Discipline Biomolecular Stereodynamics (Sarma, R. H., Ed.) Vol. I, pp 89–98, Adenine, New York.
Ts'o, P. O. P. (1974) in *Basic Principles of Nucleic Acid Chemistry* (Ts'o, P. O. P., Ed.) Vol. 1, p 466, Academic, New York.
Valcour, A. A., & Woodworth, R. C. (1986) *J. Magn. Reson. 66*, 536.
Weast, R. C., Ed. (1983) *CRC Handbook of Chemistry and Physics*, p F-40, Chemical Rubber Publishing Co., Cleveland, OH.
Wemmer, D. E., & Reid, B. R. (1985) *Annu. Rev. Phys. Chem. 36*, 105.
Wilson, R. W., & Bloomfield, V. A. (1979) *Biochemistry 18*, 2192.

Coherence Transfer in Deoxyribose Sugars Produced by Isotropic Mixing: An Improved Intraresidue Assignment Strategy for the Two-Dimensional NMR Spectra of DNA†

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ABSTRACT: Two-dimensional isotropic mixing spectroscopy has been used to confirm assignments of the deoxyribose sugar protons in the ^{1}H NMR spectrum of the DNA oligonucleotides d(CGCGTTTTCGCG) and [d(GCCGTGGCCACGGC)]$_2$. The broad-band decoupling sequence MLEV-16 was applied during the mixing period to induce isotropic coupling within the spin systems, resulting in net transfer of coherence throughout the coupled spin networks. Nearly all 1′, 2′, 2″, 3′, and 4′ protons of a given nucleotide could be identified on the basis of through-bond scalar connectivities. In addition, in the hairpin, a number of connectivities to 5′/5″ protons were found. The dependence of cross-peak intensity on the length of radio-frequency irradiation for several different coherence transfer orders is presented, and implications for optimization are discussed.

Two-dimensional NMR spectroscopy (2D NMR)[1] has become a valuable tool for investigating the solution structure of biological macromolecules. The success of the 2D NMR method lies in its ability to systematically assign the ^{1}H resonances in relatively complicated NMR spectra to specific atoms in the biopolymer; the high-resolution ^{1}H NMR spectra of DNA and proteins are typically too complex to be assigned by ordinary one-dimensional NMR methods. In addition to the resonance assignments, the 2D NMR method can yield interproton distances from the nuclear Overhauser effect (NOE) intensities, and such distances have been used to obtain qualitative solution structures in DNA and proteins [for a review, see Wemmer and Reid (1985)]. Trial structures based on model building or distance geometry matrix embedding methods can be generated and refined on the basis of these NOE-derived interproton distances to yield more quantitative three-dimensional structures (Kaptein et al., 1985; Havel & Wüthrich, 1985; Braun & Gō, 1986; Hare & Reid, 1986; Hare et al., 1986a,b).

The assignment strategy for synthetic right-handed double-helical DNA oligonucleotides has been developed by a number of independent laboratories (Feigon et al., 1983; Hare et al., 1983; Scheek et al., 1983). It consists of first identifying the resonances within a given deoxyribose sugar by their scalar coupling to adjacent vicinal protons with two-dimensional correlated spectroscopy (COSY). The isolated scalar-coupled spin systems are then associated with a particular nucleotide residue by establishment of sequential interresidue connectivities with two-dimensional nuclear Overhauser effect spectroscopy (NOESY). In practice, it is often difficult to delineate the entire coupled spin system of an isolated sugar ring with the COSY experiment because the 3′, 4′, 5′, and 5″ proton resonances fall within a narrow chemical shift range; thus, their cross-peaks fall close to the diagonal and are often difficult to resolve.

Relayed coherence transfer spectroscopy (RELAY) has been used in conjunction with the COSY experiment to extend the spin connectivity network to include 1′H–3′H and 2′H–4′H connectivities (Chazin et al., 1986; Hare & Reid, 1986). The RELAY experiment generates coherence between two protons that are not directly J coupled but are each coupled to a common spin. In practice, one can optimize the RELAY experiment to obtain all of the 1′H to 3′H cross-peaks, but it has proven to be much more difficult to observe all of the 2′H to 4′H cross-peaks with this experiment. The 2″H to 4′H cross-peaks in DNA are generally not observed by this method because of the extremely weak J coupling between 2″H and 3′H in the sugar conformations near the C2′-endo range of puckering. Cross-peaks between the 3′ and the 5′/5″ protons are likewise rarely seen, presumably because of weak scalar coupling between 3′H and 4′H or between 4′H and the 5′H, or both. Chazin and co-workers (Chazin et al., 1986) have been able to directly determine the 3′H–4′H connectivities by using a double-quantum (2Q) experiment (Braunschweiler et al., 1983). Although it should be possible in principle to establish the 1′H to 4′H and 2′H to 5′H/5″H scalar con-

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[1] Abbreviations: 2D NMR, two-dimensional nuclear magnetic resonance; 2Q, double quantum; COSY, two-dimensional autocorrelated spectroscopy; RELAY, two-dimensional relayed coherence transfer spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy.

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nectivities with multiple-step relayed coherence transfers (Eich et al., 1982; Bax & Drobny, 1983), such effects have not been observed in DNA oligonucleotides. Thus, in most cases the assignment of the 3′H and 4′H resonances are based in large part on the NOESY spectrum, and the 5′ and 5″ protons are usually not even assigned. Because the NOESY experiment is based on through-space dipolar coupling, the assignment of resonances by this method implicitly invokes some structural assumptions. Ideally, one would like to minimize the number of assignments that rely on structural assumptions; it is desirable and more rigorous to derive most of the assignments from COSY or other coherence transfer experiments since these are based on through-bond scalar coupling and are thus less ambiguous.

