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Studies on the Interaction of Cupric Isonicotinohydrazide with DNA†

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ABSTRACT: The interaction of cupric isonicotinohydrazide ($Cu^{II}INH$), an antiviral compound, with calf thymus DNA was investigated by circular dichroism (CD) and nuclear magnetic resonance (NMR). Gel electrophoresis of DNA incubated with $Cu^{II}INH$ showed cleavage of DNA to various extents. This cleavage was found to be time and concentration dependent. In the presence of $Cu^{II}INH$ the positive CD band at 274 nm disappeared and the negative band at 246 nm showed a decrease in the mean residual ellipticity value, indicating binding of $Cu^{II}INH$ to DNA. ^{31}P NMR studies indicated that the binding of copper in $Cu^{II}INH$ is to the phosphate oxygen of the DNA backbone. The binding of $Cu^{II}INH$ was also found to be reversible. Addition of ethylenediaminetetraacetic acid to the $Cu^{II}INH$–DNA complex resulted in breaking of the complex and restoring the original structural features of the B family of DNA in the resulting fragments. At the concentration level of $Cu^{II}INH$ employed, both $CuSO_4$ and INH independently did not show any interaction with DNA.

Copper complexes of chelating agents like 1,10-phenanthroline and thiosemicarbazones exhibit antiviral activity by their interaction with the nucleic acid templates and inhibit proviral DNA synthesis (Pillai et al., 1977; Sigman et al., 1979; Downey et al., 1980). Cupric ion is known to exhibit a high affinity for DNA reversibly under appropriate conditions (Eichhorn & Shin, 1968; Pezzano & Pato, 1980). Isonicotinohydrazide (INH),[1] which was found to be effective against tuberculosis (Hillerband et al., 1975), is known to form a complex, $Cu^{II}INH$, with copper ions resulting in a decrease in copper level in blood and liver (Albert, 1956; Sodikov & Gaponko, 1971). It has been reported that $Cu^{II}INH$ inhibits the multiplication of avian myeloblastosis virus by blocking the process of reverse transcription (Vasudevachari & Antony, 1985a). This inhibition was shown to be due to preferential binding of $Cu^{II}INH$ to the enzyme reverse transcriptase (Vasudevachari & Antony, 1982). Recently, it has been shown that $Cu^{II}INH$ cleaves pBR322 form I DNA into smaller fragments (Vasudevachari & Antony, 1985b). We report here evidence to show that $Cu^{II}INH$ interacts with DNA leading to cleavage of DNA based on circular dichroism (CD) and ^{31}P nuclear magnetic resonance (NMR) studies.

Materials and Methods

Materials. Calf thymus DNA (sodium salt, A grade) was obtained from Calbiochem or Sigma Chemical Co. (type I). Tris(hydroxymethyl)aminomethane, isonicotinohydrazide (INH), ethylenediaminetetraacetate (disodium salt), agarose [low electroendosmosis (EEO)], and ethidium bromide were from Sigma Chemical Co. Unless otherwise stated, incubation experiments involving calf thymus DNA (80 μg/mL) dissolved in 20 mM Tris-HCl buffer (pH 7.6) were carried out at 37 °C for 2 h. Horizontal agarose gel electrophoresis was carried out with 0.7% agarose in Tris–acetate–EDTA (pH 8.3) (Hayward & Smith, 1972).

Calf thymus DNA was sonicated as follows. One hundred milligrams of calf thymus DNA (Sigma, type I containing 5.1% Na) was dissolved in 15 mL of degassed sodium cacodylate buffer (10 mM sodium cacodylate, 0.1 M NaCl, pH 7.0) and kept overnight at 5 °C. Nitrogen gas was bubbled into the DNA sample for 15 min, and it was sonicated for 1 h by using a Bronson Model B-15 Sonifier at 0–5 °C. A $^1/_4$-in.

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[1] Abbreviations: INH, isonicotinohydrazide; CD, circular dichroism; NMR, nuclear magnetic resonance; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kb, kilobase(s).

