NMR STUDIES OF CONFORMATIONAL STATES AND DYNAMICS OF DNA

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I. INTRODUCTION

A number of exciting recent discoveries in the field of nucleic acid structure, sequence, and function have generated a renewed interest in the structural and dynamic properties of DNA. One of the major factors affecting research in this area has been the development of techniques for rapidly sequencing DNA[1,2] and the consequent outpouring of sequence data, particularly of regions involved in gene regulation.[3,4] Mutation,[5] chemical modification,[6] and UV irradiation[7] studies have generated an interesting set of observations about those sequences involved in gene regulation, but the physical basis for their function remains obscure. If more were known about the physical properties of DNA and how they are affected by sequence and chemical or enzymatic modification, perhaps some of these biochemical observations could be explained. A second important factor which has facilitated physical chemical studies of DNA has been the development of techniques for synthesizing large quantities of low molecular weight DNA of defined sequence, and the isolation of large quantities of natural DNA (plasmids and restriction fragments,[12] random sequence DNA[13-15]). A third factor contributing to the rapid expansion of DNA research has been the crystallization of various short DNA duplexes[16-20] and the unexpected discovery by Wang et al.[16] that d $(GC)_3$ in the crystalline form adopts a left-handed double-stranded configuration (termed Z-DNA). Drew et al.[18] subsequently showed that $d(CGCG)_2$ also adopts a Z-like conformation in crystals. The crystallographic observations confirmed the earlier suggestion of Pohl and Jovin,[21] based on circular dichroism (CD) measurements, that poly(dG-dC) · poly(dG-dC) might adopt a left-handed helical structure in high salt solutions. The results of the X-ray diffraction studies have necessarily raised questions about the extent to which the crystallographic structures might apply to solution state DNA, but spectroscopic studies subsequently provided evidence that poly(dG-dC) can adopt a Z-like conformation in solution.[22,23] Plasmid DNAs containing short segments of $d(GC)_n$ have been used to explore the effect of supercoiling on the B- to Z-interconversion[24] and antibody staining techniques have provided experimental evidence for Z-DNA in chromosomal DNA.[25,26] The effect of methylation of C residues on the B- to Z-conversion has been explored and possible relation to gene regulation considered.[27] The discovery of Z-DNA and the subsequent chain of experiments it triggered is a dramatic illustration of how information derived from studies on isolated DNA molecules can contribute to understanding of some of the important unanswered questions regarding the properties

of DNA in vivo. The Z-DNA studies are especially important because they greatly expanded our thinking about the range of conformational states that DNA can adopt and have reemphasized the important role that sequence plays in determining the physical properties of DNA. The discovery of Z-DNA was soon followed by the crystallization of the first right-handed DNA double helix d(CGCGAATTCGCG)$_2$ and analysis of the structure of this molecule has produced the first detailed information about the structure of the B-DNA helix in the crystalline state.[17] It is largely as a result of these and other studies that there is much current interest in the structural and dynamic properties of DNA and, fortunately, an impressive array of new tools is available for studying the properties of DNA molecules. In studies of the dynamic and structural properties of DNA nuclear magnetic resonance (NMR) spectroscopy is certainly the most powerful solution-state technique known.

For over 25 years, X-ray diffraction studies on fibers and crystals have been our major source of detailed information about DNA structures. As a result of recent technological breakthroughs it now appears that much of this same information can be obtained on DNA molecules in solution using nuclear magnetic resonance (NMR) relaxation techniques. The NMR relaxation techniques may permit DNA structures in solution to be probed at a level of detail previously reserved only for the X-ray diffraction studies. Moreover, the dynamic properties of DNA molecules in solution can be studied by the NMR methods. In this review, we focus on the major new developments that have occurred during the past 3 years in the application of new NMR techniques to DNA. Early NMR studies of DNA, starting about 1970 and continuing until now, developed the techniques and laid the foundation for the current research. (For reviews of some of the earlier work see References 28 to 32.) In these studies the standard NMR parameters of linewidth, chemical shift, and coupling constants were used to evaluate various aspects of the solution-state properties of DNA. In work carried out during the past 3 years, however, there has been a greater use of relaxation methods, including new two-dimensional NMR techniques.[33-38] The application of the two-dimensional NMR techniques is particularly exciting because it permits the structural properties of DNA molecules in solution to be explored at an unprecedented level of detail.

In this review, we limit our considerations to contributions that NMR has made during the past 3 years to our understanding of the physical chemical properties of DNAs including:

- Average conformational states of DNAs with different sequences
- Interconversions between different conformational states
- Local internal motions (amplitudes and frequencies)
- Flexibility and sequence effects on flexibility
- Base pair opening rates (breathing mechanisms and proton exchange)
- Motions in solid complexes
- Interactions with metal ions
- Supercoiling effects on DNA properties
- Effects of mismatched bases on DNA properties

Most of this review is organized according to nuclei studied (^{1}H, ^{31}P, ^{13}C) and NMR methods used. We begin by describing the parameters that are used to characterize NMR spectra, and summarize the results of recent NMR studies of DNAs using conventional spectral measurements. We then review the basic ingredients of NMR relaxation measurements and discuss their application to investigations of DNA. A considerable amount of space has been devoted to the relaxation methods because they are the ones that are currently yielding, and will continue to yield, the most detailed information about the solution-state structural and dynamic properties of DNA. In this sense, the review also attempts to anticipate the future of NMR research on DNA.

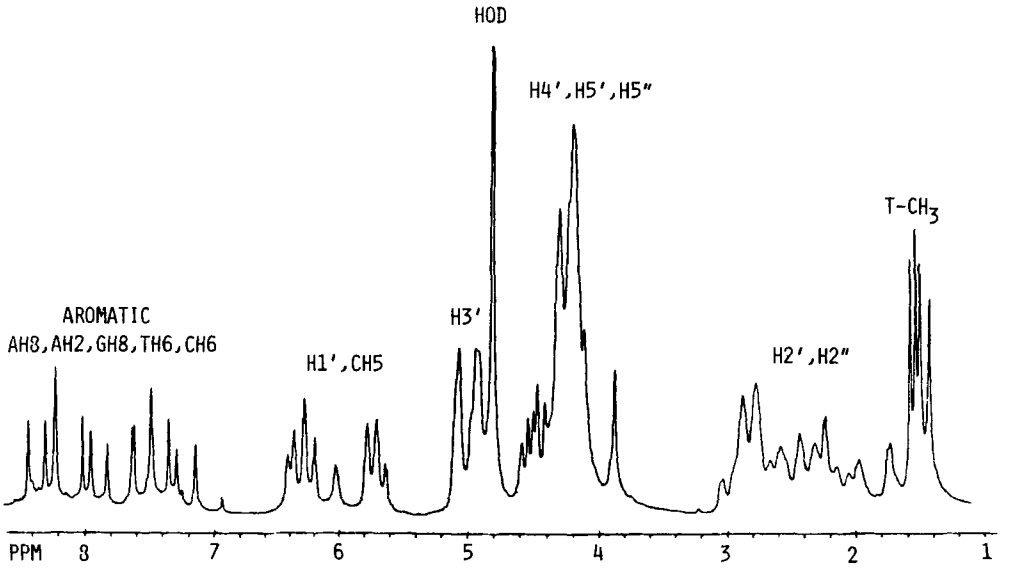

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FIGURE 1. (A) A 500-MHz ^{1}H NMR spectrum of the nonexchangeable proton resonances in d(ATATCGATAT)$_2$ at 27°C. Assignments to proton type are given above the appropriate spectral regions.[130,132] (B) A 300-MHz ^{1}H NMR spectrum of d(ATATCGATAT)$_2$ in the lowfield spectral region at 6°C. Resonances in this region are due to the imino protons of thymine and guanine residues in Watson-Crick base pairs. At this temperature, 6°C, the resonances from two terminal base pairs are broadened due to rapid exchange with solvent protons.[117] (C) The 80.7-MHz ^{31}P NMR spectrum of poly(dA-dT) at 25°C in a solution with a 0.1 *M* NaCl and 10 m*M* cacodylate buffer at pH 7. This spectrum was kindly provided by N. Assa-Munt and J. Granot. (D) The natural abundance 50.2-MHz carbon-13 NMR spectrum of double stranded calf thymus DNA (~40 bp) at 36°C in 0.2 *M* NaCl and 10 m*M* phosphate buffer at pH 7. This spectrum was kindly provided by T. A. Early and J. Feigon.

II. CONVENTIONAL NMR STUDIES OF DNA

A. Basic NMR Properties

Some examples of the ^{1}H, ^{31}P, and ^{13}C NMR spectra of double helical samples of DNA are presented in Figure 1, and the standard Watson Crick base pairs and sugar residue are shown in Figure 2. In conventional NMR studies of DNA, the following spectral properties are of interest.

1. Chemical Shift

The location of a resonance is usually specified in terms of displacement, in parts per million (ppm) from a resonance of a reference compound (usually tetramethyl silane [TMS] for ^{1}H and ^{13}C and 85% phosphoric acid for ^{31}P). Nuclei in similar chemical environments have similar chemical shifts. Methyl protons typically resonate around 1.5 ppm downfield from TMS, whereas aromatic base protons (adenine H8, guanine H8, thymine H6, cytidine H6) resonate between 8.5 and 7.0 ppm downfield. Chemical shifts are, therefore, useful in classifying resonances as to general type, but additional information is usually required to make specific assignments because exact chemical shifts are sensitive to a number of different factors. For base protons, there are large nearest neighbor effects arising from ring current effects and atomic contributions to the magnetic anisotropy of the bases.[30-32] Hydrogen bonding also has pronounced effects on the resonance positions of exchangeable imino and amino protons. Chemical substitution on neighboring atoms can affect resonance positions of base protons, and

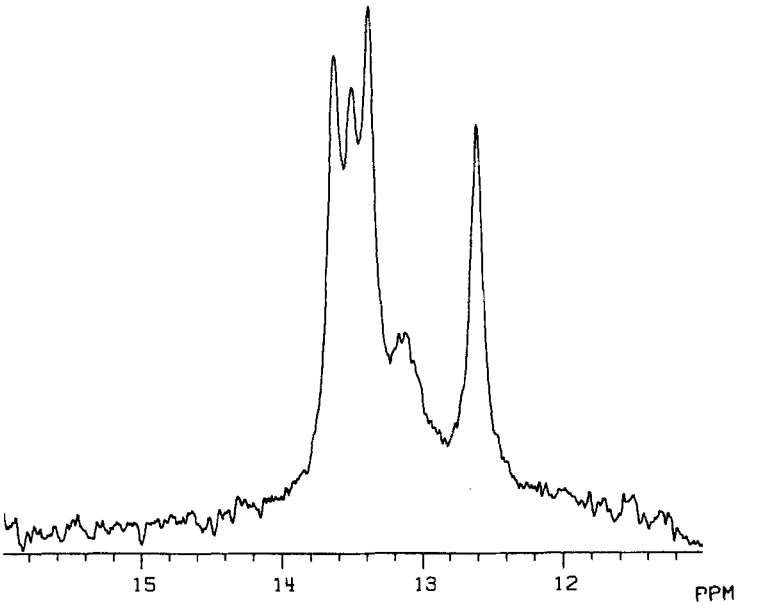


FIGURE 1B

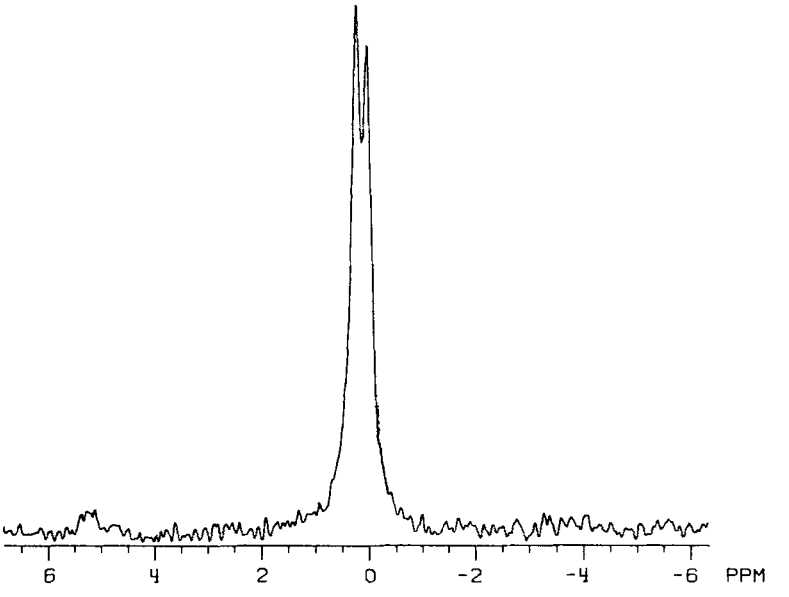


FIGURE 1C

the sugar proton resonances are sensitive to both conformation and the nature of the attached base.[39,40] The ^{31}P chemical shifts are sensitive to the backbone conformation and to hydration.[28,29]

2. Intensity

The integrated intensity of a resonance is directly proportional to the number of protons in the molecule giving rise to that resonance. A methyl resonance, for example,

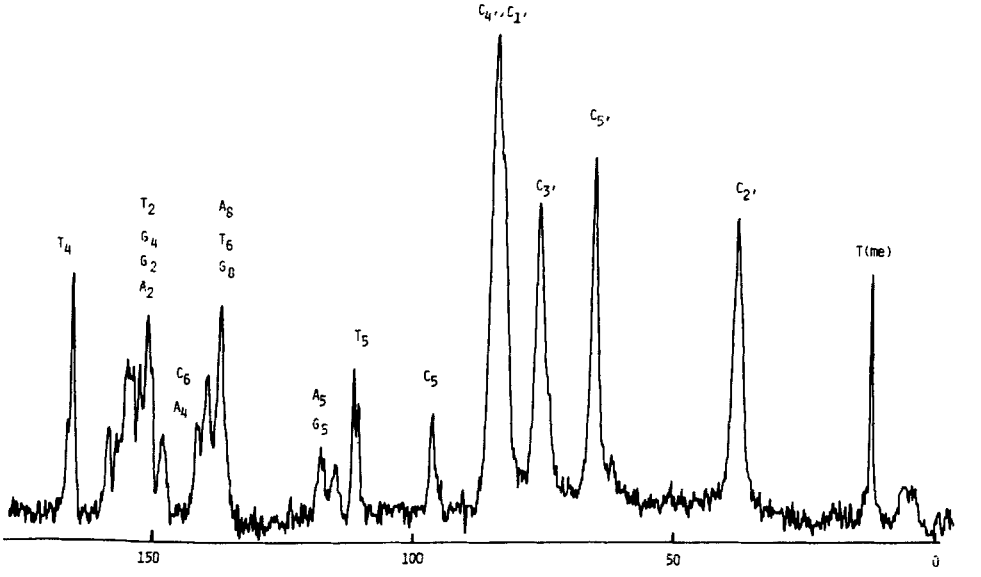


FIGURE 1D

has three times the *integrated intensity* as does the resonance from a single proton. This quantitative relation between peak intensity and number of contributing nuclei is one feature which makes NMR spectra particularly valuable for structural and identification purposes.

3. Line Width

The contribution to $\Delta\nu_{\frac{1}{2}}$, the line width of a resonance (full width at half height), due to spin-spin relaxation of a nucleus is

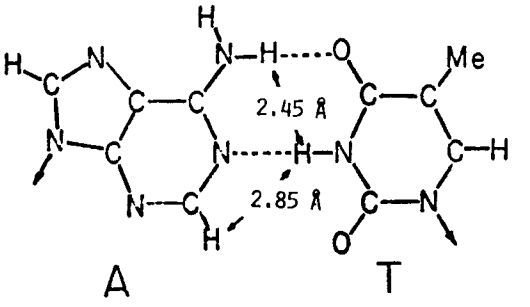
$$\Delta\nu_{\frac{1}{2}} = (\pi T_2)^{-1} \qquad (1)$$

where T_2 is the spin-spin relaxation time. If there are contributions (inhomogeneous magnetic field, chemical shift dispersion) to the line width, the observed line width will be even larger than that predicted by Equation 1.

4. Scalar Interactions

Through-bond magnetic interactions between pairs of magnetic nuclei cause the resonances from interacting nuclei to be split. In cytidine, for example, the mutual interaction of the H5 and H6 protons causes a 7.5 Hz splitting of each of the C-H5 and the C-H6 resonances (e.g., peaks at ~5.6 and ~7.5 ppm in Figure 1A). When more than two nuclei interact, even more complex splittings are observed, as in the case of the H1′ sugar protons which are split by interactions with both the H2′ and H2″ sugar protons. The magnitudes of these splittings are of interest because they can be directly related to the average conformation of the sugar. Thus, the splitting of the H1′ resonance in deoxyribonucleotides due to interactions with the 2′ and 2″ protons is expected to range from 7 to 15 Hz depending upon whether the sugar is in an N (3′-endo) or S (2′-endo) conformation, respectively (see Figure 2).[39,40] Other conformations result in intermediate values of the splitting.

Measurements of chemical shifts, splittings, and intensities were mainstays of NMR studies of DNA carried out during the period from 1970 up to 1979,[28–32] and they provided a solid foundation for the application of the various types of one- and two-



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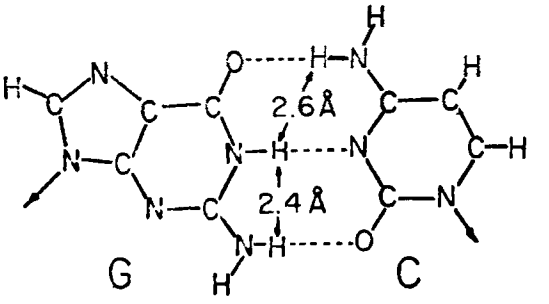

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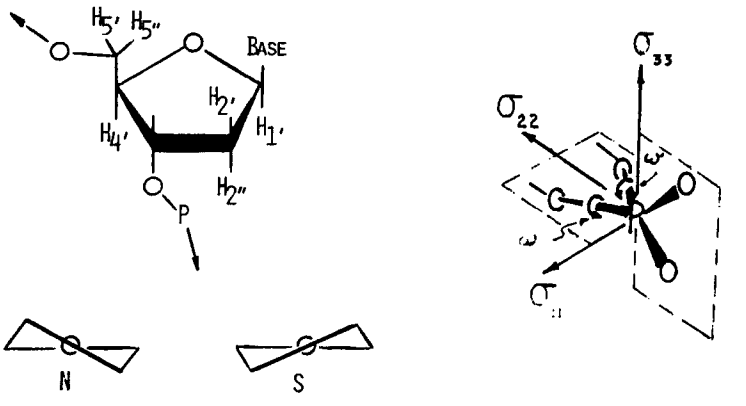

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FIGURE 2. (A) The standard Watson-Crick base pairs are shown along with selected interproton distances. (B) The deoxyribose sugar ring is shown along with the numbering system used in assigning spectra. A schematic diagram showing the pucker of the ribose ring for the S and N conformations is also presented. (C) The structure of the phosphate group is shown schematically. The orientations of the principal axes of the magnetic shielding tensor (σ_{11}, σ_{22}, and σ_{33}) relative to the symmetry axes of the phosphate group are also indicated.

dimensional relaxation methods that will be a major topic of this review. However, before considering the NMR relaxation experiments, we first discuss recent experiments in which ^{1}H, ^{31}P, and ^{13}C chemical shifts have been used to study various conformational

properties of DNA and explore the present state of understanding of the relationship between chemical shifts and structure.

B. Proton NMR Studies of Oligonucleotides

Improvements in the large-scale chemical synthesis of short oligonucleotides[8-11] are having a major impact in DNA research, in general, and in NMR studies of DNA, specifically, and in this section we discuss some of the NMR work on synthetic double helical oligonucleotides. A number of oligonucleotide sequences which have been studied during the past 5 years, but only those molecules which contain interesting or unusual features, are discussed below.