In this paper, we describe the application of two-dimensional isotropic mixing coherence transfer experiments (Braunschweiler & Ernst, 1983) to an investigation of the scalar coupling network of the synthetic DNA oligomer d-(CGCGTTTTCGCG). This oligonucleotide has been shown to exist as a *hairpin* structure in solution (Hare & Reid, 1986) and thus might be particularly susceptible to erroneous assignment of sugar resonances on the basis of NOESY spectra. We have also applied this method to the self-complementary 14-mer $[d(GCCGTGGCCACGGC)]_2$, which has a B-form structure in solution. We have found that the spectra produced with isotropic mixing can provide straightforward assignments of sugar proton resonances including, in some cases, 5′ and 5″ protons. We demonstrate that, when applied to synthetic DNA oligonucleotides, isotropic mixing experiments constitute a considerable improvement over combinations of COSY, RELAY, and 2Q experiments for determining the extended coupling networks in deoxyribose rings.

Materials and Methods

Sample Preparation. The two DNA samples used in this study were synthesized by the solid-phase phosphite triester method on an Applied Biosystems 380A DNA synthesizer. Each sample was synthesized on a 10-μmole scale, yielding over 800 A_{260} units of crude material. The crude DNA was purified by gel-exclusion chromatography on a 120 × 2 cm column of superfine Sephadex G-25 eluted with distilled water as described by Kintanar et al. (1987). Approximately 25 mg of each purified sample was dissolved in 0.4 mL of buffer at pH 7. The buffer for the hairpin sample contained 10 mM sodium phosphate, and the buffer for the self-complementary 14-mer contained 50 mM sodium phosphate and 100 mM sodium chloride. The samples were annealed by being heated to 75 °C and cooled slowly. Each DNA sample was then lyophilized to dryness twice and redissolved in 99.96% D_2O. The samples were finally lyophilized, redissolved in 0.4 mL of 99.996% D_2O, and transferred into 5-mm NMR tubes.

NMR Spectroscopy. All NMR experiments were performed on a home-built 500-MHz NMR spectrometer (Gladden and Drobny, unpublished results). Absolute-magnitude COSY and RELAY spectra were collected with 1024 complex t_2 points and 400 points in t_1. Isotropic mixing experiments were recorded in the phase-sensitive mode with time-proportional phase incrementation (Drobny et al., 1978; Bodenhausen et al., 1980; Marion et al., 1983), at several mixing times ranging from 50 to 250 ms. These spectra were acquired with 1024 complex points in t_2 and 300 points in t_1. An MLEV-16 sequence (Levitt et al., 1982) was used to induce long-range coherence transfer by isotropic mixing within the scalar-coupled spin systems. Effective radio-frequency fields of up to 25 kHz were employed with no adverse effects to the probe or sample.

Following collection, the data were processed with software written by Dennis Hare. Data obtained from isotropic mixing, COSY, and RELAY experiments were apodized with a phase-shifted sinebell function and then Fourier transformed. Noise ridges in the t_1 dimension were attenuated by multiplying the first row of the spectrum by one-half prior to transformation in t_1 (Otting et al., 1986).

Results and Discussion

Coherence Transfer by Isotropic Mixing. Several comprehensive discussions regarding the theory of coherence transfer by isotropic mixing have previously been published (Müller & Ernst, 1979; Chingas et al., 1981; Braunschweiler & Ernst, 1983; Chandrakumar & Subramanian, 1985; Bax & Davis, 1985), and we will therefore cite only the prominent aspects and predictions of the theory here. The most notable feature of the isotropic mixing experiment as used in this study involves the application of radio-frequency (rf) pulse trains during the mixing period. The applied field produced by the rf pulses reduces the magnitude of the chemical shift interactions so that the effective chemical shift differences are nearly equal to the spin-coupling constants for a system of coherently coupled spins. Under these circumstances, NMR energy transitions can no longer be attributed to a single nucleus and instead must be considered to result from the full set of all coupled spins. In this *strong-coupling limit*, coherence transfer can occur between pairs of spins throughout the coupled spin system, even when there is no direct coupling between a given pair of spins. It is therefore possible with this pulsed method to observe long-range coherence transfers in the normally weakly coupled spin systems of deoxyribose sugars.

The major requirement of the isotropic mixing method is that, during the mixing period, the generated average Hamiltonian must be dominated by isotropic spin–spin coupling. In the deoxyribose moieties of DNA, this mandates the reduction of chemical shift differences of ca. 2 kHz (δ 1′H–δ 2′H) to values near the 1′H–2′H coupling constant, $J_{1'H,2'H} \simeq 10$ Hz. This requirement apparently poses no serious difficulties, and in practice, methods that produce effective broad-band decoupling should be capable of generating the required isotropic coupling. In our hands, we find that the MLEV-16 sequence (Levitt et al., 1982) works quite well, although there is no reason why the Waltz-16 sequence (Shaka et al., 1983) would not also be effective.

An interesting prediction of the theory of coherence transfer by isotropic mixing is that the process is oscillatory (Bertrand et al., 1978; Müller & Ernst, 1979; Klevit & Drobny, 1986). For a two-spin system in the limit of strong rf field, the theory predicts that the cross-peak amplitude generated in the isotropic mixing experiment varies with a $\sin^2(\pi J \tau_m)$ dependence, where J is the strength of the scalar coupling and τ_m is the mixing time. Therefore, the intensity of a given cross-peak will oscillate as a function of τ_m with maxima occurring at $(n + 1)/(2J)$ (Klevit & Drobny, 1986). For an extended network of spins such as a deoxyribose sugar, the functional dependence of the cross-peak intensity will be much more complicated and in general will depend on τ_m and several J-coupling constants.

