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A 300- and 600-MHz Proton Nuclear Magnetic Resonance Investigation of a 12 Base Pair Deoxyribonucleic Acid Restriction Fragment: Relaxation Behavior of the Low-Field Resonances in Water†

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ABSTRACT: High-resolution (300- and 600-MHz) proton NMR spectra and 300-MHz relaxation data on the exchangeable imino protons of a 12 base pair (bp) restriction fragment are presented. Analysis of these results permits conclusions on the nature of internal motions in this short DNA helix, the nature of the various interactions which are responsible for the observed relaxation rates, and the mechanism of opening of A·T base pairs. By combining information on the thermal stability and the chemical shifts of the resonances with rudimentary ring current shift calculations, all A·T and G·C resonances in the low-field spectrum can be identified and tentatively assigned to specific base pairs in the molecule. Spin–spin (R_2) and spin–lattice (R_1) relaxation rates of the low-field resonances have been measured at a number of temperatures by using the long-pulse method. The low-temperature relaxation behavior of the low-field resonances can be accounted for theoretically in terms of a model in which the molecule is treated as a rigid rotor and in which the relaxation is entirely attributed to proton–proton and proton–nitrogen dipolar interactions. At 21 °C, we find good quantitative agreement between theory and experiment, and various intercomparisons of the observed and calculated relaxation rates and relaxation rate ratios serve to test different aspects of the theory. Under these conditions, theory predicts that the proton–nitrogen dipolar interaction makes no contribution to the spin–lattice relaxation rates but does contribute significantly to the spin–spin relaxation rates. At higher temperatures, transient opening of the base pairs, with the onset of exchange with the solvent protons, introduces an additional pathway for relaxation. At 38 °C, the highest temperature studied, the exchange mechanism dominates the relaxation of the A·T resonances and is responsible for half of the R_1 observed for the G·C resonances. Both the chemical shift data and relaxation measurements indicate that the opening of the A·T base pairs is faster than that of the interior G·C base pairs. The exchange rates for all of the A·T resonances are faster than for neighboring G·C base pairs, indicating that at 38 °C the T imino proton exchange with solvent occurs *without* opening of the neighboring G·C pairs. This implies that the transient opening of A·T base pairs is independent of the DNA length and sequence for any A·T base pairs not located at or near (within 3 bp) the helix termini.

High-resolution proton NMR has been widely used to investigate the properties of DNA molecules in solution, but most of these studies have been limited to measurement of the standard NMR parameters of chemical shift, intensity, and line width (Patel, 1975, 1978; Patel & Hilbers, 1975; Patel & Canuel, 1976; Kearns, 1977; Selsing et al., 1978; Early et al., 1977; Early & Kearns, 1979; Kan et al., 1975; Rill et al., 1980). Such studies are consequently limited in terms of the detailed structural information they can provide because of uncertainties in the theories used to interpret chemical shifts, the conformational state of DNA in solution, and the role of conformational fluctuations. Proton relaxation measurements, although virtually unused in this area of research, appear to have great promise in providing the detailed information about DNA structure and conformational fluctuations now required (Bolton & James, 1980; Hogan & Jardetzky, 1980; Early et al., 1980a,b; Broido & Kearns, 1980). In those cases where dipole–dipole interactions dominate, the relaxation rates vary

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as the inverse sixth power of the internuclear separations and therefore can be used to accurately determine a number of key interproton distances in the DNA molecule and, hence, its structure.

We recently presented (Early et al., 1980b) the results of a preliminary investigation of the relaxation behavior of the nonexchangeable and exchangeable protons in the 12 base pair (bp) restriction fragment with the sequence d(CCGCACTGATGG)·d(CCATCAGTGCGG). In the present paper, we have used the long-pulse method (Early et al., 1980) to carry out extensive measurements of both the spin–lattice and the spin–spin relaxation rates of the imino protons in this 12-bp fragment over a range of temperatures. The resonances arising from the imino protons in Watson–Crick base pairs are located in the low-field region (10–15 ppm) (Kearns, 1977), and in this short DNA, these resonances are well resolved. The resonances from A·T and G·C base pairs can be identified and tentatively assigned to specific base pairs in the sequence. Analysis of the relaxation behavior of imino protons is reasonably straightforward because all of the important internuclear distances are more or less fixed by the hydrogen-bonding geometry. Furthermore, the overall tumbling of the molecule is sufficiently fast that it dominates the effects of internal motion on the relaxation process, and these would have greatly complicated the analysis had they been important. Finally, the spatial separation between the imino protons and the sugar protons is large, and therefore conformational fluctuations which only involve the backbone do not influence the relaxation behavior of the low-field resonances.

The various processes which do affect the relaxation behavior of the low-field resonances have been examined, and we have developed a theory which quantitatively accounts for most of our observations (after correcting for exchange contributions) in terms of magnetic dipolar interactions. These results confirm our earlier suggestion (Early & Kearns, 1979) that conformational fluctuations involving reorientation of the base-pair planes are slower than 10 ns. A study of the temperature dependence of the relaxation provides insight into both the overall motions of the molecule in solution and, more importantly, into the mechanism of transient opening of base pairs in the DNA helix. This study lays the groundwork for more detailed studies of exchange mechanisms and the various factors (sequence, ionic strength, and pH) which affect proton exchange. These measurements also provide an important test of the theory which is currently being extended to treat the relaxation behavior of nonexchangeable protons in this and other molecules for the purpose of characterizing in more detail the structure of DNA in solution (Early et al., 1980a,b, 1981).

