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NMR Investigation of DNA Conformational Changes on Base Protonation: Use of Cu^{2+} and Pyrazole as Probes[†]

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ABSTRACT: In order to evaluate models for the acid denaturation of DNA and to assess the potential importance of protonated bases in mutations and gene expression, an NMR investigation of DNA and nucleotides in the pH range 7–2 has been conducted. The changes in the imino proton spectral region are readily observed and quite dramatic on lowering pH. At pH 7.0, calf thymus DNA has imino proton signals for AT (13.6 ppm, 56% area) and GC (12.6 ppm, 44% area) base pairs but no peaks in the 10–12 ppm region. At pH 5 a broad peak(s) between 10 and 11 ppm was (were) observed, and it narrowed and shifted to 10.9 ppm at pH 3.2. The original GC area was lost by pH 3.2 while the AT area was reduced by 50%. Below pH 3 the remainder of the AT signal was lost, and the area of the 10.9 ppm peak increased. Over this pH range the aromatic proton signals of DNA sharpened, and the cytosine amino proton signals in DNA narrowed and shifted downfield. Addition of pyrazole in the pH 4–6 range caused broadening of the new resonance but had very little effect on the original signals. Addition of Cu^{2+} in the pH 4–6 range resulted in a large loss in area of the GC and the new upfield peak(s). However, at lower pH, the upfield peak was not totally broadened by Cu^{2+}. At pH below 7, the broad ^{31}P signal of calf thymus DNA shifted slightly downfield and sharpened. This last result is inconsistent with a previously proposed model in which the deoxyguanosine adopts a syn conformation. The new results on DNA are explained by an alternate model in which initial protonation of C in the pH 5–7 range leads to the formation of non-base-paired CH^+ and G. For such a disrupted base pair, the G imino proton resonates at approximately 10.6 ppm, and the G is probably stacked in the double helix but with enhanced breathing motions. The pK_a of C in the DNA is 3.7. *At pH below 5*, there is continued protonation of C, initiation of protonation of A (pK_a = 3.2), and shifting of the new peak from 10.6 to 10.9 ppm, indicating more severe disruption of the duplex.

Deoxyribonucleic acid (DNA)[1] can adopt several conformations depending on environmental conditions such as salt, temperature, pH, and solvent [Saenger (1984) and references cited therein; Bloomfield et al., 1974]. Considerable effort has been expended to characterize these conformational states, to elucidate their interactive effects with binding agents, and to determine their biological significance.

Thermal denaturation of DNA is generally well understood as a transition from double-helical to single-stranded DNA (Bloomfield et al., 1974). The effects of increasing salt concentration and GC base pair composition are known to stabilize the helix and raise the melting temperature (T_m) at pH 7. The effects of acid-induced unfolding of DNA are less well understood than, for example, the salt-induced B to Z transition and the thermal denaturation of DNA. Spectrophotometric studies have shown that addition of acid to neutral DNA solutions (pH 3–5) results in a partially protonated helix (Cavalieri & Rosenberg, 1957; Dove et al., 1959; Peacocke & Preston, 1958; Geiduschek, 1958). The T_m of the protonated helix is decreased and much less sensitive to GC content than the T_m of the helix at pH 7 (Zimmer & Venner, 1966).

It is important to investigate the nature of the protonated DNA duplex for several reasons: (1) pH is an important effector and probe of DNA structure; (2) low pH has been used in numerous physical studies of DNA and its interactions with other molecules (Lyamichev et al., 1985; Banville et al., 1985; Chen, 1984, 1983, 1986; Atwell et al., 1985; Capelle et al., 1979); (3) the H^+ species is a simple and well understood electrophile that has proved useful for comparisons with other electrophiles such as alkylating agents and metal species (Marzilli et al., 1980); (4) protonation of the bases is one of the principal initiators of depurination and hence of mutation (Drake et al., 1983); (5) protonation appears to be involved in formation of mutations inducing A-C mismatched base pairs (Sowers et al., 1986). Chen (1984), for example, has shown that the kinetics of the B to Z transition are facilitated at low pH with a rate about 1 order of magnitude faster at pH 3.8 than at pH 7.0. Also, DNA binding studies of agents such as diacridines have been carried out in a pH 5.0 solution to enhance solubility and binding to DNA (Capelle et al., 1979). In the case of pyrene binding to DNA, at pH <7, the base pair specificity is altered (Chen, 1983). Finally, it is known that long homopurine–homopyrimidine sequences often flank genes in eucaryotic DNA, and they are thought to play a role in gene structure and expression (Lyamichev et al., 1983). Recently, it has been found that these sequences undergo a transition to a new conformation (H conformation) (Lyamichev et al., 1983). Unlike the B to cruciform (Lyamichev et al., 1983) and B to Z interconversion (Lee et al., 1979; Chen,

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[1] Abbreviations: CD, circular dichroism; CMP, cytidine 5′-monophosphate; dGMP, deoxyguanosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; HHW, NMR signal line width at half-height; DNA, deoxyribonucleic acid; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; PIPES, 1,4-piperazinediethanesulfonic acid; UV, ultraviolet.

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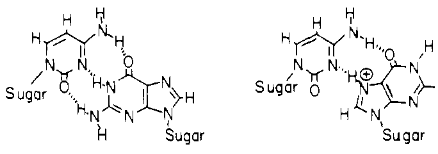
Chart I



Watson & Crick Hoogsteen

1984), the B to H transition is dramatically enhanced by lowering the pH (Lyamichev et al., 1985). Protonation of cytosine appears to be essential for this transition, which may be involved in the regulatory control of these sequences even at pH 7 (Lyamichev et al., 1983).

Some controversy remains over whether the DNA double helix can affect the site and order in which the bases of helical DNA are protonated (Chen, 1984; Zimmer et al., 1968; Courtois et al, 1968; O'Connor et al., 1981). For example, using CD and ORD measurements of DNAs differing in base composition and of the mononucleotides, Guschlbauer and co-workers (Courtois et al., 1968) argued that the pK_a values from the mononucleotides could not be the same as those for the bases in DNA. They based this argument on a comparison of CMP with one pK_a value to poly(C), which has several apparent pK_a values for protonation of N-3. Their hypothesis was that, in DNA, G(N-7) was first protonated followed by conversion of G from the anti to syn conformation and subsequent formation of a Hoogsteen base pair with C (Chart I). Since CMP has a pK_a of about 4.2 at N-3, they argued that this Hoogsteen base-paired GH$^+$C was stabilized through sharing of the G(N-7) proton with C(N-3).

