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Interaction of actinomycin D, ethidium, quinacrine daunorubicin, and tetralysine with DNA: ^{31}P NMR chemical shift and relaxation investigation

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ABSTRACT

The binding of actinomycin D, ethidium, quinacrine, daunorubicin, and tetralysine to DNA has been investigated using ^{31}P NMR. Titration of DNA with actinomycin yields a new downfield peak or overlapping peaks as would be expected from the slow dissociation kinetics of this compound. The other intercalators shift the DNA ^{31}P signal downfield as a single exchange averaged peak. Tetralysine causes a slight upfield shift. The chemical shift titration curves for the intercalators are sigmoid curves suggesting that cooperative processes or competing effects on the chemical shift are being observed. The magnitude of the chemical shift change at saturation of DNA with the compounds is found to vary significantly and to be linearly related to the DNA base pair unwinding angle for the compounds. Analysis of ^{31}P spin lattice relaxation times (T_1) and linewidths as a function of temperature (below Tm) and titration with the above compounds indicates that T_1 does not change significantly while linewidth increases with decreasing temperature and increasing bound intercalator. One interpretation of these results is that in both cases the overall motion of DNA becomes slower while the internal motion is not greatly affected.

INTRODUCTION

NMR studies on DNA were initially confined to denatured or low molecular weight model systems (1-3). Recently, it has been shown that sonicated or nuclease treated short double helical DNA samples can be analyzed by NMR (see for example 3-9). Because of the potential sensitivity of ^{31}P chemical shifts to phosphate bond and tortional angles, this method is a valuable addition to techniques for monitoring conformational changes in DNA (9). We have shown that the DNA chemical shift is sensitive to temperature (4), interaction with simple metal species (10), platinum antitumor agents (10), and intercalating drugs (11, 12). Temperature (4) intercalating drugs (11, 12), platinum antitumor agents (10), and $HgCl_2$ (10) cause downfield shifts in the DNA ^{31}P signal while magnesium and calcium ions cause upfield shifts (10). The DNA ^{31}P linewidth and T_1 values also are sensitive to temperature and complexing agents (4, 10-12).

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The intercalating drugs, $HgCl_2$, and simple metal ions shift the DNA ^{31}P signal as a single peak and do not give any new signals, suggesting that these small molecules are in fast or perhaps intermediate exchange among DNA phosphate binding sites. The covalent binding antitumor agent cis-dichloro diamine-platinum (II), however, gives a new downfield peak, in addition to slight shifts of the main ^{31}P signal, on reaction with DNA (10).

In the work reported here we extend our ^{31}P NMR observations on the interaction of intercalating drugs with DNA to include effects of drug to DNA ratio, drug structure, temperature, exchange rate, and base pair composition of the DNA.

MATERIALS AND METHODS

Ethidium bromide (11, 12), quinacrine (13), and daunorubicin (14) were prepared and characterized as previously described. Actinomycin D from Sigma and tetralysine from Vega were used as obtained. Calf thymus DNA was from Worthington while M. lysodeikticus DNA was obtained from Sigma. Preparation of low molecular weight (approximately 200 base pair) DNA by sonication has also been previously described (12). Briefly this involves high salt-low temperature pulsed sonication, filtration, and ethanol precipitation of the DNA. This DNA is redissolved, phenol and ether extracted, and reprecipitated with ethanol. The redissolved, dialyzed sample is then characterized by polyacrylamide gel electrophoresis against ϕX 174 and λ restriction fragments, Tm, spectrophotometric and NMR measurements. Sonication for short periods gives very heterogeneous samples but sonication to low molecular weight under our conditions gives more homogeneous preparations as the low molecular weight limit is approached. Further fraction of this sample by gel exclusion chromatography to produce a more homogeneous molecular weight distribution had little effect on the ^{31}P NMR results (12). All samples were dialyzed against one of the following buffers: PIPES 00 (0.01 M piperazine-N,N'-bis[2-ethanesulfonic acid]; 0.001 M EDTA; pH 7); PIPES 10 (PIPES 00 with 0.1 M NaCl); or PIPES 20 (PIPES 00 with 0.2 M NaCl).