Coherence Transfer in d(CGCGTTTTCGCG). Identical expanded regions of the two-dimensional NMR spectra of d(CGCGTTTTCGCG) obtained with COSY, RELAY, and isotropic mixing experiments are shown in Figures 1, 2, and 3, respectively. These spectra were obtained under very similar conditions.

In the COSY spectrum (Figure 1), only sugar protons that are directly coupled through three bonds give rise to cross-

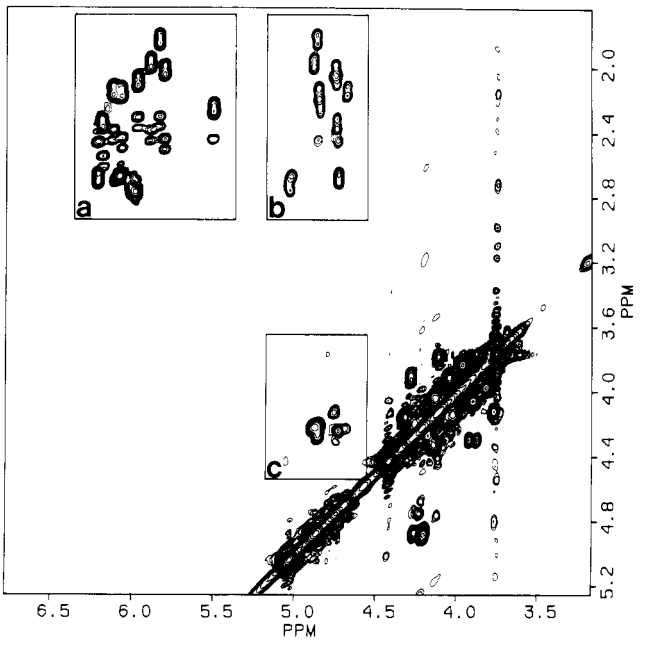

FIGURE 1: Contour plot of an expanded region of the 500-MHz absolute-magnitude ^1H COSY spectrum of d(CGCGTTTTCGCG) in D_2O at 25 °C showing the cross-peaks between sugar protons. The distinct cross-peak types are enclosed in boxes and indicated by letters: (a) 1′H–2′H and 1′H–2″H cross-peaks; (b) 2′H–3′H and 2″H–3′H cross-peaks; (c) 3′H–4′H cross-peaks.

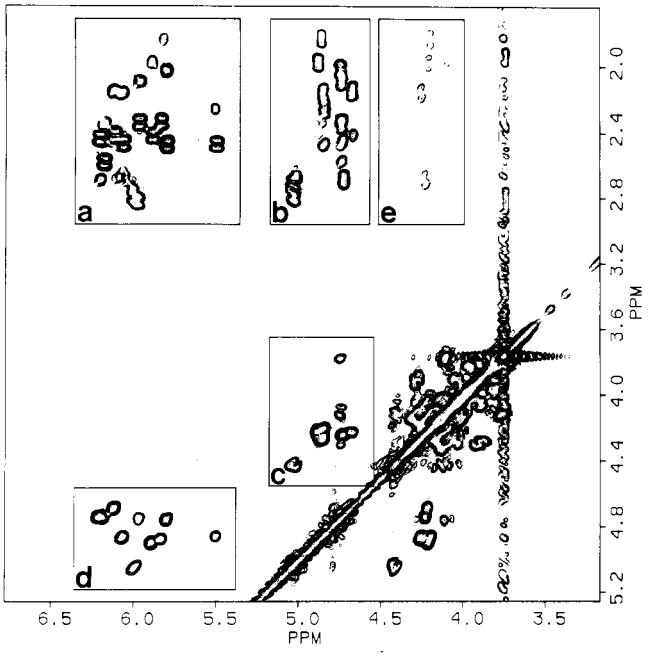

FIGURE 2: Expanded region of the 500-MHz absolute magnitude ^1H RELAY spectrum of d(CGCGTTTTCGCG) at 25 °C showing the cross-peaks between sugar protons. The COSY cross-peaks present in boxes a–c of Figure 1 appear together with several additional COSY cross-peaks and the RELAY cross-peaks: (d) 1′H–3′H cross-peaks; (e) 2′H–4′H cross-peaks.

peaks. These occur between 1′H and 2′H/2″H (box a), between 2′H and 3′H (box b), between 3′H and 4′H (box c), and between 4′H and 5′H/5″H (close to the diagonal in the 3.8–4.4 ppm region). Cross-peaks between 2″H and 3′H are either extremely weak or absent, probably due to weak scalar coupling between these protons. Note the extremely poor resolution of the cross-peaks in the 3′H to 4′H region due to the narrow chemical shift dispersion of both these types of protons. Furthermore, several cross-peaks in this region are missing, again suggesting a weak scalar coupling between 3′H and 4′H (weak 2″H–3′H and 3′H–4′H coupling is to be expected if the sugar is in the C2′-endo conformation). Clearly for the majority of sugar spin systems, the COSY experiment yields useful connectivities only between 1′, 2′/2″, and 3′ protons.