Several other groups attributed the UV, CD, and ORD changes on lowering the pH primarily to initial protonation of C(N-3) and with subsequent protonation of A(N-1) (Dove et al., 1959; Zimmer et al., 1968; Zimmer & Venner, 1966). Similar conclusions were reached by O'Connor et al. (1981) in a recent study using Raman difference spectroscopy. Unlike the UV, CD, and ORD methods, Raman spectroscopy can differentiate spectral changes due to base protonation from those due to base stacking and DNA conformational changes. O'Connor et al. (1981) compared the Raman difference spectra of calf thymus DNA (CT DNA) with that of four mononucleotide bases and concluded that C and A of CT DNA are protonated first, with apparent transition pH values of about 4.1 for C and 3.3 for A. Protonation of the DNA at C and A was accompanied by a conformational change away from the typical B DNA. By pH 4.09, no B-form DNA was detected. Additional DNA denaturation was observed at pH <3, which coincided with the protonation of G(N-7) with an apparent pK_a of 2.6.

To address these questions of DNA base protonation and associated conformational changes, we have prepared NMR-size DNA (ca. 200 bp) (Wilson et al., 1985) and carried out the first ^{31}P and ^{1}H NMR studies of DNA at acidic pHs. ^{31}P NMR spectroscopy allows us to follow conformational changes in the phosphate backbone of DNA (Chen & Cohen, 1984) and to address questions such as whether the guanosine unit of protonated DNA is in the syn or in the anti conformation. ^{1}H NMR spectroscopy of the exchangeable imino protons of DNA provides a direct probe of base pairing and an indirect probe of base stacking and base protonation. Such studies can provide information on the degree of acid denaturation, on the sequence of base pair protonation, and on the type of the hydrogen bonding of protonated base pairs (e.g., Hoogsteen vs. Watson–Crick). Our major ^{1}H NMR observation is that a new upfield imino ^{1}H signal is present in the spectrum of CT DNA at pH values below 6. As a consequence, we have carried out the first systematic study on the chemical shifts and exchange characteristics of the imino protons of TMP and cyclic GMP as a function of pH. We have also explored the use of Cu^{2+} and pyrazole as paramagnetic and exchange-broadening agents, respectively.

EXPERIMENTAL PROCEDURES

Materials. For the NMR studies, calf thymus DNA (CT DNA) from Worthington was sonicated to about 200 base pairs as described previously (Wilson et al., 1985) and dialyzed against a PIPES buffer (0.01 M PIPES and 0.001 M EDTA at pH 7.0) with 0.10 M NaNO$_3$ (PIPES 10) or without salt (PIPES 00). The 2′-deoxynucleotide 5′-monophosphates dAMP, dCMP, and TMP in the free acid form and guanosine cyclic 2′,3′-monophosphate (cGMP) in the sodium form were from Sigma.

Stock solutions of 0.01 M Cu(NO$_3$)$_2$ were prepared in dilute HNO$_3$. The pH of the Cu^{2+} solution was adjusted with NaOH prior to addition to DNA. Pyrazole (Aldrich) was dissolved in deionized water and the pH adjusted with HNO$_3$ to that of the DNA solution.

Methods. The nucleotides were dissolved in deionized water for a final concentration of ca. 0.04 M, and the pH was typically adjusted to 7.0. Both the CT DNA (0.04 M phosphate) and the nucleotide NMR solutions contained either 10% D$_2$O or 100% D$_2$O. The pH of both the mononucleotide and the DNA solutions was lowered by addition of dilute HNO$_3$, and the pH determination was followed immediately by acquisition of the ^{1}H NMR spectra. The duration of each ^{1}H NMR experiment was ca. 1 h for each of the four mononucleotide acid titrations and ca. 8 h for the high- and low-salt CT DNA acid titrations. All proton chemical shifts were determined with respect to an internal reference of TSP [3-(trimethylsilyl)propionic acid sodium salt]. In addition, the DNA ^{1}H NMR samples contained an external reference of *trans*-bis(dimethylglyoximato)ethyl(4-*tert*-butylpyridine)cobalt(III) (CoDMG) in CCl$_4$ with an O–H–O proton signal at 18.39 ppm. The external reference was contained in a sealed capillary tube, which was centered in a 5-mm NMR tube with a Teflon plug. ^{31}P NMR samples of CT DNA (0.025 M phosphate) in PIPES 10/100% D$_2$O were contained in a 10-mm NMR tube with a volume of about 2 mL.

To determine the changes in signal intensity during each titration, an integration was employed, and then normalized areas could be obtained at each pH by the following procedure. First, the integrated area of the signal of the external reference was instrumentally set to 100. Next, the area of an AT + GC signal for a particular DNA solution at pH 7 was integrated. The value obtained, typically 600–700 under our conditions, is defined as the normalized area. At lower pH, the total imino proton area (AT + GC + upfield signal) was integrated, and as a check, those of the overlapping AT and GC signals (AT + GC) and the upfield (10–11 ppm) signal were integrated separately. The relative percent of each imino proton signal (AT:GC:upfield signal) was determined, from a curve deconvolution program from the Nicolet 1280 software. To obtain the normalized areas of the signals, the relative percent values were multiplied by the normalized total imino proton area.

Instrumentation. Measurements of pH were made with an Orion Model 701A pH meter equipped with an Ingold microelectrode designed to fit inside a standard 5-mm NMR tube.

^{1}H NMR spectroscopy was carried out on a Nicolet 360 NB FT spectrometer (1280 software) using a 14084-Hz spectral window and either a standard one-pulse sequence or

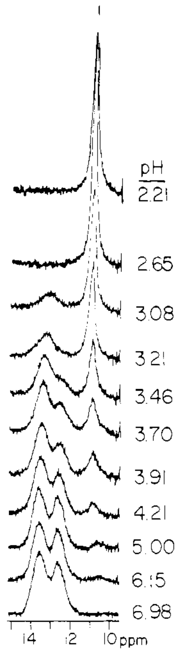


FIGURE 1: Effect of lowering pH from 6.9 to 2.2 on the imino ^{1}H NMR spectrum of CT DNA (PIPES 10). Each spectrum is plotted on the same vertical scale relative to an external reference (CoDMG in CCl_4). Spectra in all figures were obtained with 90% H_2O–10% D_2O as solvent unless specified otherwise.

a modified ^{1}H–Redfield 21412 pulse sequence (Redfield et al., 1975). For the mononucleotide solutions the parameters are 8K data points, 1-Hz line broadening, a carrier frequency of 10.0 ppm, and 44 transient FID's at 23 °C. For the DNA solutions the parameters are 4K data points, 1-Hz line broadening, a carrier frequency of 15.7 ppm, and 2000 transient FID's at 30 °C.