1. RNA-DNA Hybrids

There have been only a couple of studies of RNA-DNA hybrids.[41,42] **$(dG)_n \cdot (rC_{11}dC_{16})$** — This molecule, which contains an RNA-DNA hybrid covalently linked with a DNA-DNA section, was designed to determine whether or not the RNA-DNA hybrid portion of the molecule adopts an RNA-like geometry, and if so, whether it affects the contigous DNA-DNA portion of the molecule.[41] A comparison of the spectra of $(dG)_n \cdot (rC)_n$, $(rG)_n \cdot (rC)_{22}$, and $(dG)_n \cdot (dC)_{22}$ demonstrated that the spectra of $(dG)_n \cdot (rC)_n$ and $(rG)_n \cdot (rC)_n$ are virtually identical. This confirms that a free DNA-RNA hybrid adopts an RNA-like (A-form) geometry and that this geometry is different from that exhibited by $(dG)_n \cdot (dC)_n$ (presumably B-form). The spectrum of $(dG)_n \cdot (rC_{11}dC_{16})$ was found to be identical with that expected for a sum of $(dG)_n \cdot (rC)_{11}$ and $(dG)_n \cdot (dC)_{16}$. In the lowfield spectrum, two G-imino resonances of the proper relative intensities were observed at the same positions as those observed in the DNA · DNA and RNA · DNA molecules separately. Likewise, the aromatic spectrum was clearly a composite of the $(dG)_n \cdot (rC)_n$ and the $(dG)_n \cdot (dC)_n$ spectra with the expected intensity ratios. From this, it was concluded that both RNA-like and B-DNA conformations can coexist within a single helix with little perturbation of base pairs at the junction region.

d(CTTTTG)·r(GAAAAAC) — Pardi et al.[42] compared the conformations of this RNA-DNA hybrid molecule with the corresponding DNA-DNA and RNA-RNA helices of the same sequence. The spectrum d(CTTTTTG)·r(GAAAAAC) was intermediate between that of corresponding DNA · DNA and RNA · RNA molecules, suggesting a conformation intermediate between A- and B-form. However, all of the H1′ ribose protons in the hybrid oligonucleotide r(CAAAAAG) · d(CTTTTTG) were found to have a 1′-2′ coupling constant $J_{1',2'} \leq 15$ Hz, indicating that the ribose sugar ring pucker is ~85% N-type (3′ endo).[39,42] The sugar conformations on the deoxy strand could not be determined. Based on these limited NMR studies, it appears that the DNA-RNA hybrids do adopt an RNA-like geometry, consistent with the results of other physical chemical studies.

2. Non-Watson-Crick Oppositions

NMR has been especially useful in elucidating the nature of non-Watson-Crick oppositions and their effect on the DNA helix.

CGTGAATTCGCG / GCGCTTAAGTGC — This duplex contains two G · T oppositions and the nuclear Overhauser effect (NOE) between the G-imino lowfield NMR spectrum and the T-imino proton confirms the formation of a hydrogen bonded G · T wobble base pair.[43] In a 12-mer which contained G · C instead of G · T pairs, the T_m is elevated by about 20° C, although there is no change in the transition enthalpies. This indicates that entropic differences are responsible for the different stabilities of the two molecules.

CCAAGATTGG / GGTTAGAACC — In this molecule, Kan et al.[44] investigated the effect of G · a oppositions on the base pairing structure of a short DNA decanucleotide. In the lowfield

NMR spectrum (at −10 and −5°C), they observed an extra lowfield resonance, as compared with d(CCAAGCTTGG)$_2$ resonances, which they attribute to GA base pairs oriented in the *anti*-conformation. The *anti*-conformation is indicated by the observation of an NOE between the G imino proton and the A-H2 proton. This arrangement is somewhat surprising since the *anti* G · syn-A conformation would have been more easily accommodated without distortion of the double helix.

d(ATCCTATAGGAT)$_2$ and d(ATCCTATTAGGAT)$_2$ — Haasnoot et al.[45] have compared the lowfield NMR proton NMR spectra of a self-complementary 12-mer and the analogous 13-mer containing an additional T-T opposition. Incorporation of the T-T pair destabilizes the rest of the helix, but, interestingly, there is a resonance (intensity of two protons at ~10.2 ppm) which can be clearly attributed to the T-imino proton located about 1 ppm upfield from the position observed (11.2 ppm) from "free" T in the low temperature spectrum of d(TTTT). Since the bases in d(T$_4$) are not paired, the low temperature value of 11.2 ppm can be taken as the resonance position for a T-imino proton hydrogen bonded to water, but in slow exchange because of stacking interactions, steric effects, and low temperature. Thus, the observation of a T-imino resonance in a double helix does not necessarily mean that the T is hydrogen bonded with another base. The fact, however, that the T-imino resonance in the 13-mer is observed 1 ppm upfield from 11.2 indicates that the T bases are more or less stacked within the double helix. In d(T$_4$), the T-imino resonance is badly broadened around 0°C, whereas in the DNA helix the T-T resonance is still present at 34°C indicating that the exchange rate of the T-imino protons is greatly slowed by incorporation in the double helix. Surprisingly, they found that the resonances from the T-imino in the adjacent A · T base pairs "melt" between 10 to 15°C, or almost 15°C *below* the T-T pair, suggesting that differential stacking on the adjacent A residues may play an important role in controlling the exchange rates. If so, there should be differences between T-imino exchange rates in ApT, TpA, and other molecules.

The above NMR studies demonstrate that a variety of non-Watson-Crick oppositions can be accommodated within "regular" DNA helices, albeit with some destabilization of the helix. These include the G·T wobble base pair proposed by Crick, the purine-purine opposition G · A which forms a hydrogen-bonded base pair, and the T-T pyrimidine-pyrimidine opposition which may or may not actually form a hydrogen-bonded base pair. Since both bases in the G · A base pair have antinucleotide conformations, it would appear that virtually any opposition of bases can be accommodated in the DNA helix.

3. Effect of Extra Bases

d[ATCCTA(T)$_n$TAGGAT] — With this series of molecules Haasnoot et al.[46] examined the effects of loop formation. For n = 1, only intermolecular dimer formation was observed, but for n = 2, the spectral data obtained at different concentrations and temperatures were consistent with the presence of both dimers and hairpins. For n = 3, 4, or 5, it was concluded that only hairpins were present. Since loops containing only two bases are believed to be sterically impossible, the authors suggest that the adjacent A · T base pair in n = 2 hairpins are disrupted to form a loop of four bases.

d(CGCAGAATTCGCG)$_2$ — This 13-mer can form a duplex with 12 Watson-Crick base pairs, but it contains two extra A residues.[47] By comparing the spectral properties of this molecule with the corresponding 12-mer lacking the extra A, it was concluded that the extra A residues are stacked in the duplex, rather than forming a single base bulge. As expected, however, incorporation of the extra A destabilized the duplex to strand transition by about 20°C. The adjacent base pairs are not disrupted by A stacking, but they are destabilized with regard to proton exchange. Another interesting feature observed in the ^{31}P spectrum is the slightly downfield-shifted phosphorus resonance (at 3.48 ppm), which is attributed to the extended dC$_4$(3′-5′)dG$_3$ phosphodiester linkage on the partner strand *opposite* the mismatched dA residue.

C. Proton Studies of DNA Restriction Fragments

The first NMR studies on a DNA restriction fragment were reported in 1980,[48] and since then measurements on at least five other fragments have been reported. Early et al.[48-50] also reported the first extensive spin-lattice and spin-spin relaxation measurements on small DNA restriction fragments (vide infra). Extensive lowfield relaxation measurements at various temperatures were carried out on a 12-mer as well as two other restriction fragments of 43 and 69 base pair in length, and used to study the mechanism of proton exchange and length effects on flexibility in these molecules.

Nick et al.[51] prepared and studied the lowfield NMR spectrum of the *lac* operator DNA isolated from a plasmid by *Eco* RI restriction. One 40 base pair fragment they examined contained AATT sticky ends resulting from the restriction digestion, and this gave a lowfield spectrum that was relatively broad and devoid of structure (very similar to the random sequence DNA fragments studied earlier by Early and Kearns[13]). However, when the sticky ends were removed by S1 nuclease digestion, some structure appeared in the lowfield spectra, and in the 600 MHz spectrum they were able to count about 25 to 30 protons at 20°C (34 were expected, assuming that resonances from the two base pairs at either end are missing at this temperature).

Zuiderweg et al.[52] studied a 14 base pair synthetic fragment and its two component 7-base pair fragments which comprise part of the *lac* operator sequence and proposed assignments based on the temperature dependence of the spectra, chemical shift considerations, and comparison of the spectra of the three different fragments. Analogous to the earlier work of Early et al.,[49] they noted that the chemical shifts of the A · T resonances were more temperature sensitive than those from the G · C base pairs and suggest that this might be due to increased propellering of the A · T pairs at higher temperatures. A 51 base pair restriction fragment containing the *lac* operator has also been compared with the 14 base pair fragment and used to make partial assignments.[52]

D. Attempts to Deduce Structures from Proton Chemical Shifts in DNA

Proton chemical shifts are sensitive to polynucleotide conformations, and by monitoring these chemical shifts it is possible to follow various conformational transitions.[30,32] Recently there have been serious attempts to use ^{1}H chemical shift data to make more quantitative deductions about certain structural features of the DNA.[53-56]

It was recognized in earlier NMR studies of nucleic acids that vertical stacking of the bases causes upfield shifts of the proton resonances due to induced magnetic fields generated by "ring currents" in the neighboring bases. Geissner-Prettre and Pullman[57] developed a set of semiempirical equations to calculate these shifts, and these have been very valuable and widely used in the interpretation of proton NMR spectra.[53-57] However, because of the sensitivity of the calculated shifts to the detailed electronic structure of the molecule, the results can, at best, be considered to be semi-quantitative. Morever, Giessner-Prettre and Pullman[58] subsequently modified their initial calculations to include local atomic contributions to the shielding. These are even more difficult calculations since they include contributions from the diamagnetic anisotropy of all atoms in the framework as well as the effects of polarization or electric field effects. Because a multitude of contributions enter into the computation of the total magnetic shielding of each proton in a DNA helix, it is virtually impossible to critically test the importance of any specific contribution, let alone test the validity of the final results which require known structures. Trends may be qualitatively predicted, but certain parts of the calculation cannot be trusted quantitatively.

Despite the limitations and possible shortcomings of the semi-empirical shift theories, various authors have used them to account for observed magnetic shielding constants in terms of assumed solution-state structures. The most extensive testing of this approach has been carried out by Mitra et al.[53,59] who computed magnetic shielding constants for protons in a number of different DNAs using a range of assumed structures. Their

Table 1
A COMPARISON OF THEORETICALLY COMPUTED MAGNETIC SHIELDING CONSTANTS[53] AND THOSE EXPERIMENTALLY OBSERVED FOR POLY(dG-dC)·POLY(dG-dC) IN HIGH SALT[60]

	Shielding (ppm)				
Model	**CH_5**	**CH_6**	**$CH1'$**	**GH_8**	**$GH1'$**
B-DNA	0.85	0.73	0.58	−0.15	0.25
Z_1-DNA	0.95	0.51	0.91	0.18	0.17
Z_2-DNA	0.75	0.45	0.77	0.22	0.20
Experimental value[60]	1.05	0.62	0.69	0.30	0.15

analysis of the spectra of poly(dG-dC) is especially interesting since the crystal structure of $d(CG)_3$ has been determined at atomic resolution. On the basis of their results (see Table 1), it was concluded that the best fit of the data is provided by the Z1 structure described by Wang et al.[16] (average deviation between experimental and calculated of ±0.13 ppm). Although this might appear to be a rather good fit, it should be realized that the full range of shifts observed for many protons is often less than 1.0 ppm. The average deviation for the Z2 structure was slightly less than for Z1, but this structure was rejected because of 0.3-ppm disagreement in the values for the C-H5 resonance. We note, however, that the calculated upfield shift of this particular resonance is due largely to ring current shifts from neighboring G residues and if these are not correctly predicted by the semiempirical theory, this might account for the discrepancy. While there is convincing evidence that poly(dG-dC) in high salt solutions is in a left-handed helical conformation,[22,23,60] it is instructive to compare the observed shifts with those calculated using the *right*-handed *B*-DNA structure. For the most part, the agreement between calculated and observed values is remarkably good. In fact, the spectra of the high and low salt forms of $d(GC)_n$ are sufficiently similar that Patel et al.[60] initially interpreted their NMR data on the left-handed form in terms of an alternating right-handed B-form structure. There is a serious discrepancy (0.45 ppm) only for the G-H8 resonance where a large (0.38 ppm) contribution from the sugar phosphate group was added to account for the assumed change in the glycosidic bond angle, χ, on going from mononucleotide to the polynucleotide conformation. However, as pointed out by Cruz et al.,[61] Mitra et al.[53] based their calculations on a value of χ obtained from nuclear Overhauser effect (NOE) measurements in guanosine monophosphate (GMP), and this gives an unrealistic weighting to the range of possible conformational states because the largest NOEs arise from a limited set of conformations. Note, also, that for all of the standard structures (A, B, C, D, alternating B), the predicted shifts of G-H8 are in the *opposite* direction from that observed for B-form poly(dG-dC). This indicates that there may be something wrong in the procedures used to evaluate the effect of χ on the shift of base protons. While we may agree that, all factors considered, the best fit of the poly(dG-dC) data in high salt is obtained with Z1 or perhaps Z2 structure, it is difficult to have confidence in the results in view of the multitude of untested parameters needed to calculate all the various shift contributions. Conformational fluctuations could also affect the agreement between values calculated for static structures and those measured experimentally. One is, therefore, left with an uneasy feeling about the results of these calculations which will probably not be removed until the structure of some DNA molecule in solution is rigorously determined by some other approach (relaxation measurements?).

E. ^{31}P NMR Studies of DNA

^{31}P chemical shifts in DNA are very sensitive to the conformation and environment of the phosphodiester group[62-77] and this sensitivity has been widely used to investigate the properties of DNA molecules in solution, DNA drug complexes, metal binding, high molecular weight complexes such as chromatin and nucleosome core particles, and orientational effects on the ^{31}P spectra in solid DNA. In this section, we consider the various factors which influence ^{31}P chemical shifts and discuss their applications to studies of DNA structure.

F. Conformational Effects on ^{31}P Chemical Shifts

Gorenstein et al.[62,63] first noted there is a strong correlation between ^{31}P chemical shifts in a variety of phosphate compounds and their phosphate ester O—P—O bond angles, and, subsequently, related ^{31}P chemical shifts to the charge density on phosphorus.[64,65] From molecular orbital charge density calculations on simple phosphate compounds, they developed a conformation map relating chemical shift to the torsional angles ω and ω' (see Figure 2C[64,65]). The authors emphasized that limited knowledge of torsional angles and deficiencies in the charge density calculations prevented quantitative application of the theory, but suggested that relative shifts might reasonably be obtained from such maps. Prado et al.[66] have carried out more extensive *ab initio* calculations of the magnetic shielding tensor of the phosphate group.

To test their calculations, Gorenstein and Luxon[67] used their theory and tRNA crystallographic data to predict the ^{31}P NMR spectrum of $tRNA^{phe}_{yeast}$. Since the observed spectrum contains a collection of resonances spanning almost 7 ppm, whereas the predicted spread of resonances was only 2.6 ppm, they concluded that some factor(s) other than torsional angles was responsible, and suggested that changes in the ester RO—P—OR bond angle might also be involved. Hydrogen bonding is another contribution that may be especially important in the tRNA molecules, and Salemink et al.[68] have specifically suggested this as a possible contribution to the wide range of observed shifts.

Hydrogen bonding effects appear to have been discounted in most analyses of ^{31}P spectra of polynucleotides, but theory[66] and experiment indicate they are not negligible and this is directly demonstrated by the studies on solvent effects on the ^{31}P chemical shifts of cyclic nucleotides.[69] An example of this work is presented in Figure 3 where it is seen that addition of organic solvents to aqueous solutions of the cyclic mononucleotides can produce large (up to 3.0 ppm) shifts of the ^{31}P resonance.[69] Since cyclic nucleotides are relatively rigid, the most straightforward interpretation of these results is that there are substantial hydrogen bonding effects on ^{31}P chemical shifts, and that a reduction in the number of solvent molecular hydrogen bonded to the phosphate (through addition of organic solvents) causes large upfield shifts. We must, therefore, conclude that a quantitative understanding of the relation between ^{31}P chemical shifts to phosphodiester conformation is yet to be achieved. Despite these uncertainties ^{31}P NMR has been usefully applied in a number of interesting studies of DNA molecules.

G. ^{31}P NMR Evidence for Conformational Differences in DNA

In random sequence 150 bp DNA, the chemical shift of the ^{31}P NMR signal appears at about 1.85 ppm upfield from 85% H_3PO_4, and observed the line width is over 1 ppm (70 to 72). This large line width evidently arises from chemical shift dispersion caused by heterogeneity in the backbone conformation, base effects on the chemical shift, and/or hydrogen bonding effects.[70-72] Evidence for this heterogeneity is provided by synthetic polynucleotides and short oligonucleotides which exhibit resolved resonances split by as much as 0.8 ppm.[73-75]

In the ^{31}P spectrum of low molecular weight (~55 bp) poly(dA-dT) shown in Figure 1C

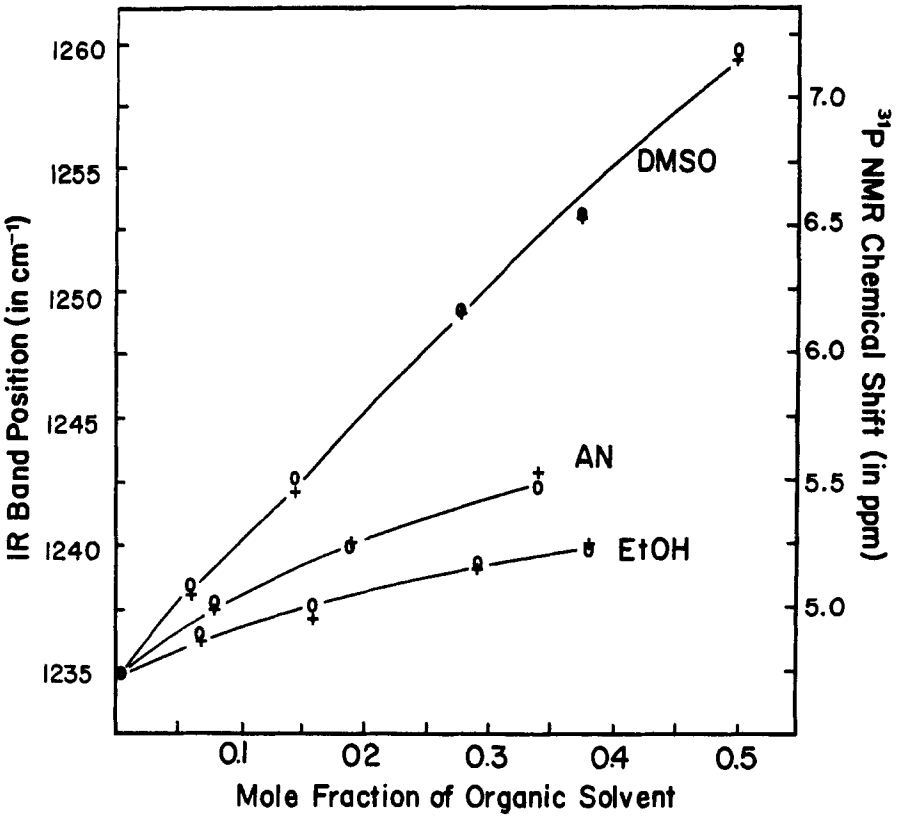

FIGURE 3. A comparison of solvent effects on the ^{31}P NMR chemical shift (+) and the position of the PO_2^- antisymmetric stretch (o) of 3′, 5′cyclic adenosine monophosphate in solvents consisting of various mole fractions of water and dimethyl sulfoxide (DMSO), acetonitrile (AN), and ethanol (EtOH). The measurements were carried out at 26°C (From Lerner, D. B., Bectel, W. J., Goodman, M., and Kearns, D. R., unpublished results. With permission.)