Materials and Methods

Instrumentation. Spectra (300 MHz) were obtained with a home-built FT spectrometer which utilized a Varian HR 300 magnet and cross-coil probe. In order to observe the low-field resonances from DNA and overcome the dynamic range problem arising from the intense water peak, we used the long-pulse FT method in which a weak (~151 mG) RF pulse was applied at 13.15 ppm (between peaks e and f in Figure 1) (Early et al., 1980a). At 300 MHz, the water resonance is located about 2500 Hz from the RF carrier, so a 90° pulse was about 380 μs and a 180° pulse was about 760 μs. The residual water signal was further attenuated with a Rockland Model 442 low pass filter in the audio stage of the receiver. Quadrature phase detection was used with a sweep width of ±2500 Hz to prevent the residual water signal from folding into the low-field spectral region. Resonance positions are in parts per million (ppm) downfield relative to the standard DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

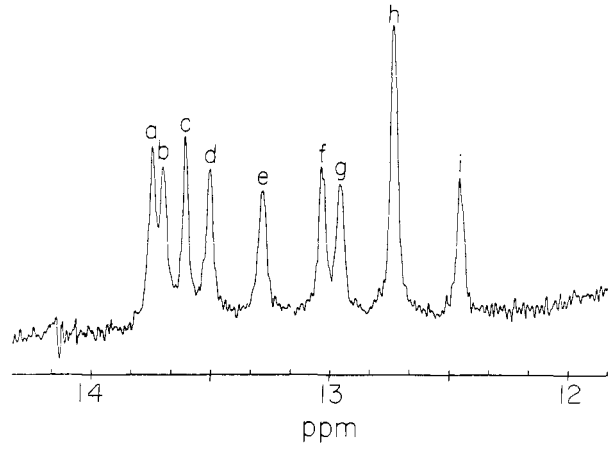

FIGURE 1: Low-field proton spectrum (600 MHz) of the 12-bp restriction fragment in H_2O. The sample contained 1.5 mg of DNA in 0.12 mL of water containing 0.35 M NaCl and 85 mM phosphate at pH 7.3.

Spin–lattice relaxation rates ($R_1 = 1/T_1$) were measured by using the standard (180°–τ–90°) pulse sequence whereas spin–spin relaxation rates ($R_2 = 1/T_2$) were determined by using the Hahn spin–echo method with a 90°–τ–180°–τ pulse sequence. For these experiments, 1000 FIDs were accumulated with a Nicolet 1180 computer with a pulse recovery time of ~2.0 s. In most cases, 80%–90% inversions were obtained for the T_1 experiments. The 600-MHz spectra were obtained at the Regional Instrument Center at Carnegie-Mellon Institute.

Sample. The preparation of the 12 base pair restriction fragment was described in the preceding paper in this issue (Hillen et al., 1981). For the NMR measurements, 1.5 mg was dissolved in 0.12 mL of solution containing 350 mM NaCl and 85 mM sodium phosphate at pH 7.3.

Results

(1) Low-Field (15–10 ppm) Spectrum at 600 MHz. The 600-MHz spectrum of the 12-bp fragment is shown in Figure 1. At 25 °C, this spectrum contains nine well-resolved resonances (labeled peaks a–i) with peak h containing twice the intensity of each of the other peaks. If each of the smaller peaks corresponds to a single proton, then peak h corresponds to two protons, and the total intensity corresponds to 10 protons. Since 12 resonances were expected for this 12-bp fragment, it is evident that resonances from two of the base pairs are absent at this temperature, and this is confirmed by a study of the temperature dependence of the 300-MHz low-field spectrum.

(2) Temperature Dependence of the Low-Field Spectrum at 300 MHz. The temperature dependence of the low-field spectrum obtained at 300 MHz is shown in Figure 2. With the assumption that peak i corresponds to one proton per molecule, the 5 °C spectrum corresponds to a total intensity of 12 protons per molecule (peak e contains 3.0 proton resonances). As the temperature is increased from 5 °C, most line widths decrease due to a reduction in viscosity and an increase in molecular tumbling rates, although a couple of resonances broaden on warming. At 18 °C, peak e has decreased from three proton resonances to one proton resonance, and by 38 °C, peak e and peak g have totally "melted", leaving seven resonance lines (eight proton resonances). The temperature dependences of the chemical shift of the nine resolved resonances are plotted in Figure 3. Since the 5 and 28 °C data showed a systematic error in chemical shifts (probably due

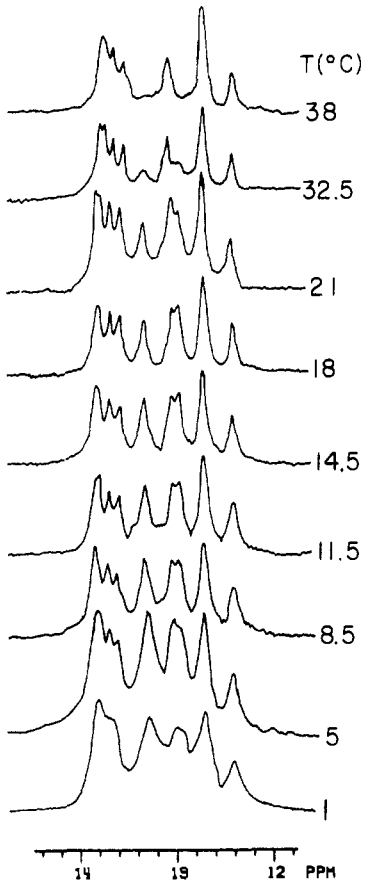

FIGURE 2: Temperature dependence of the low-field proton spectrum of the 12-bp fragment illustrating the selective loss of resonances. No attempt was made to adjust the relative intensity of the spectra to normalize the peak integrated area. The temperature, in degrees centigrade, is indicated to the right of each spectrum.

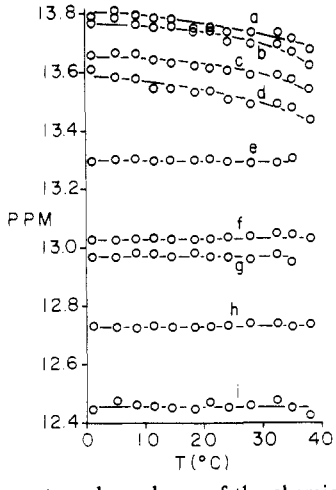

FIGURE 3: Temperature dependence of the chemical shifts for the nine low-field resonances of the 12-bp fragment. Chemical shifts are measured relative to DSS.

to an error in measuring the magnetic field offset), these data were corrected by shifting all values by 0.05 ppm to bring peaks e and g in line with the rest of the data. The four lowest field resonances (peaks a–d) show a gradual upfield shift with increasing temperature while the chemical shifts of the remaining resonances (peaks e–i) are independent of temperature.