^{31}P NMR spectroscopy was carried out on a JEOL FX 60 Q (24.15-MHz) NMR spectrometer. Trimethyl phosphate was added as a standard. Data were collected with 8192 time domain points, 1-Hz line broadening, a 90° pulse with a $5T_1$ delay time, and proton decoupling at 30 °C.

RESULTS

^{1}H NMR Spectrum of CT DNA

Effect of pH on Imino H Signals of CT DNA in PIPES 10. At pH 7.0, the signals of the hydrogen-bonded imino protons of the AT and GC base pairs are typically at 13.6 and 12.6 ppm, respectively (Figure 1). The percent areas and HHW values of the AT and GC signals were estimated by curve deconvolution to be 56% and 296 Hz for AT and 44% and 282 Hz for GC. This correlates well with the relative base pair composition of CT DNA of 58% AT and 42% GC (Setlow, 1976).

Lowering the solution pH has a pronounced effect on the imino ^{1}H NMR spectrum of DNA (Figure 1). From pH 7 to pH 4 there were no significant changes in the chemical shifts of the AT and GC imino proton signals (Figure 2). However, at approximately pH 6, a new signal was observed at ca. 10.6 ppm (Figure 1). On lowering of the pH below 4, the AT signal shifted upfield by 0.4 ppm to 13.2 ppm at pH 3, while the GC signal shifted upfield by less than 0.1 ppm (Figure 2). In contrast, the new upfield signal shifted downfield on lowering the pH, and at pH 3.2 it was at 10.9 ppm, where it remained to pH 2.2 (Figure 2). From pH 7.2 to pH 3.7, the line shape of the new upfield area suggests the presence of two broad overlapping signals (Figure 1). The chemical shifts of the AT and GC and the average chemical shift for the new upfield signal are plotted at the top of Figure 2 for reference.

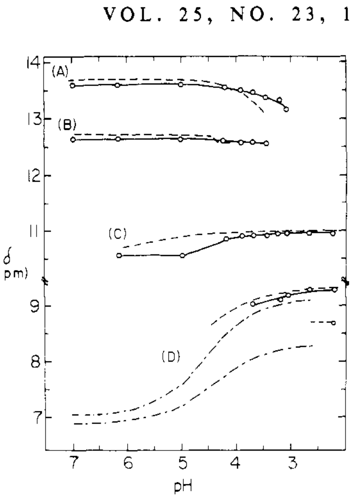


FIGURE 2: Plot of chemical shift vs. pH for (A) AT imino proton signal, (B) GC imino proton signal, (C) new upfield signal, and (D) one cytosine amino proton signal of CT DNA in PIPES 10 (—) and PIPES 00 (--). The chemical shifts of the two amino proton signals of dCMP (not in PIPES buffer) were also included for comparison (---).

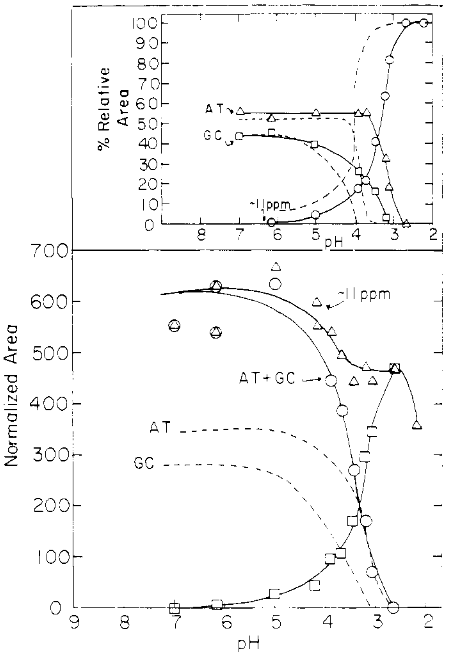


FIGURE 3: Plots of normalized areas vs. decreasing pH of CT DNA in PIPES 10 for AT + GC signals (○), new upfield signal (□), and total of these signals (△). Additionally, plots of the relative normalized areas (relative area of AT or GC signal × total normalized area) of AT and GC signals of CT DNA (--). (Inset) Relative areas of the three imino ^{1}H NMR signals of DNA are plotted as a function of decreasing pH. Symbols have the same significance as for the main plot. The dashed line indicates results for a similar experiment in PIPES 00.

From pH 7 to pH 5 there were no significant changes in the total normalized areas of the DNA imino proton signals (Figure 3) relative to the CoDMG O–H–O external standard. At pH 6.1 the new upfield signal represented 1% of the total normalized imino proton area, and this increased to 4% of the total normalized area at pH 5.0. Due to the experimental error of about 10% of the total normalized area of AT plus GC (AT + GC), it is not possible to determine if the area increase in the 10.6 ppm signal at pH 5.0 correlated with a decrease in the area of either AT or GC signals.

The normalized areas of the AT + GC signals decreased significantly over the pH 5–3 range (Figure 3), and decreases in the area of the GC signal were particularly large. At pH

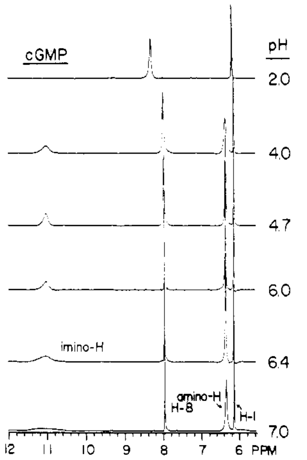


FIGURE 4: Influence of lowering pH from 7.0 to 2.0 on the ^{1}H NMR spectrum of cGMP. Selected spectra are illustrated.

3.8, the normalized GC signal was only 50% as large as the normalized GC signal at pH 7. This 50% area decrease was not obtained for the AT signals until pH 3.15, a pH at which no GC signal was observed. By pH 2.65, the AT signal was unobservable. These changes are visually apparent in Figure 1 and are plotted quantitatively in Figure 3.

The normalized area of the new upfield signal increased sharply, corresponding to the sharp decrease in AT + GC signals. On lowering the pH to 3.2, the total normalized imino proton area is only 75% of that at pH 7. The area of the upfield signal is 48% of the total normalized area at pH 7, while the AT signal area accounts for 25% and the GC signal area for the other 2%. It should be noted that some decrease is seen in the total normalized area from pH 7 to pH 2.65 (Figure 3), and this can probably be accounted for partially by the nonlinear power profile created by the Redfield pulse.

