Copper(II) Complexes Containing N,N-Donor Ligands and Dipeptides Act as Hydrolytic DNA-Cleavage Agents

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Copper(II) complexes are known to play a significant role in both naturally occurring biological systems and pharmaceutical agents. Recently, Cu^{II} complexes have gained importance in DNA cleavage essential for the development of anticancer drugs and chemotherapeutic agents. Therefore, we have designed small molecules, consisting of a metal ion, N,N-donor ligands, and dipeptides, to probe their DNA-cleaving potential. Accordingly, the interaction of Cu^{II} with ethylenediamine, histamine and the dipeptides histidylglycine, histidylalanine, and histidylleucine has been investigated. The binding modes, stabilities, and geometries of these complexes were determined by various physicochemical techniques. Their DNA-binding abilities were probed by absorption and fluorescence spectroscopy, and their DNA-cleavage potential was tested by electrophoresis.

Introduction. – Metal complexes with N,N-donor ligands or with dipeptides containing side chains with potential donors are extremely important. In particular, Cu^{II} complexes are known to play a significant role in biological systems and as pharmaceutical agents [1–3]. Among suitable dipeptides, those containing histidine (His) are particularly interesting, since the imidazolyl group ionizes at biological pH. Further, the interest in the design, synthesis, and study of these metal complexes has been spurred by their ability to act as DNA- or RNA-conformational probes, footprinting reagents, and as models to understand the selective recognition of these biopolymers [4–7]. Recently, DNA-binding metal complexes with basic peptide ligands have been developed by *Long* and co-workers [8]. Suitable peptide ligands enable a metal complex to present to a nucleic acid target the same functional groups employed by proteins for the selective recognition of DNA and RNA.

Even though quite a few reagents have been successfully applied to hydrolytic RNA cleavage [9], only few were successful with DNA [10] because of its relatively high hydrolytic stability [11]. Hydrolytic-cleavage agents have an advantage over oxidative-cleavage agents: they do not require co-reactants. Metal ions are known to play an important role in cleavage reactions [12], but the precise role in the hydrolytic mechanism is unclear [13].

Sigman and co-workers [14] have shown that $[Cu^{+}(phen)]$ (phen = [1,10]phenanthroline) was the precursor in the efficient cleavage of DNA in the minor groove. $[Cu^{+}(phen)_2]$ was also used as a footprinting reagent and as a probe for DNA and RNA secondary structures [15]. Some Cu-based ligand systems [16] have also been used to promote oxidative degradation of DNA.

Lanthanide ions [17] and their macrocyclic complexes [18] were used to catalytically hydrolyze activated phosphate esters. However, because of their high lability, the

cleavage was affected. Another species that rapidly hydrolyzes supercoiled DNA in the presence of H_2O_2 (or O_2) and a reducing agent is 'diiron' [19].

Recent studies involving Cu^{II} pertains to the hydrolytic cleavage of phase DNA [20]. The hydrolysis of phosphodiesters by Zn complexes of His-containing peptides was also reported [21].

Due to the presence of the imidazole ring, His exhibits metal-binding and DNA-binding activities different from those of other amino acids [22–24]. Cu^{II} Complexes of His showed hydrolytic cleavage activity for both plasmid DNA and a dinucleotide at physiological pH and temperature [25]. Cu^{II} Complexes of peptides exhibit different modes of binding to DNA depending on the position of the His residue in the peptide [26–27]. *Nagane et al.* [28] reported that a tetrapeptide with a C-terminal Leu induces a considerable change in the behavior of the complexes when binding to DNA. It was also reported that ternary Cu^{II}–dipeptide complexes with 1,10-phenanthroline (phen) molecules show significant differences in their nuclease activity, depending on the nature of the peptide moiety [29].