(3) Temperature Dependence of the Relaxation Rates. Spin–lattice and spin–spin relaxation rates were measured by using the long-pulse method (Early et al., 1980). An example of the relaxation measurements carried out at 18 °C is shown

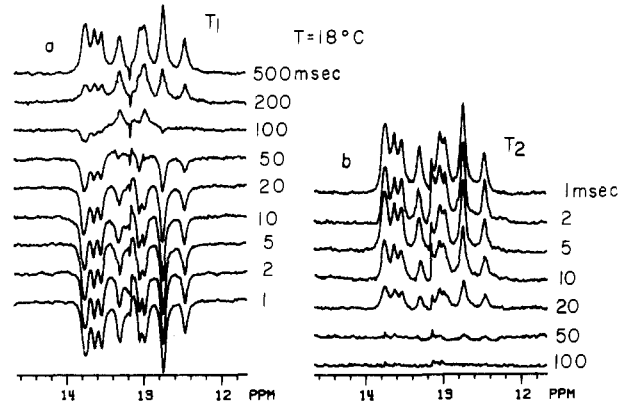

FIGURE 4: An example of spin–lattice and spin–spin relaxation measurement on the imino proton resonances in the 12-bp molecule is shown at 18 °C. For a discussion of experimental conditions, see the text.

Table I: Spin–Lattice Relaxation Rates (s^{-1}) for the Nine Low-Field Resonances of the 12-bp Fragment Given for 12 Different Temperatures[a]

	peak[b]								
temp[c]	a	b	c	d	e	f	g	h	i
1		11.8			40		18	16.1	14.5
5	7.3		8.0	8.4	27	14	13	12.2	13.3
8.5	5.7		6.4	7.1	18	9.2	10	8.8	9.8
11.5	5.0		5.7	6.2	15	8.7	10	7.9	8.4
14.5	4.5		4.9	5.9	13	7.4	9.4	6.6	6.0
18	4.3		5.2	5.8	14	7.3	11	6.3	5.7
21	4.2	4.4	5.4	5.9	17	7.0	13	5.4	5.1
24	4.2	4.9	6.3	6.5	25	6.8	19	5.0	4.7
28	4.7	5.9	7.6	7.4	30	6.6	32	4.7	4.1
32.5	7.2	8.6	10	11	90	7.8	55	4.6	3.9
35	8.6	13	14	15	125	7.1	100	4.8	3.9
38	12	13	18	18		7.8		5.1	3.9

[a] The observed values have an error limit of ±10%. [b] See Figure 1 and Table III. [c] Temperatures, in degrees centigrade, were measured before and after the relaxation measurements and were within 1 °C.

Table II: Summary of the Spin–Spin Relaxation Rate (R_2) Observed for the 12-bp DNA Fragment at Three Temperatures[a]

		A·T				G·C				
temp (°C)	peak:[b]	a	b	c	d	e	f	g	h	i
18		30	30	29	32	42	30	34	33	32
21		25	22	21	25	34	27	29	25	26
38		16	20	21	22		17		12	9.5

[a] See text for sample conditions. [b] See Figure 1 and Table III.

in Figure 4, and the rates obtained at various temperatures are summarized in Tables I and II. Spin–lattice relaxation rates (R_1) were measured for all nine resonances at 13 different temperatures, and these results are presented in Figure 5. The four lowest field resonances (a–d) have T_1 maxima close to 20 °C. The T_1 data of the remaining resonances (e–i) are shown in Figure 5B. The "early melting" peaks e and g have a T_1 maximum at about 15 °C, but the remaining peaks (f, g, and i) have T_1 maxima at about 35 °C. It is important to note that at low temperatures the four lowest field peaks a–d have longer T_1 values than the remaining peaks while above 30 °C they have a shorter T_1 than peaks f, h, and i. Figure 6 shows the same data plotted in the form of ln R_1 vs. the reciprocal of the absolute temperature, and from these curves it is possible to estimate the activation energies for processes which are involved in the high-temperature relaxation mechanism. Spin–spin relaxation rates (R_2) were measured at

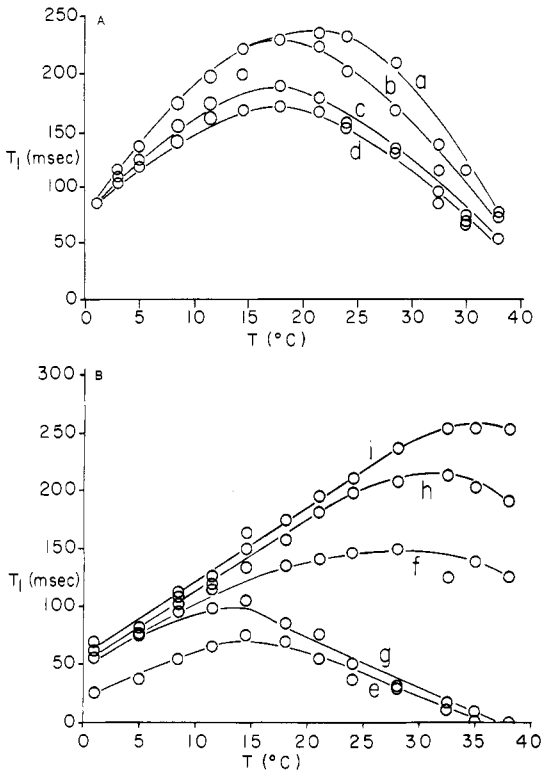

FIGURE 5: (A) T_1 values of the four lowest field (A·T) resonances a–d (see Figure 1) plotted vs. temperature. (There is no theoretical significance to the curves drawn through the points; however, for a discussion of the observed behavior, see the text.) (B) The T_1 values of the five G·C resonances e–i (see Figure 1) are plotted vs. temperature.

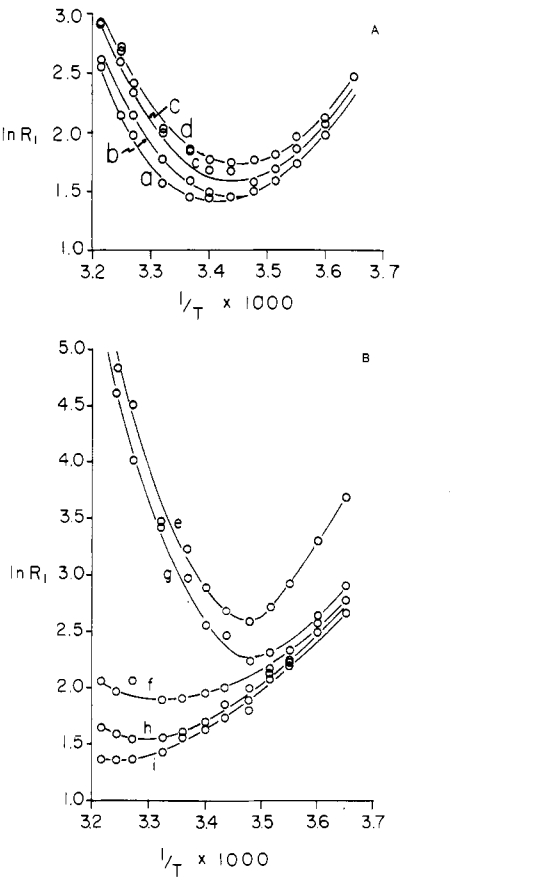

FIGURE 6: (A) In this representation, the natural log of R_1 of the A·T resonances a–d is plotted vs. the reciprocal of the absolute temperature. The limiting slope of the curves in the high-temperature region of the plot corresponds to an activation energy for exchange of 16 ± 1 kcal/mol. (B) The natural log of the R_1 of the G·C resonances e–i is plotted vs. the reciprocal of the absolute temperature.

several temperatures, and these data are summarized in Table II.