No large changes were observed in the HHW's of AT and GC signals from pH 7.0 to pH 4.7. Below pH 4.7, the HHW of both AT and GC increased. In contrast, the HHW of the upfield signal, which could be measured quantitatively, appeared to decrease on lowering the pH from 6.1 to 2.6 (Figure 1). Initially at pH 6.1, the HHW was ca. 300 Hz but decreased to its lowest value of 94 Hz at pH 2.6.

Effect of Salt. The effects of using lower salt (PIPES 00) are compared with the higher salt conditions (PIPES 10) in Figures 2 and 3 (inset). The overall chemical shift changes are similar for all three imino signals under both low- and high-salt conditions, but the changes occur at higher pH in the low-salt buffer (Figure 2). Below pH 5 under low-salt conditions, the AT signal starts to shift upfield to 13.1 ppm at pH 3.5. At high salt, a similar upfield shift of AT was not observed until pH 3.1. The GC signal at low salt shifted upfield within a small pH range from 12.7 ppm at pH 4.45 to 12.5 ppm at pH 4.40. Under both salt conditions, the GC signal shifted no further upfield than 12.5 ppm. Under low-salt conditions, the upfield signal shifts downfield much more gradually than under high-salt conditions. At pH values below 3.5, this signal is at 11.0 ppm in low salt and 10.9 ppm in high salt. The relative imino proton area changes of AT and GC signals also occurred at higher pH under the low-salt conditions (Figure 3, inset). The relative AT area, for example, decreased by 50% at pH 3.1 in high salt and at pH 3.8 in low salt. The AT signal was unobservable by pH 2.7 in high salt and by pH 3.1 in low salt. A 50% reduction of the GC relative area occurred at pH 3.7 in high salt and at pH 4.3 in low salt. The GC signals were unobservable at pH 3.1 in high salt and at pH 3.8 in low salt.

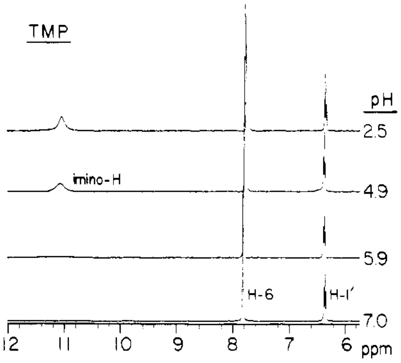
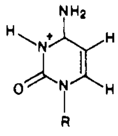
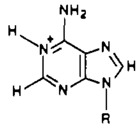
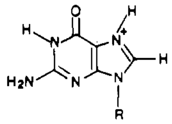


FIGURE 5: Effect of decreasing pH from 7.0 to 2.5 on the ^{1}H NMR spectrum of TMP.

Table I: Summary of pK_a Data

base	mono-nucleotides, $\mu = 0.05^a$	CT DNA (Raman study), $\mu = 0.004^b$	CT DNA (^{1}H NMR study)^c $\mu = 0.015$	$\mu = 0.115$
 dC(N-3)	4.25	4.1	4.3	3.7
 dA(N-1)	3.77	3.3	3.8	3.1–3.2
 dG(N-7)	2.27	2.6		

^{a}Izatt et al. (1971) and references cited therein. ^{b}O'Connor et al., 1981. ^{c}Our results.

Comparison of Mononucleotide ^{1}H NMR Signals with Those of CT DNA under Low-pH Conditions

The ^{1}H NMR spectra (6–12 ppm) of dAMP, cGMP, dCMP, and TMP were studied and compared with that of CT DNA in the pH range of 7–2 [Figures 4 and 5, supplementary material Figures 2 (see paragraph at end of paper regarding supplementary material], and Table I]. All of the aromatic (CH) and amino proton assignments for the four mononucleotides were made by comparison with earlier literature [Kearns (1976) and references cited therein]. Since there are no comparative pH studies on the imino NH region of mononucleotides, we have included our findings here.

Imino NH Region of G and T. The imino NH signal of cGMP and TMP is very broad until pH values below neutrality (Figures 4 and 5). At pH 6.7, the imino NH signal of cGMP appears first as a broad signal (HHW ≃ 270 Hz) with a chemical shift of 10.97 ppm. By pH 4.7, the NH signal is its sharpest (HHW ≃ 60 Hz) with an essentially unchanged chemical shift. Below pH 4.7, along with protonation of N-7, the NH signal again broadens until it is no longer observable at pH values below 3.0. The line width of the TMP NH signal decreased continuously over this pH range, from ca. 290–300 Hz at pH 5.96 to 45 Hz at pH 2.50. The chemical shift of this signal is essentially constant at 11.04 ppm from pH 4.93 to pH 2.5. In summary, the imino NH chemical shifts of TMP and cGMP are nearly identical at ca. 11.0 ppm, while their

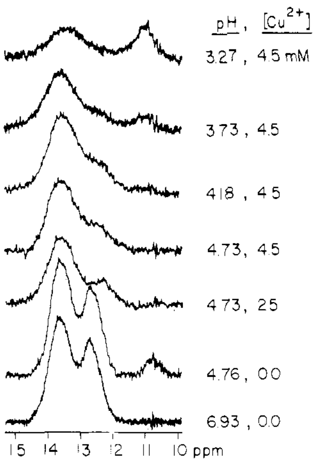


FIGURE 6: Influence of Cu^{2+} on the imino ^{1}H NMR spectrum of CT DNA in PIPES 10 at low pH.

HHW's differ significantly as a function of pH.

In a comparison of the new imino NH region of CT DNA (Figure 1) with that of cGMP and TMP (Figures 4 and 5) at pH <7, the most striking parallel is the signal at ca. 11 ppm present in spectra from all three samples. This result together with the observation that the double-helical structure of CT DNA unfolds upon protonation (O'Connor et al., 1981) suggests that the new upfield signal of CT DNA is a variable mixture of both G and T imino NH signals. To explore this possibility further, we have included studies of Cu^{2+} and pyrazole as probes of the G and T contribution to the new upfield signal of CT DNA.

Effect of Cu^{2+} on Imino Proton Signals of DNA, TMP, and cGMP. Previous studies have shown that the addition of Cu^{2+} to DNA causes a large broadening of the GC imino proton signal (Banville et al., 1985). Cu^{2+} also broadens the imino signal of cGMP and eliminates its CH-8 signal (supplementary material Figure S3A). Similar results for CH signals were observed in other studies (Marzilli, 1977) and have been explained by the binding of Cu^{2+} to G(N-7). No Cu^{2+} to base binding has been demonstrated for 5′-TMP and thymidine (Eichhorn, 1973). At pH 4.7, Cu^{2+} was added to a PIPES 10 solution of CT DNA up to an effective concentration of 3.5 mM (Figure 6). The Cu^{2+} caused a small loss in the intensity of the AT signal and a large loss in the intensity of the GC and 10.7 ppm signals. On lowering the pH of this solution to 4.18, where the normalized AT area of DNA had decreased by 10% with no added Cu^{2+}, a small upfield signal is observed. At pH 3.7, this upfield signal is slightly larger. At pH 3.3 with Cu^{2+} added, ca. 60% of the area is due to AT and 40% due to the upfield signal. In the absence of Cu^{2+} at pH 3.2, the relative areas are 33% AT signal, 64% upfield signal, and 3% GC signal.