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flat tip at 50% power was employed on a continuous mode with the sonicator on for 20 s and off for the same duration of time. The sample was centrifuged at 10000 rpm for 30 min at 5 °C, and the supernatant was ethanol precipitated and dissolved in the buffer. This was repeated 3 times, and the sample was then freeze-dried and stored at 5 °C. A 2% agarose gel prepared in Tris–acetate–EDTA (50, 20, and 1 mM, respectively) at pH 7.8 with *Hin*fI digest of λ-DNA as the standard indicated that the sonicated DNA on an average consisted of fragments of size 120 ± 80 base pairs.

The $Cu^{II}INH$ was prepared according to the method reported earlier (Srivastava et al., 1977). Elemental analysis of the complex carried out at CIBA-Geigy research center, Bombay, gave the following result: Anal. Calcd for $Cu^{II}INH$: C, 21.66; H, 3.31; N, 12.63; S, 9.62; Cu, 19.1. Found: C, 21.64; H, 3.69; N, 12.2; S, 9.87; Cu, 20.4. The complex is a blue powder, insoluble in water, Me_2SO, and majority of organic solvents, but readily soluble in HCl (solubility 50 mg/mL). It melts around 225–230 °C, probably with decomposition. Stock solutions were prepared by dissolving the complex in a minimum volume of 1 N HCl (about 50–100 μL) and then making it up to the required volume with the above-mentioned buffer.

Methods. CD spectra of DNA were obtained by using a JASCO J-20 automatic recording spectropolarimeter operating at 20 °C. The region between 210 and 310 nm was scanned for each sample. Mean residual ellipticity values were calculated according to the formula

$$[\theta]_\lambda = 330\theta_{deg}/Cl$$

where $[\theta]_\lambda$ is the mean residual ellipticity value at a particular wavelength expressed in deg·cm^2·dmol^{-1}, C the concentration in grams per 100 mL, l the length of the cell in dm, θ_{deg} the observed rotation in degrees, and 330 the mean molecular weight of a deoxyribonucleotide. This equation is obtained from the basic equation for circular dichroism (Zacharias et al., 1982). An equation of similar type has been used to calculate mean residual ellipticity values in proteins and DNA.

Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra were obtained with a Varian FT-80A spectrometer operating at 32.2 MHz for phosphorus at a probe temperature of 20 °C. Proton-decoupled spectra were obtained for the sonicated DNA in Tris-HCl buffer (pH 7.6). About 2000–10000 scans were accumulated for each sample. Line broadening function used was 3 Hz, and all signals were measured with reference to phosphoric acid external reference. A 10 mg/mL concentration of sonicated DNA was used for each run.

Cyclic voltametry setup consisted of a platinum button working electrode and a platinum wire counter electrode. Saturated calomel electrode was used as a reference electrode. Sodium perchlorate as a supporting electrolyte was present in all samples at 0.2 M. Scan rate employed was 50 mV/s with 240 mg/mL of DNA in 20 mM Tris-HCl buffer at pH 7.6.

A Varian X-band spectrometer (operating at 9.05 GHz) was used for recording the electron spin resonance spectra. IR spectra were recorded in Nujol on a Perkin-Elmer spectrophotometer.

Results

Structure of $Cu^{II}INH$. From the elemental analysis, the molecular formula of the complex can be inferred to be $Cu(INH)SO_4·2H_2O$. The insolubility of the complex in water and other organic solvents indicates that the structure of the complex may be either the one with the sulfate oxygen coordinated to the cupric ion or a polymeric one involving coordination through the pyridine nitrogen (from other ligand moieties) to Cu^{2+}, besides the carbonyl oxygen and the hydrazide nitrogen. ^{1}H NMR studies of INH on gradual addition of Cu^{2+} showed broadening of the 2H and 6H protons along with the hydrazide NH_2 and NH (Cu^{2+} with d^9 configuration of electrons is paramagnetic and hence causes broadening of NMR signals in its proximity) (Hillerband et al., 1975). But the 3H and 5H protons did not show any appreciable broadening in the concentration range studied, indicating the binding of pyridine nitrogen to Cu^{2+} ion. This leads to the conclusion that the structure of the resulting complex may be polymeric. The broadening of IR bands of $Cu^{II}INH$ in the region 3100–3300 cm^{-1} and the shift of carbonyl band at 1650 cm^{-1} confirmed the NMR data that the hydrazide NH_2 and keto oxygen is coordinated to Cu^{2+}. The presence of additional water molecules in the polymeric form is indicated by the broad absorption band in the region 3400–3600 cm^{-1}.