Discussion

(*1*) *Identification of the Low-Field Resonances.* The 600-MHz spectrum of the 12-bp molecule in the low-field region from 15 to 11 ppm downfield of DSS is shown in Figure 1. Resonances from A·T base pairs are generally located at lower fields than resonances from G·C base pairs (Early & Kearns, 1979; Early et al., 1977), and each type of resonance is displaced upfield from its intrinsic unshifted position (A·T° = 14.5, G·C° = 13.6 ppm; Kearns, 1977) by several factors, the most important of which is ring current shifts from neighboring bases. The 600-MHz spectrum at 25 °C contains nine individual resonances, and by inspection it is obvious that peak h corresponds to an intensity of two protons, if other peaks in the spectrum correspond to one proton. The total intensity in the low-field region therefore corresponds to 10 protons, instead of 12 as expected for a 12-bp helix. Since it is known from previous NMR studies (Patel & Hilbers, 1975; Kan et al., 1975) of short (4 and 6 bp) DNA and RNA helices that resonances from terminal base pairs are subject to "early melting" due to fraying at the ends of the helices, we attribute the missing two resonances to the two terminal G·C base pairs which are "melted out" at this temperature. This interpretation of the 600-MHz spectrum is confirmed by a study of the temperature dependence of the 300-MHz low-field spectrum. When the temperature is lowered to 5 °C, new intensity appears at 12.93 and 13.25 ppm, and an internal integration of the spectrum yields an integrated intensity corresponding to 12 protons as required (see Figure 2).

Tentative assignments of the low-field resonances to specific base pairs can be made on the basis of the temperature dependence of the spectrum and very rudimentary considerations of ring current shifts. Since melting of the helix should initiate at the two ends, the three proton resonances in peak e and one resonance in peak g, which are the first resonances to be lost on heating from 1 to 38 °C, are assigned to the two G·C base pairs at each end of the molecule (base pairs 1, 2, 11, and 12). Because base pairs 1 and 12 have only one nearest neighbor base pair, they are expected to resonate at lower fields than 2 and 11 and are therefore assigned to peak e. Base pair 2 has two G·C neighbors while base pair 11 has one A·T and one G·C neighbor. On this basis, we assign the higher field resonance g to base pair 11 and base pairs 1, 2, and 12 to the lower field resonance e (intensity 3 at 5 °C). Resonances a–d are too far downfield to be assigned to G·C resonances (intrinsic position 13.6 ppm) and therefore must be from the four A·T base pairs 5, 7, 9, and 10. Resonances from two of these A·T base pairs (9 and 10) are expected to be at higher fields because each has an A neighbor and, hence, experiences larger ring current shifts from the A base (Arter & Schmidt, 1976). Because the imino proton of thymine is displaced from the base-pair dyad axis, neighbor bases on the same strand as the thymine are the most important contributors to its total ring current shift in B form DNA. This implies that the resonance from base pair 10 is the most upfield-shifted A·T resonance (peak d) since T_{10} is located between an A and a G base. Similarly, base pair 9, which has an A neighbor to the T, is assigned to peak c. The least ring current shifted A·T base pair should be 7 (T is between G and C), leaving resonance b assigned to base pair 5 (this T is between two G's).

These assignments of the A·T base pairs seem to be sub-

Table III: Summary of the Proposed Assignments of Resonances in the Low-Field Proton NMR Spectrum of CCGCACTGATGG / GGCGTGACTACC

peak[a]	a	b	c	d	e	f	g	h	i
relative intensity	1	1	1	1	3	1	1	2	1
base-pair assignment	7	5	9	10	1, 2, 12	3	11	4, 6	8

[a] Peak designation in Figure 1.

stantiated by the 28 °C relaxation data (Table I) where we find $R_1(d) \simeq R_1(c) > R_1(b) > R_1(a)$. At this elevated temperature, exchange of the imino proton of A·T base pairs is beginning to dominate the T_1 process (see below), and therefore T_1 should be correlated with base-pair stability. With the assumption that the molecule melts from the ends (fraying), the more stable base pairs will be located further from the helix ends, and these base pairs should have longer T_1 values at the high temperature. This leaves peaks f and i and two resonances in h to be assigned to base pairs 3, 4, 6, and 8. Of these four base pairs, only base pair 3 has no A·T neighbor, and it is assigned to peak f, the lowest field resonance from an internal G·C base pair. This assignment is also substantiated by the fact that it has the shortest T_1 of the interior G·C pairs at 30 °C and is the earliest melting of the four G·C resonances. Peak i is the highest field G·C resonance observed and therefore assigned to base pair 8 since it is the only G·C base pair with two A·T neighbors. This leaves peak h which is assigned to base pairs 4 and 6. A summary of the proposed assignments is presented in Table III. Although the A·T and G·C resonances can be identified, the proposed assignments to specific base pairs should be considered tentative.

(2) *Temperature Dependence of the Chemical Shifts.* The effect of temperature on the chemical shifts of the low-field resonances is shown in Figure 3. The G·C resonances are invariant over this temperature range whereas all A·T resonances shift upfield by about 0.15 ppm. The fact that the chemical shifts of the G·C resonances do not change indicates that the average conformational state of the helix is little changed over this same temperature range. Since A·T base pairs are intrinsically less stable than G·C pairs, the temperature effect on the A·T resonances could be due to a temperature effect on the average hydrogen bonding properties of the A·T base pairs. Specifically, if the imino hydrogen bond to T were transiently disrupted without formation of a new hydrogen bond with water, the T imino resonance would shift upfield to perhaps 7.5 ppm or higher (Katz & Penman, 1966). If we assume that the interconversion between the fully hydrogen-bonded state and the "cracked" state is very rapid, then the equilibrium between the closed and the "cracked" state will determine the chemical shift of the A·T resonances. To the extent that this process only involves single base pairs, one would expect to find similar temperature effects on the chemical shifts of A·T resonances in other DNAs. If this interpretation is correct, then the temperature effects on the chemical shifts of A·T resonances may provide a monitor of the very early stages in premelting.