For NMR experiments DNA samples were lyophilized and redissolved in the same volume of 99.8% D_2O containing 0.01% trimethylphosphate as an internal standard. Spectra were accumulated on a JEOL FX 60 Q NMR spectrometer with quadrature detection at 24.15 MHz with 0.025 M DNA phosphate using 10 mm NMR tubes. Typically 5000 scans were obtained with fast Fourier transformation of 8192 time domain points, a 90° pulse, 13 sec delay time, broad band proton decoupling, and 0.5 Hz line broadening. Temperature was maintained with a

JEOL NM 5471 variable temperature controller. Spin-lattice relaxation times were determined by the inversion recovery method and data were analyzed by standard linear least-squares methods with software supplied with the instrument by JEOL. Nuclear Overhauser effects (NOE) were determined using the gated method to maintain proton decoupling (8). Other conditions are described in the Figure legends.

RESULTS

Ethidium Bromide. The ^{31}P NMR chemical shift and line width of our calf thymus DNA preparation do not change significantly from PIPES 00 to PIPES 20 buffer at 30° C. The effect of ionic strength on the ethidium induced ^{31}P NMR chemical shift of calf thymus DNA is shown in Figure 1. As can be seen ionic strength changes, at this DNA concentration, have little affect on the titration curve. It is interesting that the chemical shift titration curve is somewhat sigmoid in shape. The ethidium binding constant in all of these

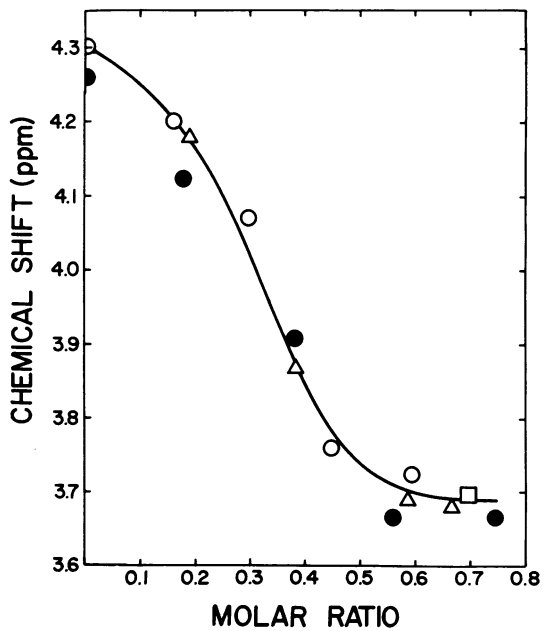

Figure 1: The effects of ethidium bromide on the ^{31}P chemical shift of calf thymus (open symbols) and M. lysodeikticus (filled circles) DNA samples are shown as a function of the molar ratio of ethidium to DNA base pairs. The buffers are PIPES 10, △ and ●; PIPES 20, ○, and a saturation point is shown for PIPES 00, □.

buffers is greater than 10^5 (15) indicating that up to a molar ratio of 0.4 essentially all of the added ethidium is bound to DNA at these concentrations. The curvature and leveling of the plot at higher ratios is due to an increasing amount of free ethidium as the saturation limit of 0.5 is reached. The fact that all ionic strengths give approximately the same limiting chemical shift at high molar ratio suggests that outside binding is not having any significant effect on these results. Increasing or decreasing the DNA concentration by a factor of two had no significant effect on the results shown in Figure 1. Results are also shown in Figure 1 for a similar titration of sonicated Micrococcus lysodeikticus DNA with ethidium. The initial chemical shift of this high G·C DNA shows a reproducible downfield shift of approximately 0.05 PPM with respect to calf thymus DNA samples prepared in an identical manner. The effect of ethidium on the linewidth of both DNA samples is also quite similar and shows an approximately linear increase to approximately 45 to 50 Hz at near saturation levels at 30°C.