two peaks of nearly equal intensity split by ~0.20 ppm are observed,[75,76] and similar splittings are observed in poly(dA-dbr^{5}U) and poly(dI-dC).[77] While this splitting demonstrates that there are two environments for the phosphodiester groups in these molecules, it does not indicate how they differ. One possibility is that the two peaks arise from two conformationally distinct regions within the helix. This possibility is rejected because the lifetime of such states would have to be much greater than about 50 msec, and the equilibrium constant between the two states would have to remain constant (near unity) over a wide range of temperatures and ionic strengths.[77] The effects of ring current shifts on the two phosphate groups (ApT and TpA) were calculated to be negligible.[77] An alternative interpretation, and the one that is widely accepted, is that the two ApT and TpA phosphate groups exist in different conformational states.[76] In support of this, Shindo noted that one of the two ^{31}P signals in poly(dA-dT) is broadened more in the nonproton decoupled spectrum and suggested that this might indicate a different sugar conformation for each of the phosphate environments.[76] If local phosphodiester conformational effects are assumed to dominate the ^{31}P shifts, then the shift of one resonance to lower field can be simply interpreted as a shift from g,g toward g,t conformation.[77] In this regard, Cohen et al.[77] note that poly(dA-dT) is preferentially cut by DNAse I every other phosphodiester bond,[78] yielding products of the type (ApT) with a 3′—OH adenyl residue and that the crystalline structure of (dA-dTdA-dT) has an alternating sugar pucker and phosphodiester bridge conformation.[20] In the enzymatic

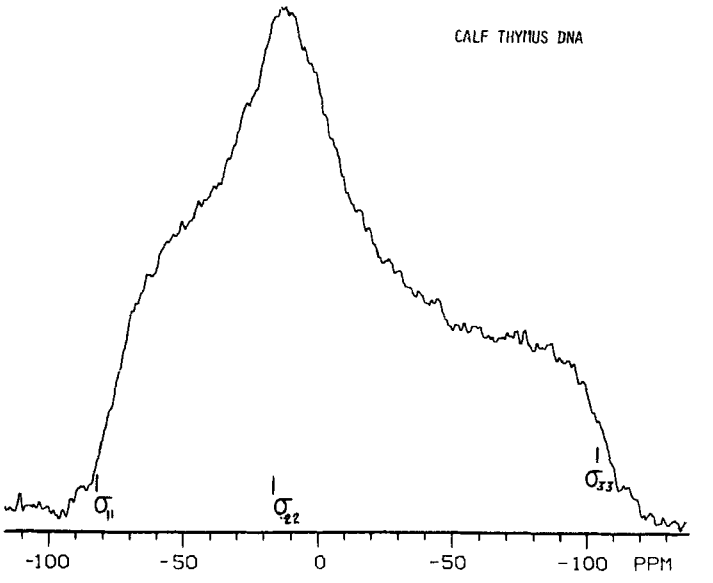

FIGURE 4. The proton decoupled ^{31}P NMR spectrum (80 MHz) of solid calf thymus DNA at ~10% relative humidity and 25°C. The principal elements of the chemical shielding tensor are $\sigma_{11} = -83$, $\sigma_{22} = -18$, and $\sigma_{33} = +104$ ppm (relative to 85% phosphoric acid). (From Cushley, R. and Kearns, D. R., unpublished results. With permission.)

digestion of poly(dG-dC) by DNAse I, the same preferential cleavage pattern is also noted,[78] yet no splitting of the ^{31}P peaks is observed.[77] If there is direct correlation between ^{31}P chemical shifts and backbone conformation, then one would predict that poly(dI-dC), which exhibits a 0.12-ppm splitting in ^{31}P, would be intermediate between poly(dA-dT) and poly(dG-dC) in its digestion behavior. With regard to the significance of the DNAse I digestion patterns, Dickerson and Drew[79] note that the selectivity in the digestion of the dodecamer d(CGCGAATTCGCG)$_2$ observed by Lomonossoff et al.[78] correlates with the twist angle of base pairs at the cleavage site.

In summary, there is convincing evidence that the phosphate groups in different DNAs do exist in different *environments,* but this could mean different conformational states and/or different states of hydration. Conformational effects will probably turn out to be the dominant factor, but changes in hydration may be responsible for some of the more subtle differences observed.

H. ^{31}P NMR Structural Studies of DNA Films and Fibers

Since X-ray diffraction studies on fibers and crystals of DNA have been our major source of structural information on DNA molecules, it is desirable to have spectroscopic techniques which can be used on both solids and solution-state samples. Fortunately, ^{31}P NMR can be used on solid-state samples of DNA without resorting to the more specialized solid-state techniques of solid-state NMR (magic angle spinning, cross polarization, side-band suppression). These studies rely on the fact that the ^{31}P chemical shift anisotropy is quite large (~190 ppm) and (with proton decoupling) dominates the appearance of the solid-state spectra.[80] In solution, the observed chemical shift is averaged over all possible orientations. However, in a solid, where the molecules are held in fixed positions, each phosphate group gives rise to a resonance with a unique position that is determined by the angle the applied magnetic field makes with the molecular axes of the phosphate group (see Figure 2). As an example of this effect, the spectrum of a solid sample of DNA is shown in Figure 4. The overall spectral width encompasses almost 200

ppm and the unusual shape of the spectrum results directly from the chemical shift anisotropy. A study of barium diethyl phosphate crystals has provided an approximate determination of the principal axes of the shift tensor relative to the symmetry axes of the phosphate group.[81] Therefore, if the alignment of a phosphate group relative to the magnetic field is known, the resonance position can be predicted. For example, when the magnetic field is aligned along the σ_{11} axis (see Figure 2) the ^{31}P resonance is observed at −83 ppm, whereas the most upfield resonance (+104 ppm) is observed when the field is aligned along the σ_{33} axis.[82,83] For other field alignments, the resonance position is intermediate between these two extremes. The key point is that if all the phosphate groups in a molecule were aligned identically with respect to the applied field, a single, relatively narrow line would be observed. In an oriented fiber of DNA, all of the phosphate groups the DNA backbone should be identically aligned with respect to a magnetic field applied along the helix axis. The first ^{31}P NMR experiments on oriented DNA fibers were reported by Shindo et al.[82] who observed partial narrowing of the spectrum (to 53 ppm) when the fiber axis was aligned along the magnetic field. The most successful studies to date were carried out by Nall et al.[83] who obtained highly oriented samples of calf thymus in the A-form (as shown by X-ray diffraction) which exhibited a single, narrow (21 ppm) resonance when properly aligned. In analyzing their spectra, Nall et al.[83] assumed no motional averaging and that the orientation of the principal elements of the chemical shielding tensor corresponded to the local (assumed) symmetry about the phosphodiester group (see Figure 2). With these major assumptions, they obtained an excellent fit of spectra observed using a wide range of orientations of the helix axis relative to the magnetic field. Several important conclusions follow from these observations. First, the fibers studied by Nall et al.[83] must have been highly ordered, otherwise it would have been impossible to find an orientation in which the ~200-ppm-wide spectrum collapsed to a single 21-ppm-wide resonance. The fact that Shindo et al.[82] obtained a broad (~50 ppm) resonance from A-DNA when the fiber axis was aligned along the magnetic field was probably due to incomplete ordering of the DNA molecules in the fiber. Second, the backbone must be approximately regular with respect to orientation of the phosphate group relative to the helix axis, although small chemical shift differences are allowed. Certain alternative models for the A-form, including the Rodley side-by-side model,[84] can be eliminated since they give rise to a quite different spectrum.

I. Evidence for Molecular Motion in the Solid State

^{31}P NMR can also be used to study molecular motion in solid complexes of DNA. In the hydrated B-form, randomly oriented solid DNA gives the characteristic asymmetric ^{31}P chemical shift pattern shown in Figure 4; however, rather surprisingly, DiVerdi and Opella[85] found that upon warming the sample above 20°C there is a narrowing of the spectrum and by 50°C it is reduced to a single, symmetric line (halfwidth of about 25 ppm at 60.9 MHz) located at the position of the isotropically averaged line. These observations clearly demonstrate that in the hydrated fibers of B-form DNA there is substantial motion of the phosphate groups in the backbone which occurs more rapidly than 10^4 Hz. in dehydrated DNA, they[85] found no evidence for motion at 25°C, and we have extended these measurements all the way up to 80°C without observing significant changes in the spectrum (Cushley and Kearns, unpublished results[85a]). It may, therefore, be concluded that hydration of the DNA is essential for backbone motion in fibers at elevated temperatures. We have also explored the behavior of solid samples of DNA prepared by condensation from solution containing spermine or spermidine and find that partially hydrated samples (75% relative humidity) also exhibit increased backbone mobility at elevated temperatures. The major difference with the spermidine-containing samples is that the temperature at which the ^{31}P resonance narrows is increased by about ~10°C. Evidently, the addition of polyamines does not seriously affect the high temperature

flexibility of the backbone of the DNA in the hydrated fibers. Upon dehydration (< 10% relative humidity) of the spermine complexes, a powder pattern spectrum is observed at all temperatures.

The increased flexibility in the DNA backbone at elevated temperatures indicated by the ^{31}P spectrum is, however, not manifested in either the proton spectrum or in the deuterium spectrum of DNA specifically deuterated in the purine-8 position. DiVerdi and Opella[85] observed a powder pattern in the deuterium spectrum over the entire ranges and Cushley and Kearns[85a] (unpublished results) find that the proton spectrum of hydrated DNA is similarly unchanged. The interesting conclusion to be drawn from these studies is that substantial internal motion in the phosphate backbone occurs *without* corresponding motion in other parts of the molecule.

J. ^{31}P NMR Studies of Metal Ion Binding to DNA

There is an extensive literature on the role metal ions play in biological processes involving nucleic acids, but in most cases the specific functions of the metals are incompletely understood.[86-90] Certainly a major role of divalent metal ions is to stabilize the secondary structure of DNA and RNA, but the literature is conflicting on the relative importance of different modes of binding to the polynucleotides which include direct coordination of the metal to the phosphate groups, outer-sphere complexation with the phosphate via a water-bridge, interaction with electron-donor sites on the heterocyclic bases, and diffuse atmospheric binding.[91-94] ^{31}P NMR studies are beginning to resolve some of these uncertainties.

Wilson et al.[95] observed that the binding of certain divalent metal ions causes small, but measurable shifts of the ^{31}P resonance of DNA. Both Ca^{2+} and Mg^{2+} produce 0.3-ppm upfield shifts of the ^{31}P resonances, whereas Sr^{2+} causes less than a 0.1-ppm shift, and Ba^{2+} is without effect. The authors interpreted these differences in terms of differences in the site binding of the metals to the phosphate sites on the DNA; however, differences in hydration of the phosphate group induced by the metals could account for the small changes that are observed. Since Mn^{2+} and Ba^{2+} have virtually identical binding constants, even though Ba^{2+} is without effect on the phosphate resonance, these NMR studies suggest that site binding to the phosphate groups (i.e., inner-sphere binding of the metal directly to the phosphate oxygens) makes little contribution to the total DNA binding strength of these metals.

The above studies demonstrate that ^{31}P spectral measurements can be very useful in monitoring various aspects of DNA structure, internal motions, and its interaction with other ions and molecules. While there are still important limitations in using chemical shifts to deduce structure, further theoretical studies in conjunction with new structural data may overcome these limitations. A discussion of ^{31}P relaxation measurements on DNA is deferred until after consideration of the basic relaxation equations.

K. ^{13}C NMR Studies

Because of low natural abundance (1.1%) and intrinsically low sensitivity there have been few ^{13}C NMR studies of DNA.[96-102] The sensitivity limitations can, to some extent, be overcome by use of large samples (hundreds of milligrams) and extensive signal averaging. The first ^{13}C spectra of double helical samples of DNA were reported by Rill et al.[96] who compared spectra of native and denatured DNA at 37.7 MHz and at 67.9 MHz, and an example of a ^{13}C NMR spectrum of random sequence DNA is shown in Figure 1B. In subsequent studies, Hogan and Jardetzky[97] reported a series of ^{13}C NMR measurements at 25.1 MHz in which they measured T_1, line width, and NOE on overlapping resonances from various carbon atoms in the DNA, and Bolton and James[98] have reported the results of preliminary studies on DNA at 50.3 MHz.

There have been several recent studies on alternating polymers. Shindo[101] measured the

natural abundance ^{13}C NMR spectra of 145 bp poly(dA-dT) and found definitive evidence that the sugar rings exist in two different conformational states. At least three of the deoxyribose ring carbon atoms, C2′, C3′, and C1′ or C4′, are split by about 1.8 ± 0.2 ppm in the double helical state and this is attributed to two distinct conformations of the sugar rings. These observations add to the evidence from both ^{1}H and ^{31}P NMR that the phosphodiester backbone of poly(dA-dT) has an alternating conformation. The intriguing question as to precisely what these differences are remains unanswered, but Chen et al.,[102] who also measured the ^{13}C NMR spectrum of poly(dA-dT), have proposed that the more downfield shifted resonances are due to thymine sugar in the C2′-endo conformation, and the less downfield shifted resonances to adenine sugars in the 3′-endo conformation. The ^{13}C line widths and the T_1 values for the 145-bp fragment of poly(dA-dT) are smaller than the values that would be predicted using a rigid rod model with a length corresponding to 145 bp. For the base carbon atoms, the line widths (between 140 and 177 Hz) are consistent with a rigid rod model of DNA with effective length only 95 bp, whereas for the sugar carbons, an effective length of only 30 to 45 bp is indicated. The fact that the thymine C6 resonance is narrower (140 Hz) than the resonance for adenine C8 (177 Hz) was interpreted to indicate that the thymine residues are more mobile than the adenine. Chen et al.[102] also measured the ^{13}C spectra of poly(dG-m^{5}dC) in low salt (presumably in the B-form) and in the high salt (presumably Z-form). Only a single resonance was observed from the C2′ of poly(dG-m^{5}dC) in low salt, thus, giving no evidence for an alternation in the conformation of the sugar residues. However, in the ^{31}P NMR spectrum of this molecule, a splitting was observed, indicating some alternation in structure. In the high salt form (presumably Z-form), the resonance from C2′ appeared to be a doublet while the C3′ resonance remained broad and unresolved, again indicating an alternating structure. From these data, it was concluded that the sugars of poly(dG-m^{5}dC) exists in only one conformation in the right-handed B-form, but in two distinct conformations in the left-handed Z-form, presumably the 2′-endo and 3′-endo ribose conformation.

L. ^{15}NMR

The first ^{15}N NMR studies of DNA appeared only recently.[103-105] Spectra of DNA enriched in ^{15}N were obtained and $^{15}\{^{1}H\}$ NOEs were observed for resonances from protonated ^{15}N atoms.[103] In order to account for the negative NOEs that were observed in certain peaks, a correlation time for internal motions of less than 5 ns was required, whereas positive NOEs were attributed to protonated nitrogens with internal motion correlation times longer than 5 ns.

Cross et al.[104] and Di Verdi and Opella[105] used solid-state techniques to obtain the ^{15}N spectra of uniformly labeled DNA. From these measurements they were able to determine the size of the ^{15}N-^{1}H dipolar coupling and, hence, imino N—H bond distance.[105] Within experimental error, the hydrated and low humidity forms of DNA have a G and T N—H bond length of 1.13 Å. This is to be compared with a value of ~1.1 Å deduced earlier[49] from relaxation rate measurements on the imino protons in DNA restriction fragments, and the recent studies[106] on poly(dA-dT) which yield a value of 1.15 ± 0.05 Å (vide infra).

III. APPLICATION OF RELAXATION TECHNIQUES TO THE INVESTIGATION OF DNA STRUCTURES AND DYNAMIC PROPERTIES

A. General Considerations

The application of one- and two-dimensional NMR relaxation methods to studies of DNA is of recent origin, and the number of studies completed relatively few. However, it

is likely that relaxation methods will dominate much of the future research in this area because they provide one of the few methods for quantitatively determining internuclear distances in molecules in solution. Moreover, relaxation measurements can be used to obtain information about the overall tumbling and specific internal motions in the molecule. The two-dimensional relaxation techniques can provide a complete map of all short (between 2.0 and 3.5 Å) interproton distances in the molecule in a single experiment, and this information is often sufficient to set important restrictions on the possible conformational states of DNA molecules.

When the magnetization of a collection of nuclei is disturbed by application of a radio frequency (RF) pulse, the system returns to equilibrium by a number of different processes including redistribution of magnetization among the spins within the molecule, transfer of magnetization between DNA nuclei and solvent nuclei, and exchange of energy in the spin system with rotational and translational energy.[107] For the imino and amino protons, chemical exchange with solvent molecule protons can be a major pathway for relaxation[106,108,109] and for ^{31}P and ^{13}C, fluctuations in the local magnetic susceptibility can also contribute to the relaxation.[110,111] Two different types of relaxation measurements are typically carried out. In the first type (spin-lattice relaxation), the recovery of magnetization along the static magnetic field (the longitudinal or z-component) is measured, and the corresponding rate constant, R_1, is referred to as the spin-lattice relaxation rate $[R_1 = (T_1)]^1$. In the second class of experiments, transient magnetization in a plane perpendicular to the applied static field is created by application of a perturbing RF pulse and the decay of this in-plane (transverse) magnetization, characterized by a relaxation time T_2 and corresponding rate constant $R_2 = (T_2)^{-1}$, is referred to as spin-spin relaxation. We now summarize the basic relaxation equations and discuss some of the factors which are needed to understand the application of one- and two-dimensional relaxation techniques to studies of DNA. Relaxation caused by magnetic dipolar interactions will be considered first and at the end contributions from chemical shift anisotropy (CSA) will be discussed.

B. Theoretical Consideration

1. Spin-Lattice Relaxation in a Two-Spin System

The relaxation of a two-spin system containing a proton, H, and some other spin ½ nucleus, I, will be treated first and then the results will be extended to a multispin system.

a. Selective Inversion

The rate at which the longitudinal magnetization recovers depends upon the precise way in which the spin system has been perturbed.[107,112] In the special case that the magnetization of only one of the two spins is selectively perturbed the initial rate of recovery is given by the following expression[107]

$$R_1^{H-1}(s) = \hbar^2 \frac{\gamma_H^2 \gamma_I^2}{2r^6} \{J_0(\omega_H - \omega_I) + 3J_1(\omega_H) + 6J_2(\omega_H + \omega_I)\} = \rho \qquad (2)$$

where γ_H and γ_I are the gyromagnetic ratios for the H and I spins, r is the H-I internuclear separation and ω_I is the Larmor precession frequency of I. This expression applies to the mutual relaxation of protons ($\omega_I = \omega_H$), the relaxation of ^{13}C by protons ($\omega_I = \omega_C$), and proton-induced relaxation of ^{31}P ($\omega_I = \omega_P$). From the standpoint of structural studies, it is important to note that the relaxation rate varies with the inverse sixth power of the internuclear separation. Information about the molecular dynamics is contained in the spectral density terms, $J_n(\omega)$, which occur in all the relaxation rate expressions. We, therefore, digress at this point to discuss these $J_n(\omega)$ factors in more detail.

Relaxation is usually induced by high frequency electromagnetic fields generated by through-space magnetic dipolar interactions between the nuclei which are modulated by random overall molecular motions and by internal motions.[107] The power spectrum of the electromagnetic radiation generated by modulation of these magnetic interactions is described by the spectral density, $J(\omega)$, and for an isotropic rigid rotor, characterized by a single rotational correlation time, τ_c, $J(\omega)$ has the simple form

$$J(\omega) = \left(\frac{1}{5}\right)\frac{\tau_c}{1+\omega^2\tau_c^2} \tag{3}$$

For rigid cylindrical molecules, two different rotational correlation times, τ_ℓ and τ_s, are required to characterize rotational diffusion about the long and short axes of the molecule, respectively, and if there is internal motion (usually characterized by τ_{int}) a third time has to be included. In this case the special density can often be expressed as sums of terms analogous to Equation 3:

$$J(\omega) = \sum_i A_i \frac{\tau_i}{1+\omega^2\tau_i^2} \tag{4}$$

where A_i = constant and $\tau_i = f(\tau_\ell, \tau_s, \tau_{int})$.[113] One should note that different conventions for normalizing $J(\omega)$ are used by other authors, and this leads to different numerical factors in the various rate constant expressions. The equations in this review are based on the normalization implied by Equation 3.

b. Nonselective Relaxation Measurements on Protons

In a two-proton system ($\gamma_H = \gamma_I$) where *both* spins are initially inverted, the recovery of the magnetization is characterized by the following rate constant:[107,112]

$$R_1^{H\cdots H}(ns) = \frac{^3\gamma_H^{4\hbar^2}}{2r^6}\{J_1(\omega_H) + 4J_2(2\omega_H)\} = (\rho + \sigma) \tag{5}$$

where ρ is defined in Equation 2 and

$$\sigma = \frac{\gamma_H^{4\hbar^2}}{2r^6}\{-J_0(O) + 6F_2(2\omega_H)\} \tag{6}$$

and r in this case is the interproton separation. Note that the expression for the nonselective rate constant lacks a $J_0(\omega_H - \omega_H) = J_0(O)$ term that appeared in the expression for $R_1(s)$. The absence of this term arises from the fact that if both spins are inverted they cannot mutually relax each other by exchanging magnetization. For an isotropic rotor, we see from Equation 3 that $J_0(O) = \tau_c/5$ and if $\omega\tau_c << 1$, then $J_0 > J_1 > J_2$. In this case the *selective* relaxation rates, $R_1(s)$, are predicted to be much larger than the corresponding nonselective rates, $R_1(ns)$.