Additional information appears in the RELAY spectrum (80-ms mixing time) of this hairpin-forming DNA oligonucleotide (Figure 2). Cross-peaks between 1′H and 3′H and between 2′H and 4′H are seen in boxes d and e, respectively. The latter are much weaker than the former; in view of the strong 2′H–3′H coupling, the weak 2′H–4′H RELAY peaks indirectly point to a weak 3′H–4′H coupling in the majority of the sugars. Also, some cross-peaks between 2″H and 3′H now appear (box b), and more 3′H to 4′H cross-peaks are observed (box c). Only one cross-peak between a 3′H and a 5′H/5″H is seen. The 1′H to 3′H relayed connectivities are easily and directly determined in box d, where 11 of the 12 expected cross-peaks are observed (two of the peaks contain double cross-peaks). The 12th cross-peak in this region, corresponding to G4, appears when a shorter mixing time (50 ms) is used (data not shown). In box e, only 5 or 6 of the 12 expected 2′H to 4′H cross-peaks are observed. Many of the missing cross-peaks in this region were not observed even when several other mixing times were used. For these sugars, one must rely on the cross-peaks in box c in order to make the connectivity from 2′H to 4′H via the 3′H, and as we noted earlier, there are many redundant chemical shifts in this region.

A two-dimensional spectrum of d(CGCGTTTTCGCG) produced via isotropic mixing is shown in Figure 3. The total mixing period of 125 ms was assembled from 182 MLEV-16 cycles. All cross-peaks appearing in the RELAY and COSY spectra can be found in the isotropic mixing spectrum. In addition, an entirely new region of cross-peaks between 1′ and 4′, 5′, and 5″ protons is observed. Almost all of the cross-peaks between 2′/2″ protons and 4′ protons are now seen, and there are several more cross-peaks between 3′ and 5′ protons. Close inspection of Figure 3 reveals slight downfield shifts in the resonance positions relative to the COSY and RELAY spectra (Figures 1 and 2). This is attributable in large part to the slightly higher temperature setting in the isotropic mixing experiment (30 vs 25 °C). Comparison of several 2D NMR spectra taken at different temperature settings indicate a residual shift in the isotropic mixing spectrum, which is probably due to sample heating of not more than 5 °C by the rf irradiation during the mixing period.

The abundance of cross-peaks in the isotropic mixing spectrum confers a high degree of reliability to the assignment of the coupled spin systems. All of the information is at least doubly redundant. One can easily determine the connectivity within individual deoxyribose sugars by carrying out a so-called "box walk". Beginning from a 1′H to 3′H cross-peak, we draw a vertical line upward, picking up cross-peaks from 1′ to 4′, 5′/5″, and 2′/2″ protons. From each pair of cross-peaks between the 1′ and the 2′/2″ protons, we draw horizontal lines to the right, picking up cross-peaks from the 2′/2″ protons to the 3′, 4′, and sometimes 5′/5″ protons. From each set of cross-peaks across the top, we draw lines down toward the diagonal, picking up cross-peaks between 3′H and 4′H and between 4′ and 5′/5″ protons. We can then "close the box" by drawing horizontal lines to the left, picking up the 1′H to 4′H cross-peak and the 1′ to 5′/5″ proton cross-peak(s). Several representative examples of these box walks are drawn in Figure 3.

Using this technique, we were able to unequivocally establish the complete scalar connectivity network from 1′H to 4′H for 10 of the 12 sugar ring systems. The only exceptions had