The effect of Cu^{2+} on the imino proton signal of TMP is smaller than that on cGMP (supplementary material Figure S3). At pH 4.7, addition of 0.0015 M Cu^{2+} to a 0.04 M TMP solution broadened the imino proton signal from a HHW value of 47 Hz to one of 87 Hz. Further addition of Cu^{2+} (to 0.0035 M) at pH 4.7 increased the HHW value to 115 Hz. Upon lowering the pH from 4.7 to 2.6, the HHW values decreased to 36 Hz. This value is similar to the HHW value (pH 2.8) in the absence of Cu^{2+} (supplementary material Figure S1).

At pH 4.7, addition of 0.0015 M Cu^{2+} to a 0.04 M cGMP solution (supplementary material Figure S3A) eliminated the H(8) signal and broadened the imino proton signal to 115 Hz. Addition of Cu^{2+}, at pH 4.7, up to a concentration of 0.0035 M, increased the HHW value to 217 Hz. Upon decreasing the pH of the 0.0035 M Cu^{2+} solution to 4.1, the HHW value of the imino proton signal of cGMP decreased to 162 Hz, a value ca. 65 Hz above the pH 4.0 HHW value without Cu^{2+} present (supplementary material Figure S1). However, a further decrease in pH first to 3.7 and then to 3.0 increased the HHW value to 180 and 347 Hz, respectively. Without Cu^{2+} present, the imino proton signal of cGMP did not reach a HHW value of ca. 180 Hz until pH 3.2 and a HHW value of ca. 347 Hz until pH 3.0.

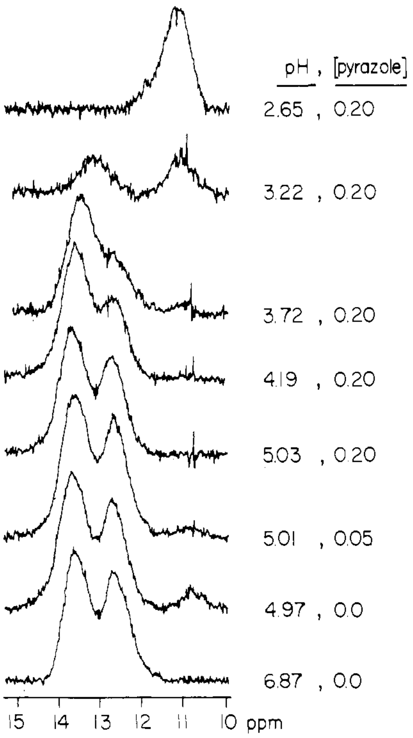


FIGURE 7: Influence of pyrazole on the imino ^{1}H NMR spectrum of CT DNA in PIPES 10 at low pH.

These results support the idea of initial protonation of DNA GC base pairs as pH is lowered below neutrality. Before total protonation of GC base pairs, AT base pairs begin to be protonated, and the DNA is significantly destabilized.

Effect of Pyrazole on Imino Proton Signals of DNA, TMP, and cGMP. The purpose of this experiment is to study the pyrazole-catalyzed solvent exchange of imino protons. Up to 200 mM pyrazole was added to a DNA/PIPES 10 solution at pH 5.0. The original AT + GC imino signals were not significantly affected by the exchange catalyst, but the upfield signal was completely broadened (Figure 7). On lowering the pH to 4.2, the upfield signal remained unobservable, and the intensity of the GC signal significantly decreased. At pH 3.7, a small upfield signal had reappeared (10.8 ppm). On lowering the pH to 3.2 and then to 2.6, a decrease at AT signal intensity and an increase in the upfield signal intensity can be observed. However, pyrazole (pK_a = 2.5) is substantially protonated at these two pH values and is less effective at catalyzing exchange.

Pyrazole was added to TMP and cGMP to quantitate its potential as a low-pH catalyst of imino proton exchange (supplmentary material Figure S4). At pH 4.70, addition of pyrazole (200 mM) increased the HHW of TMP from 79 to 332 Hz and the HHW of cGMP from 82 to 448 Hz. On lowering the pH to 4.17, the HHW of TMP remained essentially unchanged while that of cGMP increased further to 556 Hz. Finally, by pH 3.05 the HHW of TMP decreased to 274 Hz (in contrast to the 45 Hz observed with no pyrazole). Since these pH conditions are near the pK_a of pyrazole (pK_a

$\sim$ 2.5), the decrease in the NH signal of TMP may be attributed to a decrease in the amount of pyrazole in the unprotonated form. The imino signal of cGMP with pyrazole was predictably unobservable at pH 3.05, since the HHW of the cGMP imino proton signal without pyrazole was ca. 330 Hz at this pH. These results illustrate that imino protons exposed to solvent even under fairly low-pH conditions (pH 4) have exchange reactions that are readily catalyzed by pyrazole.

Effect of Low pH on Aromatic CH and Amino Region of CT DNA. At pH 7, the double-helical structure of DNA leads to relatively broad unresolved signals in the 5.8–8.5 ppm region (supplementary material Figure S5). As the pH was lowered, the signals began to sharpen, and at pH 2.1 relatively sharp signals are observed. This sharpness is consistent with a relatively flexible species that is essentially single stranded. The apparent doublet at 7.6–7.7 ppm is tentatively assigned to the H(6) doublet of C by comparions to the CMP results. In the D_2O titration, it is observable at pH 4.4 and below. On lowering the pH to 2.2, two signals can be observed at 9.3 and 8.7 ppm. Similar downfield shifts were observed only in the case of dCMP (supplementary material Figure S2), where the two amino proton signals were at 9.10 and 8.25 ppm by pH 2.6. A similar low-pH experiment with DNA in PIPES 00/100% D_2O demonstrated that these two downfield signals were absent at pH $\sim$2. On the basis of these results, the two downfield signals at 9.3 and 8.7 ppm (pH 2.2) can be assigned to the cytosine amino protons of DNA.