(3) *Analysis of the Relaxation Rates.* The temperature dependence of the spin–lattice relaxation times and rates for all nine peaks in the low-field spectrum are shown in Figures 5 and 6, respectively. Qualitatively, one observes that as the temperature of the sample is raised from 1 °C, the T_1 for most resonances initially increases (R_1 values decrease), reaches a maximum at some temperature which depends upon the specific resonance under consideration, and then finally decreases as the temperature approaches 40 °C. The temperature dependence of the relaxation rates can be qualitatively understood in the following manner. Well below the melting temperature of the helix, the proton relaxation rates are determined by magnetic dipolar interactions, and these rates decrease with increasing temperature because of a decrease in solvent viscosity and an increase in the molecular tumbling rate. However, at higher temperatures, the imino proton becomes susceptible to exchange with the water due to transient opening of the base pair, with the result that its relaxation rate increases as exchange with solvent begins to dominate the relaxation. At the temperature where T_1 is maximum (R_1 is minimum), the contributions to relaxation from dipole–dipole interactions (magnetic interactions) and exchange are comparable. For this 12-bp molecule, the T_1 maxima for A·T and G·C base pairs occur at about 20 °C and above 35 °C, respectively. We now present a brief review of the theory describing the magnetic contributions to the relaxation rates as it applies to this system and compare the theoretical predictions with the experimental results.

(a) *Theory of Magnetic Dipole Contributions to Relaxation.* Consider the relaxation behavior of two spins, I and S. For a *selective* spin relaxation measurement in which a π pulse is applied to just the I spins, the *initial* rate for the relaxation of I spins, R_1, due to proton–proton dipolar interactions with S is (Abragam, 1978)

$$R_1 = \frac{\gamma_I^2\gamma_S^2\hbar^2S(S+1)}{12r^6}[J_0(\omega_I - \omega_S) + 18J_1(\omega_I) + 9J_2(\omega_I + \omega_S)] \quad (1)$$

where r is the I–S internuclear separation, γ_I and γ_S are the magnetogyric ratios of the I and S spins, and $J_n(\omega)$ is the spectral density at frequency ω resulting from molecular motions which modulate the I–S interaction dipolar interaction. If more than one spin contributes to the relaxation of the I spin, the total relaxation rate will be the sum of contributions from each spin (assuming no cross-correlation for their motion).

The analogous equation for the dipolar contributions to the spin–spin relaxation rates is

$$R_2 = \frac{\gamma_I^2\gamma_S^2\hbar^2S(S+1)}{24r^6}[4J_0(0) + J_0(\omega_I - \omega_S) + 18J_1(\omega_I) + 36J_1(\omega_S) + 9J_2(\omega_I + \omega_S)] \quad (2)$$

To apply these equations, it is necessary to specify all relevant internuclear vectors and to develop a model to describe the way in which neighboring nitrogen and proton nuclei move relative to the proton of interest. Initially we make the following assumptions. (i) The overall motion of the DNA helix can be characterized by two rotational correlation times, τ_s, the correlation time for axial rotation, and τ_1, the correlation time for end-over-end tumbling, and we assume these can be calculated by the Perrin formulas (Perrin, 1934). (ii) The important interproton and proton–nitrogen distances are taken to be those shown in Figure 7. Interactions between imino protons in adjacent base pairs are neglected because they make negligible contributions to R_2 for A·T base pairs and small (~10%) contributions to R_2 for G·C base pairs. (iii) Internal motions of the base pairs are assumed to be of sufficiently small amplitude and low frequency that the relaxation processes can be treated by a rigid rotor model. (iv) Chemical shift anisotropy and scalar interactions are assumed to make negligible contributions to the relaxation of the imino protons.

With these assumptions, the theory developed by Woessner (1962) can be used to calculate the spectral densities, $J_n(\omega)$,

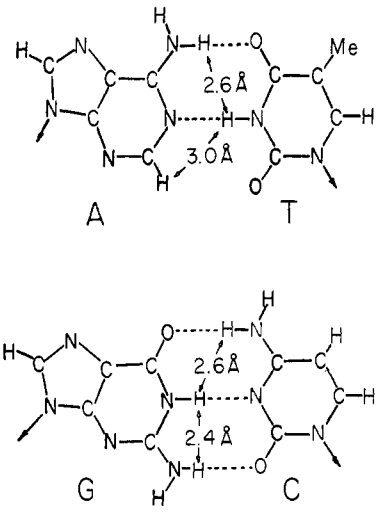

FIGURE 7: The A·T and G·C base-pair geometry is illustrated, and the protons which are closest to the imino proton of the T and G base are indicated. The interproton distances (in angstroms) were measured from a Dreiding model.

needed in eq 1 and 2 and the corresponding relaxation rates. The final expressions for the spectral densities are

$$J_n(\omega) = 2K_n\left[A\frac{\tau_a}{1+\tau_a^2\omega^2} + B\frac{\tau_b}{1+\tau_b^2\omega^2} + C\frac{\tau_c}{1+\tau_c^2\omega^2}\right] \quad (3)$$

where

$$\tau_a^{-1} = 6D_1 \qquad \tau_b^{-1} = D_s + 5D_1 \qquad \tau_c^{-1} = 4D_s + 2D_1$$
$$(\tau_1 = 1/6D_1,\ \tau_s = 1/6D_s)$$

and

$$A = \tfrac{1}{4}(3\cos^2\theta - 1)^2 \qquad B = 3\cos^2\theta\sin^2\theta$$
$$C = \tfrac{3}{4}(\cos^2\theta - 1)^2$$

