sensitivity and accuracy. Clearly any departure from $\Delta \sim 1/(2J_{\text{CH}})$ will result in a very significant sensitivity loss. Although not predicted by theory, we have noted a slight improvement in the agreement between ^{13}C T_2 values measured via direct detection and measured by the REVINEPT sequence if the value of Δ' is decreased to $1/(8J_{\text{CH}})$. (See Table 1, columns *b*, *c*, and *d*). A choice of $\Delta' = 1/(8J_{\text{CH}})$ in the REVINEPT experiment results in a 30% sensitivity loss. For macromolecules the extent of the improvement in using the REVINEPT sequence with $\Delta' = 1/(4J_{\text{CH}})$ versus $\Delta' = 1/(8J_{\text{CH}})$ will vary depending on the spin-flip rate of the methyl protons. As the spin-flip increases relative to the transverse relaxation rates of the ^{13}C multiplet components the differences in the relaxation rates of the multiplet components will become smaller and the differences in T_2 values measured using REVINEPT-based schemes with $\Delta' = 1/(4J_{\text{CH}})$ or with $\Delta' = 1/(8J_{\text{CH}})$ may become negligible or not substantial enough to warrant a 30% loss in sensitivity. In a recent study of internal motion in the protein SNase, ^{13}C T_2 values were measured for the C^δ -leucine methyl carbons by the REVINEPT experiment with $\Delta' = 1/(8J_{\text{CH}})$ and via ^{13}C -direct-observe methods (for the eight carbons whose shifts were sufficiently well resolved). Very good agreement was found between the T_2 values measured by direct observation and by REVINEPT with $\Delta' = 1/(8J_{\text{CH}})$ with the ^{13}C T_2 values for seven of the eight carbons differing by less than 5% and the largest difference between measured values using the two different methods of $\sim 10\%$.

CONCLUSIONS

The major results of this work can be summarized as follows: (1) In the measurement of heteronuclear relaxation rates using polarization-transfer schemes it is important that the transfer of magnetization from individual heteronuclear transitions to observable ^1H magnetization be analyzed in detail. For AX_3 spin systems, this transfer depends critically on the values of θ and $2\pi J_{\text{CH}}\tau'$ used in REVDEPT- and REVINEPT-based experiments, respectively. For θ and $2\pi J_{\text{CH}}\tau'$ set to 54.7° (0.955 rad), and in the absence of relaxation during the REVDEPT or REVINEPT pulse sequences, magnetization from individual ^{13}C transitions is transferred equally to ^1H magnetization and identical relaxation times are obtained from ^{13}C -direct-observe and from polar-

ization-transfer experiments. Similar conclusions have been reported previously by Palmer *et al.* (11).

(2) In general, setting θ and $2\pi J_{\text{CH}}\tau'$ to 54.7° (0.955 rad) is a necessary but insufficient condition for the measurement of accurate relaxation rates of ^{13}C methyl groups in macromolecules. This is because individual ^{13}C transitions are not transferred equally to all individual ^1H transitions and the ^1H transitions relax at very different rates during the refocusing delays in the REVDEPT and REVINEPT sequences. These effects are predicted to be less severe for REVINEPT-based experiments than for REVDEPT-based experiments and the trends in the experimental data support this conclusion. Slightly better agreement between REVINEPT-based experiments and direct-detection experiments can be obtained by shortening Δ' with modest losses in sensitivity.

(3) In order to minimize the effects of cross correlation between ^1H - ^{13}C dipolar interactions and cross-correlation effects between ^1H - ^{13}C dipolar interactions and CSA on measured transverse relaxation rates, ^1H 125° pulses are applied every 5 ms, yet at a rate fast compared to the relaxation rate of the fastest-decaying component of ^{13}C magnetization. In this limit the decay of ^{13}C transverse magnetization in methyl groups is biexponential, with the fastest-decaying component decaying at a rate five times faster than the slowly decaying component (for macromolecules) and making up 50% of the magnetization (in the limit of very short relaxation delays, T). Although schemes based on the application of ^1H 180° pulses can also be used to eliminate the effects of cross correlation between ^1H - ^{13}C dipolar interactions and CSA, cross correlation between ^1H - ^{13}C dipolar interactions is not affected. In this case, the fastest-decaying component of transverse ^{13}C magnetization makes up only 25% of the total magnetization and decays at a rate nine times faster than the slowly relaxing fraction of magnetization (for macromolecules). For the case where ^1H 180° pulses are applied (versus 125° pulses) it is therefore more difficult to extract reliable relaxation times from the initial decay of the signal.