In a comparison of the dCMP amino signals and the DNA–cytosine amino signals, differences in chemical shifts exist as a function of pH. Both of the two DNA–cytosine signals are consistently more downfield than the two corresponding dCMP signals over the pH range for which the DNA–cytosine signals do not overlap extensively with the CH signals. For example, at pH 2.6, the downfield signals of dCMP (9.1 ppm) and DNA–cytosine (9.3 ppm) differ by 0.2 ppm. The upfield signals of dCMP (8.3 ppm) and DNA–cytosine (8.7 ppm) differ by 0.5 ppm. Under higher salt conditions (PIPES 10), similar changes in chemical shift were observed for the two DNA–cytosine signals.

On lowering the pH from 3.8 to 2.6, the HHW's of both dCMP signals are less than the HHW's of the downfield DNA–cytosine signals. The difference in HHW between the downfield signals of dCMP and DNA–cytosine decreases as the pH is lowered. At pH 3.8, for example, the HHW's for the downfield signals of dCMP and DNA–cytosine differ by 29 Hz, and at pH 2.6 they differ by only 7 Hz. Since the upfield amino NH signal of cytosine in DNA overlapped considerably with the DNA CH signals, the HHW could not be determined.

Effect of pH on ^{31}P NMR Spectra. The effects of pH on the ^{31}P NMR spectrum of CT DNA in PIPES 10 were studied from pH 7.2 to pH 3.3. At pH 7.2, a solution of CT DNA typically gives a single ^{31}P signal at –4.29 ppm. On lowering the pH to 5.7, there is no significant shift of this signal. By pH 3.8 the ^{31}P signal is shifted slightly downfield to –4.26 ppm. Finally, at pH 3.28, the ^{31}P signal is further downfield at –4.19 ppm. Small downfield shifts of the ^{31}P signal have previously been observed in the case of thermally denatured and recooled DNA (Mariam & Wilson, 1979).

DISCUSSION

The protonation of DNA (pH 5–3) has been described by two general models, both of which involve GC base pairs as the initial protonation site. In model I, protonation is at N-3 of C followed by protonation at N-1 of A then N-7 of G (O'Connor et al., 1981). In model II, initial protonation is at N-7 of G resulting in the conversion of guanosine from the anti to syn conformation and the formation of a Hoogsteen base pair between G and C (Courtois et al., 1968). These models are based on UV, CD, ORD, and Raman spectroscopic analysis of the effects of protonation on DNA (Cavalieri & Rosenberg, 1957; Dove et al., 1959; Peacocke & Preston, 1958; Geiduschek, 1958; Zimmer & Venner, 1966; Courtois et al., 1968; O'Connor et al., 1981). We have conducted NMR spectroscopic studies on DNA at low pH, and the information provided by NMR has allowed us to differentiate between these two models and to provide additional information concerning the protonation of DNA.

The pK_a values of the mononucleotide constituents of DNA below pH 7 are as follows: C(N-3), A(N-1), and G(N-7) (Table I). Model I requires no change in the order of base protonation. Apparent pK_a's of the bases in DNA, determined by Raman spectroscopy (O'Connor et al., 1981), are not very different from those of the mononucleotides (Table I). Model II requires that the pK_a of G(N-7) is increased to above that of C(N-3) in double-helical DNA (Courtois et al., 1968), but individual pK_a values could not be determined by the CD method.

From the ^1H NMR data in Figure 3, apparent pK_a's of DNA bases were determined from the pH at which the AT or GC imino signal area is reduced to half the pH 7 value (Table I). At low salt (μ = 0.015 M), these apparent pK_a values are essentially identical with those of the mononucleotides (μ = 0.05 M). At high salt (μ = 0.115 M), the apparent pK_a values are about 0.5 unit below the low-salt values. This result is expected since higher salt concentrations both stabilize the DNA helix and suppress the protonation of the DNA bases. Also, protonation of the negatively charged polymer and subsequent counterion release should be less favorable in high salt.

A characteristic feature of the imino ^1H NMR region of DNA at pH <6 is the appearance of a third signal at ca. 11 ppm. The loss of AT + GC signal area parallels an increase in area of this third signal as the pH is lowered (Figures 1 and 3). According to model I, this signal must be primarily the imino signal of non-base-paired G. According to model II, it would be the imino signal of a protonated Hoogsteen-type GC base pair.

To our knowledge, the imino ^1H signal of non-base-paired G in H_2O has not been reported. To evaluate whether this one new upfield signal is consistent with model I or II, the ^1H NMR spectra of mononucleotides were studied. The imino protons of cGMP and TMP are in fast exchange with H_2O at pH 7, and therefore, the signals are not observable. However, under acidic conditions (pH <6) both the cGMP(N-1) and TMP(N-3) imino protons have observable signals (Figures 4 and 5) with identical chemical shifts (11 ppm), and this shift is similar to that of the new DNA upfield signal. The imino signal of G is broadened by N-7 binding both by Cu^{2+} and by H^+. The imino signal of T is less severely broadened by Cu^{2+}, and at low pH, the signal can be observed even in the presence of Cu^{2+}.

According to model I, protonation at C should be accompanied by a loss of the GC imino signal and the appearance of the imino signal of non-base-paired G at ca. 11 ppm and of the two amino signals of C at ca. 8.7 and 9.2 ppm as observed. This assignment of initial protonation to GC is additionally supported by the fact that there is no significant change in the normalized area of the AT signal on lowering the pH from 7 to 4.7. Similarly, the protonation of A at the lower pH should lead to an additional contribution of the imino signal of non-base-paired T to the 11 ppm signal. In fact, by pH 2.6, at least 75% of the original AT + GC imino proton area of DNA at pH 7 can be accounted for in the area of the upfield signal. Hence, both T and G imino protons must contribute to the 11 ppm signal at pH 2.6.

Furthermore, the addition of 4.5 mM Cu^{2+} to DNA at pH 4.7 resulted in a total loss of the upfield area at 10.7 ppm and supports the assignment of this initial upfield signal to the imino proton of non-base-paired G. On lowering the pH below 4.7 in the presence of Cu^{2+}, the upfield signal responded in a fashion consistent with its assignment to coincident G and T imino signals.

At pH 3.27 (slightly above the apparent pK_a of A) the ratio of the AT area to the upfield area is 1:0.7 with Cu^{2+} present (Figure 6) and 1:1.3 without Cu^{2+} present (Figure 1). Since Cu^{2+} will selectively minimize the contribution of G to this 11 ppm signal, the approximately 50% relative decrease in this signal can be attributed to the loss of the G imino signal. These results agree qualitatively with the mononucleotide NMR data as a function of pH. On lowering the pH below 7, the relative contribution of T to the signal should increase and plateau, and the relative contribution of G should increase to a maximum and then decrease to zero below pH 3 as N-7 is protonated (supplementary material Figure S1).