and

$$K_0 = \tfrac{4}{5} \qquad K_1 = \tfrac{2}{15} \qquad K_2 = \tfrac{6}{15}$$

θ is the angle between the unique axis of the molecule and the internuclear vector. Since only the closest nuclei are an important source of relaxation, due to the r^{-6} dependence of the relaxation rates, there will be only three significant contributions to the nuclear relaxation rate of G·C imino protons, including two hydrogen-bonded amino protons in the same base pair at 2.4 and 2.6 Å and the guanine imino nitrogen at 1.0 Å. The closest magnetic nuclei to an imino proton of an A·T base pair are a hydrogen-bonded amino proton at 2.6 Å, a carbon-bound A (H-2) proton at 3.0 Å, and a thymine imino nitrogen at 1.0 Å. For B-form DNA, all of these interactions will have $\theta = 90°$. Note, however, that when τ_1 and τ_s are close in magnitude (i.e., approaching isotropic rotor), the calculated relaxation rates are insensitive to the value of θ. For spin–spin relaxation rates, there will be additional contributions from interactions with adjacent imino protons in neighboring base pairs. The exact distance between two imino protons of neighboring bases in DNA is not known due to the uncertainties of the helical geometry of the DNA, but they cannot be closer than 3.4 Å at $\theta = 0°$. This represents the upper limit to the magnitude of the imino–imino interaction. It is probably much less for A·T imino protons because the imino proton is located off from the dyad of the base pair, and this increases the imino–imino distance beyond 3.4 Å and makes θ nonzero.

Table IV: Comparison of Predicted and Observed Relaxation Rates for a 12-bp DNA Fragment

rate	obsd[a]	calcd
R_1(A·T)	5.0 (2.0 ± 0.5)	1.5
R_1(G·C)	5.3 (4.3)	3.0
R_2(A·T)	23.3 (19.8)	21
R_2(G·C)	25.3 (24)	25
R_1(G·C)/R_1(A·T)	2.3 ± 0.5	2.0
R_2(G·C)/R_2(A·T)	1.2	1.2
R_2(A·T)/R_1(A·T)	~12	14
R_2(G·C)/R_1(G·C)	5.7	8.5

[a] Measurements are at 21 °C. Values in parentheses have been corrected for exchange with water and are uncertain due to the large exchange contribution (3 ± 0.5 s^{-1}) for A·T and ~1 s^{-1} for G·C. A·T values were averaged from data for peaks a–d while G·C values were the average of peaks f, h, and i.

(*b*) *Comparison between Theory and Experiment.* The theory outlined above only applies to magnetic dipolar contributions to the relaxation rates, yet exchange of the imino protons with solvent can make significant contributions, particularly at higher temperatures. Therefore, for comparison of the theoretical and experimental relaxation rates, the experimental rates have to be corrected for the exchange contribution. At 21 °C, for example, we estimate (see below) that the exchange contribution to relaxation of the A·T imino proton relaxation is about 3.5 s^{-1} whereas, for the G·C imino proton, it is on the order of 1 s^{-1}. Since a range of relaxation rates was observed for the different A·T and G·C resonances at any given temperature, we used average values to compare with theory, and these results are shown in Table IV for data obtained at 21 °C.

A number of interesting comparisons between theory and experiment are possible, and each tests different aspects of the theory. Since all of the relaxation rates vary approximately linearly with the rotational correlation times, the ratio of any pair of rate constants should be approximately independent of the absolute value of τ_1 and τ_s. The prediction that R_1(G·C)/R_1(A·T) $\simeq$ 2 results from the fact that two protons are located closer to the imino proton in the G·C base pair than in the A·T base pair, and experimentally we find R_1(G·C)/R_1(A·T) $\simeq$ 2.0 ± 0.5. The much smaller value predicted for the ratio R_2(G·C)/R_2(A·T) (theory = 1.2, experiment = 1.2) is due to the fact that the nitrogen dipolar contribution to the relaxation is large in both cases. Other relaxation rate ratios also show good agreement between experiment and theory. For prediction of the absolute magnitudes of the relaxation rates, the rotational correlation times need to be specified. For the 21 °C data, we find that use of an apparent solvent viscosity of 1.35 cP corresponding to τ_1 = 6 ns and τ_s = 4 ns leads to an excellent agreement between experiment and theory.

The 24 °C relaxation data can be accounted for by using a viscosity of 1.2 cP corresponding to τ_1 = 5.2 ns and τ_s = 3.6 ns. Since the simple rigid rotor model which we have used to compute relaxation rates gives a good account of the 20–25 °C data, there is no need to include any contribution from internal motions of the base-pair planes. For example, if there were large amplitude (±20°) fast (1 ns) motions of the planes of the base pairs, this would have reduced the effective rotational correlation times to about 3 ns (Baldo et al., 1979). The fact that the apparent rotational correlation times are much larger at low temperatures (see below) is even stronger evidence against internal motions of the base pairs. The present results therefore support our earlier suggestion that the motions of the base-pair planes are relatively restricted on the 10-ns time scale (Early & Kearns, 1979).

One final point to be noted is that the good agreement between theory and experiment depends critically upon the use of the hydrogen-bonding distances shown in Figure 7. As we noted, spin–spin relaxation rates are primarily determined by dipolar interactions with the attached nitrogen atom, and if a value significantly (10%) different from r_{NH} = 1.0 Å is used, the predicted nitrogen contribution would change by almost a factor of 2. This does not, however, rule out the possibility of tautomeric states in which there is a double proton transfer between bases involving both the imino proton of one base and an amino proton of the complementary base. However, if unusual tautomeric states of the bases were involved, Figure 7 indicates that the imino proton in an A·T pair would be located only 2.4 Å away (instead of 3.0 Å) from the A H_2 and, hence, would be predicted to have a spin–lattice relaxation rate greater than for the normal G·C tautomer. If the G·C pair were also in an unusual tautomeric state, its relaxation rate would be even smaller. The experimental results rule out both these unlikely possibilities and support the widely held belief that the bases are in their usual tautomeric states.

(*c*) *Temperature Dependence of the Relaxation Rates.* (*i*) *Evidence for Intermolecular Association at Lower Temperatures.* Upon cooling from 21 to 1 °C, all spin–lattice relaxation rates increase. If we assume the DNA geometry remains unchanged over this temperature range, as is indicated by the absence of any temperature effect on the chemical shifts of the G·C resonances, then the large increase in the relaxation rates observed going from 21 to 1 °C has to be attributed to an increase in the rotational correlation times. A small part (~7%) of this change is due to the intrinsic temperature dependence of the rotational correlation time in a solution of constant viscosity (Perrin, 1934), but a more important contribution arises from the ~2-fold increase in the intrinsic viscosity of water between 21 and 1 °C (Hardy & Cottingham, 1949). In this way, about 30% of the observed low-temperature effect on the relaxation rates can be accounted for. The remainder of the temperature effect on the relaxation rates is undoubtedly due to temperature effects on the intermolecular associations which are more pronounced at the lower temperatures. At the relatively high concentrations used in the NMR experiments, it is not surprising to find evidence for intermolecular association of short DNA helices.