(4) Although at first glance it may appear that for the application to the measurement of relaxation properties of methyl groups double-polarization-transfer schemes are more sensitive than single-polarization-transfer experiments, the sensitivity difference between the two classes of experiments is marginal. In principle, with $2\pi J_{\text{CH}}\tau'$ (θ) set to 54.7° , the double-polarization-transfer experiments are a factor of $3.3/(^1\text{H}$ - ^{13}C NOE) more sensitive than the single-polarization-transfer experiments (19). However, the ^1H - ^{13}C NOE is substantial for methyl groups in proteins, with typical values of 2.5. Thus the double-polarization-transfer experiments are predicted to be at most a factor of 1.3 more sensitive than their single-transfer counterparts. However, the double-polarization-transfer experiments are complicated by an increase in the number of pulses (and pulse imperfections) and an increase in the number of delays in the sequences relative to single-polarization-transfer experiments. Given that the decay times for certain components of the magnetization are on the order of the delays in the polarization-transfer sequences, it seems prudent to use sequences with the smallest number of delays. In addition, for methyl groups in macromolecules, the ^{13}C T_1 values are often shorter than the methyl proton T_1 values, which allows for a faster repetition rate in single polarization transfer experiments. For these reasons we strongly prefer single-polarization-transfer experiments for measuring ^{13}C relaxation times of

methyl groups in macromolecules. For other applications, however, such as measurement of ^{13}C relaxation times in AX spin systems, the double-polarization-transfer approach is preferred.

Finally, it should be mentioned that polarization-transfer schemes for measuring heteronuclear T_1 relaxation times in macromolecules are less sensitive to the types of errors discussed above than are T_2 measurements. This is because ^1H spin flips between protons attached to the heteroatom and neighboring protons occur on a time scale that is faster than the longitudinal decay rates of the individual ^{13}C transitions and thereby average differences in these rates. Nevertheless, the procedures indicated in this paper for the measurement of accurate transverse relaxation rates also improve the accuracy of T_1 measurements.

APPENDIX

The effect of an X ϕ pulse on the A lines of the 3/2 manifold in the AX₃ spin system is given by

$$\nu = \mathbf{R}\nu^0, \quad [\text{A1}]$$

where the vectors $\nu^0 = (\nu_1^0, \nu_2^0, \nu_3^0, \nu_4^0)$ and $\nu = (\nu_1, \nu_2, \nu_3, \nu_4)$ consist of the density elements for the four lines of the A quartet (ν_1 and ν_4 are the outer lines) before and after the ϕ pulse and \mathbf{R} is the 4×4 rotation matrix given by

$$\begin{bmatrix} (1+C)^3/8 & 3(1+C)S^2/8 & 3(1-C)S^2/8 & (1-C)^3/8 \\ 3(1+C)S^2/8 & (1+C)^3/8 - CS^2 & (1-C)^3/8 + CS^2 & 3(1-C)S^2/8 \\ 3(1-C)S^2/8 & (1-C)^3/8 + CS^2 & (1+C)^3/8 - CS^2 & 3(1+C)S^2/8 \\ (1-C)^3/8 & 3(1-C)S^2/8 & 3(1+C)S^2/8 & (1+C)^3/8 \end{bmatrix} \quad [\text{A2}]$$

where $C = \cos \phi$ and $S = \sin \phi$.

It is possible to rewrite Eq. [A1] in a form where \mathbf{R} is diagonal. One obtains

$$\nu' = \mathbf{R}'\nu'^0, \quad [\text{A3}]$$

where $\nu' = (\psi_1, \psi_2, \psi_3, \psi_4)$, $\nu'^0 = (\psi_1^0, \psi_2^0, \psi_3^0, \psi_4^0)$, $\psi_1 = (\nu_1 + \nu_2 + \nu_3 + \nu_4)/2$, $\psi_2 = (\nu_1 - \nu_2 - \nu_3 + \nu_4)/2$, $\psi_3 = (3\nu_1 + \nu_2 - \nu_3 - 3\nu_4)/(2 \times 5^{1/2})$, $\psi_4 = (\nu_1 - 3\nu_2 + 3\nu_3 - \nu_4)/(2 \times 5^{1/2})$, and \mathbf{R}' is a diagonal matrix whose nonzero elements are

$$\begin{aligned} R_{11} &= 1 \\ R_{22} &= [1 + 3 \cos 2\phi]/4 \\ R_{33} &= \cos \phi \\ R_{44} &= \cos \phi [1 - 5 (\sin^2 \phi)/2]. \end{aligned} \quad [\text{A4}]$$

ACKNOWLEDGMENTS

We thank A. Palmer for making available preprints of his work on the measurement of relaxation times. This work was supported by the Intramural AIDS Antiviral Program of the Office of the Director of the National Institutes of Health. L.E.K. acknowledges financial support from the Medical Research Council of Canada in the form of a Centennial Fellowship.

REFERENCES

1. A. ALLERHAND, D. DODDRELL, AND R. KOMOROSKI, *J. Chem. Phys.* **55**, 189 (1971).
2. L. E. KAY, M. IKURA, R. TSCHUDIN, AND A. BAX, *J. Magn. Reson.* **89**, 496 (1990).