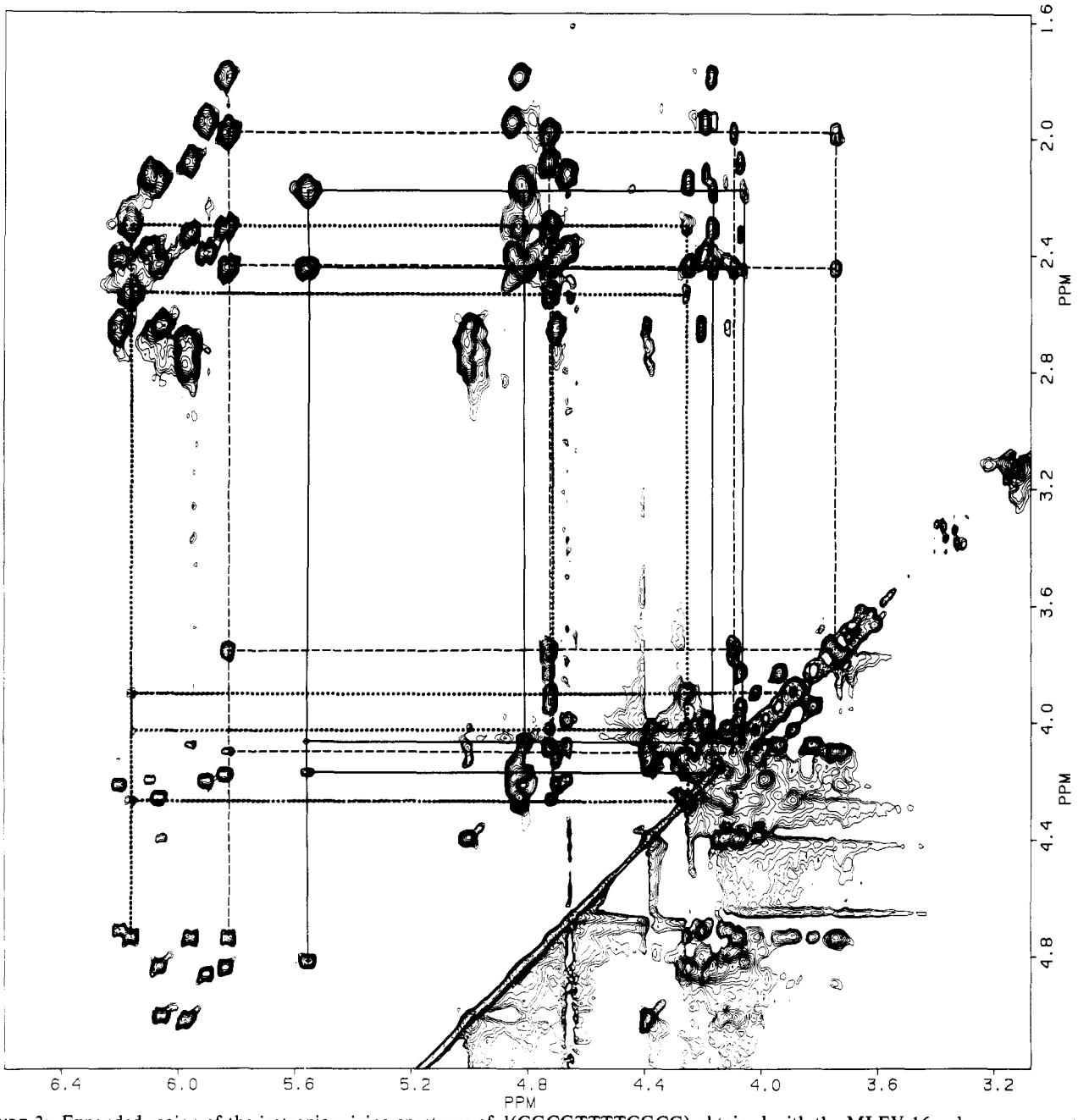

FIGURE 3: Expanded region of the isotropic mixing spectrum of d(CGCGTTTTCGCG) obtained with the MLEV-16 pulse sequence at 30 °C. The region shown is equivalent to those in Figures 1 and 2. This spectrum was obtained by applying 182 MLEV-16 cycles for a total mixing period of 125 ms. Lines connect cross-peaks of deoxyribose protons forming box walks (see text), which demonstrate the extensive connectivities made apparent with this technique: C1 (--); T8 (···); C9 (—).

chemical shift overlaps in *all* regions of the spectrum, and so the ambiguities in the assignment could not be resolved. These undetermined spin systems were later identified as G2/G10 after establishing sequential connectivities from the NOESY spectrum (data not shown). These results are summarized in Table I; no discrepancies were found compared to the NOE assignments of Hare and Reid (1986).

An encouraging result of this study was the observation of long-range coherence transfers involving the 5′H/5″H resonances. Using isotropic mixing, we have observed several long-range correlations between 1′H or 2′H/2″H and 5′H/5″H resonances. We have confirmed 1′H–5′H/5″H connectivities in the deoxyribose rings of residues C1, C9, and G12 and connectivities between the 1′H and both the 5′H and the 5″H of residues T6 and T8. In addition, we have observed 2′H/2″H to 5′H or 5″H connectivities in residues C1, C9, and G12. Finally, several more connectivities to 5′/5″ protons may be determined from the 4′H to 5′H/5″H cross-peaks near the diagonal. These data are also included in Table I.

Note that in the isotropic mixing spectrum, in cases where connectivity to both 4′H and 5′/5″ protons was observed, it was tacitly assumed that the 4′H resonance was the farthest downfield. However, these resonances may be assigned more rigorously with the isotropic mixing experiment by investigating the time dependence of coherence transfer (vide infra).

Although it is possible to identify all the coupled spins in a sugar moiety by this method, one cannot (a priori) differentiate between the 2′H and 2″H or between the 5′H and the 5″H. In fact, none of the ^{1}H 2D NMR methods that utilize only coherence transfer (COSY, RELAY, isotropic mixing) can distinguish between 2′H and 2″H or between 5′H and 5″H in deoxyribose without making some assumption about the

Table I: Assignment of the Sugar Proton Resonances of d(CGCGTTTTCGCG) Derived from Isotropic Mixing Experiments (Chemical Shifts in ppm)[a]

1 2 3 4 5 6 7 8 9 10 11 12
5′-C-G-C-G-T-T-T-T-C-G-C-G-3′

residue[b]	1′H	2′H	2″H	3′H	4′H	5′/5″H
C1	5.88	2.05	2.50	4.80	4.15	3.81
G2						
C3	5.91	1.86	2.37	4.23	4.90	
G4	6.13	2.70	2.70	5.06	4.46	
T5	6.14	2.21	2.50	4.89	4.31	
T6	6.17	2.19	2.44	4.72	4.25	4.06, 4.16
T7	6.03	2.14	2.38	4.79	4.12	
T8	6.24	2.37	2.60	4.79	4.32	3.95, 4.09
C9	5.60	2.27	2.50	4.88	4.23	4.12
G10						
C11	5.97	2.14	2.45	4.96	4.25	
G12	6.27	2.72	2.48	4.77	4.27	4.17

[a] Derived from spectra collected at 25 °C. Solution conditions are discussed in the text (see Materials and Methods). The 2′/2″ protons were differentiated on the basis of coupling constants assuming C2′-endo conformation for the sugars. [b] After the coupling network was established, the spin systems were identified from the sequential assignments with a NOESY spectrum.

conformation of the sugar. In practice, additional information from NOESY data is needed to identify the 2″H [this proton is almost always closer to the 1′H than is the 2′H, and the NOESY cross-peak to the former (1′H to 2″H) is more intense—the single exception would be for the 1′-endo,2′-exo twist conformation in which the 2′H and 2″H are equidistant from the 1′H]. The coherence transfer 2D NMR methods (COSY, RELAY, isotropic mixing) are useful in corroborating these 2′H from 2″H assignments since these experiments provide a measure of the scalar couplings to adjacent spins; for the most common conformations of deoxyribose sugars in DNA, the 2′H and 2″H resonances have characteristic J coupling constants.