2. Spin-Lattice Relaxation in a Multiproton System

In the above analysis we only considered two interacting spins. When many protons interact directly or indirectly with one another, the decay kinetics are more complicated and the following set of coupled differential equations apply:[112,114]

$$\frac{dI_{z_i}}{dt} = -\sum_j \rho_{ij}(I_{z_i} - I_{o_i}) - \sum_j \sigma_{ij}(I_{z_j} - I_{o_j}) \tag{7}$$

where I_{zi} = magnetization of spin i, I_{oi} = equilibrium value of the magnetization of spin i, and ρ_{ij} and σ_{ij} are as defined in Equations 2 and 6, with $r = r_{ij}$ and $\gamma_H = \gamma_I$. From analysis of the two-spin systems it is clear that experimental relaxation rates depend upon the way in which the spin system is initially prepared. By choosing appropriate experimental conditions the experimental relaxation rates can be directly related to simple theoretical expressions containing structural and dynamic quantities of interest. If all N spins in the system are initially inverted (i.e., $I_{zi} = I_{zj} = -I_{oi}$) by application of a nonselective 180° RF pulse, then for the case $J_0(O) >> J_i(\omega), J_2(\omega)$ the nonselective relaxation rates for the different spins will become nearly equal and be given by the expression

$$R_1^{H-H}(ns) = \frac{3}{2}\frac{\gamma_H^4 \hbar^2}{N}\sum_{i,j}\left(\frac{1}{r_{ij}}\right)^6 \{J_1(\omega_H + 4J_2(2\omega_H)\} \tag{8}$$

Because the nonselective relaxation rate is an average of many rates it is virtually impossible to isolate the individual interactions which may be responsible. It may be possible to obtain some information about the frequencies of molecular motion that are present, but it will, in general, be difficult to assign these to specific internal motions of the molecule. Nevertheless, if the structure of the molecule is known (or can be assumed) various models for internal motion can be examined and possibly some eliminated.

If a selective 180° RF pulse is applied only to spin-i ($I_{zi} = -I_{oi}$ and $I_{zj} = I_{oj}$, initially) the *initial* relaxation rate is given by:

$$R_1^0(s) = \sum_{j \neq i} \rho_{ij} \tag{9}$$

Finally, if two spins, *i* and *j*, are selectively inverted ($I_i = -I_{oi}$, $I_j = -I_{oj}$, $I_{zk} = I_{ok}$, initially) the semiselective rates R_1^0 (ss:ij) and R_1^0 (ss:ji) for spins i and j, respectively, are

$$R_1^0(ss{:}ij) = \sum_{j \neq i} \rho_{ij} + \sigma_{ij} \tag{10}$$

$$R_1^0(ss{:}ji) = \sum_{i \neq j} \rho_{ij} + \sigma_{ij} \tag{11}$$

In the above analysis we considered only three special cases out of many possible initial conditions which could be generated. Even in the simple case where, initially, the magnetization of only one type of proton is perturbed, spin diffusion, via zero quantum processes, spreads the magnetization throughout the spin system and eventually leads to a more or less uniform distribution of spin polarization throughtout the system. At this point the decay would be more appropriately described by the expressions for a nonselective polarization. These considerations indicate that nonexponential decay behavior is to be expected following selective, or even bi-selective, excitation of the spins.

3. Nuclear Overhauser Effect (NOE) Measurements

From the above discussion of spin-lattice relaxation mechanisms it is evident that perturbation of the magnetization of one set of nuclei will disturb the equilibrium magnetization of other nuclei with which they interact. Changes (increase or decrease) in the intensity of one resonance resulting from the irradiation of a second resonance are referred to as the nuclear Overhauser effect (NOE).[112]

a. Heteronuclear NOE

In DNA, continuous irradiation of proton resonances usually results in an increase in the intensity of ^{31}P and ^{13}C resonances from those nuclei which interact with the protons.

The fractional change in the intensity of nonirradiated nuclei (I) is given by[112]

$$\eta = \frac{\gamma_H}{\gamma_S}\left\{\frac{6J_2(\omega_I + \omega_H) - J_0(\omega_H - \omega_I)}{J_0(\omega_H - \omega_I) + 3J_1(\omega_I) + 6J_2(\omega_H + \omega_I)}\right\} \quad (12)$$

Since the enhancement factor, η, depends upon the difference between J_2 and J_0, it can be either positive or negative. Note that since $J_0(\omega_H - \omega_S)$ depends upon motions which generate power at the frequency $(\omega_H - \omega_S)$, for ^{13}C and ^{31}P the quantity $(6J_2 - J_0)$ is usually positive since for $\omega_I << \omega_H$, $(\omega_H + \omega_I) \simeq (\omega_H - \omega_I) \sim \omega_H$ and $6J_2(\omega_H) > J_0(\omega_H)$.

b. Homonuclear NOE

With the proton NMR of DNA there are complications due to rapid transfer of magnetization between spins (spin diffusion).[48,106] In this case continuous irradiation of one resonance will often lead to saturation of all, or most, of the other resonances in the proton spectrum. To overcome this limitation various transient NOE experiments have been devised.[115,116] In one type of experiment a single resonance is selectively inverted by application of a 180° RF pulse, and at some subsequent time a nonselective 90° RF pulse is used to measure the entire spectrum and determine the effects of initial selective pulse.[115] A second type of experiment involves the selective irradiation of a single resonance for some relatively short period of time (usually comparable to the selective relaxation time) followed, either immediately or after a suitable delay, by observation of the entire spectrum.[116] The kinetics of the NOE experiments are, therefore, useful in identifying pairs of protons in the molecule that are physically close together since the resonances first affected are from protons physically close to the irradiated proton. This follows from Equations 2 and 9 which indicate that the relaxation of a proton which has been selectively irradiated is very sensitive to the interproton separation from neighbor protons.

For slowly tumbling molecules $J_0(O) > J_1(\omega_H) > J_2(2\omega_H)$ and relaxation initially occurs via zero-quantum transitions and, hence, a *reduction* in the intensity of other proton resonances in the molecule (negative NOE) is observed because $(-J_0(O) + 6J_2(2\omega))$ is negative. This is to be contrasted with heteronuclear NOEs which tend to be positive because $J_0(\omega_H - \omega_I) \simeq J_2(\omega_H + \omega_I)$.

By carrying out a series of experiments in which each individual resonance in the molecule is selectively irradiated and the effect on the rest of the spectrum measured, it would be possible to identify all short interproton distances in a molecule, and when there are a limited number of resonances to be considered these experiments are quite feasible. However, when the number of resonances is large this type of experiment is quite time consuming. During the past several years a number of elegant two-dimensional NMR techniques for obtaining the same information in a single experiment have been developed and their application to DNA is discussed in a later section.

4. Spin-Spin Relaxation in Spin ½ Systems

By application of a 90° RF pulse, magnetization initially along the Z-axis can be rotated into the x-y plane (ie., perpendicular to the static applied field). The decay of this in-plane (transverse) magnetization is referred to as spin-spin relaxation and for a two-spin system consisting of a proton H and a second spin I it is given by the following expression:[107]

$$R_2 = \frac{\hbar^2\gamma_H^2\gamma_I^2}{4r_{HS}^6}\{4J_0(O) + J_0(\omega_H - \omega_I) + 9J_1(\omega_H) + 6J_2(\omega_H + \omega_I)\} \quad (13)$$

Various spectral densities appear in this expression, but for a slowly tumbling molecule where $J_0(O) > J_1(\omega) > J_2(\omega)$, we obtain the simplified expression ($I \neq H$)

$$R_2 = \frac{\hbar^2 \gamma_I^2 \gamma_H^2}{r_{HI}^6} J_0(O) \qquad (14)$$

for the contribution of spin I to the relaxation of the proton H. If several nuclei interact with spin H, the above expression should be summed over all contributing nuclei. For slowly tumbling molecules in the limit $J_0(O) > J_1(\omega) > J_2(\omega)$ we predict $\frac{R_2}{R_1\,(s)} = 2.5$ for relaxation involving proton-proton interactions only. However, in the fast motional limit where $\omega\tau << 1$, $(J_1 = J_2 = J_0)$ the more familiar small molecule result $\frac{R_2}{R_1\,(s)} = 1.0$ is obtained.[107] We note that nitrogen-proton dipolar interactions can also make large contributions to R_2, if the N-H distance is short enough (~1.1 Å). For example, the relaxation caused by a nitrogen atom (^{14}N) separated by 1.0 Å from a proton is comparable to that caused by the interaction of two protons separated by 2.4 Å because the shorter N-H distance compensates for the smaller magnetic moment of ^{14}N.

The Equations 2 and 13 which describe the spin-lattice and spin-spin relaxation of a proton by some other nucleus, I, apply equally to the relaxation of I nuclei by a proton. The only difference is that the relevant frequencies, ω_I, in the expressions for $J_n(\omega)$ have to be changed.

5. CSA Contributions to the Relaxation of ^{13}C and ^{31}P

In addition to dipolar relaxation, both ^{13}C and ^{31}P can be relaxed by a contribution from the chemical shift anisotropy (CSA). In this case the appropriate expressions are[107,110,111]

$$R_1^{CSA} = \frac{4}{3} \gamma_S^2 H_0^2 \Delta\sigma^2 J_1(\omega_S) \qquad (15)$$

$$R_2^{CSA} = \frac{1}{4} \gamma_S^2 H^2 \Delta\sigma^2 \{4J_0(O) + 3J_1(\omega_S)\} \qquad (16)$$

where

$$\Delta\sigma = \sigma_{33} - \frac{1}{3}(\sigma_{11} + \sigma_{22}\sigma_{33}) \qquad (17)$$

For phosphorus,[80] $\Delta\sigma \simeq 100$ ppm and the approximate orientations of Δ_{ii} are shown in Figure 2C. Note that the CSA contribution to the relaxation rates contains a factor which depends upon the square of the applied magnetic field, H_0, and, hence, is expected to be more important at higher magnetic fields. This field dependence can also be used to distinguish CSA relaxation from other contributions.[110]

The above analysis indicates how relaxation rates, obtained by different preparations of the spin system, are related to internuclear distances, r_{ij}, and to factors which depend upon the overall molecular motion and internal molecular motions. When the relevant internuclear distances are known, the relaxation data can be used to obtain information about molecular motions. Conversely, when the time constants for molecular motion are known (e.g., overall tumbling times) relaxation rates can be used to extract structural data. When both the internuclear distances and the molecular motions are unknown, the relaxation data can be used in conjunction with various models to set limits on the range

of structural parameters and motional parameters that might apply. To resolve ambiguities that often arise in such analysis, measurements must be done over a range of magnetic fields, and on different nuclei.

With this brief summary of the basic factors controlling relaxation rates we now discuss the various relaxation experiments which have been carried out on DNA.

IV. APPLICATION OF RELAXATION MEASUREMENTS TO DNA

A. Relaxation of the Imino Protons

The hydrogen-bonded imino protons of guanine and thymine (see Figure 2A) give rise to resonances in the lowfield (10 to 15 ppm) region of the proton spectrum (Figure 1B). Their relaxation behavior is especially interesting because they are relaxed both by magnetic dipolar interactions involving neighboring protons and nitrogen atoms, and by exchange with the solvent molecule protons. Consequently, relaxation experiments can provide information about base pairing structure, N—H bond distances, and breathing of base pairs in the DNA.

1. Magnetic Dipolar Contributions to Relaxation

At low temperatures where exchange with solvent molecule protons is slow, the relaxation of imino protons in Watson-Crick G·C and A·T base pairs is caused by through-space magnetic dipolar interactions, and analysis of this relaxation behavior can provide structural and dynamic information. The first relaxation measurements on lowfield imino resonances of DNA in water were reported in 1980[108] and, subsequently, a variety of DNA systems have been studied, including restriction fragments,[48-50] synthetic polynucleotides,[106] and short synthetic oligonucleotides.[117,118]

a. Poly(dA-dT)

To illustrate the way in which relaxation measurements can be used to probe various molecular properties, first consider the relaxation behavior of poly(dA-dT) (see Figure 5), since it is the most thoroughly studied system.[106,119,120] In the experiments shown in Figure 5A, a "selective" 180° pulse was applied at 13.2 ppm, to invert the lowfield resonance from the T imino proton, and in Figure 6 the data for the receovery of the longitudinal magnetization in a 1°C experiment are plotted in a semilog plot. The highly nonexponential decay, first observed in a DNA with poly(dA-dT),[119] is due to the effects of spin diffusion. The value of the initial rate, 23 s^{-1}, is primarily sensitive to zero-quantum transitions (see Equation 9) which depends upon the end-over-end molecular tumbling time. After the initial rapid decay, there is equilibration of the spin polarization among the four strongly interacting protons located within each A·T base pair (the imino proton, the A-H2, and the two amino protons) and the slow component of the decay is best described by Equation 8 which is more appropriate for a uniform distribution of spin polarization. In this case, the relaxation is mainly due to one- and two-quantum transitions which are induced by high frequency internal motions, although some zero-quantum transitions involving interactions with distant sugar protons also contribute. In contrast with the nonexponential recovery of the longitudinal magnetization, the decay of the transverse magnetization at this temperature is exponential and characterized by a rate constant of 125 s^{-1} (see Figure 6). If, as a first approximation, a rigid rod model of the DNA is used to describe the DNA motions, then the initial values of the longitudinal relaxation rate and the transverse relaxation rate are both predicted to be too large by about a factor of 2, and the predicted value of the slow component of the longitudinal relaxation rate is about a factor of 50 too small.[119,120] However, we could account for all of the relaxation data, using the interproton distances shown in Figure 2 if we assumed

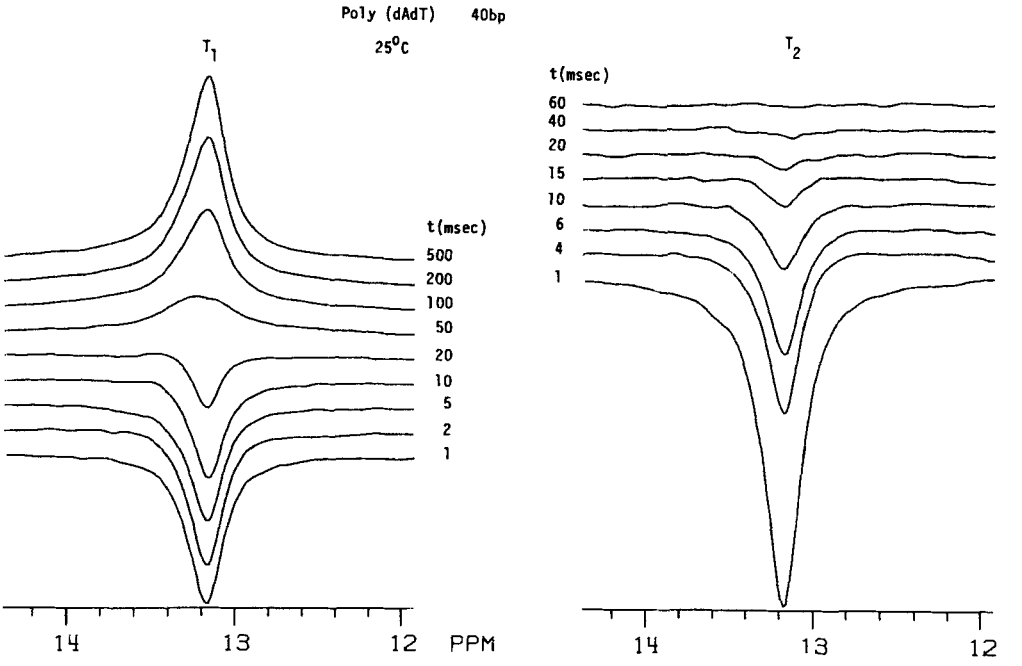

FIGURE 5. Examples of a spin-lattice (T_1) and a spin-spin (T_2) relaxation measurement on the lowfield thymine imino resonance in poly(dA-dT) sample containing 53 ± 5 bp. The sample was at 25° C and dissolved in 0.1 *M* NaCl containing 10 m*M* Cacodylate buffer at pH 7.[119]

that the planes of the base pairs undergo substantial (~±20° relative to the helix axis) high frequency (0.3 to 1.0 ns) motions[119,120] (see Table 2). In-plane torsional motions are relatively ineffective because motion about the helix axis is already fairly rapid. We also find that an imino N—H distance of 1.15 ± 0.05 Å is required to fit the observed ratio R_2/R_1 (s).[120] (Note that by comparing the ratio $\frac{R_2}{R_1\,(s)}$ we eliminate uncertainties in the spectral density factors [assuming $J_0 > J_1, J_2$].) While there is some latitude in choosing the other interproton distances, the N—H distance is very much restricted to the range between 1.1 and 1.2 Å. These relaxation results indicate, therefore, that there is considerable internal motion in poly(dA-dT) which involves out-of-plane motions of the bases with a correlation time of ~0.5 ns.

b. Poly(dG-dC)

It is interesting to compare the relaxation properties of poly(dA-dT) with those of poly(dG-dC), and these results given in Table 2 illustrate several points.[121] First, the values of R_1s and R_2 for a sample with an average size of ~60 bp cannot be accounted for by a rigid rod model for the DNA motions, analogous to the poly(dA-dT) results which required some degree of internal motion. Second, there is a very substantial deuterium isotope effect on the relaxation rates, as required by the assumed mechanisms of relaxation.[131] Quantitative comparison is difficult because 50% deuteration produces many different partially deuterated species in solution, each with different relaxation behavior. Third, in order to account for the $\frac{R_1}{R_2}$s ratio using the interproton distances given in Figure 2 it is again necessary to assume that the imino N—H distance in a G·C base pair is 1.15 Å and that the planes of the base pairs are oriented approximately perpendicular to the helix axis.

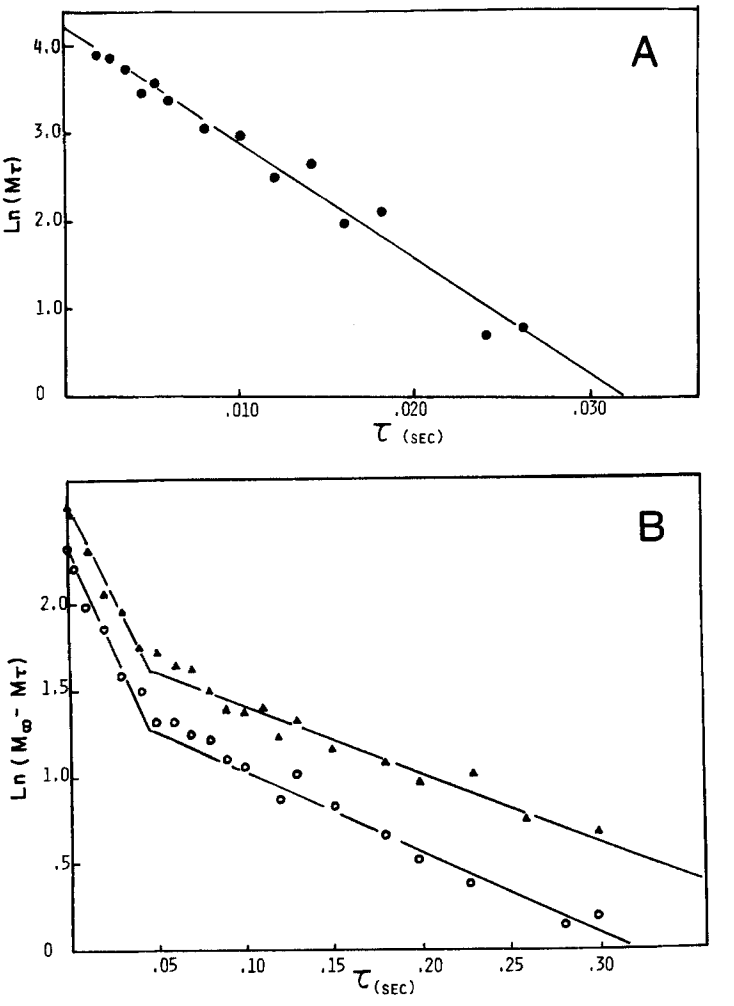

FIGURE 6. Semilog plots of the recovery of the transverse (top) and longitudinal magnetization (bottom) of the lowfield resonance of poly(dA-dT) in H_2O at 1°C. The experiments were carried out at 300 MHz.[119]

c. Restriction Fragments

In earlier studies of DNA restriction fragments, Early et al.[50] found that the relaxation behavior of 43- and 69-bp DNA could not be accounted for by a rigid rod model and that some type of internal motion or flexibility had to be introduced. They interpreted their results in terms of a reorientation of the base pairs arising from worm-like motions of the DNA, although their data could equally well be interpreted in terms of more localized motions of the planes of the bases. In view of the above observations, it would be interesting to have data on other synthetic DNAs and natural DNA with varying AT/GC content to see if there are differences in stiffness which correlate with GC content.