As shown in Figure 2, increasing the temperature of a saturated ethidium-

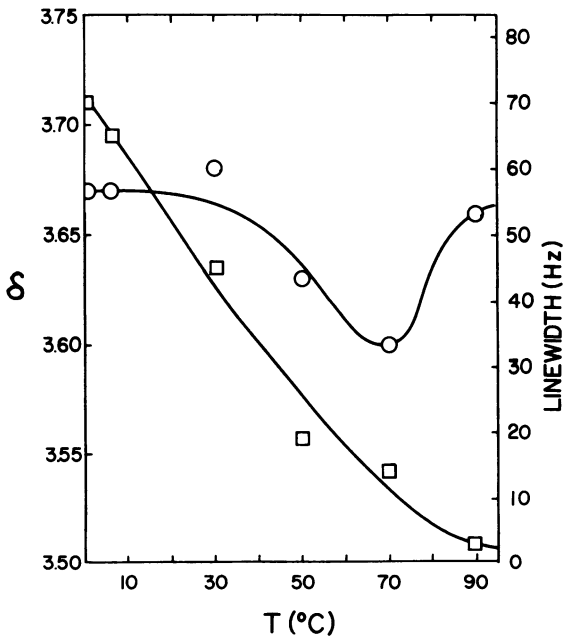

Figure 2: The effects of temperature on the ^{31}P chemical shift, δ (○), and linewidth (□) are shown for a saturated ethidium-DNA complex (molar ratio = 0.7) in PIPES 00.

DNA complex has a very small effect on the chemical shift. Since denatured species of DNA formed at high temperature have chemical shifts near those of the ethidium-DNA complex (4), this is to be expected. The relatively constant chemical shift over this temperature span indicates that under these conditions, ethidium remains intercalated up to the point of denaturation of DNA. The linewidth of this complex is also shown in Figure 2 as a function of temperature. There is a dramatic and continuous decrease in linewidth of this sample up to approximately 90° C.

The ^{31}P spin lattice relaxation time (T_1) of DNA first decreases slightly, then increases markedly as the denaturation temperature is approached. The ^{31}P T_1 of our DNA sample, for example, is 2.2, 1.9, and 3.9 sec at 30, 50, and 70° C respectively. The saturated ethidium-calf thymus DNA complex at those same three temperatures has a T_1 of 2.3, 2.0, and 1.7 sec respectively. The Tm of the DNA alone under the low ionic strength NMR conditions is approximately 65° C while the saturated ethidium-DNA complex has a Tm near 80° C. These results show that the ^{31}P T_1 of both free DNA and the ethidium-DNA complex gradually decrease as the temperature is increased below the Tm. As the DNA begins to unfold, the mobility of the single strands is much greater than the double helix and the T_1 increases markedly. Adding ethidium at 30° C in a titration to saturation, as in Figure 1, gave no change in the DNA ^{31}P T_1 within experimental error (±10%). The NOE of the DNA phosphate groups in this sample at 30° C is 1.3 (also ±10%) and also did not change within this error limit in a titration to saturation with ethidium.

Quinacrine, Daunorubicin, Tetralysine.

As shown in Figure 3, the intercalating drugs quinacrine and daunorubicin also produce downfield shifts in the DNA ^{31}P signal but the magnitude of the shifts is quite different from each other and from the shift produced by ethidium (reproduced in Figure 3 for reference). The titration curve for quinacrine is sigmoid in shape as for ethidium. Because of the smaller total shift produced by daunorubicin, it is more difficult to specify the curve shape but as seen in Figure 3, it also seems to be somewhat sigmoid. Both daunorubicin and quinacrine produce line broadening which increases as a function of intercalator concentration in a manner very similar to ethidium. The DNA ^{31}P NMR linewidth at 30° C increases approximately linearly in a titration and is 45 to 50 Hz at saturating levels of all three of the intercalating compounds. The DNA ^{31}P T_1 and NOE values, as with ethidium, do not change significantly on titration to saturation with quinacrine and daunorubicin at 30° C. In addition, increasing the temperature of the

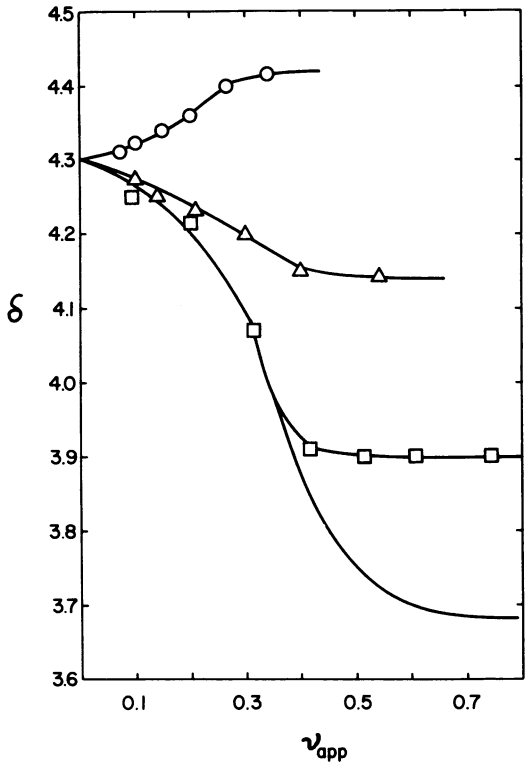

Figure 3: The ^{31}P chemical shift of DNA is plotted as a function of molar ratio (ν_{app}) of compound added per DNA base pair for tetralysine (○), daunorubicin (△), and quinacrine (□). The curve for ethidium (lower solid line) from Figure 1 is reproduced for reference.