It is at least equally difficult to differentiate between the 5′ and 5″ protons since in the preferred conformation about the C4′–C5′ bond (sc^+), it is relatively common to find 4′H–5′H and 4′H–5″H couplings that are nearly equal. Therefore, assignment of the 5′H as opposed to the 5″H may have to rely on the intensity of the appropriate NOESY cross-peaks combined with structural information derived from the other sugar proton couplings. The fact that we observe long-range coherence transfer to 5′/5″ protons in some spin systems and not in others is undoubtedly a reflection of subtle differences in J coupling constants and may be indicative of different conformations of the deoxyribose sugar.

Application to Other DNA Oligonucleotides. The application of the isotropic mixing technique is not limited to relatively small hairpin-forming DNA oligonucleotides. We have successfully applied this method to larger B-form DNA molecules. For example, the two-dimensional spectrum of $[d(GCCGTGGCCACGGC)]_2$, a self-complementary 14-mer, is shown in Figure 4. Again, there are a large number of cross-peaks in the various spectral regions as was observed in Figure 3. However, there are fewer cross-peaks between 4′ and 2′/2″ protons (13 cross-peaks); interestingly, these cross-peaks are derived from only 7 of the 14 coupled spin systems. Apparently about half of the sugars show both 2′H–4′H and 2″H–4′H cross-peaks, whereas the other half show neither of these cross-peaks. Also, no long-range coherence transfers to 5′/5″ protons were observed, with the exception of the 3′H to 5′H/5″H cross-peak of residue G1. Nevertheless, many more connectivities could be determined from this spectrum than from the corresponding COSY and

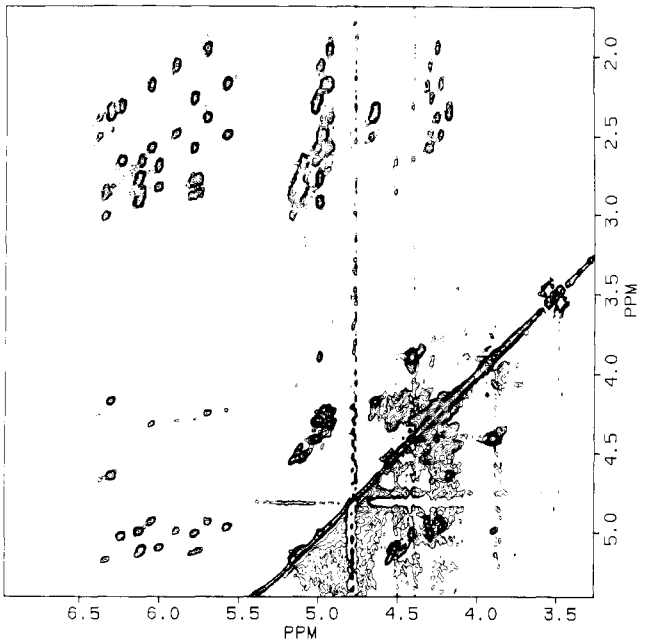

FIGURE 4: Isotropic mixing spectrum of $[d(GCCGTGGCCACGGC)]_2$ obtained at 25 °C with the MLEV-16 pulse sequence with a total mixing time of 100 ms. The region shown is equivalent to those presented in Figures 1–3.

RELAY spectra (data not shown). The lack of all possible coherence transfers in some of the spin systems of this B-form DNA molecule is likely a consequence of small intervening J coupling constants dictated by the conformation of the particular sugar moieties. This problem may perhaps be alleviated by utilizing longer mixing periods (vide infra). However, the longest mixing time attempted on this sample was 150 ms, and no additional 5′H/5″H connectivities were observed.

Time Dependence of Coherence Transfer by Isotropic Mixing. We have investigated the oscillatory nature of coherence transfer induced by isotropic mixing by obtaining two-dimensional spectra of both the hairpin-forming and B-form DNA oligonucleotides at various mixing times. Our intent was not to attempt a full quantitative analysis of these data but rather to gain a feel for the coherence transfer process in DNA oligonucleotides so as to be better able to empirically optimize the experiment. As expected, the cross-peak intensities in different regions oscillated as a function of mixing time. This can be seen in Figure 5, where the variation in intensity of several representative cross-peaks is shown as a function of mixing time for d(CGCGTTTTCGCG). For example, the intensity of the 2′H–2″H COSY-type cross-peak (Figure 5a, filled triangles) is high at 50 ms, decreases to a local minimum between 100 and 125 ms, is at a local maximum at 150 ms, and then levels off above 200 ms as the amplitude of the oscillation becomes damped. On the other hand, the intensities of 1′H to 2′H and 2′H to 3′H cross-peaks (open triangles and crosses) are likewise high at a mixing time of 50 ms, but the oscillations appear to be "out of phase" with the previous example before becoming damped at longer mixing times. In contrast, the 1′H to 3′H RELAY-type cross-peak (squares) is relatively weak at 50 ms, reaches a maximum at 75 ms, and finally oscillates "with the same phase" as the 2′H–2″H cross-peak.