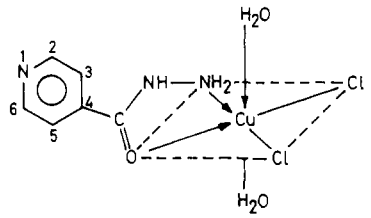

FIGURE 1: Structure of cupric isonicotinohydrazide complex.

Since $Cu^{II}INH$ dissolves in small amounts in HCl, it can be surmised that addition of HCl breaks down the polymeric structure leading to dissolution of the complex. Addition of small amounts of HCl did decrease the broadening of the 2H and 6H protons to a small extent in the proton NMR. They become slightly sharp. Hence, in the presence of HCl its structure may be given as shown in Figure 1, which is similar to the one obtained by X-ray studies of $Cu(INH)Cl_2·HCl$ (Hanson et al., 1981). In buffer at pH 7.6 the pyridine nitrogen will not be protonated. Cu^{2+}, as shown in Figure 1, may exhibit a distorted octahedral structure since it is well known that Cu^{2+} forms distorted octahedral complexes due to the Jahn–Teller effect (Cotton & Wilkinson, 1972).

Cleavage of DNA by $Cu^{II}INH$. Fragments obtained by incubation of calf thymus DNA with varying concentrations of $Cu^{II}INH$ when studied by agarose gel electrophoresis indicated the cleavage of DNA to various extents (Figure 2). Appropriate amounts of the $Cu^{II}INH$ solution in HCl when added to the DNA solution in Tris-HCl buffer (pH 7.6) did not change the pH of the buffer solution. It was shown that calf thymus DNA of size 1–10 kb gave rise to fragments of size 150–1600 base pairs on incubation with 300 μM $Cu^{II}INH$. This cleavage was observed at concentration levels of 200 μM $Cu^{II}INH$ and above. Similar studies with $CuSO_4$ and INH did not show any fragmentation of DNA.

Circular Dichroism Studies. The CD spectrum of calf thymus DNA exhibits a positive band at 274 nm and a negative band at 246 nm, typical of those obtained for B-DNA (Figure 3). While the former band is due to base stacking (corresponding UV maxima at 258 nm), the latter may be due to the helicity observed in the DNA (Ivanov et al., 1973). Calf thymus DNA incubated with $Cu^{II}INH$ at 37 °C showed different mean residual ellipticity values for both positive and negative bands from those observed for the unincubated sample (Table I). The CD spectra of samples of DNA incubated with $Cu^{II}INH$ showed a decrease in the mean molar ellipticity values for both the positive and negative bands with increase

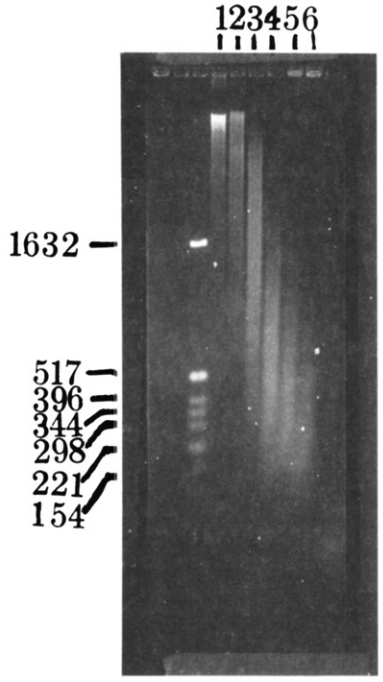

FIGURE 2: Gel electrophoresis of DNA samples incubated with various concentrations of $Cu^{II}INH$: calf thymus DNA (80 μg/mL in Tris-HCl buffer, pH 7.6) incubated with (1) 0, (2) 50, (3) 100, (4) 200, (5) 300, and (6) 400 μM $Cu^{II}INH$.