In the light of the above, we planned to develop stable small molecules as models to improve our knowledge of protein/nucleic acid and small-molecule/nucleic acid recognition. The present manuscript deals with the interaction of Cu^{II} with N,N donor ligands, *i.e.*, ethylenediamine, histamine and dipeptides (histidylglycine, histidylalanine, histidylleucine), and their potential to cleave DNA.

Results and Discussion. – Histidylglycine, histidylalanine and histidylleucine (HisGly, HisAla, and HisLeu, resp.) contain two N-atoms in the imidazole ring of the His moiety, one of which is protonated in the biologically relevant pH range of 5–7. This N-atom can coordinate to metal ions, especially when His is a part of a protein chain. HisGly, HisAla, and HisLeu may coordinate to metal ions through carboxylate, imidazole, and amino groups, ethylenediamine (=ethane-1,2-diamine; EN) and histamine (=2-(1*H*-imidazol-3-yl)ethane-1-amine; Hist) through amino and amino/imidazole groups, respectively. The dissociation constants for HisGly, HisAla, HisLeu, EN, and Hist (H^+ abstraction from COOH, NH^+, NH_3^+ and NH_3^+, NH^+, NH_3^+, resp.), are known [30][31]. However, they were redetermined under experimental conditions to avoid discrepancies that might affect the complex stabilities. Gratifyingly, there was an excellent agreement between our values (*Table 1*) and those reported [30][31]. The complexation constants K of Cu^{II} 1:1 complexes with EN, Hist, and HisGly [32],

Table 1. *Acidity of Protic Groups of Selected Ligands.* Conditions: aqueous solution of the free ligand, at 35°. For comparison, literature values [29–31] are given in parentheses. Abbreviations: EN, ethylenediamine; HisAla, histidylalanine; HisGly, histidylglycine; HisLeu, histidylleucine; Hist, histamine; Im, 1*H*-imidazol-4-yl.

Ligand (L)	pK_a		
	COOH	ImH^+	NH_3^+
EN	–	–	9.15 (9.70)
			7.07 (6.93)
Hist	–	5.89 (6.01)	9.42 (9.57)
HisGly	2.80 (2.32)	5.80 (5.39)	7.16 (7.15)
HisAla	3.39 (3.24)	5.91 (5.82)	7.46 (7.62)
HisLeu	3.60 (3.51)	5.85 (5.61)	7.42 (7.54)

respectively, were also redetermined to minimize the errors in the evaluation of Δ (log K) values.

The titration curves of Cu^{II} with HisGly, HisAla, and HisLeu (*Fig. 1*, curve *D*) showed an inflection at $m = 3$, indicating simultaneous dissociation of three protons[1]). The corresponding K_{ML} and K_{MA} values[2]) were calculated according to *Eqns. 1* and *2* (see the *Exper. Part*) and are presented in *Table 2*.