The remaining panels in Figure 5 show the behavior of the RELAY-type and multiple-RELAY-type cross-peaks. The variation in intensity as a function of mixing time for the 2′H/2″H to 4′H cross-peaks and 1′H to 4′H cross-peaks is shown in panels b and c of Figure 5, respectively. The intensity

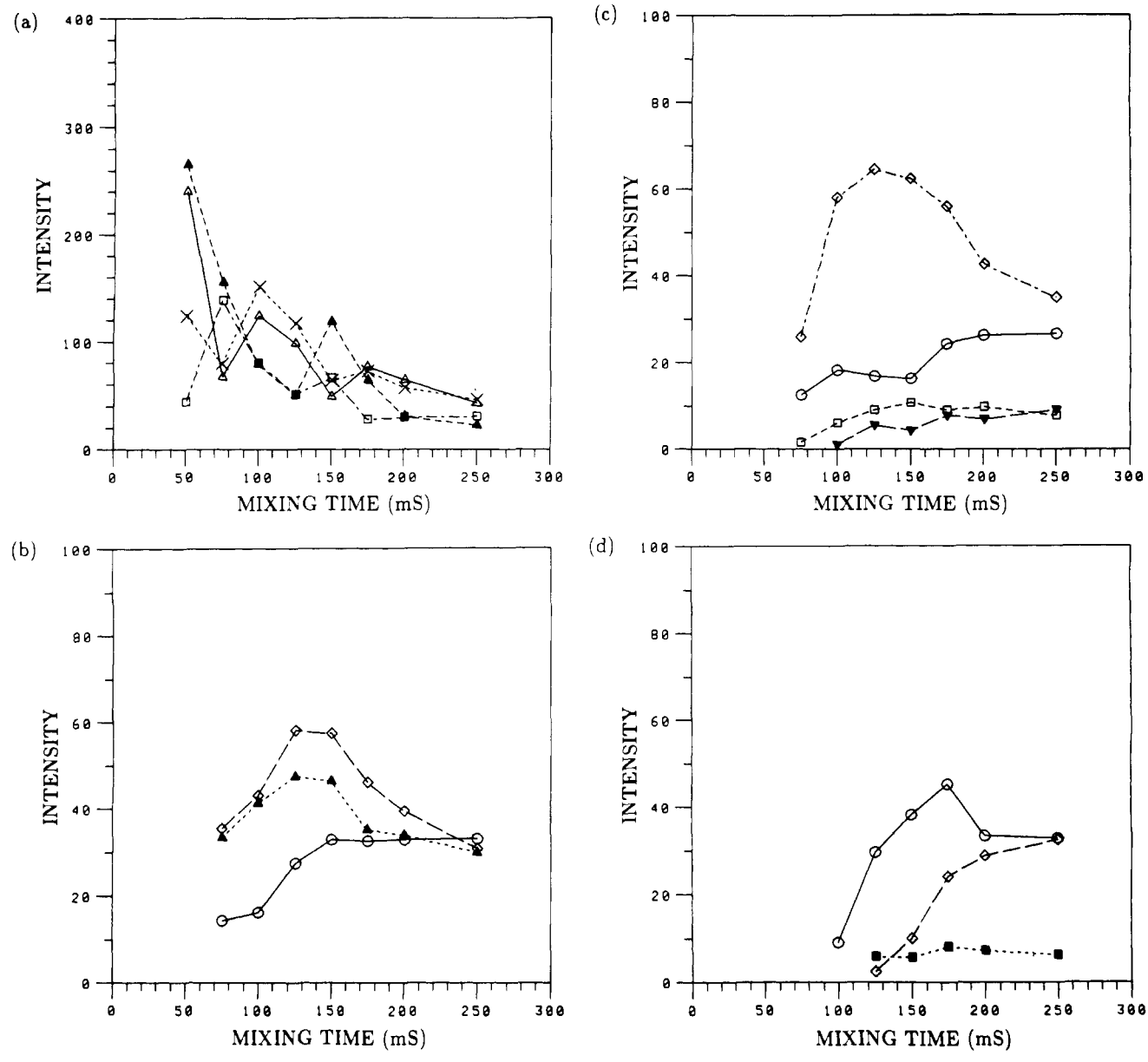

FIGURE 5: Plots of the integrated intensity (in arbitrary units) of cross-peaks in the isotropic mixing spectra of d(CGCGTTTTCGCG) as a function of the experimental mixing time. Individual measured cross-peak intensities were obtained from complete two-dimensional isotropic mixing experiments with mixing times of 50, 75, 100, 125, 150, 175, 200, and 250 ms: (a) 1′H–2′H connectivity for residue C3 (▲), 2′H–2″H connectivity for residue C3 (▲), 2′H–3′H connectivity for residue C11 (×), and 1′H–3′H connectivity for residue C9 (□); (b) 2′H–4′H connectivities C1 (○), C3 (▲), and G12 (◇); (c) 1′H–4′H connectivities C1 (○), T7 (▼), C9 (□), and G12 (◇); (d) 1′H–5′H/5″H connectivities C1 (○), T8 (■), and G12 (◇).

of these cross-peaks shows two kinds of behavior. Intensity begins to appear at about 75 ms and then either passes through a broad maximum at about 150 ms or rises gently to a plateau value at long mixing times. These differences may be a consequence of different conformations or motional characteristics of nucleotides at the ends of the stem or in the loop region of the hairpin. Figure 5d shows the behavior of 1′H to 5′H/5″H cross-peaks. Again, the functional variation of the cross-peak amplitude may well reflect the position of the nucleotide in the DNA structure.

A similar pattern is found for the time-dependent, isotropic mixing spectra of the B-form self-complementary 14-mer, except that the amplitude of oscillation may be even greater (data not shown). For example, the spectrum obtained with a mixing time of 80 ms has strong 1′H to 2′H and 2″H to 3′H cross-peaks, and nearly all the 1′H to 3′H cross-peaks appear. However, the 2′H to 3′H cross-peaks are almost completely absent. This spectrum is quite unlike the COSY-like spectrum we expect to see at very short mixing times. Increasing the mixing time to 100 ms leads to interesting changes in the spectrum; the 2′H to 3′H cross-peaks recover some intensity, and a large number of 2′H/2″H to 4′H and 1′H to 4′H cross-peaks appear. At the same time, the 2′H to 2″H cross-peaks near the diagonal become severely attenuated. At 150 ms, the cross-peaks between 2′ and 2″ protons recover some intensity, and several more 2′H to 4′H and 1′H to 4′H cross-peaks appear. However, unlike for the hairpin sample (Figure 5d), no long-range connectivities to 5′/5″ protons were observed at this long mixing time.