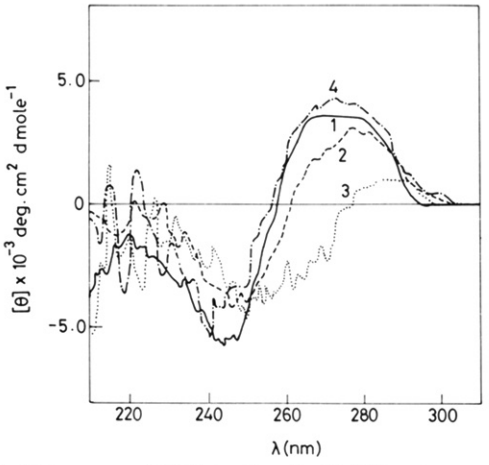

FIGURE 3: CD spectra of DNA incubated with different concentrations of $Cu^{II}INH$: calf thymus DNA (80 μg/mL in Tris-HCl buffer, pH 7.6) incubated with (1) 0, (2) 100, and (3) 300 μM $Cu^{II}INH$ at 37 °C for 2 h and (4) sample 3 containing 5 mM EDTA.

in the concentration of $Cu^{II}INH$ (Figure 3 and Table I). The negative band also showed significant broadening. The ellipticity values given in Table I are the average values of two to three experiments. Above 400 μM $Cu^{II}INH$ concentration it was difficult to observe any change in the CD spectra because of the increase in noise level due to absorption of $Cu^{II}INH$. When the experiment was repeated with sonicated DNA (incubated with $Cu^{II}INH$) (fragment size 120 ± 80 base pairs), the observations were very similar. From Table I, it can be seen that the same experiment carried out with various concentrations of $CuSO_4$ or INH alone did not produce the effect observed by $Cu^{II}INH$.

The changes observed at 274 nm are not due to induced CD by $Cu^{II}INH$. The UV spectrum of $Cu^{II}INH$ does not show any absorption around 260 nm at the concentration level of $Cu^{II}INH$ used in our experiments. CD scanning of $Cu^{II}INH$ alone does not exhibit any band around 260 nm. However, DNA itself exhibits this band which has been shown to be

Table I: CD Parameters of Calf Thymus DNA–$Cu^{II}INH$ Interactions[a]

sample	mean residual ellipticities (deg·cm²·dmol⁻¹) $[\theta]_{274}$	$[\theta]_{246}$
DNA (unincubated)	4263 (3)	−5170 (3)
DNA (incubated)	3630 (2)	−5693 (2)
DNA + 50 μM $Cu^{II}INH$	3245 (3)	−4620 (3)
DNA + 100 μM $Cu^{II}INH$	2585 (3)	−4675 (3)
DNA + 200 μM $Cu^{II}INH$	2585 (3)	−3850 (3)
DNA + 300 μM $Cu^{II}INH$	529 (5)	−3300 (5)
DNA + 400 μM $Cu^{II}INH$	495 (3)	−3245 (3)
DNA + 300 μM $Cu^{II}INH$		
0 min	−5115 (2)	−2145 (3)
15 min	−4868 (2)	−3465 (2)
30 min	−2475 (2)	−3465 (2)
60 min	−962 (3)	−3438 (3)
120 min	529 (5)	−3300 (5)
DNA + 200 μM INH	3465	−3548
DNA + 300 μM INH	3630	−5155
DNA + 400 μM INH	3135	−3548
DNA + 200 μM $CuSO_4$	4290	−3548
DNA + 300 μM $CuSO_4$	3053	−4950
DNA + 400 μM $CuSO_4$	3053	−4125
DNA + 300 μM $Cu^{II}INH$ + 5 mM EDTA	4455	−3383
DNA + 400 μM $Cu^{II}INH$ + 5 mM EDTA	3548	−4373

[a] Calf thymus DNA, 80 μg/mL in 0.02 M Tris-HCl buffer at pH 7.6, was used throughout. All samples were incubated at 37 °C for 2 h (unless otherwise stated). The values in parantheses indicate the number of experiments from which the tabulated average values were arrived at.

affected by high salt concentration (Ivanov et al., 1973). Induced CD bands can also occur at wavelengths other than 274 nm. The region from 300 to 700 nm was subjected to CD scanning for this purpose, but no satisfactory indication of induced CD could be detected.