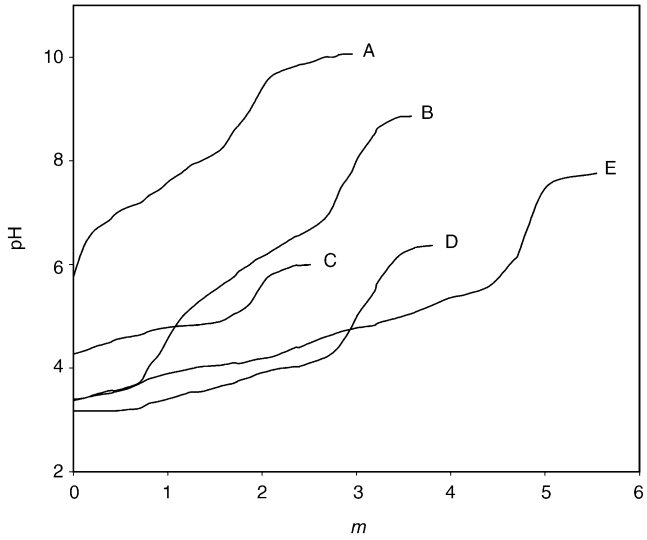


Fig. 1. *Potentiometric titration curves of free EN* (curve *A*), *free HisLeu* (curve *B*), *[Cu^{II}(EN)] 1:1 complex* (curve *C*), *[Cu^{II}(HisLeu)] 1:1 complex* (curve *D*), *and [Cu^{II}(EN)(HisLeu)] 1:1:1 complex* (curve *E*). Conditions: aqueous 0.1M KNO_3 solution, at 35°. The horizontal axis (*m*) states the number of (molar) equivalents of base added relative to Cu^{II}. For abbreviations, see text and *Table 1*.

Table 2. *Formation Constants for Selected Binary Cu^{II} Complexes.* Conditions: 0.1M aqueous KNO_3 solution, at 35°. For abbreviations, see *Table 1*.

Complex (1:1)	log *K*	Complex type[1])	Donor atoms involved
[Cu^{II}(EN)]	10.48	ML	N, N
[Cu^{II}(Hist)]	9.43	ML	N, N
[Cu^{II}(HisGly)]	8.05	MA	N, N, O
[Cu^{II}(HisAla)]	8.48	MA	N, N, O
[Cu^{II}(HisLeu)]	9.51	MA	N, N, O

The mixed-ligand titration curves of [Cu^{II}(EN)(HisGly)], [Cu^{II}(EN)(HisAla)], [Cu^{II}(EN)(HisLeu)] (*Fig. 1*, curve *E*) and [Cu^{II}(Hist)(HisGly)], [Cu^{II}(Hist)(HisAla)] and [Cu^{II}(Hist)(HisLeu)] (*Fig. 2*, curve *E*) showed inflections at $m = 1$ and $m = 5$. The titration curves between $m = 0$ and $m = 1$ exactly coincided with those of the free ligand curves, indicating no interaction in that region. Thus, ternary-complex formation was

[1]) The term *m* (see *Fig. 1*) represents the number of (molar) equivalents of base added relative to Cu^{II}.

[2]) The terms ML and MA stand for 'metal/ligand' and 'metal/amino acid', resp., the latter actually refering to 'dipeptide' rather than 'amino acid'. MLA represents, thus, mixed ternary complexes.

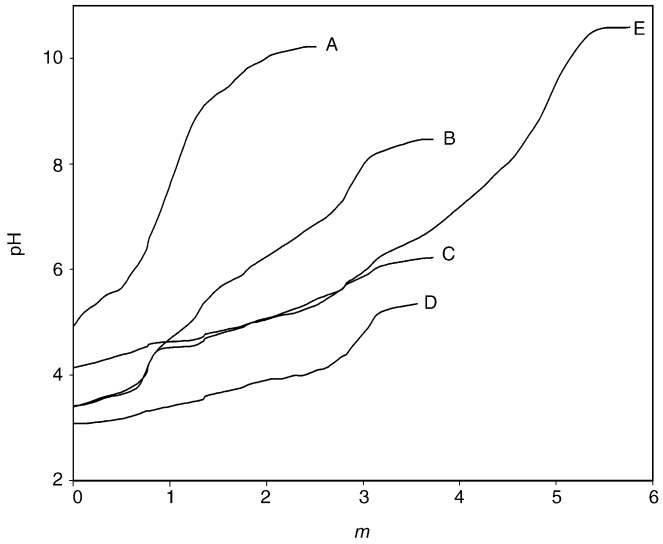


Fig. 2. *Potentiometric titration curves of free Hist* (curve *A*), *free HisLeu* (curve *B*), *[Cu^{II}Hist] 1:1 complex* (curve *C*), *[Cu^{II}(HisLeu)] 1:1 complex* (curve *D*), *and [Cu^{II}(Hist)(HisLeu)] 1:1:1 complex* (curve *E*). For conditions and abbreviations, see *Fig. 1* and *Table 1*, resp.