quinacrine and daunorubicin-DNA complexes results in significant line narrowing as with ethidium. Simple lysine peptides are known to bind strongly to DNA at low ionic strength, primarily through an electrostatic interaction with the DNA phosphate groups (16, 17). The addition of tetralysine to DNA causes a slight upfield shift of the DNA ^{31}P NMR signal as shown in Figure 3. Over this concentration range, tetralysine did not significantly affect the T_1 and linewidth of the DNA phosphate groups. Similar effects are seen on titrating DNA with magnesium ions (10).

Actinomycin

It is known that the kinetics of interaction of actinomycin derivatives with DNA are much slower than for other intercalating compounds (18, 19). On adding actinomycin D to calf thymus DNA, a separate broad downfield ^{31}P NMR

signal (or signals) appears. The main phosphate signal does not shift significantly but does broaden extensively on addition of actinomycin. These effects are illustrated in Figure 4 with a spectrum for an actinomycin-DNA complex at 30° C and a molar ratio of 0.27. Uncomplexed DNA and a saturated ethidium-DNA complex are included in Figure 4 for reference. At this ratio of actinomycin to DNA, the actinomycin binding sites are essentially saturated (18). The main (largest) peak in the actinomycin-DNA spectrum has a chemical shift of 4.31 PPM and a linewidth of approximately 36 Hz at 30° C. On heating the sample, this peak shifts downfield and narrows. For example its chemical shift is 4.28, 4.16, and 4.11 PPM at 40, 50 and 60° C respectively. The linewidths are 30, 23, and 20 Hz at these same three temperatures. The low-field shoulder at 30° C resolves into a separate broad peak or probably overlapping peaks centered near 2.4 PPM at 60° C.

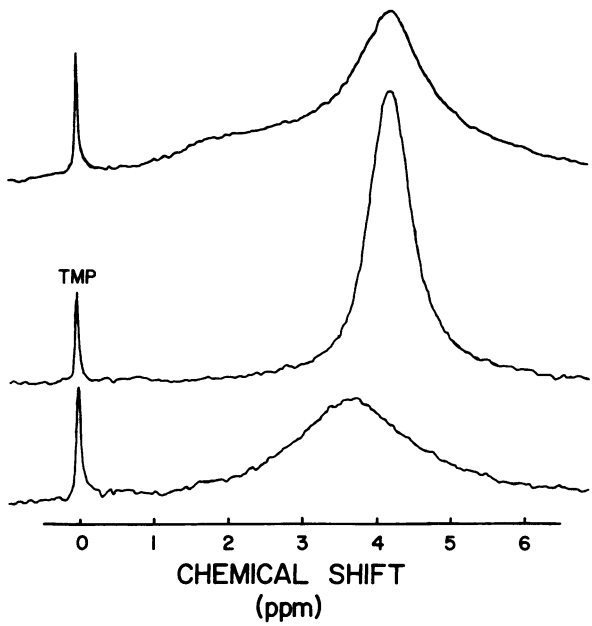

Figure 4: A ^{31}P NMR spectrum for an actinomycin-DNA complex (ν_{app}=0.27) in PIPES 00 at 30° C (top curve) is compared to spectra obtained under similar conditions for DNA alone (middle spectrum) and an ethidium-DNA complex (ν_{app}=0.7, lower spectrum). Chemical shifts are upfield from trimethylphosphate (TMP).