3. S. W. FESIK, H. L. EATON, E. T. OLEJNICZAK, E. R. P. ZUIDERWEG, L. P. MCINTOSH, AND F. W. DAHLQUIST, *J. Am. Chem. Soc.* **112**, 886 (1990).
4. A. BAX, G. M. CLORE, AND A. M. GRONENBORN, *J. Magn. Reson.* **88**, 425 (1990).
5. V. SKLENAR, D. A. TORCHIA, AND A. BAX, *J. Magn. Reson.* **73**, 375 (1987).
6. L. E. KAY, T. JUE, B. BANGERTER, AND P. C. DEMOU, *J. Magn. Reson.* **73**, 558 (1987).
7. N. R. NIRMALA AND G. WAGNER, *J. Am. Chem. Soc.* **110**, 7557 (1988).
8. N. R. NIRMALA AND G. WAGNER, *J. Magn. Reson.* **82**, 659 (1989).
9. L. E. KAY, D. A. TORCHIA, AND A. BAX, *Biochemistry* **28**, 8972 (1989).
10. G. BODENHAUSEN AND D. J. RUBEN, *Chem. Phys. Lett.* **69**, 185 (1980).
11. A. G. PALMER III, P. E. WRIGHT, AND M. RANCE, *Chem. Phys. Lett.* **185**, 41 (1991).
12. L. G. WERBELOW AND D. M. GRANT, in "Advances in Magnetic Resonance" (J. S. Waugh, Ed.), Vol. 9, p. 189, Academic Press, San Diego, 1977.
13. R. L. VOLD AND R. R. VOLD, *Prog. NMR Spectrosc.* **12**, 79 (1978).
14. M. GOLDMAN, *J. Magn. Reson.* **60**, 437 (1984).
15. L. G. WERBELOW AND A. MARSHALL, *J. Magn. Reson.* **11**, 299 (1973).
16. D. M. DODDRELL, D. T. PEGG, AND M. R. BENDALL, *J. Magn. Reson.* **48**, 323 (1982).
17. M. R. BENDALL, D. T. PEGG, D. M. DODDRELL, AND J. FIELD, *J. Magn. Reson.* **51**, 520 (1983).
18. G. A. MORRIS AND R. FREEMAN, *J. Am. Chem. Soc.* **101**, 760 (1979).
19. D. P. BURUM AND R. R. ERNST, *J. Magn. Reson.* **39**, 163 (1980).
20. P. H. BOLTON, *J. Magn. Reson.* **41**, 287 (1980).
21. L. K. NICHOLSON, L. E. KAY, D. BALDISSERI, J. ARANGO, P. E. YOUNG, A. BAX, AND D. A. TORCHIA, *Biochemistry* **31**, 5253 (1992).
22. R. R. ERNST, G. BODENHAUSEN, AND A. WOKAUN, "Principles of Nuclear Magnetic Resonance in One and Two Dimensions," p. 38, Clarendon Press, Oxford, 1986.
23. H. Y. CARR AND E. M. PURCELL, *Phys. Rev.* **94**, 630 (1954).
24. S. MEIBOOM AND D. GILL, *Rev. Sci. Instrum.* **29**, 688 (1958).
25. A. BAX, M. IKURA, L. E. KAY, D. A. TORCHIA, AND R. TSCHUDIN, *J. Magn. Reson.* **86**, 304 (1990).
26. L. E. KAY, L. K. NICHOLSON, F. DELAGLIO, A. BAX, AND D. A. TORCHIA, *J. Magn. Reson.* **97**, 359 (1992).
27. J. W. PENG, V. THANABAL, AND G. WAGNER, *J. Magn. Reson.* **95**, 421 (1991).
28. R. R. VOLD AND R. L. VOLD, *J. Chem. Phys.* **64**, 320 (1976).
29. A. G. REDFIELD, in "Advances in Magnetic Resonance" (J. S. Waugh, Ed.), Vol. 1, p. 1, Academic Press, San Diego, 1965.
30. D. E. WOESSNER, *J. Chem. Phys.* **36**, 1 (1962).
31. R. S. NORTON, A. O. CLOUSE, R. ADDLEMAN, AND A. ALLERHAND, *J. Am. Chem. Soc.* **99**, 79 (1977).
32. J. BOYD, U. HOMMEL, AND I. D. CAMPBELL, *Chem. Phys. Lett.* **175**, 477 (1990).
33. A. G. PALMER III, N. J. SKELTON, W. J. CHAZIN, P. E. WRIGHT, AND M. RANCE, *Mol. Phys.* **75**, 699 (1991).
34. O. W. SØRENSEN, G. EICH, M. H. LEVITT, G. BODENHAUSEN, AND R. R. ERNST, *Prog. NMR Spectrosc.* **16**, 163 (1983).
35. N. MULLER, G. BODENHAUSEN, AND R. R. ERNST, *J. Magn. Reson.* **75**, 297 (1987).
36. L. E. KAY AND J. H. PRESTEGARD, *J. Am. Chem. Soc.* **109**, 3829 (1987).
37. A. ABRAGAM, "Principles of Nuclear Magnetism," Oxford University Press, 1961, p. 296.
38. L. E. KAY AND D. A. TORCHIA, *J. Magn. Reson.* **95**, 536 (1991).
39. D. MARION AND K. WÜTHRICH, *Biochem. Biophys. Res. Commun.* **113**, 967 (1983).