considered in the buffer region $m = 1-5$ by assuming simultaneous dissociation of four protons. The constants K_{MLA} were determined according to *Eqn. 3* (*Exper. Part*). All constants were subjected to refinement considering all possible species in solution. The extent of stabilization in ternary complexes was quantified in terms of $\Delta(\log K)$, *i.e.*, the difference between the stabilities of the ternary and the sum of the binary systems. In all cases studied, positive values of $\Delta(\log K)$ were found, indicating stabilization in ternary complexes (*Table 3*). The percentages of various species present in solution were computed as a function of pH to identify the most stable species at biological pH. Interestingly, 1:1:1 ternary complexes are the major species present at biologically significant pH (*Fig. 3*).

In all the systems investigated, the $\Delta(\log K)$ values increased with increasing length of the amino-acid side chain (Gly → Leu) of the peptides. This may be attributed to hydrophobic interactions.

As can be seen from *Table 3*, the EN systems are more stable than the Hist systems. This may be due to the formation of five-membered chelates in the former systems.

Table 3. *Formations Constants for Selected Ternary Cu^{II} Complexes of Type MLA*[1]). Conditions: 0.1M aqueous KNO_3 solution, at 35°. For abbreviations, see text and *Table 1*.

Complex (1:1:1)	log*K*	Δ(log *K*)	Donor atoms involved
[Cu^{II}(EN)(HisGly)]	20.83	2.22	N, N, N, N
[Cu^{II}(EN)(HisAla)]	21.60	2.64	N, N, N, N
[Cu^{II}(EN)(HisLeu)]	22.73	2.74	N, N, N, N
[Cu^{II}(Hist)(HisGly)]	19.72	1.29	N, N, N, N
[Cu^{II}(Hist)(HisAla)]	19.91	1.73	N, N, N, N
[Cu^{II}(Hist)(HisLeu)]	21.45	2.24	N, N, N, N

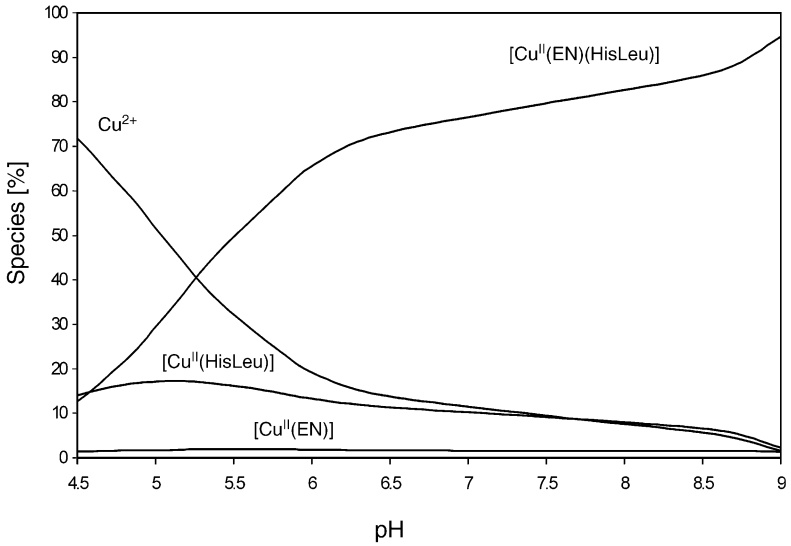


Fig. 3. *pH-Dependent distribution of species involved in the formation of the ternary 1:1:1 complex [Cu^{II}(EN)(HisLeu)]*

The ^{1}H-NMR spectra of the ligands in the absence and presence of Cu^{II} are shown in *Figs. 4* and *5*. The assignment of the free-ligand resonances are based on literature comparison [33][34] and simulation (*ChemDraw Ultra 7.0*). There was excellent agreement between our values and those reported.