In contrast, the upfield signal of DNA cannot be easily explained with model II. Protonation at G(N-7) would increase the acidity and, hence, solvent exchange rate of the G imino proton. Thus, it is unlikely that the imino proton signal of an N-7-protonated G in DNA would be observable. Futhermore, the formation of a Hoogsteen base pair would effectively block G(N-7) from Cu^{2+} binding. From pH 4.7 to pH 3.3, the Cu^{2+} binding dramatically reduced both the GC imino proton area and the 11 ppm area (Figure 6). These results agree qualitatively with the mononucleotide NMR data (supplementary material Figures S3 and S4) and strongly suggest that a non-base-paired G contributes to the 11 ppm signal, not a Hoogsteen $GH^{+}C$ base pair.

Another means of assessing the utility of models I and II is with the cytosine NH and CH signals. In model I, the protonated C should not be involved in base pairing. Therefore, we would expect NH signal chemical shifts similar to those of dCMP, as observed (Figure 2). The line widths are somewhat broader in the CT DNA experiments, but this is expected from residual stacking and the size of the molecule. Model II would require coincidental canceling of effects (downfield shift due to H bonding and upfield shift due to "less" protonation) to account for the similarity of shifts with those for dCMP. If model II were correct, we would expect that the H(6) doublet of C would be broad due to base pairing and restricted motion, and it is not.

From pH 7 to pH 3, the ^{31}P NMR spectrum of DNA changes to qualitatively resemble the spectrum of DNA thermally denatured at pH 7 and then cooled to 30 °C (Mariam & Wilson, 1979). Model II requires a change in a C–O–P–O torsion angle to accommodate guanosine in the syn conformation and the formation of a Hoogsteen base pair ($GH^{+}C$). Such a syn conformation should lead to a significant change in the ^{31}P spectrum. For example, the ^{31}P spectrum of Z DNA, with G in the syn conformation, contains a downfield signal at ca. −2 ppm (Cohen et al., 1981). Model I predicts a protonation-induced partial denaturation of DNA first at GC and then at AT base pairs. This would lead to (1) an increase in the amount of single-stranded DNA with a decrease in the ^{31}P correlation time and a subequent decrease in the HHW (Bolton & James, 1979) and (2) a gauche,gauche to gauche,trans transition of the phosphate backbone causing a downfield shift (Chen & Cohen, 1984). Since our results are similar to those observed for thermally denatured DNA at 30 °C, they are most consistent with model I.

Full acid denaturation of DNA has been described as a two-step process:

$$\mathrm{ds} \xrightarrow{H^+} \mathrm{ds}^+ \rightarrow \mathrm{s}^+/\mathrm{s}$$

An equilibrium between the double-stranded DNA (ds) and its partially protonated form (ds^+) is followed by denaturation to partially protonated single-stranded form (s^+/s) at pHs below 3 (Cavalieri & Rosenberg, 1957; Peacocke & Preston, 1958; Geiduschek, 1958). Our results suggest that at pH values between 6 and 5, the principal forms of ds^+ contain a small amount of non-base-paired CH^+ and G. The upfield signal, corresponding to stacked but not paired G, appears first at 10.6 ppm. On lowering the pH below 4 (near the apparent pK_a of C), this signal shifts further downfield by 0.3 ppm. In comparison, solvents of decreasing hydrophilic character shift the imino signal of G further upfield to ca. 10.5 ppm. The upfield position of the 10.6 ppm signal indicates that non-base-paired G (Kearns, 1977) is not fully exposed to solvent. As the pH is lowered, the DNA structure will be further disrupted. This disruption could lead to the increased solvation of non-base-paired G, which would account for the 0.3 ppm downfield shift of the upfield signal. The addition of 200 mM pyrazole caused elimination of the upfield signal at pH 4.7. However, this result does not eliminate the possibility that the imino proton with the 10.7 ppm signal is partially shielded from solvent. The local helix opening rate in the protonated regions could be sufficient to give fast exchange with base. In summary, in the initial ds^+ species, the G imino proton either is exposed to solvent or is in partially shielded stacked regions undergoing fast "breathing" motions or other exchange reactions.

At pH 3.8, corresponding to the apparent pK_a of A and where 50% of the AT imino signal remains, there is essentially no GC imino proton signal observed in both high- and low-salt conditions. At this point, the AT signal begins to shift upfield, suggesting that only AT-rich regions are left intact since imino signals for terminal base pairs and for base pairs flanked by AT are to higher field than signals of base pairs flanked by GC base pairs. The overall stability of the AT base pairs without any observable GC present may be partially accounted for by the relatively large stacking energy of partially protonated A. For example, the dinucleotide ApA^+ has a stacking energy in solution similar to that of the unprotonated species ApA (Geraldes & Williams, 1982). But no stacking was observed for the fully protonated species A^+pA^+. Such A,A^+ stacking as well as increased solvent exchange will influence the shift of the AT imino signals. At pH 3.8, ds^+ can be viewed as a species with ca. 50% of the original AT base pairs intact. The remainder have been protonated at N-1 of A with disruption of AT hydrogen bonding. The AH^+ may be stacked to some extent with the intact AT base pairs. The GC base pairs are disrupted with G exposed to solvent, and the protonated C bases have considerable flexibility.

Examination of the CH signals in D_2O also supports model I. A doublet signal at 7.8 ppm in the pH 2.1 spectrum (supplementary material Figure S5) is very likely cytosine H(6). This signal begins to emerge from the very broad CH signals of double-helical DNA at pH 4.4 and is clearly observed at lower pH. This behavior is consistent with relatively lower mobility of protonated C at the higher pHs but greater flexibility at lower pH. If model II were correct, the signal should remain broad since C should be held rigidly in the protonated GC Hoogsteen base pair.

At pH 2.6 (below the apparent pK_a of A) the original AT signal is unobservable, and only the 11 ppm signal remains. Contrary to the mononucleotide results with cGMP, the G imino proton signal must contribute to the 11 ppm signal of DNA at a pH below 3. The implication is that the G imino proton of single-stranded DNA (s^+/s) is not as freely exposed to solvent as that of cGMP or, more likely, it is more difficult to protonate G(N-7) in a DNA already protonated at C(N-3) and A(N-1). However, at pH 2.2, the normalized area of the DNA upfield signal decreased to about 55% of the original AT + GC signal area. G(N-7) of DNA should be ca. 50% protonated at pH 2.2, and this may contribute to the loss of the G imino proton signal at 11 ppm.