Note that since coherence transfer from 3′H to 4′H always occurs before any transfer to 5′/5″ protons, the time dependence of isotropic mixing may be used to rigorously distinguish between the 4′H and the 5′H/5″H, all of which have similar chemical shifts.

Summary and Conclusions. In summary, we have shown that the isotropic mixing experiment is an excellent method

for assigning the extended coupled spin system of deoxyribose sugars in oligonucleotides. Moreover, the technique appears to be generally applicable to any oligonucleotide that may be studied by other 2D NMR experiments. An obvious advantage is that with a single experiment one can reliably establish the connectivity from 1′H to 4′H, and in favorable cases, one can extend the network to the 5′/5″ protons. Unfortunately multiple coherence transfer between 5′H/5″H and either 2′H or 1′H appears to be restricted to terminal residues, loop residues, or loop–stem junction residues in "abnormal" DNA structures such as hairpins. Apparently the range of sugar conformations available in normal duplex DNA (and in the helical stem region of the hairpin) hinders long-range coherence transfer to 5′/5″ protons. This is probably due to 3′H–4′H torsional angles that are always close to 90°, resulting in weak scalar coupling between these protons, although we cannot at present rule out other possible explanations such as motion of the flexible DNA backbone. Nevertheless, above a certain threshold (75 ms for the hairpin, 125 ms for the 14-mer duplex), the experiment will work well over a wide range of mixing times as the oscillations in coherence transfer rates (and cross-peak amplitudes) become damped. Finally, a study of the time dependence of coherence transfer by isotropic mixing may be an alternative way to extract information about J coupling constants in the sugar spin system, and these in turn may be correlated to the sugar conformation. We are currently undertaking a quantitative investigation along these lines to determine if the isotropic mixing method may be used to obtain such important structural information in DNA oligonucleotides.

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Registry No. d(CGCGTTTTCGCG), 103639-63-0; [d(GCCGTGGCCACGGC)]$_2$, 112319-63-8; deoxyribose, 533-67-5.

REFERENCES

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Bax, A., & Drobny, G. (1985) *J. Magn. Reson. 61*, 306–320.
Bertrand, R. D., Moniz, W. B., Garroway, A. N., & Chingas, G. C. (1978) *J. Am. Chem. Soc. 100*, 5227–5229.
Bodenhausen, G., Vold, R. L., & Vold, R. R. (1980) *J. Magn. Reson. 37*, 93–106.
Braun, W., & Gō, N. (1985) *J. Mol. Biol. 186*, 611–626.
Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521–528.
Braunschweiler, L., Bodenhausen, G., & Ernst, R. R. (1983) *J. Mol. Phys. 48*, 535–560.
Chandrakumar, N., & Subramanian, S. (1985) *J. Magn. Reson. 62*, 346–349.
Chazin, W. J., Wüthrich, K., Hyberts, S., Rance, M., Denny, W. A., & Leupin, W. (1986) *J. Mol. Biol. 190*, 439–453.
Chingas, G. C., Garroway, A. N., Bertrand, R. D., & Moniz, W. B. (1981) *J. Chem. Phys. 74*, 127–156.
Drobny, G., Pines, A., Sinton, S., Weitekamp, D. P., & Wemmer, D. E. (1979) *Faraday Symp. Chem. Soc. 13*, 49–55.
Eich, G., Bodenhausen, G., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 3732–3733.
Feigon, J., Leupin, W., Denny, W. A., & Kearns, D. R. (1983) *Biochemistry 22*, 5943–5951.
Hare, D. R., & Reid, B. R. (1986) *Biochemistry 25*, 5341–5350.
Hare, D. R., Wemmer, D. E., Chou, S. H., Drobny, G., & Reid, B. R. (1983) *J. Mol. Biol. 171*, 319–336.
Hare, D. R., Shapiro, L., & Patel, D. J. (1986a) *Biochemistry 25*, 7445–7456.
Hare, D. R., Shapiro, L., & Patel, D. J. (1986b) *Biochemistry 25*, 7456–7464.
Havel, T. F., & Wüthrich, K. (1985) *J. Mol. Biol. 182*, 281–294.
Kaptein, R., Zuiderweg, E. R. P., Scheek, R. M., Boelens, R., & van Gunsteren, W. F. (1985) *J. Mol. Biol. 182*, 179–182.
Kintanar, A., Klevit, R. E., & Reid, B. R. (1987) *Nucleic Acids Res. 15*, 5845–5862.
Klevit, R. E., & Drobny, G. P. (1986) *Biochemistry 25*, 7770–7773.
Levitt, M. H., Freeman, R., & Frenkiel, T. (1982) *J. Magn. Reson. 47*, 328–330.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.
Müller, L., & Ernst, R. R. (1979) *Mol. Phys. 38*, 963–992.
Otting, G., Widmer, H., Wagner, G., & Wüthrich, K. (1986) *J. Magn. Reson. 66*, 187–193.
Scheek, R. M., Russo, N., Boelens, R., Kaptein, R., & van Boom, J. H. (1983) *J. Am. Chem. Soc. 105*, 2914–2916.
Shaka, A. J., Keeler, J., & Freeman, R. (1983) *J. Magn. Reson. 53*, 313–340.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.