For the [Cu^{II}(EN)(HisLeu)] system in a ratio Cu/EN/HisLeu 1:100:100, peak broadening and downfield shifts of CH(γ), CH(ε), CH(α), CH$_2$(β), CH$_2$(1), and CH$_2$(2) were observed, indicating the involvement of the imidazole and amino N-atoms of HisLeu, and of the two amino N-atoms of EN on complexation. When the Cu^{II} concentration was increased tenfold (Cu/EN/HisLeu 1:10:10; *Fig. 4,c*), the paramagnetic effect of the metal ion was clearly observed; and the signals totally vanished for an equimolar ratio of all complex partners (*Fig. 4,d*).

A similar experiment was performed with Hist and HisLeu in the presence and absence of Cu^{II} (*Fig. 5*). The *doublets* at δ_H 7.05 and 8.05 (*Fig. 5,a*) are due to the presence of two imidazole rings. In the case of the ratio Cu/Hist/HisLeu 1:100:100, peak broadening and downfield shifts of CH(γ), CH(ε), CH(α), CH$_2$(β), CH$_2$(1), and CH$_2$(2) were observed, indicating the involvement of imidazole N-atoms, and amino N-atoms from Hist and HisLeu, respectively, upon complexation. The ^{1}H-NMR spectra, thus, clearly established a tetra coordination of Cu^{II}.

To further assign the geometries of these complexes, electronic spectra of [Cu^{II}(EN)(HisLeu)] and [Cu^{II}(Hist)(HisLeu)] were recorded. Both exhibited d $\rightarrow$ d transition bands in the visible region, with maximum absorptions at 567 and 575.5 nm, which is typical for square-planar complexes [35–39].

The most-stable systems from each complex type, *i.e.*, [Cu^{II}(EN)(HisLeu)] (**1**) and [Cu^{II}(Hist)(HisLeu)] (**2**), were selected for DNA-binding studies. On addition of increasing amounts of DNA to these complexes in solution, both hypo- and