Interaction of $Cu^{II}INH$ with DNA was also studied as a function of time. There was instantaneous binding of $Cu^{II}INH$ to DNA which was evident from the decrease in the positive band in the CD spectrum. There was gradual broadening as well as gradual decrease in the mean residual ellipticity value of the negative band as a function of time (Table I). It was also observed that when EDTA was added to the reaction mixture incubated with varying concentrations of $Cu^{II}INH$, the resultant CD spectra obtained were those of native DNA, the mean residual ellipticity values also being in the same range (Table I and Figure 3). Gel electrophoresis also showed that addition of EDTA stopped the cleavage.

^{31}P NMR Studies of $Cu^{II}INH$ Interaction with Sonicated DNA. Since calf thymus DNA gave a highly viscous solution, the ^{31}P NMR signal was too broad to obtain any meaningful result. Hence sonicated DNA was used. ^{31}P NMR could not be carried out at the concentrations used for CD, because for CD only microgram levels of sonicated DNA were required. ^{31}P NMR requires at least milligram quantities of the sample to get a reasonably good spectrum at 32.2 MHz. The phosphorus signal from the phosphate backbone of the sonicated DNA was observed at 1.1–1.3 ppm upfield to phosphoric acid. Samples of DNA incubated with varying concentrations of $Cu^{II}INH$ gave broad signals for the phosphorus, and the observed broadening increased with increasing concentrations of $Cu^{II}INH$ (Figure 4). Spectral width of the signal at half-height ($\nu_{1/2}$) was measured since it was related to the line shape and spin–spin relaxation time (T_2) by the relation $\nu_{1/2} \propto 1/T_2$. The width from the phosphoric acid signal (external reference) was used as the standard, and the observed $\nu_{1/2}$ values of the samples were normalized with respect to the former; these values are given in Table II. Since copper in

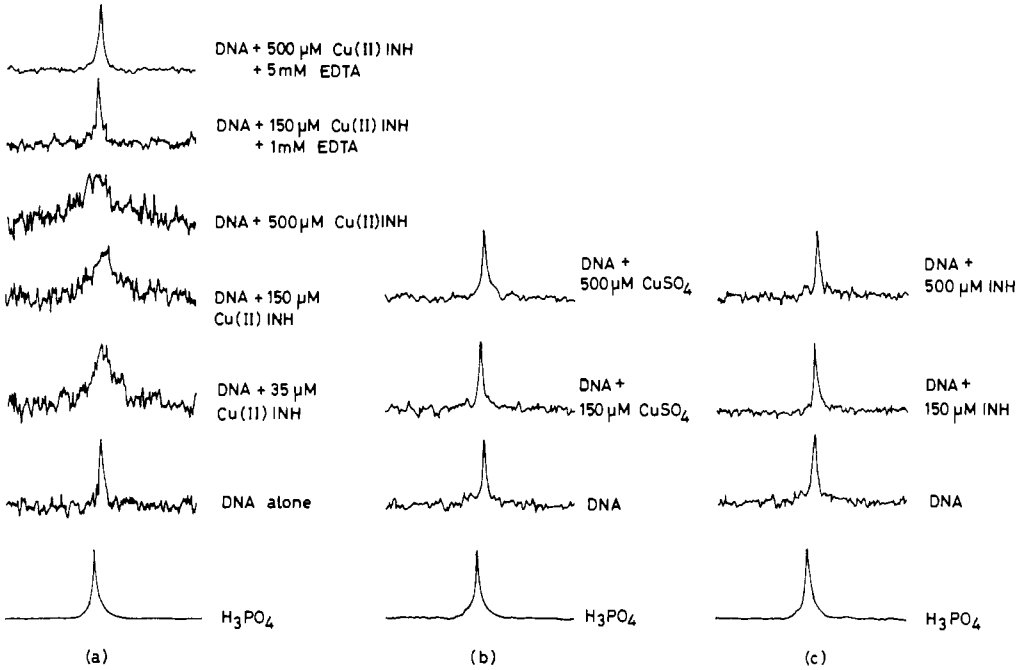

FIGURE 4: ^{31}P NMR spectra of (a) DNA samples incubated with various concentrations of $Cu^{II}INH$ including those showing the effect after the addition of different concentrations EDTA to the incubated samples and (b and c) DNA samples incubated with equivalent concentrations of $CuSO_4$ and INH, respectively.