The fact that rotational correlation times longer than 20–30 ns have to be used to account for the low-temperature relaxation data indicates that internal motions of the DNA involving reorientation of the planes of the bases relative to the helical axes either are of very low amplitude, such as to be unimportant, or are slower than 20 ns. This conclusion fits well with our earlier suggestion that substantial reorientation of the base-pair planes arising from bending of the DNA takes place in about 100 ns (Early & Kearns, 1979). It does not agree with the suggestion of Hogan & Jardetzky (1980) that the planes of the bases experience large internal motions (±20°) with a correlation time of 1 ns. A longer correlation time for the internal motion of the bases is also indicated by recent studies of higher molecular weight DNA restriction fragments (Early et al., 1981).

(*ii*) *Exchange Contributions to Relaxation.* The relaxation rates obtained at the higher temperatures are dominated by proton exchange with the solvent and therefore provide an excellent method for studying transient opening of base pairs in DNA. In previous NMR studies (Patel & Hilbers, 1975; Kan et al., 1975) of the low-field resonances of DNA and RNA helices, the kinetics of the melting process have been inferred from line-width measurements by assuming that the observed line width, $\Delta\nu_{1/2}$, is related to the relaxation rate through the expression

$$\Delta\nu_{1/2} = R_2/\pi \qquad (4)$$

Even though well-resolved resonances are obtained with the 12-bp molecule, the observed line widths (~20 Hz) are over a factor of 2 larger than those calculated by using the R_2 data. Thus, there could be errors in using the observed line-width data to compute exchange rates. Furthermore, R_1 values are much more sensitive to exchange contributions than R_2 rates because they are typically 5–10 times smaller than the corresponding R_2 values. Therefore, in the present experiments where we are able to directly measure both R_1 and R_2 for each imino proton in the molecule, we obtain a more complete description of the transient opening of the base pairs than could be obtained from line-width and chemical shift data.

When a base pair in a helix transiently opens, the imino proton responsible for one of the low-field resonances may exchange with solvent protons and, hence, shorten both the T_1 and T_2 values for that resonance (Hilbers, 1979). Whether or not exchange occurs every time a base pair opens depends upon the rate the imino protons exchange with water while the base is in an opened state and on the lifetime of the opened state (Patel & Hilbers, 1975). The recent studies (Mandal et al., 1979) of exchange in the poly(A)·poly(U) system indicate that exchange of the uridine imino protons occurs every time the base pair is in the opened state. Furthermore, NMR studies of the nucleosides indicate that the exchange of the G and U imino protons is base catalyzed with a diffusion-controlled rate constant for OH^- (McConnell, 1978). If we assume that the rate of exchange of the T imino protons in DNA is also limited by the rate of opening of A·T base pairs [as in poly(A)·poly(U)], then a measurement of T_1 under conditions where relaxation is exchange limited provides a direct measure of the rate at which A·T base pairs in the helix transiently open. In the low-temperature region where magnetic interactions dominate, the ratio $R_2/R_1 > 5$, but when exchange begins to dominate, this ratio approaches the theoretical limit of 1.0 (i.e., $R_1 = R_2$). Thus, the temperature effect on the R_1/R_2 ratio provides an additional criterion for determining when exchange makes a major contribution to R_1.

We now consider the specific behavior of selected resonances in the low-field spectra. The terminal G·C base pairs (1 and 12) and their neighboring base pairs (2 and 11) should be the first to melt, and it was on this basis that resonances e and g were assigned to these four base pairs. Resonances from the remaining four internal G·C base pairs are affected by exchange only at the highest temperature studied, 38 °C, at which point $R_2/R_1 \simeq 2.3 \pm 0.1$. The variations observed in relaxation rates at 38 °C of these four G·C resonances are attributed to the different locations of the corresponding base pairs within the helix.

The effect of temperature on the four A·T resonances is most interesting. At 38 °C, we find that the R_1 values for the four A·T base pairs are *larger* than those for the four most stable G·C base pairs which flank them. While one could argue that the assignments of the resonances to specific A·T or G·C base pairs may not be correct, the inescapable conclusion is that at 38 °C the R_1 values for the A·T base pairs (at this temperature almost entirely due to exchange) are significantly larger (factor of 4) than those for the G·C base pairs which surround them. For example, the R_1 value for $A{\cdot}T_7$ is 13 s^{-1} whereas the corresponding opening rates of the neighboring $G{\cdot}C_{6,8}$ base pairs are only 2 and 3 s^{-1}, respectively. Similar considerations apply to the three other A·T resonances—each

has a relaxation rate which is *larger* than that of either of its neighboring G·C base pairs and larger in fact than their combined rate. If we assume that exchange of imino protons occurs every time a base pair opens, we conclude that exchange of A·T imino protons in this molecule occurs via a mechanism which only involves opening of a single A·T base pair *without opening neighboring G·C base pairs*. One consequence of this conclusion is that we predict that the opening rate for A·T base pairs in all DNA should be more or less independent of sequence and helix length (for DNA larger than 12 bp) and, hence, all DNA should exhibit the same opening rates for A·T base pairs. Experiments confirming this prediction are described in the following paper in this issue (Early et al., 1981). We note that the intrinsic opening rate which we deduce for an A·T base pair at 38 °C (15–20 s^{-1}) corresponds closely with the value obtained for the poly(A)·poly(U) system using stopped-flow techniques (Mandal et al., 1979). Furthermore, the activation energy for the exchange process, which can be derived from the NMR data shown in Figure 6, is about 15–17 kcal/mol compared with the value of 15 kcal found for poly(A)·poly(U). In view of the close correspondence between the rate constant and activation energy which we measure for the transient opening of the A·T base pairs in the 12-bp fragment and the corresponding values observed for poly(A)·poly(U), we are inclined to conclude that the mechanisms are also the same. Teitelbaum & Englander (1975) had previously proposed a traveling loop model to account for the mechanism for base-pair opening, but in their later work (Mandal et al., 1979), they suggest that a mechanism involving opening of single base pairs might be reasonable. Our results on the 12-bp fragment which convincingly demonstrate that a single base opening mechanism is operative in DNA support their more recent suggestion regarding the base-pair opening mechanism. Nakanishi & Tsuboi (1978) report a value of 0.13 s^{-1} at 25 °C for poly(I)·poly(C) which, if it applied to the DNA system, would make a negligible contribution to the relaxation rate of G·C pairs at 38 °C.