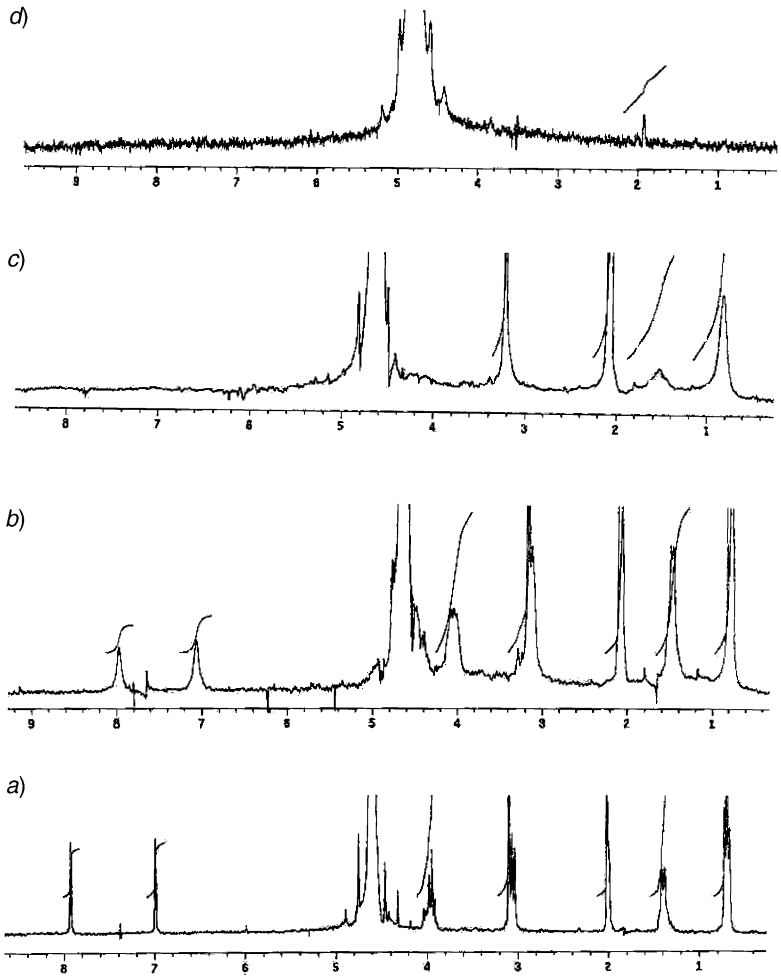


Fig. 4. *^{1}H-NMR Spectra of EN and HisLeu as a function of copper(II) concentration.* Conditions:D_2O, pD 7, 25°; [EN] = [HisLeu] = 30 mM. *a*) – *d*) [$Cu(NO_3)_2$] = 0, 0.3, 3.0, 30 mM, resp. (*i.e.*, [Cu^{II}]/[EN]/[HisLeu] 0 : 1 : 1, 1 : 100 : 100, 1 : 10 : 10, and 1 : 1 : 1, resp.). For abbreviations, see *Table 1.*

hypsochromic shifts (blue shift from 567 and 575.5 to 564 and 572.5 nm, resp.) were observed. These blue shifts and decreased absorbance intensities for complexes **1** and **2** indicate DNA binding.

Ethidiumbromide (EB)[3]) strongly fluoresces in the presence of DNA due to complete intercalation between adjacent DNA base pairs, a process that can be reversed by addition of a competing molecule (fluorescence quenching) [40]. Two mechanisms have been proposed in this context, the replacement of molecular fluorophores, and/or electron transfer [41][42]. The quenching of the fluorescence of

[3]) Systematic name: 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide.

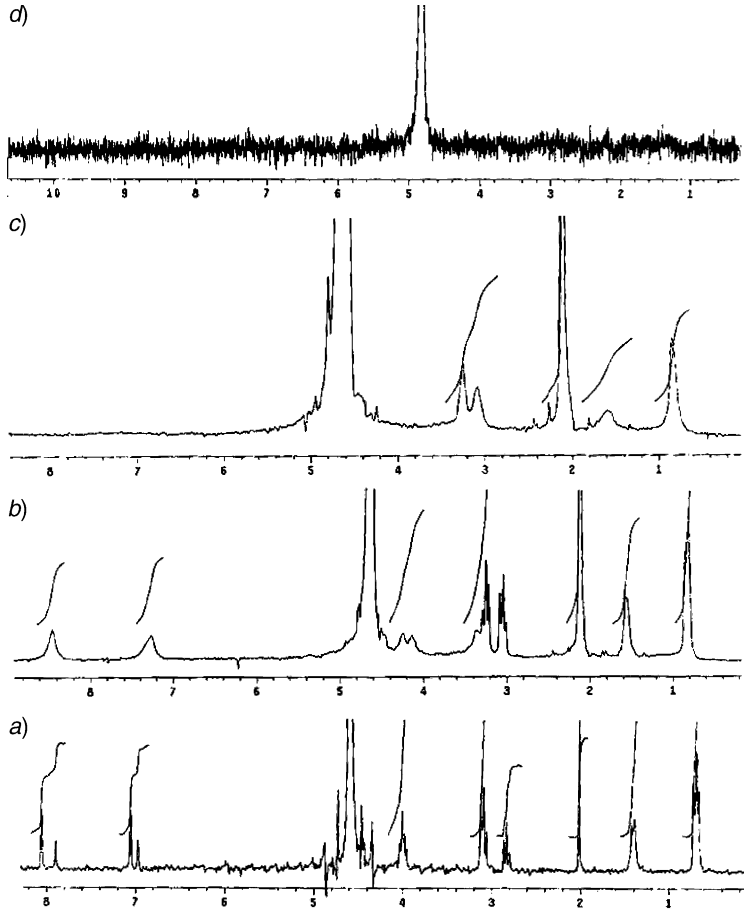
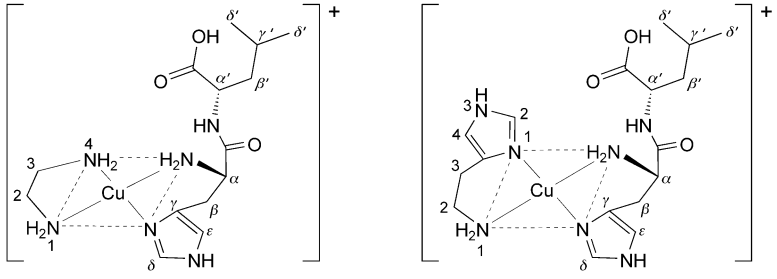