DISCUSSION

The sigmoid shape of the DNA ^{31}P NMR titration curve with intercalating ligands is quite apparent (Figure 3). This curvature at low ν values suggests that the first molecules to intercalate have a slightly different effect on the DNA structure than those added later. The smaller ^{31}P shift could indicate a reduced conformational effect of these first molecules or structural changes which result in competing changes in ^{31}P chemical shift. It is interesting to note that recently Winkle and Krugh (20) with intercalating drugs and Crothers and co-workers (21, 22) with outside binding ligands have suggested that cooperative conformational effects occur in DNA at low ν values. At higher ν values they find that DNA-ligand effects can be explained more closely by classical intercalation or outside binding predictions. It seems possible that ^{31}P chemical shifts are also sensitive to these initial structural variations which occur in binding many cationic compounds to the DNA double helix. At higher molar ratios in the ^{31}P NMR titration, the chemical shift levels off, as expected, due to saturation of binding sites. Titration of the G·C rich M. lysodeikticus DNA with ethidium yields results which are quite similar to those obtained with calf thymus DNA, suggesting that specific interactions at the DNA sugar-phosphate backbone, and not base pair composition are the significant factors in the sigmoid shape of the ^{31}P titration curves. This argument is supported by the fact that the DNA ^{31}P linewidths for ethidium, quinacrine, and daunorubicin increase approximately linearly and about the same amount in a titration. This suggests that the chemical shift depends more directly on conformation while the linewidth is sensitive to the amount of bound intercalator.

The magnitude of the DNA ^{31}P chemical shift at saturating levels of the intercalating drugs depends strongly on the ligand structure and apparently very little on the type of DNA used (Figure 1). The ligand induced ^{31}P chemical shift changes at saturation are in the same order as the DNA unwinding angles for these compounds. In Figure 5, the chemical shifts changes for these intercalators are plotted directly against unwinding angles measured using closed circular superhelical DNA titrations (23, 24). Tetralysine is included in Figure 5 as a reference for the ^{31}P chemical shift expected for site binding of a cation directly at DNA phosphate groups without intercalation. Although the unwinding angle of tetralysine is graphed as zero, some nonintercalating cations can actually cause the double helix to wind by a small amount (0.5 to 1.0°) (see 25 for example). A shift of the tetralysine unwinding angle one degree to the left (DNA winding) would not significantly

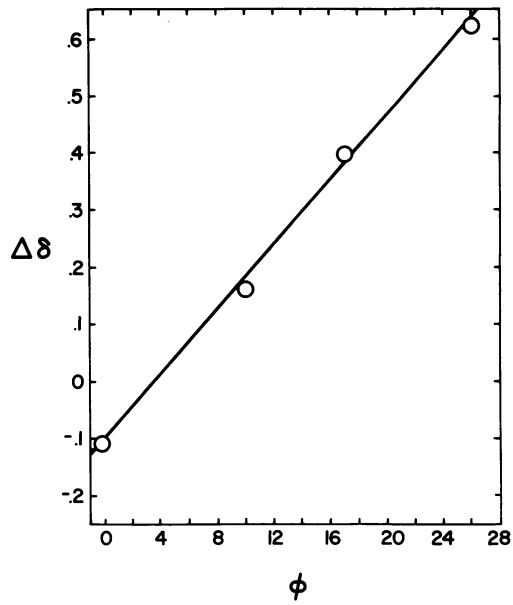

Figure 5: The chemical shift change ($\Delta\delta$) of the DNA ^{31}P signal on titration to saturation is plotted versus the unwinding angle, ϕ, for ethidium (ϕ=26°), quinacrine (ϕ=17°), daunorubicin (ϕ=10°), and tetralysine (ϕ~0°).

change the correlation of the results with intercalating drugs. Davanloo and Crothers (17) found upfield shifts of up to one PPM when adding tetralysine to $d(pA)_3pGpC(pT)_3$. At lower temperatures they found the magnitude of the upfield shift was reduced and they concluded that at least part of the upfield shift could be due to stabilizing the complementary oligonucleotide double helix relative to single strands. Lerner and Kearns (26) have pointed out that ^{31}P chemical shifts in nucleotides are quite sensitive to hydrogen bonding, with downfield shifts occurring when the phosphate groups are in better hydrogen bonding solvents. Since the phosphate groups are exposed to water in the free and intercalated state, it seems unlikely that the shifts observed are due to increases in solvent hydrogen bonding. All of these results suggest that the intercalation conformational change, resulting in unwinding of the double helix, provides the most direct explanation for the observed ^{31}P chemical shift changes.