Table II: ^{31}P NMR Parameters of $Cu^{II}INH$–DNA Interactions[a]

sample	peak width ($\nu_{1/2}$)
DNA (10 mg/mL)	0.20
DNA + 35 μM $Cu^{II}INH$	0.60
DNA + 150 μM $Cu^{II}INH$	0.75
DNA + 500 μM $Cu^{II}INH$	0.85
DNA + 150 μM $Cu^{II}INH$ + 1 mM EDTA	0.15
DNA + 500 μM $Cu^{II}INH$ + 5 mM EDTA	0.15
DNA + 150 μM $CuSO_4$	0.20
DNA + 500 μM $CuSO_4$	0.15
DNA + 150 μM INH	0.15
DNA + 500 μM INH	0.15

[a] Values were obtained at 32.2 MHz and are given in centimeters. Peak widths were normalized with respect to that of phosphoric acid, which was found to be 0.15 in most cases. DNA used was sonicated, and the average size was found to be 150 ± 75 base pairs (for the $Cu^{II}INH$–DNA) and 120 ± 80 base pairs (for the $CuSO_4$–DNA and INH–DNA interactions).

$Cu^{II}INH$ is paramagnetic, the observed broadening is indicative of the proximity of $Cu^{II}INH$ binding to the phosphate group of DNA. The cleavage as evidenced by the gel electrophoresis may result in small chemical shifts of the ^{31}P NMR signal. However, since the fragment size of the cleavage is large, appreciable shifts are not observed. Addition of EDTA to the reaction mixture restored the original peak width in the ^{31}P NMR spectrum of the native DNA (Figure 4). It was also observed that addition of $CuSO_4$ and INH at the concentration levels used for $Cu^{II}INH$ did not have any effect on the NMR spectrum of DNA (Table II and Figure 4). T_2 is dependent on temperature and viscosity. Probe temperature was maintained the same for all the samples run. Change in viscosity was not measured. However, the samples used contained the same amount of DNA. The observed effects reflect only the structural changes occurring in DNA in presence of $Cu^{II}INH$ because the original peak was restored on addition of EDTA.

Cyclic Voltametric and ESR Studies of the $Cu^{II}INH$–DNA System. Since cuprous ions are known to attack DNA randomly (Minchenkova & Ivanov, 1967), it is important to determine the state of copper in $Cu^{II}INH$. Cyclic voltametry experiments showed that both $Cu^{II}INH$ and $Cu^{II}INH$–DNA samples could be reduced to Cu^{+} by applying extra energy in the form of voltage. In the time scale of this experiment (milliseconds duration) this reaction was observed to be reversible, involving a single electron, and the redox potential for the reaction, $Cu^{2+} \rightleftharpoons Cu^{+} + e^{-}$, was determined to be 0.114 V. If Cu^{+} was present in the sample as a stable species, this reversibility would not have been noticed. Hence it can be concluded that only Cu^{2+} was present in both the samples. ESR spectra recorded in a Varian X-band spectrometer operating at 9.05 GHz were very similar for both $Cu^{II}INH$ and $Cu^{II}INH$–DNA solutions, indicating that the geometry around Cu^{2+} did not change when bound to DNA and that the state of the copper ion remained the same in both the cases. Diamagnetic cuprous ions if formed in detectable amounts would reduce the ESR signal intensity appreciably. Also, in the time scale of the experiments no stable free radicals were detected. The ESR spectra obtained were identical with those reported earlier (Hillerband et al., 1975).

Discussion

The decrease in the magnitude of the CD bands of DNA at 274 nm and at 246 nm and broadening of the band at 246 nm on addition of $Cu^{II}INH$ indicate that $Cu^{II}INH$ binds to DNA, effecting structural changes within the DNA molecule. It is known that CD is very sensitive to structural changes occurring within a DNA molecule brought about by different conditions such as solvent, temperature, pH, ionic strength, and presence of interactive molecules (Ivanov et al., 1973). From the CD studies on DNA fibers under conditions of humidity and ionic strength close to those used in X-ray studies to obtain A, B, and C forms (Tunis-Schneider & Maestre, 1970) and other studies on the CD of complementary RNA (Samejuma et al., 1968), it can be inferred that CD spectra of B family of DNA are characterized by the negative band at 246 nm and a gradual decrease in the magnitude of the positive band at 274 nm with the increase in the winding angle of the helix (Ivanov et al., 1973). Variations in structural features within this family of DNA resulting in transition to other forms have also been reported. Theoretical calculation