Fig. 5. *^{1}H-NMR Spectra of Hist and HisLeu as a function of copper(II) concentration*. Conditions: D_2O, pD 7, 25°; [Hist] = [HisLeu] = 30 mM. *a*) – *d*) [$Cu(NO_3)_2$] = 0, 0.3, 3.0, 30 mM, resp. (*i.e.*, [Cu^{II}]/[Hist]/[HisLeu] 0 : 1 : 1, 1 : 100 : 100, 1 : 10 : 10, and 1 : 1 : 1, resp.). For abbreviations, see *Table 1*.



1
$[Cu^{II}(EN)(HisLeu)]^+$

2
$[Cu^{II}(Hist)(HisLeu)]^+$

EB bound to DNA can be used to determine the DNA-binding strength of a given molecule.

The emission spectra of EB bound to DNA in the absence and presence of **1** and **2** shown in *Fig. 6*. The addition of the complexes to DNA · EB caused a reduction in fluorescence intensity, indicating that the complexes did bind to DNA. *Liu et al.* [42] previously reported that the fluorescence quenching of DNA · EB by macrocyclic tetraaza complexes is probably due to the replacement of the DNA intercalator. This

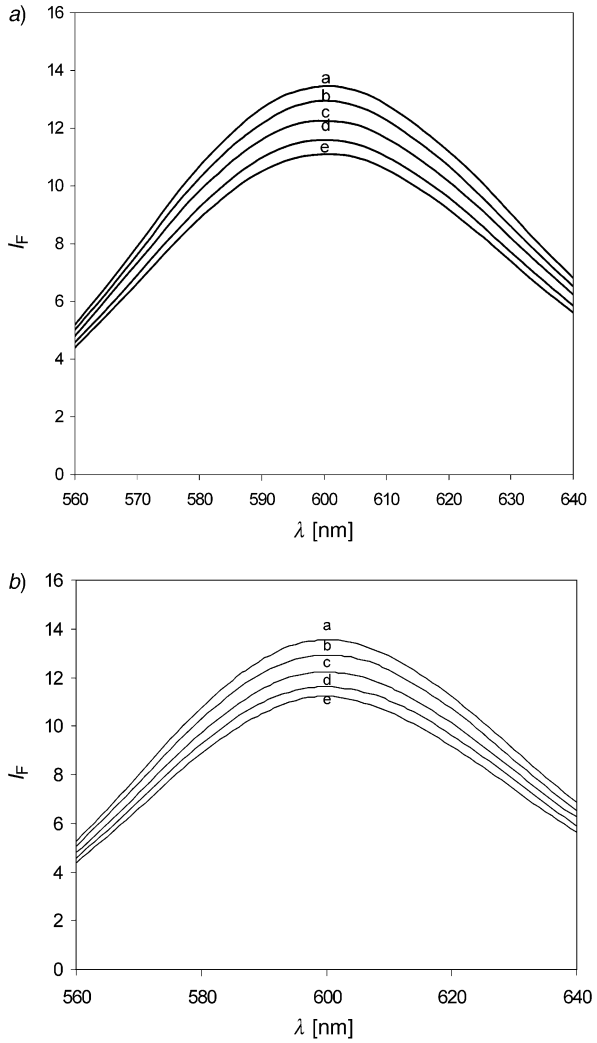


Fig. 6. *Fluorescence titration of ethidium bromide · CT-DNA* (EB · CT-DNA) *with* a) *[Cu^{II}(EN)(HisLeu)]* (**1**) *and* b) *[Cu^{II}(Hist)(HisLeu)]* (**2**) *as guests.* Conditions: 10 mM aqueous NaCl solution, initial pH 7.5; [EB = 110 μM, [CT-DNA] = 112 μM; [**1**] = [**2**] = 0, 32, 62, 90, and 117 μM, resp. The fluorescence intensity I_F decreased in both titrations with increasing concentrations (a – e) of guest added.