2. NOE Measurements

Nuclear Overhauser effects (NOEs) are another manifestation of magnetic dipole-induced relaxation processes and they have been widely used in studies of small molecules[112] and tRNA molecules.[122,123] Very recently NOE measurements have been most effectively used in DNA studies to determine assignments of resonances in the

Table 2
IMINO PROTON SPIN-SPIN (R_2) AND SPIN-LATTICE (R_1) RELAXATION RATES OBTAINED ON POLY(dA-dT) (53 ± 5 bp) AT 1°C[106,119] AND ON ~ 60 bp POLY(dG-dC) AT 21°C[121,133] (BOTH SAMPLES WERE IN WATER CONTAINING 0.1 *M* NaCl AT pH 7)

		R_1, s^{-1}		$R_{2,I}$, s^{-1}	
Sample	**Measurement**	**obs**	**calc**	**obs**	**calc**
Poly(dA-dT)	Semiselective	18		125	125[a]
(~ 53 bp)	Selective	23	23[a]		
	Nonselective	~1	1[a]		
Poly(dG-dC)	Semiselective	15		113	120[b]
(~60 bp)	Selective	20.3	22.7[b]		
(50% D_2O)	Semiselective	10		76	

[a] Calculated using ±20° fluctuation of base pair planes relative to helix axis with a correlation time of ~0.5 ns, and r_{NH} = 1.15 Å.
[b] Calculated using a rigid rod model with r_{NH} = 1.15 Å, an "effective" length of 45 bp, and interproton distances shown in Figure 2A.

lowfield spectrum and in the aromatic region. In a particularly interesting example of this method, Patel and Pardi[118] found that by irradiating one of the two lowfield resonances arising from imino guanine or thymine protons in the GT#3 pair of $\begin{matrix}\text{CGTGAATTCCCG}\\ \text{GCGCTTAAGTGC}\end{matrix}$ they observed a decrease in the intensity from the other imino resonance in the GT#3 pair. Moreover, they also observed small decreases in the intensity from imino protons of the adjacent GC#2 and the GC#4 base pairs. These observations are significant for several reasons. First, the large NOE between the G and T imino protons proves they are hydrogen bonded in a wobble base pair. Second, the observation of small NOEs on two other lowfield resonances confirms that these arise from base pairs which are adjacent to the GT pairs, and this is extremely useful information in assigning the spectra of these molecules. If appropriate kinetic experiments were carried out, it would be possible to use the transient NOEs to actually determine the distance between the imino protons in adjacent base pairs in DNA and in this way obtain crucial structural data.

3. Exchange Contributions to Relaxation

a. Mechanisms of Proton Exchange in DNA

At elevated temperatures, transient opening of base pairs in the double helix becomes sufficiently rapid that proton exchange with solvent molecules is the dominant mechanism by which the imino protons are relaxed.[31,109] Under these conditions, relaxation measurements provide an excellent method for studying proton exchange mechanisms in DNA since the opening rates of individual base pairs can be monitored. With other techniques (tritium exchange,[124] stopped flow, optical,[125,126] and infrared[127] spectra) only the collective behavior of exchangeable protons in all base pairs is measured.

The kinetic scheme used to interpret exchange contributions to the relaxation is as follows:[109,128]

$$AH^* \cdot B \underset{k_c}{\overset{k_0}{\rightleftharpoons}} AH^* + B \xrightarrow[k_{ex}]{+H_2O} AH + H^*HO \qquad (18)$$

where AH* · B represents a closed base pair in the double helix, (AH* + B) represents an open state from which exchange of the AH* proton with solvent may take place, and AH represents the situation after exchange. In the limit that k_c, the rate at which an open base pair closes, is faster than the exchange rate, k_{ex}, a base pair will open and close many times before exchange occurs. However, in the limit that k_{ex} is larger than k_c the rate of exchange is equal to k_0, i.e., every time the base pair opens, exchange occurs (referred to as open limited).[128] Since k_{ex} is affected by buffers and the pH of the solution, it is easy to adjust experimental conditions so that proton exchange from the bases is open limited.[126] With poly(dA-dT) and poly(dG-dC) these conditions are realized at pH 7, and this generally appears to be true for interior base pairs in other DNAs.[118] The opening and closing rates of terminal base pairs may, however, be sufficiently rapid that exchange is not in the open-limited region unless higher concentrations of buffer or more extreme pH values are used.[118] Exchange effects on NMR relaxation rates in DNA were first reported in 1981,[49,50] and since then a number of different molecules have been investigated. Since the behavior of the A·T and G·C base pairs is different, they are treated separately.

b. Exchange Behavior of A·T Base Pairs

Initial studies of three DNA restriction fragments[49,50] demonstrated that the thymine imino proton exchange rate in A·T base pairs is relatively insensitive to the molecular weight of the DNA and to the DNA sequence. Thus, the rate of exchange from A·T base pairs sandwiched between two G·C base pairs is, within experimental error, the same as for base pairs which are neighbors to other A·T base pairs. With these DNA systems, the average rate of exchange of A·T base pairs is described by $k = k_0 \exp(-E_a/RT)$ where $k_0 = 2 \times 10^{12}$ and $E_a = \sim 16 \pm 2$ kcal/mol. In a 12-base pair restriction the exchange rates of the different A·T base pairs differed by less than a factor of 1.5 from each other at any given temperature.[49] In the larger DNA only average values could be measured. The values previously obtained for poly(A)·poly(U) are $E_a = 15.0 \pm 0.9$ kcal and a rate constant of 1.1 s^{-1} at 20°C.[126] With poly(dA-dT) we[106,119] find an activation energy of 17 ± 2 kcal and an exchange rate of about 2.5 s^{-1} at 20°C. Since comparable exchange rates and activation energies are observed for A·T (or A·U) base pairs in different DNAs, it would seem reasonable to conclude that the same mechanism is also involved in all systems. The most straightforward interpretation of these observations is that exchange from A·T occurs by opening of individual base pairs. Alternatively, Englander et al.[129] recently proposed that exchange occurs via an open mobile unit, of approximately 10 base pairs (a soliton) which diffuses along the double helix. These open units correspond to thermally induced soliton excitations of the double helix which retain coherence by sharing the energy of a twist deformation among several base pairs. Although this is an intriguing proposal, a soliton mechanism is not consistent with the observations that the same rates and activation energies are observed for short DNA, for a series of DNA restriction fragments, and for both poly(dA-dT) and poly(rU)·poly(rA). A soliton mechanism involving 10 base pair is clearly excluded for the 12-base pair restriction fragment studied by Early et al.,[49] because the high temperature exchange rates for the G·C base pairs are much slower than the A·T base pairs sandwiched between them.[49] If a soliton mechanism were operative, both A·T and G·C base pairs would be expected to have similar exchange rates.

Recently Patel et al.[118] published relaxation data on the thymine imino protons of A·T base pairs in the dodecamer $d(CGCGAATTCGCG)_2$ which indicates that the activation energy for exchange is about 14 ± 2 kcal. This value is, within experimental error, the same as we deduced from our studies of restriction fragments and poly(dA-dT), but the magnitudes of the rates were all about a factor of three smaller than those which we observed. This suggests that there may be some small, but interesting, sequence effects on A·T exchange rates which should be explored further.

Table 3
A COMPARISON OF THE IMINO PROTON SPIN-LATTICE RELAXATION RATES (s^{-1}) FOR d(ATATCGATAT)$_2$ and d(ATATGCATAT)$_2$ AT VARIOUS TEMPERATURES

	ATATGCATAT			ATATCGATAT		
T°C	A·T$_{(3,8)}$	A·T$_{(4,7)}$	G·C$_{(5,6)}$	A·T$_{(3,8)}$	A·T$_{(4,7)}$	G·C$_{(5,6)}$
10	5.5	5.0	5.2	4.9	5.2	5.0
15	6.1	4.1	4.4	6.2	4.7	3.9
22	13.1	5.2	3.9	12.3	7.2	4.0
26				18	9.5	4.5
28	23	11	5.8	22	12	5.2
34	64	35	20	69	34	17
38	141	98	63	148	84	51
42		218	191		230	176
Apparent activation energy (kcal/mol)	36	46	64	37	48	63

We[130,131] have carried out spin-lattice relaxation rate measurements on the two decamers, d(ATATCGATAT) and d(ATATGCATAT), with the results shown in Table 3. At most temperatures studied, the exchange of the terminal AT_1 and the AT_2 base pairs was too fast to be measured. Above 30°C, exchange with solvent dominates relaxation of all the imino protons in the two molecules and from the data shown two features are unusual. First, the apparent activation energies for exchange of the thymine imino protons are all much larger than those found in the DNA restriction fragments and poly(dA-dT). Second, the exchange rates at a given temperature are also larger in the two decamers. Both of these features can be attributed to the fact that the rate of thermal denaturation of helical portions of these relatively unstable, short, AT-rich decamers is larger than the lower activation energy, single base pair opening mechanism. Although the activation energy is larger the entropy factors must be more favorable for the disruption of the helix than opening a single base pair. One must, therefore, be careful about applying exchange data obtained on short DNAs to exchange processes in larger DNA.

c. Exchange Properties of G·C Base Pairs

While the exchange rates of A·T base pairs appear to be more or less sequence independent, at least for the more stable DNA, this is not true for G·C base pairs. Limited experimental data show that the exchange behavior of G·C base pairs varies widely depending upon the DNA sequence and conformational state. For poly(dG-dC) in the B-form, we find activation energy for exchange is approximately 20 kcal/mol with a rate of $\sim 0.5\ s^{-1}$ at 25°C.[121] In the Z-form, the rate of exchange was too slow ($<2\ s^{-1}$) to be measured, even at 80°C![121] In poly(rG)·poly(rC) the activation energy for exchange was 21 ± 1 kcal, but the 25°C rate was only $0.07\ s^{-1}$. When A·T base pairs are incorporated adjacent to the G·C base pairs, the guanine imino exchange rates are considerably faster, and different results are obtained for different DNAs.[49,50,121,130,131,133] In some cases (see Table 3), the exchange of the G·C base pairs appears to involve a helix opening mechanism (as evidenced by a very high apparent activation energy and a large preexponential factor). Because of the greater stability of the G·C base pairs relative to A·T base pairs it is to be expected that, to a first approximation, A·T neighbor base pairs will affect the exchange behavior of a G·C base pair, but not vice versa. Thus, the faster

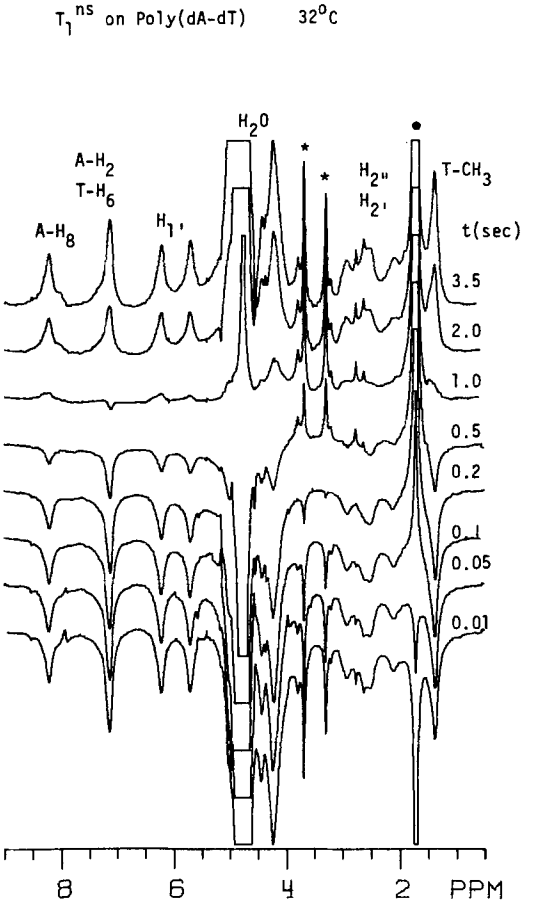

FIGURE 7. An example of the recovery of the longitudinal magnetization in poly(dA-dT) following the nonselective inversion of all nonexchangeable protons. These experiments were carried out in D_2O at 32°C and at 300 MHz.[119]

opening of an A·T base pair facilitates exchange of neighboring G·C base pair, analogous to fraying effects of base pairs located near the terminus of a helix.[128] In such a case, one would expect to find an apparent activation energy which is some weighted average of the activation energies for the various processes which contribute.[118]

These preliminary studies demonstrate that, neglecting end effects, A·T base pair opening rates are relatively, but not completely, insensitive to sequence whereas the G·C opening rates are sequence dependent. Additional sequences should be studied to characterize sequence effects on opening A·T base pairs and to develop rules for predicting G·C base opening rates. The effects of supercoiling on the opening rates and the possible influence of various other agents (methylation, metal ions, polyamines, drugs) on exchange rates remain to be explored. These studies should provide a coherent picture of the important factors which affect the base pair opening rates and permit us to evaluate their possible role in various biological processes (recognition of specific DNA sequences by proteins, regulation of genes, genetic regulation). A direct determination of opening rate constants may also be useful in various statistical mechanical treatments of local melting in DNA.

Table 4
A SUMMARY OF NONSELECTIVE ^1H SPIN-LATTICE RELAXATION MEASUREMENTS ON DOUBLE HELICAL DNA

	Spin lattice relaxation times (s)					
Length (bp)	12[a]	~70[b]	~40[c]	40[c]	260[d]	[e]
Frequency (MHz)	300	200	220	300	360	360
Resonance						
H-8	1.5	0.9	0.9	1.5	1.4	1.4
H-2	2.5	—	—	—	—	—
H-6	1.5	1.0	0.9	1.5	1.2	—
H-1′	1.5	0.8	0.9	1.3	1.2	1.4
CH_3	1.5	0.9	0.8	1.3	1.4	1.6
H-2′, 2″	1.5	—	0.65	1.1	1.2	—
					(0.13)[f]	

[a] Early et al.[48,49]
[b] Poly(dA-dT).[106]
[c] Random DNA.[51a]
[d] Random DNA.[97]
[e] Poly(dA-dT) $S_{20w} = 7.8$.[30]
[f] Value obtained at 100 MHz.

B. Relaxation Behavior of Nonexchangeable Protons

1. Nonselective Spin-Lattice Relaxation Rates [$R_1(ns)$]

The relaxation behavior of nonexchangeable carbon-bound protons can be treated using Equations 2 and 13. An example of a nonselective spin-lattice relaxation experiment is shown in Figure 7 for poly(dA-dT) and results are summarized in Table 4.[106,119,120] As expected from theory, longitudinal proton relaxation rates obtained by *non*selective inversion of all resonances are more or less the same for all protons regardless of the DNA length, sequence, or temperature. The nonselective longitudinal relaxation rates are, however, sensitive to magnetic field, and all of these features can be understood as follows. Because of rapid spin diffusion, zero quantum transitions do not contribute to the overall relaxation of the spins and the observed relaxation rate is a weighted average of all one- and two-quantum transitions which contribute to relaxation of the N interacting protons (Equation 8). Consequently, these rates are not very useful in probing the DNA structure or for identifying the important internal molecular motions, but they are useful in evaluating the frequencies, and perhaps the amplitudes, of internal motions once a reasonable model for the average structure of the molecule has been chosen.

For random sequence DNA, a large increase in the nonselective relaxation rates is observed at 100 MHz, as compared with 360 MHz, consistent with a model for internal motion with $\tau_{internal} = 10^{-9}$ s.[97] Because of their close proximity (1.8 Å) to each other, sugar protons (e.g., H2′-H2″ and H5′-H5″) are expected to make the major contributions to $R_1(ns)$. Hogan and Jardetzky[97] were able to fit their relaxation data using a model in which there are ±35° fluctuation in the H2′-H2″ axis with a time constant on the order of 10^{-9} s, but we note that they assume that cross relaxation and spin diffusion are not contributing to the relaxation. Evidently, they only considered the interacting pair of H2′, H2″ sugar protons and did not sum over the set of N (N = 8 to 10) interacting protons associated with each nucleotide unit. Carrying out this summation would rduce their calculated rates by a factor of $\frac{1}{2}$ to $\frac{2}{5}$ (note that there are four methylene protons and eight

Table 5
A COMPARISON OF THE NONEXCHANGEABLE PROTON RELAXATION RATES (s^{-1}) FOR THE B- AND Z-FORMS OF POLY(dG-dC)[121] AND POLY(dA-dT)[106,119]

		Rate			
Sample	**Proton**	**R_1 (ns)**	**R_1 (s)**	**R_2**	**R_2/R_1 (s)**
Poly(dG-dC)	G8	0.73	34.5	62.5	1.81
B-form	C6	0.68	29.7	66.6	2.34
(55 ± 5 bp,	C5	0.54	25.0	58.8	2.35
21°C)	H1′	0.69	22.2	90.9	4.09
	H1′	0.69	23.8	83.8	3.50
	H2′, 2″	0.73	23.8[a]	111.1[b]	4.66
Poly(dG-dC)	G8	0.54	27.7	90.9	3.28
Z-form	C6	0.38	33.3	125	3.75
(55 ± 5 bp,	C5	0.40		142	
21°C)	GH1′	0.59		166	
	CH1″	0.46		200	
	H2′, 2″	0.49		200[b]	
Poly(dA-dT)	A8	0.7 ± 0.2	10.5	18	1.7
(70 bp,	H1′	0.7 ± 0.2	21	25	2.5
21°C)	H2′, 2″	0.7 ± 0.2	10.2[a]	26[b]	2.5

[a] Slowed by loss of 2′, 2″ interactions.
[b] Scalar coupling effects.

to ten nucleotide protons to be relaxed). In order to bring the calculated and observed rates into agreement, it would be necessary to shorten the internal motion time to 0.3 to 0.5 ns or to further increase the amplitude of the internal motion.

2. Selective Spin-Lattice Relaxation Rates [R_1 (s)]

Combinations of selective relaxation rate measurements in conjunction with bi-selective relaxation measurements provide direct information regarding pair-wise dipolar interactions between protons.[120,133–135] At this time, only selective relaxation measurements have been carried out and only for a limited number of samples, including random sequence DNAs,[106] the synthetic DNA polymers poly(dA-dT)[119,120] and poly(dG-dC),[121,133] and some synthetic short decanucleotides,[130,131] and these results are summarized in Table 5. In contrast with the nonselective relaxation rates which were more or less independent of the DNA length, the specific protons, and the temperature, the selective longitudinal relaxation rates are sensitive to all of these parameters.