In conclusion, our results show that the order of base protonation of CT DNA is not changed relative to the mononucleotides and support model I for the protonation of DNA. These results are consistent with observations made by Raman spectroscopy and have important implications for the B to H and B to Z transitions. In the case of the B to Z transition below pH 7, the rate of interconversion is enhanced, but the equilibrium position is not changed. Hence, protonation of C effectively lowers the activation energy associated with this interconversion. For example, the rate of the salt-induced B → Z conversion of $(G\text{-}C)_2$ is enhanced at pH 3.8 (Chen, 1984). At this pH value, the B $\rightleftarrows$ Z equilibrium position is not altered. Protonation of C would increase the exposure of the G base to solvent and reduce the steric hindrance to the anti to syn conversion. It is interesting that the protonation of DNA destabilizes the GC before the AT base pairs. This is in contrast to the thermal denaturation of DNA at neutral pH where the AT base pairs are the first to melt. Hence at low pH, the stability of CT DNA is a consequence of the AT base pairs not the GC base pairs. In fact, as seen in Figure 1, at low pH some AT base pairs remain intact in the absence of any observable GC base pair hydrogen bonding. These results also show that, in 0.1 M salt at pH values as high as 5, approximately 5% of the base pairs, primarily GC, are disrupted.

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SUPPLEMENTARY MATERIAL AVAILABLE

Five figures showing full line widths at half-height plotted as a function of pH for the imino proton signal of cGMP and TMP and the amino proton signal of cGMP, ^1H NMR spectra of dCMP as a function of decreasing pH, effect of Cu^{2+} on ^1H NMR spectra of TMP and of cGMP under acidic conditions, influence of pyrazole on ^1H NMR spectra of TMP and cGMP at pH <7, and aromatic ^1H NMR region of CT DNA at pH 2.1 and 7.0 in PIPES 00/"100%" D_2O and PIPES 00/90% H_2O + 10% D_2O (8 pages). Ordering information is given on any current masthead page.

Registry No. cGMP, 7665-99-8; TMP, 365-07-1; Cu, 7440-50-8; pyrazole, 288-13-1.

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Nuclease Activity of 1,10-Phenanthroline–Copper Ion: Reaction with CGCGAATTCGCG and Its Complexes with Netropsin and *Eco*RI[†]

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ABSTRACT: The self-complementing dodecamer 5′-CGCGAATTCGCG-3′ and its complexes with the antibiotic netropsin and the restriction endonuclease *Eco*RI provide substrates of known three-dimensional structure to study the stereochemistry and mechanism of the artificial nuclease of 1,10-phenanthroline–copper ion [(OP)$_2$Cu$^+$]. Analysis of the reaction products with the 5′-^{32}P dodecamer on 20% sequencing gels has demonstrated the presence of 3′-phosphoglycolate ends in addition to 3′-phosphomonoester ends expected from previous studies. A reaction intermediate, which is a precursor to 3′-phosphomonoester termini, has been trapped; in contrast, no comparable species for the 5′-phosphomonoester termini can be detected when 3′-labeled DNAs are utilized as substrates. The reactive oxidative species formed by the coreactants (OP)$_2$Cu$^+$ and hydrogen peroxide is distinguishable in its chemistry from the hydroxyl radicals produced by cobalt-60 γ-irradiation. The freely diffusible hydroxyl radicals generated by cobalt-60 irradiation produce equivalent amounts of 3′-phosphomonoester and 3′-phosphoglycolate termini whereas the 3′-phosphomonoesters are the preferred product of (OP)$_2$Cu$^+$ and H_2O_2. On the basis of the structures of the products obtained, the principal site of attack of the coordination complex is on the C-1 of the deoxyribose within the minor groove. This conclusion is supported by the footprinting of netropsin binding to the dodecamer. Crystallographic results have demonstrated that netropsin binds to the minor groove at the central AATT residue. A clear protection of attack by the coordination complex at the deoxyriboses associated with A-5, T-6, T-7, and C-9 is fully consistent with attack from the minor groove without intercalation during the course of the cleavage reaction. *Eco*RI binds to the tridecamer TCGCGAATTCGCG by interactions in the major groove, leaving the minor groove accessible to solvent. Consistent with the proposed attack of (OP)$_2$Cu$^+$ in the minor groove, the restriction endonuclease does not protect the dodecamer from oxidative attack.

The 1,10-phenanthroline–cuprous complex[1] with hydrogen peroxide as a coreactant is an artificial nuclease that cleaves DNA by an oxidative mechanism under physiological conditions (Sigman et al., 1979). (OP)$_2$Cu$^+$ preferentially cleaves B-form DNA over A DNA; it does not cleave the left-handed Z structure or single-stranded DNA incapable of forming secondary structures (Marshall et al., 1981; Pope & Sigman, 1984). The conformational sensitivity of the nuclease action probably results from the obligatory formation of a noncovalent intermediate between the coordination complex and the DNA during the course of the reaction.

Recent studies using restriction fragments containing the *lac* control region as substrates have clearly demonstrated that (OP)$_2$Cu$^+$ reactivity depends upon primary sequence but that all bases (e.g., A, T, G, and C) may serve as hypersensitive sites (Sigman et al., 1985; Spassky & Sigman, 1985). The conserved promoter sequence (Pribnow, 1975) represents one region of preferential cutting. Single base changes in this region cause dramatic alterations in the cleavage pattern, reflecting the sensitivity of the nuclease activity to the primary sequence but not to a single base. For example, (OP)$_2$Cu$^+$ cleaves the 5′-labeled template strand of the control region of the wild-type promoter strongly at residues cytosine-13 and adenine-12. In the (OP)$_2$Cu$^+$ cleavage of the template strand isolated from the Ps variant, which differs from the wild-type by a single base change at residue C-9 (cytosine to thymidine), an additional hypersensitive band becomes apparent at adenine-10 (Sigman et al., 1985). Enhanced reactivity to the reagent implies that the single base change alters the local conformation of the DNA to provide a more productive binding site for the coordination complex.

In order to identify those features of DNA conformation that enhance its reactivity to the reagent, we studied the reactivity of the coordination complex with a B DNA of known three-dimensional structure, the self-complementing dode-

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[1] Abbreviations: OP, 1,10-phenanthroline; (OP)$_2$Cu$^+$, 2:1 1,10-phenanthroline–cuprous ion complex; (OP)$_2$Cu^{2+}, 2:1 1,10-phenanthroline–cupric ion complex; MPA, 3-mercaptopropionic acid; Tris,tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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