Summary

This paper presents the first detailed description of the relaxation behavior of the low-field resonances in a short (12-bp) DNA helix. The fact that there is good agreement between the measured rate constants and those calculated by using a rigid rotor model indicates that the major molecular motions contributing to the proton relaxation arise from the overall tumbling of the molecule and that internal molecular motions are relatively unimportant. This good agreement further indicates that we have properly accounted for the various interactions that contribute to the relaxation process and that the theory can therefore be considered calibrated. The fact that internal motions in the 12-bp DNA fragment are not important in relaxing the low-field resonances agrees with conclusions drawn from our earlier studies on line-width measurements on a series of higher molecular weight DNA (Early & Kearns, 1979) and the new results obtained on 43- and 69-bp DNA restriction fragments (Early et al., 1981). In higher molecular weight DNA, we expect to find discrepancies between theory and experiment when the amplitudes and rates of internal motion (bending and twisting) become important relative to the overall molecular tumbling rate. Measurements on the imino proton may prove especially helpful in analyzing the relaxation behavior of the nonexchangeable protons which probe other aspects of the DNA structure in solution. From measurements on the relaxation behavior of the imino protons, the overall rotational correlation time of the molecule can be determined, thereby reducing the number of unknown parameters that appear in the relaxation expressions. Furthermore, our studies demonstrate that there is relatively little motion of the planes of the bases relative to the helix axis, at least on the time scale of 10–100 ns, and this serves to limit the types of internal molecular motion that might be important in the relaxation of the nonexchangeable base protons.

In the higher temperature range (30–40 °C), exchange of the imino protons with solvent dominates the relaxation of the A·T resonances, and these relaxation data provide information on the mechanism by which the imino protons exchange with the solvent. Because two of the four A·T base pairs in this molecule are sandwiched between G·C base pairs which have slower exchange rates, we are able to conclude that the exchange of the imino protons occurs as a single base-pair phenomenon, without opening of the neighboring base pairs. An important implication of this observation is that the rate of opening of A·T base pairs in high molecular weight DNA should be similar to that observed in the short DNA, and results presented in the following paper (Early et al., 1981) confirm this expectation. In future studies, we shall report on an examination of the various factors which influence the exchange properties of the A·T and G·C imino protons of A·T and G·C base pairs in DNA.

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A 300-MHz Proton Nuclear Magnetic Resonance Investigation of Deoxyribonucleic Acid Restriction Fragments: Dynamic Properties†

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ABSTRACT: The 300-MHz relaxation behavior of the imino protons in Watson–Crick base pairs has been investigated in two DNA restriction fragments 43 and 69 base pairs (bp) in length over temperatures ranging from 18 to 59 °C. These results, in conjunction with data obtained on a 12-bp fragment, provide information on the nature of internal motions in the DNA and on the mechanism by which imino protons exchange with the solvent. Because the correlation time for axial rotation is much shorter than the correlation time for end-over-end tumbling, theory shows that the relaxation behavior of the imino protons is relatively insensitive to torsional modes but is very sensitive to end-over-end tumbling and bending modes. The "effective" end-over-end rotational correlation times required to fit the NMR relaxation data, 85 and 226 ns, are considerably shorter than the values, 150 and 490 ns, calculated for rigid cylinders 43 and 69 bp in length, respectively. The short effective rotational correlation times in these DNA are attributed to elastic bending modes which reorient the planes of the bases with respect to the helix axis. At elevated temperatures, the spin–lattice relaxation rates are dominated by exchange with the solvent, and measurements in this region can be used to investigate various aspects of the mechanism of breathing. The combined studies of the 12-, 43-, and 69-bp DNA demonstrate that the exchange of the T imino protons occurs by a single base-pair opening mechanism which is little affected by the DNA length or the neighboring base sequence. The activation energy of the process, 15.7 kcal, is relatively high and can probably be attributed to steric restraints imposed by the backbone. The close correspondence between the results obtained with the DNA restriction fragments and those obtained with poly(A)·poly(U) suggests that a common breathing mechanism is involved.

Proton nuclear magnetic relaxation techniques have been widely used in the investigation of the structures and conformational dynamics of small molecules (Noggle & Schirmer, 1971; Zens et al., 1976), but until recently they have been little used in studies of DNA. The few proton studies that have been reported (Early & Kearns, 1979; Hogan & Jardetzky, 1979, 1980) have been limited to studies of the relaxation behavior of the nonexchangeable protons using D_2O as the solvent. In recent papers (Early et al., 1980a,b), we demonstrated that the long-pulse method can be used to measure the spin–lattice and spin–spin relaxation rates of exchangeable protons in H_2O. In the preceding paper (Early et al., 1981), where we studied a 12-bp DNA restriction fragment (sequence shown in Table I), we have shown how measurements on the relaxation behavior of the imino protons in Watson–Crick base pairs can provide insight into the static and dynamic properties of the DNA double helix. The low-temperature spin–spin and spin–lattice relaxation rates in this 12-bp fragment are well predicted by using a relaxation theory in which the DNA helix is treated as a rigid rotor, indicating that insofar as relaxation of the low-field resonances is concerned, this short DNA fragment can be considered to be rigid on a time scale of 10 ns. However, in studies of larger DNA molecules (Early & Kearns, 1979; Early et al., 1980a), we found evidence, based on line-width considerations and relaxation measurements, for flexibility in the DNA indicating reorientation of the base-pair planes with time constants shorter than 200 ns. Consequently, one goal of the present study was to examine intermediate-sized molecules to determine where internal motions in the DNA begin to be important relative to the overall tumbling of the molecule.

A second point of interest in these studies concerns the mechanism by which A·T and G·C base pairs open and allow the imino protons to exchange with the solvent. These data are of particular interest in connection with theoretical studies of DNA melting (Tong & Battersby, 1979; Azbel, 1980) and may be important to the high-temperature flexibility of DNA (Godfrey & Eisenberg, 1976). Information on this point can be obtained from measurements of the relaxation rates of the imino protons obtained at sufficiently high temperatures so that exchange contributions to the relaxation are important.

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