on CD (Johnson & Tinoco, 1969) have shown a decrease in the magnitude of positive 275-nm band of the DNA spectra as the molecule is shifted to the C-type geometry. This form has a greater value for the winding angle accompanied by a decrease in the width of the narrow groove, indicating it is more wound than the B form. This enables a most tight packing of the parallel DNA threads. Monovalent cations like Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ give rise to CD spectra with a reduced 274-nm positive band, implying a C form of DNA in the presence of these metal ions (Ivanov et al., 1973). C-type geometry has also been obtained for DNA in water–ethylene glycol solution (Green & Mahler, 1971; Nelson & Johnson, 1970), at high salt concentrations (Permogrov et al., 1970), and also in nucleo–histone complexes (Bram, 1971). However, recent studies question the existence of C-form DNA in solution (Lee et al., 1981; Lee & Charney, 1982; Zimmerman, 1982).

The CD spectra of DNA in the presence of $Cu^{II}INH$ resembled those obtained in the presence of the above-mentioned monovalent cations. But from the available data reported here it is not clear whether such a transformation of DNA from the B to C form takes place when incubated with $Cu^{II}INH$. However, our CD results clearly indicate binding of $Cu^{II}INH$ to DNA. The decrease in the magnitude of the positive and negative bands is not likely to be due to cleavage because addition of EDTA restores the original spectrum.

The increase in line width of the ^{31}P NMR signal of $Cu^{II}INH$-bound DNA indicates that the binding of copper in $Cu^{II}INH$ is in the proximity of the phosphorus atom of the phosphate backbone of DNA. The most probable site of copper binding may be the phosphate oxygen.

Since very small amounts of $Cu^{II}INH$ are responsible for the reaction (DNA/$Cu^{II}INH$ ratio of 0.8 for CD and 60 for ^{31}P NMR studies), it may be inferred that the binding constant for the $Cu^{II}INH$–DNA system is on the order of 10^4 mol^{-1}. Addition of EDTA results in breaking the $Cu^{II}INH$ complex bound to DNA since EDTA can form a strong complex with Cu^{2+} ($\log K = 18.7$). The $\log K$ value of the 1:1 complex of $Cu^{II}INH$ is 8.0 (Albert, 1953). Removal of $Cu^{II}INH$ from the DNA by EDTA restores the original structure in the fragmented DNA, which is likely to be double helical resembling the DNA (Figure 3 and Table I).

From our CD and NMR studies it is evident that $Cu^{II}INH$ binds to DNA reversibly and cleaves it. At the concentration levels of $Cu^{II}INH$ employed, both $CuSO_4$ and INH individually did not interact with DNA. However, the results obtained do not give an unambiguous picture of the geometry of $Cu^{II}INH$ binding to DNA. It is possible that the intercalation of the INH moiety of $Cu^{II}INH$ into DNA bases is a likely explanation for the observed results. However, the available space of 3.4 Å between the bases in the B form of DNA is not sufficient enough to allow for such intercalation. DNA intercalators like ethidium bromide do so by unwinding the DNA helix. Our data do not conclusively support the intercalation of the INH moiety of $Cu^{II}INH$ into DNA bases. Also, the exact mechanism of cleavage of DNA by $Cu^{II}INH$ is not known. Hence, at this stage it can only be assumed that the geometry of disposition of $Cu^{II}INH$ within DNA is such that it cleaves the double helix. It is also interesting to note that while intercalating drugs such as bleomycin and phleomycin promote massive degradation of DNA under conditions that include the presence of molecular oxygen (O_2), a metal ion, and a reducing agent such as mercaptoethanol or dithreitol (Waring, 1981), $Cu^{II}INH$ does not require these conditions. While bleomycin analogues can give rise to single-stranded and double-stranded fragments, $Cu^{II}INH$ was found to give rise to double-stranded fragments (EDTA addition experiments, Figure 3 and Table I).

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