Several features of the results obtained for poly(dA-dT) are to be noted. First, the AH8 proton is relaxed primarily by interactions with the 2′ proton of its own sugar group and the 2″ proton of the sugar of the neighboring 3′ → 5′ nucleotide, and the rate constant is about 10.5 s^{-1}. The TH-6 proton interacts both with sugar protons and with the T-CH3 group and, depending upon the precise distances to the sugar protons (estimated to be ~2.2 Å), should exhibit a faster relaxation rate. Unfortunately, the resonances for the A-H2 and the T-H6 protons overlap (see Figure 7) so that an accurate value for the T-H6 relaxation could not be obtained, but it is approximately 20 s^{-1}. Since the selective relaxation rate for the three T-CH3 protons is 6 s^{-1}, we infer that the corresponding contribution of the methyl-T-H6 interaction to the relaxation might be on the order of 18

s^{-1}. (Each methyl proton is close [~2.4 Å] to the T-H6 for only a short time, but a methyl proton is always close to the T-H6 proton.) It is, therefore, not difficult to account for the faster relaxation of the T-H6 relative to the AH8 proton. A detailed analysis of the poly(dA-dT) relaxation rates has not been completed, but preliminary considerations indicate the data can be accounted for by using an overall tumbling time of 400 ns for this ~70-bp fragment coupled with a common internal motion time of ~ ns.

For random sequence DNA (50 bp) selective relaxation rates of approximately 10 s^{-1} for the aromatic resonances and 50 s^{-1} for the H5′5″ sugar protons are observed.[106] (Note that since both 5′ and 5″ sugar protons are polarized in these "selective" experiments, their mutual cross relaxation does not contribute to their overall relaxation.) Similar measurements have also been carried out on poly(dG-dC) in the B-form with the results shown in Table 5. The fact that the R_1 (s) and R_2 values for the aromatic protons of poly(dG-dC) are so large relative to the corresponding values for the H2′,2″ sugar protons suggests that there is more mobility in the sugar phosphate backbone than in the bases.

3. Spin-Spin Relaxation Rates, R_2

Spin diffusion does not complicate the measurement of transverse magnetization relaxation rates and, therefore, the standard (90°, τ, 180°, τ) pulse scheme can be used with nonselective, high power pulses to make these measurements. The results of the experiments on poly(dG-dC) and poly(dA-dT) and on other DNA are collected in Table 5. In poly(dA-dT), the values range from 20 s^{-1} to 10.5 s^{-1}), and for ~60 base pair poly(dG-dC) the values range from ~60 to 110 s^{-1}. In the slow limit where $J_0 > J_1 > J_2$, we predict $\frac{R_2}{R_{1(s)}} = 2.5$ whereas in the limit of large amplitude, high frequency (internal) motion such that $J_0(O) = J_1(\omega) \cong J_2(2\omega)$ this ratio approaches 1.0. For the H1′ and H2′,2″ protons in poly(dA-dT), the theoretical value of 2.5 is closely approached, indicating that the J_0 term dominates both the spin-lattice and spin-spin relaxation rates. In poly(dG-dC), some of the R_2/R_1 (s) ratios exceed the theoretical maximum of 2.5, and this could either be attributed to unexpectedly large R_2 or small R_1 (s) value. In the case of the 2′ and 2″ sugar protons which are located only 1.8 Å apart, there is a very large dipolar interaction which contributes to R_2, but not to R_1(s). It is not possible to selectively polarize just one of these two sugar protons in poly(dG-dC). Consequently, the measured R_1 (s) values are not true selective rates, and this may account for the apparent discrepancy. Moreover, in considering the R_2 rates, we have neglected the effects of scalar coupling. As we shall see, scalar coupling can have a pronounced effect on the decay of the transverse magnetization of the sugar protons and the CH5 and CH6 protons, but only when the true T_2 values approach 30 ms or more. It is probably safe to conclude that the measured R_2 values correspond to upper limits on the true relaxation rates that would be obtained in the absence of scalar coupling effects. In the next section we discuss the use of spin-echo experiments to measure unresolved scalar couplings of protons in DNA.

4. Modulation of Spin-Echoes-Scalar Couplings-Sugar Conformations

In early experiments on small molecules, it was discovered that scalar coupling between protons in a molecule can cause modulations in the decay of transverse magnetization.[136] When a single resonance is split by a scalar interaction into a pair of resonances separated by an amount J Hz, an inversion of the two peaks occurs in a spin-echo experiment (90°, t, 180°, t) after a time delay of $t = (2J)^{-1}$.[136] An example of this effect is shown in Figure 8 for $d(ATATCGATAT)_2$ where we note that an inverted peak at 5.7 ppm from the CH5 resonance is observed at about 60 to 70 ms.[130,131] This indicates a splitting of about 7 Hz (due to coupling with the CH6 proton), and direct measurement of

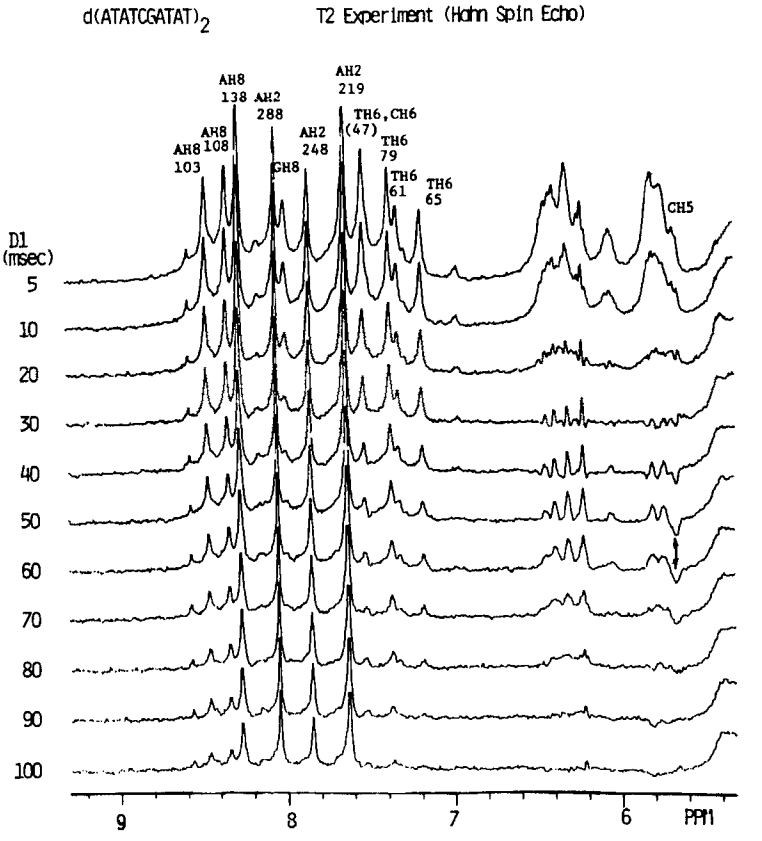

FIGURE 8. An example of a Hahn spin-echo experiment on d(ATATCGATAT)$_2$ illustrating modulation in the decay of the sugar H1′ resonances and the CH5 resonance (indicated by arrow). These experiments were carried out at 23°C and 360 MHz.[130]

the splitting in more well-resolved spectra indicates a value of 7.5 Hz. The interaction of the H1′ with its two neighboring 2′ and 2″ protons results in a pseudo triplet splitting pattern and in this case the outer pair of lines invert at $t = (2J)^{-1}$, whereas the center pair of lines remains "normal" in its decay behavior. When multiplets overlap, there is considerable cancellation of intensity at $t = (2J)^{-1}$ and the net result is that resonances which are split into unresolved triplets appear to pass through a "null" at $t = (2J)^{-2}$ and then partially recover, as the overall intensity decays with the expected exp $-\frac{t}{T_2}$ time dependence.[106,136] It is, therefore, possible to estimate the value of J by identifying the time point at which the first minimum in the spin-echo signal appears. In the results shown in Figure 8, most of the H1′ resonances appear to pass through a "null" point around 30 to 35 ms, corresponding to $J \simeq 14$ to 16 Hz. According to extensive studies of multiplet structure in various sugars, a splitting of 15 Hz corresponds to a high population of the S sugar conformation.[39,40] The results for the sequence isomer d(ATATGCATAT)$_2$ were virtually identical and similar modulations have been previously observed in a 12-base pair DNA restriction fragment.[106] The present results indicate that in d(ATATCGATAT)$_2$ and d(ATATGCATAT)$_2$ all, or most, sugar groups are primarily in an S (2′ endo) conformation since a maximum splitting of 16 Hz is expected for S-type, as compared with 9.7 Hz for N-type.[39] In cases where the resonances are sufficiently well resolved, the scalar couplings can be directly determined from the spectra, and these measurements in

the above two decamers yielded values in the range of 14 to 15 Hz, and measurements on other DNA fragments have also indicated H1′ splitting on the order of 14 Hz.[106]

In small molecule systems where the spin-spin relaxation times are quite long, several cycles of modulation can be observed before T_2 processes completely eliminate the echo and, hence, coupling constants can often be measured with great accuracy. In fact, a Fourier analysis of the echo decay can be used to determine the frequencies responsible for modulating the decay.[136–138] The result of such an analysis can be displayed as a two-dimensional spectrum with peaks at the appropriate modulation frequency, broadened by the overall exponential decay.[137,138] This type of two-dimensional spectrum, referred to as a 2D J-coupled spectrum, has yet to be reported for a DNA sample, but other 2D-NMR methods which have been used on DNA are discussed below.

5. Two-Dimensional NMR

During the past 5 years, a number of different two-dimensional NMR methods have been developed to obtain information about various internuclear interactions.[33–38,137,138] Two types of experiments that we have found useful in studies of DNA structure and dynamics are 2D J-correlated spectroscopy (COSY)[37,138] which identifies nuclei that are scalar coupled to one another, and 2D nuclear Overhauser spectroscopy (NOESY)[36,139] which can identify protons that are sufficiently close in space to each other to interact via through-space dipolar interactions.

a. 2D J-Correlated Spectroscopy (COSY)

In a 2D J-correlated experiment a 90° RF pulse is first applied, and the spins are allowed to evolve for a time t_1 (the evolution period) followed by the application of a second 90° RF pulse and immediate acquisition of the signal.[37,38] A series of experiments are conducted with t_1 incremented through a range of values. The resulting data matrix (t_1,t_2) is then double-Fourier transformed to give a two-dimensional spectrum in the frequency range (f_1,f_2). An example of this is shown in Figure 9 for $d(ATATCGATAT)_2$.[130–132] In our preceding discussion of spin-echo experiments, we noted that the scalar interaction of two protons leads to a modulation of their corresponding spin-echo intensities. In the 2D COSY experiment, the peaks along the diagonal correspond to the regular spectrum and off-diagonal peaks at positions (f_1,f_2) and (f_2,f_1) indicate that peaks on the diagonal, located at (f_1,f_1) and (f_2,f_2), are scalar coupled to one another. Thus, the useful information is contained in these off-diagonal cross peaks. In the COSY spectrum shown in Figure 9 the cross peak at (5.7, 7.5) arises from a scalar interaction between the CH6 proton (7.5 ppm) and the CH5 proton (5.7 ppm), and a large number of other cross peaks connect the various H1′ resonances with H2′ and H2″ resonances on the *same* sugar group.

The principal value of these measurements is twofold. First, the data immediately identify all resonances in the molecule from spin-coupled protons and, therefore, are valuable for assignment purposes. Second, in interpreting the 2D-NOE experiments to be discussed next, it is valuable to know in advance which resonances are spin coupled, so that through-bond scalar contributions to NOE peaks can be distinguished from through-space dipolar interactions.[140] A detailed discussion of the assignments of $d(ATATGCATAT)_2$ and $d(ATATCGATAT)_2$ using COSY spectra is given elsewhere.[130,132]

b. 2D-NOE Spectroscopy (NOESY)

In the typical (one-dimensional) NOE experiment, a specific resonance is irradiated for a suitable length of time, and (transient) changes in the magnetization at other resonance positions are monitored. The experiment can then be repeated with each resonance in the

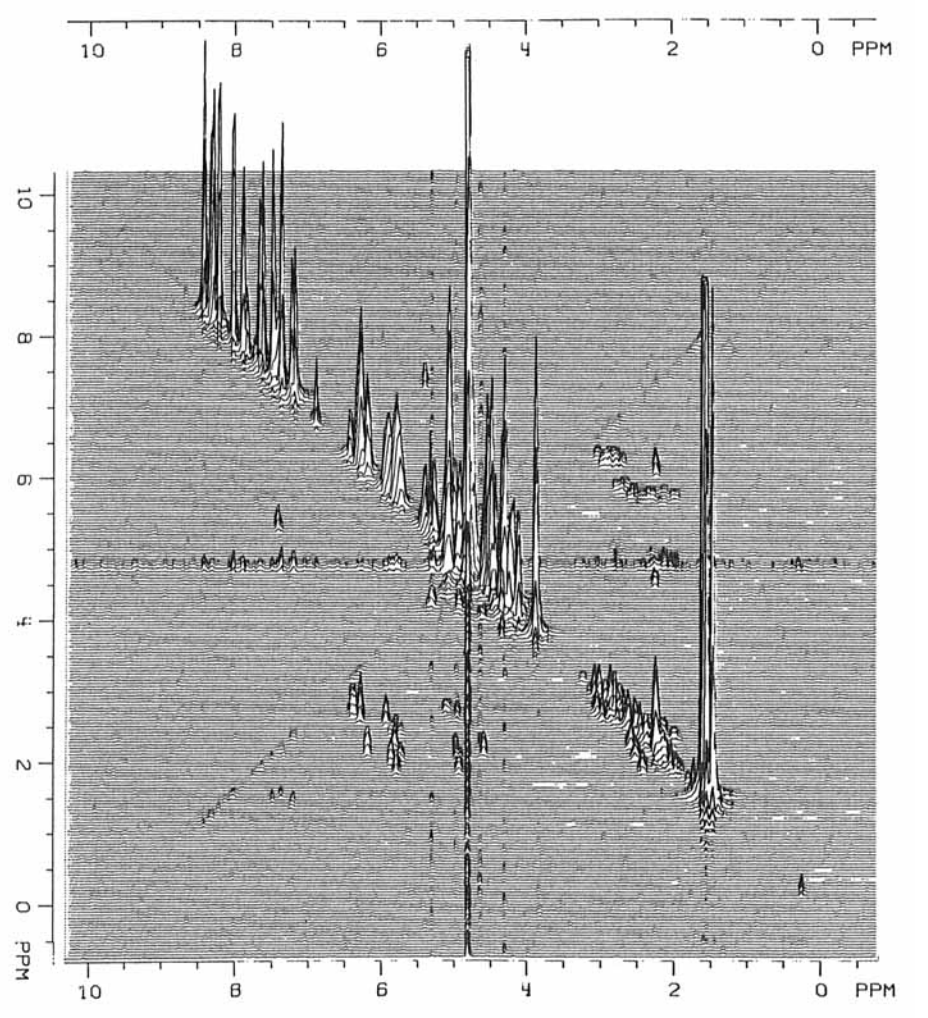

FIGURE 9. A 360-MHz COSY spectrum (stacked plot) of d(ATATCGATAT)$_2$ at 29° C. The spectral width was ±2000 Hz. The data set consisted of 1024 points in the t_2 dimension and 128 points in the t_2 dimension. Ninety-six FIDs were accumulated for each value of t_1, with a 4-sec delay between acquisitions, and the total accumulation time was ~15 hr. A 90° pulse (13.5 μs) was used for P_1, but P_2 was 9.5 μs. The resulting data matrix was processed with a phase-shifted sine-bell in both dimensions and was zero filled in the f_1 dimension. The pure absorption phase mode is used.[130,131]

molecule being irradiated in separate experiments. In the 2D-NOE method, the same information can be obtained in a single experiment.[35,36,39] The pulse sequence ($90°_x$,t_1,$90°_x$,tm,$90°_x$,t_2) is used, and the time t_1 is an evolution time during which the spins are frequency labeled. Following the second 90° pulse, the spins are allowed to exchange magnetization with each other for a fixed time, tm, and then the resulting magnetization in the system is probed by a third 90° pulse. For a given experiment, the mixing time, tm, is held fixed and t_1 is incremented in units corresponding to one half the inverse of the spectral width of interest. The results obtained with the DNA decamer (ATATCGATAT)$_2$ are shown in Figure 10. As in the COSY spectrum, the intense peaks along the diagonal of the NOESY spectrum correspond to the regular spectrum, but with distorted intensities due to partial relaxation during the delays t_1 and tm. Again it is the relatively small cross peaks located symmetrically about the diagonal that carry the

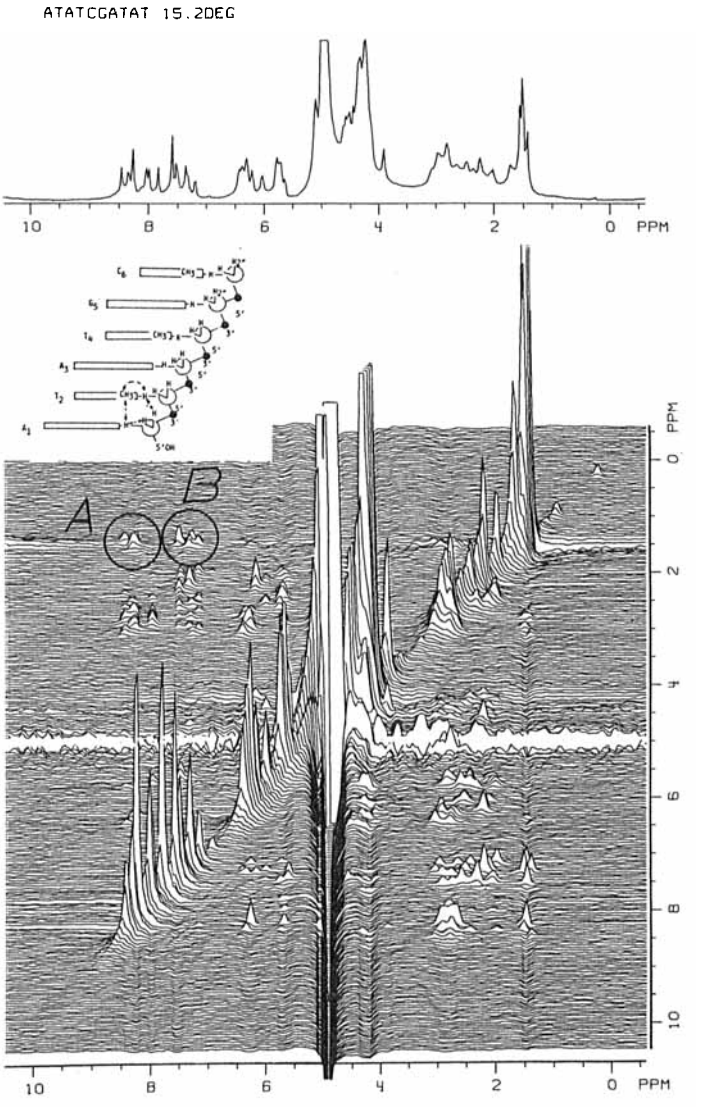

FIGURE 10. A 360-MHz NOESY spectrum (stacked plot) of d(ATATCGATAT)$_2$ at 27°C with τ_m = 350 ms. The spectral width was ±2000 Hz and the real value mode is used.[130,131] A schematic diagram illustrating some of the short interproton distances detected by the NOESY measurements is shown at the top of the figure.

information about the structure of the molecule since a cross peak at (f_1,f_2) indicates that protons giving rise to resonances located at (f_1,f_1) and (f_2,f_2) along the diagonal interact with one another. In this particular spectrum, over 60 different interactions can be discerned. At short mixing times (e.g., 30 ms), some of these interactions could be due to scalar couplings, however, with long times used in the experiment shown in Figure 10 the cross peaks undoubtedly arise from through-space dipolar interactions between protons that are spatially close together.[140] Theoretically, the cross-peak intensities vary with r_{ij}^6.[35,137] However, since other factors such as line widths and relaxation times also have important effects, it is difficult to quantitatively relate cross peak intensities, obtained using one mixing time, to internuclear separation.[36] For this purpose,

measurements should be carried out over a series of tm values to enable the build-up of the cross peak to be monitored.[141] However, for purposes of immediately detecting all of the major interproton interactions in a single experiment, the NOESY experiment is excellent.