As shown in Figure 4, actinomycin behaves quite differently than the other intercalating drugs. Since actinomycin has a high G·C specificity, the broad downfield signal or signals obtained must be due to phosphate groups at intercalated G·C base pairs. These signals are shifted farther downfield than

ethidium which has an unwinding angle similar to actinomycin (24). This result is expected since even at saturation, the chemical shift for ethidium represents the exchange-averaged value for ethidium intercalated at only half the phosphates in DNA. Since actinomycin is in slow exchange, however, the actual chemical shift for a phosphate group at the intercalation site should be observed. If this shift is twice the value for ethidium, a signal around 3 PPM would be obtained. Figure 4 suggests that with actinomycin, there may even be peaks below 3 PPM. The complex peptide chains of actinomycin, which contribute to its slow binding kinetics, may also be having an influence of the phosphate group chemical shifts. Patel (27 and references therein) has shown that actinomycin D when complexed with several short double helical oligonucleotides gives two new phosphate signals near 1.5 and 2.5 PPM (referenced to TMP). Patel has interpreted these two new peaks as representing phosphate groups on opposite sides of the intercalation site. Although he does not find significant broadening of the phosphate peaks with the double helical oligonucleotides, there is an increase in the chemical shift dispersion of the uncomplexed phosphate groups which do not show large shifts when actinomycin D binds (27).

The peak at 4.3 PPM in the actinomycin spectrum in Figure 4 represents phosphates at uncomplexed G•C and A•T base pairs which are in slow exchange or which are not exchanging (A•T/A•T sites for example) with phosphates at bound sites. The broadening of this peak at 4.3 PPM could be due to an increased dispersion of chemical shifts at uncomplexed sites due to neighbor effects, to a reduced mobility of the DNA molecule with actinomycin bound which also affects unbound phosphate sites, or to some combination of these effects. The broadening which occurs with ethidium could be due to these factors and possibly an intermediate rate of exchange among ethidium binding sites. Based on ethidium-DNA temperature-jump kinetic studies (28, 29), however, ethidium should be in fast exchange at this magnetic field strength. The reaction kinetics of quinacrine with DNA are faster than with ethidium (30) while the daunorubicin kinetics are slower (31). All three of these intercalators give similar ^{31}P line broadening of DNA, again suggesting that exchange kinetics is not an important factor in the linewidth for these complexes. This suggests that ethidium, quinacrine, and daunorubicin are in the fast exchange region while actinomycin is in slow exchange at this temperature and magnetic field strength. It, thus, seems probable that chemical shift dispersion and correlation time changes account for the line broadening obtained with all of the intercalating compounds we have examined. Even well below the Tm, the

single signal obtained with ethidium, quinacrine, and daunorubicin and the largest signal in the actinomycin-DNA complex narrow significantly as the temperature is increased. It is unlikely that significant changes in chemical shift dispersion of the DNA phosphate groups occur over this temperature range. This leaves changes in correlation time as the most significant cause of the broadening which occurs when all of these intercalators are added to DNA at temperatures below 60° C. The narrowing of the phosphate signals in these complexes as the temperature is increased would then be due to temperature induced reductions in correlation time as occurs with DNA itself (4).

There are several models which have been developed for analysis of DNA motion using relaxation rates and NOE values (reviewed in 32). Unfortunately the NMR results are not yet sufficiently detailed to provide a unique fit to a particular model (32). A characteristic feature of these models, as with simple isotropic motion, is a broad minimum in the T_1 versus correlation time plots (see 8 for example). The value of T_1 and NOE for our sample suggests that the correlation time for the ^{31}P groups in this DNA is near this minimum region. This can explain both the effects of adding ethidium and of increasing temperature on this sample. The DNA motion is reduced as ethidium is added, but the T_1 does not change significantly, as would be expected at the minimum, while the linewidth increases markedly. Although the isotropic model for internal rotation is oversimplified, it can provide a framework for evaluating these results. Near the minimum for T_1, the T_1 is fairly insensitive to changes in overall motion of the DNA molecule, while the linewidth is quite sensitive to these changes (8). Slowing of these overall molecular motions (such as bending motions) on addition of ethidium would result in an increase in linewidth with very little change in T_1. In the same manner, heating the sample below the Tm would result in increased motion with decreased linewidths and little change in T_1. It is interesting to note that Levy et al (33) have found similar effects in a ^{13}C NMR analysis of T_1 and linewidth values on heating DNA. A decrease in overall molecular motion of DNA is consistent with hydrodynamic results obtained with ethidium and other intercalating compounds on titration of the DNA (23) and is consistent with changes expected in DNA as a result of intercalation (34). Evaluation of ^{31}P relaxation in DNA-intercalator complexes at several field strengths and under a variety of conditions should provide a much better model for the double helix dynamics.

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