In the results shown in Figure 10, a number of cross peaks have been identified and used to establish certain structural features in the molecule. For example, the collection of circled peaks labeled A arise from interaction of adenine H8 protons with the thymine methyl protons, and the group of four peaks, labeled B, are due to the interaction of the thymine methyl and C-H6 protons. Since the methyl and H6 protons of a thymine group have a minimum separation of ~2.5 Å, the latter group of cross peaks provides an internal calibration of the peak intensities. Since the cross peaks between the thymine methyl and the adenine H8 resonances are of comparable intensity, this suggests that similar distances are involved. Consideration of the dimensions of the A · T base pair indicates that it is impossible for the AH8 proton to interact with the T methyl protons in the *same* base pair. Rather, the AH8-T methyl cross peaks connecting AH8 and T-methyl resonances must be due to the interactions of the AH8 protons with the methyl protons of the thymines in adjacent base pairs attached 3′-5′ (e.g., ApT 3′-5′) as indicated schematically in Figure 10. The fact that there is a cross peak connecting the AH8 resonance of the terminal A residue in $d(ATATGCATAT)_2$, and probably in $d(ATATCGATAT)_2$, with the T-methyl group in the neighboring (3′-5′) thymine residue is consistent with a right-handed B-DNA conformation.

The 2D NOESY spectra also provide useful information about the spatial location of base protons relative to the sugar protons. Moderately strong cross peaks are observed between the aromatic (AH8, GH8, T-H6, and CH6) and the 2′,2″ sugar protons, and examination of assignments of the various resonances indicates that the larger peaks are between base and sugar protons of the *same* nucleotide.[130-132] The smaller peaks involve the interaction of base protons with 2″ sugar protons of the adjacent nucleotide residue. Note, however, that the cross peaks between the TH6 protons and the H2′,2″ protons of adenine are considerably stronger than are the corresponding peaks between the AH8 protons and the sugar protons of thymine. We, therefore, conclude that the TH6 protons of thymine, while closer to its own 2′ sugar proton, is almost equidistant from an adjacent *adenine* 2″ sugar protons. The AH2 protons, on the other hand, are more asymmetrically situated, in that they are somewhat closer to their own 2′ sugar protons. In contrast to the strong cross peaks between the base protons and the 2′,2″ sugar protons, the interactions between base protons (AH8, G-H8, T-H6, C-H6) and the H1′ protons are considerably weaker, and probably arise from second-order NOE involving transfer of spin polarization from base protons to H1′ protons indirectly via the H2′,2″ protons. However, the fact that the base-H1′ interaction is so small indicates that the bases are not in a *syn*-glycosidic conformation, but rather the bases have an *anti*-conformation with respect to the sugar group.

Although the 2D techniques have been applied only to a limited number of molecules, it should be evident from the examples cited that these new techniques are extremely useful in distinguishing between various possible conformational states of DNA. When the 2D methods are further refined and used in conjunction with one-dimensional relaxation techniques, it will be possible to carry out quantitative structural studies of DNA in solution. Information about specific local interproton interactions can be obtained from spin-spin relaxation rates and from the initial values of selective spin-lattice relaxation rates. If, as is often the case, several interacting protons contribute to the relaxation of a proton of interest, then more complex relaxation experiments, such as comparison of rates measured following a selective and bi-selective relaxation measurement, are needed to measure interactions between specific pairs of protons.

Table 6
A SUMMARY OF ^{31}P RELAXATION PARAMETERS OBTAINED ON VARIOUS DNAs AT DIFFERENT FREQUENCIES

Sample size (bp)	Freq. (MHz)	Temp (°C)	T_1 (sec)	T_2 (ms)	$\Delta\nu$ (Hz)	$^{31}P\{^1H\}$ NOE $(1 + \eta)$	Ref.
~140	40.5	23	3.2	32	22	1.35	97
~260	40.5	23	3.6	13	39	1.37	
~260 (η = 5.2 cp)	40.5	23	3.8	3.1	120	1.41	97
600	40.5	23	3.9	12.3	56		147
140	40.5	8	2.8		37	1.6	72
Nucleosome core particles	40.5	8	2.8		33	1.7	72
High molecular	40.5	20	2.5		95	1.56	143
weight	40.5	40	2.3		45	1.6	143
7200							
Linear	40.5	25	2.4	19		1.3	150
Circular	40.5	25	2.5	250		1.4	150
Supercoiled	40.5	25	1.64	1170		1.45	150
190	24.1	30	2.2		16		151
1400	24.1	30	2.2		40		151
190	101.2	30	3.7		53		151
156	24.3	19	2.15		18	1.18	146
156	40.3	19			28	1.28	146
156	109.3	19	3.43		103	1.30	146

Although these latter techniques have already been used on an RNA polymer[134,135] and on other polymers,[142] they have yet to be applied to the DNA systems.

C. ^{31}P Relaxation Measurements

In a series of recent studies, ^{31}P relaxation measurements have been used to obtain information about internal motions and conformational fluctuations in the DNA backbone.[72,97,98,143-148]

1. Relaxation Rates and NOEs

The various factors which contribute to relaxation of the phosphorus nucleus are described by Equations 2, 13, 15, and 16. It is evident from the structure of these equations that the major contribution to the transverse relaxation rate, R_2, arises from the J(O) term if the overall molecular motion is slow. In this case $J_0 \sim \tau_c$ and R_2 is predicted to increase lineraly with the overall molecular tumbling time, τ_c, and, thus, the length of the DNA. R_1, on the other hand, depends on $J_0(\omega_H - \omega_P)$ and $J_1(\omega_P)$ terms which are large only if there are rapid internal molecular motions which generate RF power at frequency ω_P and $(\omega_H - \omega_P)$. Since the slow, overall motions of the molecule have relatively little effect on $J_0(\omega_H - \omega_P)$ and $J_1(\omega_P)$, R_1 is expected to be relatively insensitive to the DNA length. Similarly, we expect the $J_2(\omega_H + \omega_P)$ and $J_0(\omega_H - \omega_P)$ terms which appear in the NOE expression to be dominated by the presence of high frequency internal motions; therefore, the $^{31}P\{^1H\}$ NOE will also be relatively insensitive to the overall rate of tumbling of the molecule. The relaxation data summarized in Table 6 confirm these general expectations: the R_1 values are virtually independent of the DNA length and state (linear, supercoiled, condensed, complexed), the solvent viscosity (η), the temperature, and the line widths increase with molecular weight and with solvent viscosity. The NOEs are positive and ^{31}P relaxation parameters measured in D_2O and H_2O are the same indicating that water molecules do not

contribute significantly to the relaxation,[98] presumably because of rapid (10^{-10} sec) reorientation of the phosphate waters of hydration. The insensitivity of R_1 to the length of the DNA and to large increases in viscosity clearly demonstrate that internal motion in the DNA backbone is responsible for the relatively rapid spin-lattice relaxation rates which are observed.[97,98,143-147] At lower magnetic fields, dipolar interactions with nearby sugar protons ($H_{5',5''}$ and $H_{3'}$) are the major factors responsible for relaxation, but chemical shift anisotropy (see Equations 15 and 16) becomes increasingly important at higher fields.[110] Various detailed models for the molecular motion in the backbone have been used to interpret the ^{31}P relaxation data,[143-149] but in view of the relatively limited experimental data available (R_1, NOE, and some R_2 values) the relaxation data are probably more valuable in eliminating rather than confirming specific models for internal motion. However, quite independent of the uncertainties regarding the nature of the internal motions, it is clear from the ^{31}P NMR studies that there are substantial ($\pm 25°$), high frequency (0.5 to 2 ns) internal motions in the backbone of DNA, and this leads to a picture of DNA dynamics in which the sugar phosphate backbone is quite flexible. Additional support for this is found in proton[13,147] and ^{13}C studies.[97-101]

2. Line Widths and R_2

The ^{31}P resonance line widths have been measured in a number of different samples and at various magnetic fields (see Table 6) and two spin-spin relaxation rate measurements have been reported.[97,150] For low molecular weight samples (~150 bp), line widths (at 40 MHz) calculated from the T_2 measurement are about a factor of two to three smaller than the measured line width. This difference is reasonably attributed to chemical shift dispersion contribution to the observed line width (due to the fact that different phosphate groups in the molecule have different chemical shifts). At higher fields, chemical shift anisotropy contributions are more important.[110,151] When the viscosity of a sample of DNA of 260 base pair average length was increased by a factor of 5.2, R_1 changed little, but R_2 increased by a factor of >4, from 70 s^{-1} to 330 s^{-1}.[97] This sensitivity to viscosity arises from the fact that the slow overall motions of the molecule affect both $J_0(O)$ and R_2 (Equation 13), and these motions are expected to vary with the solvent viscosity. This points out that variation of solvent viscosity would be useful in distinguishing between localized (viscosity-independent) internal motions in the DNA and other larger-scale viscosity-dependent motions.

3. Effects of Supercoiling on ^{31}P Relaxation Parameters

Recently Bendel et al.[150] reported a most unexpected set of results for the ^{31}P relaxation of 7200 bp circular DNA (see Table 6). They found that on going from linear to supercoiled circular DNA, there is only a small change in the R_1 and NOE values, but the R_2 value decreased by almost a factor of 200! The small variations in R_1 and in the NOE indicate that local internal motions are not significantly affected by supercoiling. However, the slow reorientation of the phosphate groups in the magnetic field is very significantly affected. The authors[150] suggest that the reduction in R_2 values can be explained by a coupling of bending and higher frequency torsional motions in the supercoiled DNA which leads to an increase in the effective frequency (and amplitude) of bending motions in the supercoiled DNA. A similar narrowing of the proton spectrum in the supercoiled DNA has now been observed (James, private communication[153a]). Obvious experiments that need to be carried out include examination of the effect of degree of supercoiling, drug binding, sequence, and temperature on R_2 in supercoiled DNAs. These new results on supercoiled DNA suggest that it will now be possible to extend, to a much higher molecular weight range than previously expected, the size of DNA that can be usefully studied by ^1H and ^{31}P NMR. This area has not previously been explored by NMR and appears to be a very fertile subject for study.

4. ^{31}P NMR Studies of Divalent Metal Ion Binding

In a recent study, ^{31}P NMR relaxation has been used to probe the interaction of three divalent metal ions, Mg^{2+}, and Mn^{2+}, and Co^{2+}, with the phosphate groups of DNA.[152] Mg^{2+} is the major naturally occurring divalent metal ion and Mn^{2+} and Co^{2+} are paramagnetic and can replace Mn^{2+} in many biochemical reactions.[86,93,153,154] Because ^{31}P NMR was used, the measurements were sensitive primarily to those paramagnetic ions in close association with the phosphate groups (i.e., inner-sphere association).

Mg^{2+} has only a small effect on the ^{31}P NMR parameters (chemical shift, line width, T_1, T_2, and NOE) over a range of concentrations (from 20 to 160 mM),[95] but the two paramagnetic ions Mn^{2+} and Co^{2+} significantly increase ^{31}P relaxation rates, even at very low concentrations.[152] From an analysis of the paramagnetic contributions to the spin-lattice and spin-spin relaxation rates, the effective internuclear metal-phosphorus distances were found to be 4.5 ± 0.5 and 4.1 ± 0.5 Å for Mn^{2+} and Co^{2+}, respectively.[152] This corresponds to only $15 \pm 5\%$ of the total bound Mn^{2+} and Co^{2+} being directly coordinated to the phosphate groups (inner-sphere complexes). The total extent of Mn^{2+} binding per DNA phosphate was 0.43 ± 0.04 (~86% neutralization) and virtually independent of the metal ion concentration in the range from 2.8×10^{-5} to 2.1×10^{-3} M Mn^{2+}, in reasonable agreement with the prediction of Manning's polyelectrolyte theory.[155,156] The lifetimes of the inner-sphere complexes were 3×10^{-7} s and 1.4×10^{-5} s for Mn^{2+} and Co^{2+}, respectively, and the rates of formation of the inner-sphere complexes with the phosphate were found to be about two orders of magnitude slower than the rate at which waters of hydration exchange on the metal ions.[152] Competition experiments were used to demonstrate that binding of Mg^{2+} ions to DNA is three to four times weaker than the binding of either Mn^{2+} or Co^{2+}. Since direct inner-sphere coordination with the phosphate contributes little (~10%) to their DNA binding strength, the stronger binding of Mn^{2+} and Co^{2+} may be due either to base binding or to formation of stronger outer-sphere metal-phosphate complexes. Complementary ^1H relaxation studies also indicate little disturbance of the hydration sphere of Mn^{2+} and Co^{2+} upon binding to DNA, consistent with a small degree of inner-sphere coordination.[157] The binding constants of Mn^{2+} to DNA, at metal concentrations approaching zero, are found to be inversely proportional to the second power of the salt concentration, which is also in agreement with the prediction of Manning's polyelectrolyte theory.[155,156] At high levels of divalent metal ions the fraction of inner-sphere phosphate binding increases and by 3.1 M Mg^{2+} the inner-sphere $\rightleftharpoons$ outer-sphere equilibrium is shifted toward ~100% inner-sphere binding. In general, then, there was relatively good agreement between the predictions of Manning's condensation theory and those obtained from solution of the generalized Poisson-Boltmann equation regarding the extent of divalent ion binding to polyelectrolytes, although some discrepancies were noted.[158]

There have been suggestions that Mn^{+2} preferentially binds to G·C base pairs[159-161] and, if true, one might expect to find a dependence of binding on the A·T to G·C ratio. However, in competition experiments employing AT- and GC-rich DNA, no preferential binding to either DNA was found,[162] despite previous suggestions to the contrary.[161]

D. ^{13}C NMR Relaxation Studies

The few ^{13}C relaxation measurements that have been carried out include spin-lattice relaxation, proton-carbon nuclear Overhauser effect measurements, and line width measurements.[96-101,163] For carbon atoms bearing one or more protons, the ^{13}C relaxation is due almost exclusively to ^{13}C-^1H dipolar interactions (Equations 2 and 13). Since the C—H bond distances are all fixed and known, the ^{13}C NMR relaxation studies are not very useful for providing structural information, but they are valuable for studying internal molecular motions.

The results of some recent ^{13}C NMR relaxation measurements on DNA are

Table 7
^{13}C NMR PROPERTIES OF DNA AT 32°C AND 67.9 MHz

Carbon no.	T_1s.		Line widths (Hz)		NOE
	160 bp[a]	Poly(dA-dT)[b]	160 bp[a]	Poly(dA-dT)[b]	Poly(dA-dT)[b]
C1′ (4′)	0.54	0.35	266 ± 14	83	1.26
C2′	0.27	0.19	260 ± 20	99	1.28
C3′	0.48	0.29	248 ± 23	79	1.28
C4′ (1′)	0.54	0.35	266 ± 14	83	1.26
C5′	0.23	0.16	154 ± 37	(171)	(1.0)
T(Me)	0.90	0.70	83	24	1.43

[a] 160-bp calf thymus DNA.[99,100]
[b] 145-bp poly(dA-dT).[101]

summarized in Table 7. Although a complete model for the DNA motion has not been developed, certain conclusions can be drawn from the relaxation data. The positive NOEs in conjunction with relatively small line widths and long T_1 values require the presence of substantial (±25° to ±35°) internal motions in the double-stranded DNA with correlation times of ~1 ns. The equivalence of the NT_1 (N = number of attached protons) and the NOE values for both the sugar and protonated base carbon atoms further indicates similar dynamic behavior for both parts of the DNA.[101] Specific motional models have been proposed to account for the relaxation rates, but the data are too limited for any one model to be considered established.[164] On melting of the DNA the NOEs increase and the line widths decrease[99] as expected for a single-stranded, nonordered polymer. The fact that there is virtually no change in the T_1 values on melting could be due to an accidental symmetrical disposition of the correlation times for internal motion about the T_1 minimum, a nonexponential autocorrelation function describing the internal motions, or a more complex dependence of T_1 and overall motions.[99]

^{13}C NMR has not been used extensively in studies of DNA, but it has certain virtues and will undoubtedly find applications in future studies.

V. Z-DNA STUDIES

In previous sections we focused on specific nuclei and particular types of measurements. In this section we depart from this approach to summarize the various contributions that NMR has made to our understanding of Z-DNA and the factors affecting the B to Z transformation. The discovery of Z-DNA by Wang et al.[16] in 1979 is perhaps the most unexpected finding in DNA structure in the past 25 years. It unequivocally demonstrated that DNA molecules can adopt a remarkably wide range of conformational states and recent studies using antibody techniques have provided very suggestive evidence that Z-DNA regions exist in chromatin and may play an important role in gene regulation.[25,26] It also set off a search for other DNA sequences that can adopt the Z-structure and this ultimately lead to the finding that poly(dG-me^{5}C),[23,27] poly(me^{7}dG-dC),[165] and poly(dI-br^{5}dC)[60] can all be converted to the Z-form, and that ions such as $Co(NH_3)_6^{3+}$, spermidine, spermine, and some organic solvents facilitate conversion to the Z-form.[27,166,167] A variety of experimental techniques have been used to study the properties of Z-DNA and the interconversion of B- and Z-DNA, and in this section we summarize contributions NMR has made to this area.

A. ^{31}P and ^{1}H NMR Spectral Studies

The first ^{1}H and ^{31}P NMR study on the interconversion of the low salt (B) and high salt (Z) forms of poly(dG-dC) was carried out by Patel et al.[60] With the exception of one of the H1′ sugar protons which shifted downfield, the chemical shifts of most resonances in the proton spectra were surprisingly little shifted on conversion from B to the Z conformation. The ^{31}P spectrum of the low-salt B-form was normal (single peak at ~4.2 ppm); however, the spectrum of the high-salt (Z-form) was unusual in that one resonance was observed in the normal DNA position (~4.35 ppm) and a second resonance was observed about 1.5 ppm downfield at 2.85 ppm. On the basis of these data, Patel[60] proposed that poly(dG-dC) adopts an "alternating B-DNA" conformation in high salt.

After the work of Wang et al.[16] appeared, Simpson and Shindo[168] reexamined the ^{31}P NMR of poly(dG-dC) and reinterpreted the high-salt data in terms of Z-DNA structure. Although subsequent work[22,23] has confirmed this suggestion of Simpson and Shindo,[168] the point to be made here is that it simply is not possible to deduce DNA structures from ^{1}H and ^{31}P chemical shift data alone. Structural features can be determined using NMR relaxation measurements, and in more recent studies Patel[23] used the nuclear Overhauser effect to provide the most definitive solution-state evidence that poly(dG-dC) exists in the Z-form in high salt. One of the unique structural features of Z-DNA is that all guanine residues exist in a *syn*-conformation relative to the sugar placing the G-H8 proton in close proximity (2.2 Å) to its own H1′ sugar proton.[16] Because of this close proximity, there is a large nuclear Overhauser effect between the G-H8 and the H1′ protons. In the more usual *anti*-conformation, the H1′ distance is 3.75 Å and only a small, or negligible, Overhauser effect between the C-H6 and the C(H1′) sugar protons is seen.[23] The unusually strong NOE between the guanine H8 proton and the H1′ proton of its own sugar, therefore, provides confirmation of a key structural feature of Z-DNA. This observation in conjunction with the highly unusual ^{31}P NMR spectrum, the unusual shift of the H_1, resonance of the guanine sugar, and Raman spectra[23,169] provides convincing evidence that the high salt conformation of poly(dG-dC) in solution is the same, or similar to, the Z-DNA found by Wang in the crystal.[16] We anticipate that additional NOE or 2D-NOE studies will soon provide some of the other details of the solution-state structure of the Z-form of poly(dG-dC).

Klysik et al.[24] have investigated the B to Z transitions of blocks of $(dC\text{-}dG)_n$ contained in nonalternating DNA sequences. They found, both by CD and ^{31}P NMR, that the $(dG\text{-}dC)_n$ regions in a restriction fragment, composed of a 95-bp region of *E. coli lac* operator-promotor flanked by segments of $(dC\text{-}dG)_n$ 26 and 32 bp in length, can be converted to the Z-form in high salt. In the ^{31}P spectrum of this 153-bp fragment, approximately 20% of the intensity in the phosphorus spectrum was shifted by 1.45 ppm lower fields. Since the (dC-dG) segments comprised about 37% of the DNA, this result indicates that virtually all of the $(dC\text{-}dG)_n$ tracts were converted to the Z conformation (note that only half of the ^{31}P resonances in the Z-form are shifted to lower field). Interestingly, the CD measurements indicate that a lower fraction of the (dC-dG) segments were converted to the Z-form.

In order to explore the B-Z interconversion in a biologically more relevant system, experiments were then conducted on a supercoiled plasmid DNA containing a 58-bp segment of (dC-dG). Zacharias et al.[170] found electrophoretic evidence for the conversion of the $(dC\text{-}dG)_n$ segments in the supercoiled plasmid DNA under conditions which favored Z-formation, and the extent of the relaxation in supercoiling was consistent with the amount expected for a B to Z transition in the 58-bp (dC-dG) segment. Similar studies by Peck et al.[171] have shown that the $(dG\text{-}dC)_n$ regions flip from right to left under physiological conditions when the negative super helix density is larger than 0.03. In view

of the observation of Bendel et al.,[150] it appears that ^{31}P could be used to examine the B to Z transition in these plasmids, despite their high molecular weight (~4500 base pair).

The important conclusions to be drawn from these experiments are that $(dC\text{-}dG)_n$ segments flanked by random sequence DNA can be converted to the Z-form by a large number of different cations, and that negative supercoiling facilitates this conversion. These studies add significantly to the growing body of experimental evidence that Z-DNA does have an important biological role.

In addition to detecting Z-DNA, NMR has also been used to characterize various properties of Z-DNA, and these results are summarized below.

1. Backbone Flexibility

^{31}P relaxation has been used to compare the backbone flexibility of B- and Z-DNA. At 80 MHz, the relaxation data for the low salt (B-form) and $R_1 = 0.5\ s^{-1}$ and $R_2 = 10\ s^{-1}$ and in the high-salt Z-form the rates increase slightly to $0.6 \pm 0.1\ s^{-1}$ and $14\ s^{-1}$.[121,133] These results do not indicate any important changes in the flexibility of the poly(dG-dC) backbone on conversion from the B- to the Z-form, since the increase in R_2 can be almost entirely accounted for in terms of an increase in solvent viscosity. $^{31}P\{^1H\}$ NOE measurements have not been reported for poly(dG-dC).

2. Proton Exchange in B- and Z-DNA

We recently measured the temperature dependence of the spin-lattice and spin-spin relaxation rates of G-imino protons poly(dG-dC) in both the B and Z conformations and some of these results are displayed in Figure 11.[121,133] In the B-form, magnetic dipolar interactions dominate the relaxation at low temperatures, but above 50° C exchange of the imino protons with solvent makes a major contribution. From the high temperature data, we obtained an activation energy of 20.1 ± 0.4 kcal for the exchange process, suggesting that it involves a single base pair opening mechanism.[133] The behavior of the Z-DNA is quite different from B-DNA in that the spin-lattice relaxation rate is virtually temperature independent from 20° to 85° C. Even at 85° C, no contribution from proton exchange with solvent to the spin-lattice relaxation rate is observed.[133] This observation of an unusually slow exchange rate is consistent with a previous study of Ramstein and Leng[172] who used IR and tritium exchange techniques, and found that proton exchange from Z-DNA is greatly retarded compared with that observed for B-DNA. Evidently, the Z-DNA conformation greatly hinders the opening of individual base pairs, but the precise factors involved in this are unknown. This suggests that at low temperature Z-DNA is stiffer than B-DNA, although the ^{31}P NMR data indicate flexibility in the backbone in both.[121,133]

3. Sequence Effects

It is interesting to note that all short (10 base pairs or less) oligo d(G-C) systems that have been demonstrated to adopt a Z conformation, either by X-ray diffraction[16,18,173] or by NMR criteria,[23,168] have a $d(C\text{-}G)_n$ repeat rather than $d(G\text{-}C)_n$ repeat. For example, both $d(C\text{-}G)_3$ and $d(C\text{-}G)_2$ crystallize in the Z-form, and $d(C\text{-}G)_8$ has been shown by NMR to adopt the Z-form in high salt solution.[60] Uesugi et al.[174] have presented CD and NMR evidence which suggests that the hexamer d(CGCGCG) and possibly the tetramer d(CGCG) also adopt a Z conformation at 4 *M* NaCl, but proof of this must await NOE studies. Surprisingly, there are no reports in the literature for the conversion of $d(G\text{-}C)_n$, for $n \leq 5$, to the left-handed form. It might be inferred from Pohl's earlier optical studies that $d(G\text{-}C)_n$ oligonucleotides,[175] produced by DNase I digestion of poly d(G-C), as short as $n = 3$ are converted to the Z-form. It should be noted, however, that Lomonossoff et al.[78] found that DNase I produces primarily $d(C\text{-}G)_n$ oligomers, and, thus, the oligomers studied by Pohl[175] probably contained mainly the $d(C\text{-}G)_n$ sequences. We recently

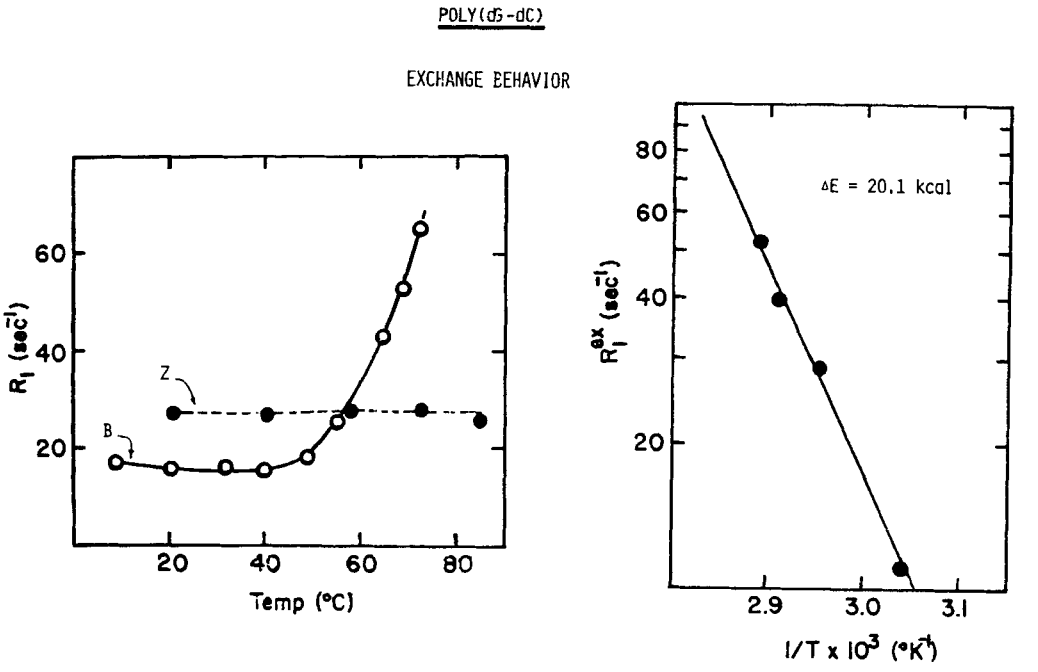

FIGURE 11. A comparison of the effect of temperature on the spin-lattice relaxation rates of the guanine imino protons in the B- and Z-forms of poly(dG-dC). The measurements on the B-form were obtained in a solution containing 0.1 *M* NaCl, pH 7, whereas the Z-form data were obtained using solutions containing 4 *M* NaCl, pH 7.[121,133]

studied the NMR properties of d(G-C)$_5$ and found it to be quite normal in low salt (0.1 *M*) conditions.[176] Even in high salt (5 *M*), this molecule appears to remain in the B-form even though longer and shorter sequence isomers, d(C-G)$_n$, adopt a Z-like conformation in high salt. Gorenstein et al.[177] noted that, by ^{31}P criteria, d(G-C)$_4$ does not convert to Z, and Breslauer et al.[178] found, by CD criteria, that d(G-C)$_3$ cannot be converted to Z-DNA, even at 5 *M* NaCl. This surprising difference in the behavior of the sequence isomers d(C-G)$_n$ and d(G-C)$_n$ indicates that terminal base pairs can have pronounced effect on the conformation of short oligonucleotides.

4. Other Sequences Which Adopt a Z Conformation

From an analysis of the Z-DNA crystal structure, it appears that alternating purine-pyrimidine sequences are probably required for a DNA to convert to the Z-form.[16,18] Poly(dA-dT) is, therefore, a reasonable candidate for such a conversion. Kypr et al.[179] and Patel et al.[180] have used both ^{1}H and ^{31}P NMR to investigate the effect of CsF on the conformation of poly(dA-dT). Under high salt conditions there is a splitting in the ^{31}P NMR, but in the *opposite* direction from that observed for poly(dG-dC), the higher field resonance in the low salt spectrum is displaced upfield by ~0.4 ppm. The behavior of the sugar H1′ protons in poly(dA-dT) is also different from that observed with poly(dG-dC), in that there is an upfield shift of one of the sugar resonances, whereas in poly(dG-dC) the salt-induced shift is in the opposite direction. The nature of the high salt structure of poly(dA-dT) is unknown, but it may be concluded on the basis of the above data that it is quite different from the Z-form of poly(dG-dC).[180]

Patel et al.[60] reported that poly(dI-br^{5}dC) exhibits an inverted CD spectrum at high salt, similar to that observed with poly(dG-dC), and suggested that this polymer also can adopt a Z conformation. Additional support for this was provided by Hartmann et al.[181]

who used CD, IR, and ^{31}P NMR. In particular, the ^{31}P spectrum of poly(dI-br^{5}dC) in low salt is a single line at 4.2 ppm, whereas in 3.9 *M* NaCl, a second resonance appears 1.6 ppm downfield from the original resonance position. The exchange of protons from the high salt form of poly(dI-br^{5}dC) is also dramatically slowed as it is in poly(dG-dC).[132,172] This collection of observations convincingly demonstrates that poly(dI-br^{5}dC), like poly(dG-dC), adopts a left-handed conformation in high salt.

Since the dinucleotide sequence m^{5}dC-dG frequently occurs in eukaryotic DNA, and since d(CpG) sequences are the major sites of methylation,[182] Behe and Fensenfeld[27] examined the effect of salt on the CD of poly(dG-m^{5}dC). They found that there is an inversion of the CD spectrum, similar to that observed with poly(dG-dC), but the transition occurs at lower salt levels (0.7 *M* instead of 2.5 *M*). Patel et al.[23] studied the NMR spectra of poly(dG-m^{5}dC) and observed changes in both the ^{31}P spectrum and proton spectra similar to those observed with poly(dG-dC) on going from 0.2 to 4.0 *M* NaCl. Furthermore, an NOE between guanosine H-8 and the H1′ sugar proton was observed in 1.5 *M* salt, consistent with a *syn*-glycosidic torsion angle at the guanosine residue. Chen et al.[102] used ^{31}P NMR to examine the effects of various salts ($MgCl_2$, NaCl, CsF, and $Co(NH_3)_6Cl_3$) on poly(dG-m^{5}dC) and found a good correspondence between the midpoints of the cooperative B to Z transition measured both by NMR and CD. In particular, they found that 0.1 m*M* $Co(NH_3)_6Cl_3$ is effective in converting 50% of this DNA to the Z-form, whereas slightly higher levels of $MgCl_2$ (0.5 m*M*) and much higher levels of NaCl or CsF (almost 1 *M*) were required to achieve 50% conversion from B to Z. In view of the correlation between gene activity and methylation at the C5 position[182] and the Z-antibody studies,[25,26] there is the real possibility that conversion to Z-DNA or other left-handed DNA structures may be important in gene regulation. A search for other sequences that can adopt a left-handed conformation and a delineation of the various factors which promote or disfavor the B to Z transition would be most worthwhile.

Arnott et al.[183] find from X-ray diffraction studies that fibers of poly d(dG-dC) occasionally adopt a conformation that they conclude is essentially the same left-handed double-stranded conformation observed in single crystal studies. They have also obtained a diffraction pattern with a sample of poly d(dG-dT)·poly d(dA-dC) which is similar to that obtained with poly d(dG-dC), indicating that it, too, may adopt a left-handed structure. Vorlivkova et al.[184] and Zimmer et al.[185] found that poly(dA-dC)·poly (dG-dT) exhibits a negative long wavelength CD band in the presence of high concentrations of CsF, and Zimmer et al.[185] demonstrated that an analogous transition can be induced in 60% ethanol and low ionic strength in the presence of micromolar amounts of Ca^{2+} ions. The suggestion that this might reflect a transition to a Z-like confirmation remains to be confirmed by NMR and other methods. One reason for not immediately accepting the CD evidence for a transition to Z-DNA is the disquieting observation of Vasmel and Greve[186] who found that the calculated CD spectrum of Z-DNA is opposite in sign to the experimental CD spectrum for authentic Z-DNA.

VI. CONCLUDING REMARKS

Most of this review was organized according to NMR techniques and nuclei, rather than particular systems or problems. It is, therefore, appropriate to summarize some of the important results which have come out of NMR studies of DNA during the past 3 years.

A. Internal Motions

NMR has helped changed our view about the flexibility and internal motions in DNA. Whereas studies of the macroscopic hydrodynamic behavior of DNA lead to a picture of

a DNA molecule which is relatively stiff over dimensions approaching 200 bp,[187-194] the persistence length, NMR, which is able to probe local internal motions, yields a somewhat different view. A combination of ^{13}C, ^{1}H, and ^{31}P studies all indicate that there are substantial (± 20 to $\pm 35°$), high frequency (0.3 to 2 ns) motions in different parts of the molecule (base planes, ribose group, phosphate group).[72,97-101,106,111,143-149,164] Proton NMR experiments on the exchangeable protons,[48-50,106,117-121,131,133] on the other hand, have provided information on the dynamics on a much longer time scale (ms) in the form of proton exchange rates with the solvent. There are, undoubtedly, motions with correlation times in the ns to ms range, but the particular NMR experiments carried out to date are not especially sensitive to them. If relaxation experiments were carried out at much lower magnetic fields even this time regime could be probed.[195] Comparison of the dipolar contributions to the lowfield relaxation properties of poly(dA-dT)[106,119] and poly(dG-dC)[121,133] does not indicate that there are pronounced sequence effects on the flexibility of DNA.

B. Base Pair Breathing

The NMR relaxation studies of proton exchange have also provided new insight into the mechanisms of base pair breathing in DNA and the factors affecting breathing rates. For A · T base pairs, the thymine imino exchange rates are primarily controlled by the rate of opening of the individual A · T base pairs.[48-50,106] This rate, which is more or less independent of the DNA sequence, is given by the expression $k_{ex} = 2 \times 10^{12} \exp(-16{,}000/RT)$. An apparent exception to this generalization is the observation that the rate of exchange of A · T base pairs in $d(CGCGAATTCGCG)_2$ is about three times slower than that which we would predict.[118] In very recent studies Patel et al.[196] report that the A · T exchange rates in the sequence isomer d(CGCGTATACGCG) are three times faster than in d(CGCGAATTCGCG). Although they[196] suggest this might be of some significance with regard to the biological role of the TATA box, we note that the observed rate for d(CGCGTATACGCG) is what we would consider "normal". In contrast with A · T pairs, the opening of G · C base pairs depends on the DNA sequence since opening of the weaker A · T base pairs affects the opening of adjacent G · C pairs. It is, therefore, not possible to write down a simple expression for the G · C opening rates which would apply to most DNA sequences.

A comparison of the proton exchange behavior of the B- and Z-forms of poly(dG-dC) illustrates the dramatic effects of conformation on exchange rates,[121] and this suggests that it would be very worthwhile to study A·T and G·C proton exchange rates in a variety of different systems.

C. Supercoiling Effects on DNA

^{31}P NMR studies of plasmid DNA have provided evidence for a remarkable coupling of torsional and bending motions in negatively supercoiled DNA which may have important biological implications.[150] Moreover, the substantial narrowing of both the ^{1}H and ^{31}P spectra that occurs on supercoiling may make it possible to extend many types of NMR measurement to DNA containing as many as 10,000 bp with resolution comparable to that obtained with 200-fold shorter, linear DNA. This would make it possible to examine the effects of supercoiling on various DNA properties such as base pair opening rates, metal ion binding, and drug-DNA interactions.

D. DNA in the Solid State

^{31}P NMR measurements on solid complexes of DNA have provided evidence for considerable motion in the DNA backbone at high temperatures in partially hydrated DNAs.[85] In dehydrated DNA, however, the backbone motions are frozen, even at high temperatures. The orientation of the phosphate groups in fibers of A-form DNA have

also been determined by ^{31}P NMR measurements and used to eliminate certain structures which have been proposed for the DNA.[82,83]

E. Non-Watson-Crick Pairs in DNA

NMR measurements, particularly NOE experiments on oligonucleotides,[118] have been especially revealing with regard to the effects of incorporating mismatched bases in the DNA helix. For example, NMR has been used to demonstrate that hydrogen-bonded G · T,[43] G · A,[44] and T · T[45] base pairs can exist within a regular double helix. Both the G · A and the T · T pairs are unexpected since they both require moderate perturbation of the helix to be accommodated. In every case, however, it is found that the incorporation of a non-Watson-Crick base pair, or extra bases, leads to a destabilization of the helix. It would now be interesting to apply the full range of relaxation techniques to some of these DNAs which contain non-Watson-Crick base pairs to see how they affect the details of the DNA structure.

F. Z-DNA

The ^{31}P and ^{1}H NOE[23] experiments along with Raman[22] experiments have provided the most definitive solution-state evidence for the existence of Z-DNA conformations in poly(dG-dC) and in $d(GC)_n$ segments imbedded within contiguous DNA regions which retain a right-handed conformation.[24] The NOE measurements demonstrating a *syn*-conformation of the G residues and the unusual lowfield peak in the ^{31}P spectra are two unmistakable signatures of Z-DNA.[23] The absence of these features in poly(dA-dT) and in poly(dA-dC) · poly(dG-dT) under high salt conditions indicates that neither of these two sequence adopts a Z conformation, although CD and X-ray diffraction data on fibers would seem to suggest otherwise.[179,183] The NMR data quite convincingly demonstrate, however, that poly(dA-dT) has an alternating conformation with a dinucleotide repeat.[180]

G. 2D NMR Studies of Decanucleotide

The application of two-dimensional NMR techniques to double helical DNA was first reported early in 1982.[130–132] The most extensive one- and two-dimensional NMR relaxation studies have been carried out on d(ATATCGATAT) and d(ATATGCATAT) with the following results. Modulations in the Hahn spin-echo experiments and direct measurements of splittings in well-resolved spectra established that most of the sugar residues in the molecules are predominantly in the S conformation. The NOESY experiments established that the A-H8 protons are located very close (comparable to the TH6-methyl distance) to methyl groups on adjacent (3′-5′) thymine residues, and that the purine H8 protons are located close to the 2′ sugar proton of the same nucleotide unit, but somewhat distant from the 2″ sugar proton of the adjacent (5′-3′) nucleotide. The pyrimidine H6 proton, however, is situated more nearly symmetrically between the 2′ sugar proton of the same nucleotide unit and the 2″ sugar proton of the adjacent (5′-3′) nucleotide. The absence of a first-order NOE between the aromatic protons (AH8, GH8, TH6, and CH6) and the H1′ sugar protons at relative short mixing times established that all bases are in the *anti*-conformation about the glycosidic bond. These features are all consistent with a B-like conformation, but not an A conformation.

Several other groups reported the results of preliminary 2D NMR studies on various DNA oligonucleotides at a recent meeting on NMR in Biology and Medicine,[196–198] and this work will presumably be published in the near future. The work of Hilbers[197] and Haasnoot[198] is especially notable since they were able to conduct NOESY experiments in H_2O solutions and were, therefore, able to monitor the exchangeable protons as well as the nonexchangeable protons.

Much of the information discussed in this review was obtained within the past year or so. In view of the availability of adequate amounts of short DNAs of various sequence, further improvements in NMR technology (pulse sequences, clever applications of older methods, instrument developments), and the application of a variety of new solid-state NMR techniques, there is every reason to expect extraordinary progress during the next couple of years. A more precise delineation of the conformations of individual sugar groups in DNAs containing ten or more base pairs should be available shortly. Qualitative information on internuclear separations in DNA are beginning to emerge. NOE and NOESY experiments place severe restrictions on the possible conformational states of the DNA. When quantitative distance determinations, obtained from selective and bi-selective relaxation experiments, are combined with information on sugar and phosphate conformations, it may be possible to deduce numerous structural features of the DNA. Perhaps then we will be able to find out what it is that is special about the various promotor sequences, the TATA box, and other sequences that are important in gene regulation.

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