means that the complexes interact with the same DNA sites as EB does. The structures of compounds **1** and **2** are similar to those of *Liu*'s macrocyclic complexes, thus, similar conclusions could be drawn on the quenching abilities of these complexes.

Fluorescence *Scatchard* plots for the binding of EB to CT-DNA (calf-thymus DNA) in the presence of metal complexes were obtained as described previously [43]. The binding isotherms of EB and DNA in the absence and presence of complexes **1** and **2** were determined experimentally and are presented in *Fig. 7*. As can be seen, the slopes and intercepts of the plots are decreased in the presence of the copper complexes, which strongly indicates intercalation with CT-DNA. These results suggest interactions between the copper coordination sphere with the basic sites of DNA, most likely N(7) of the purine moieties. Similar observations have been made earlier [44].

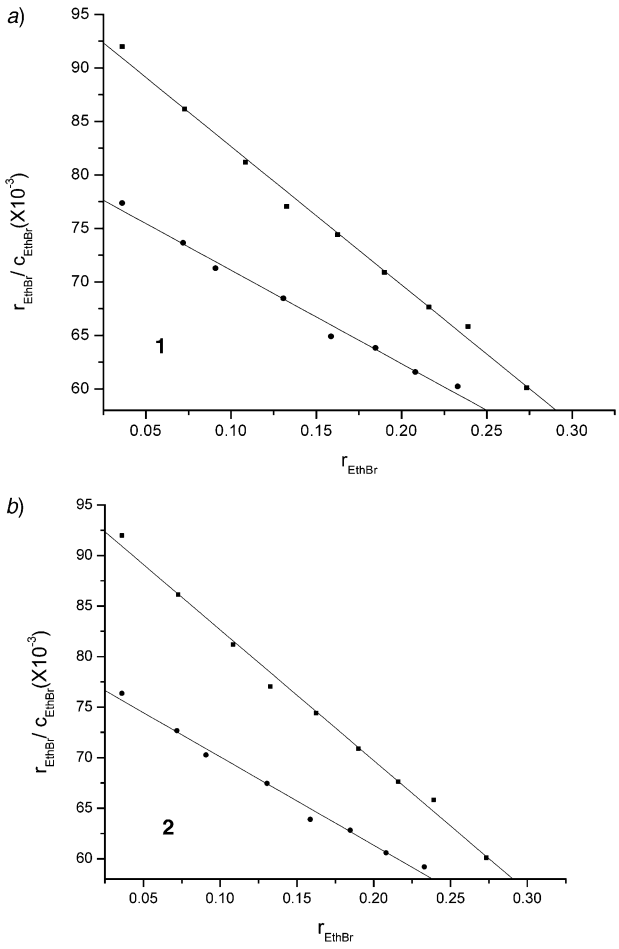


Fig. 7. *Fluorescence* Scatchard *plots for the binding of ethidium bromide* (EB) *to CT-DNA in the absence* (upper line) *and presence of* a) *[Cu^{II}(EN)(HisLeu)]* (**1**) *and* b) *[Cu^{II}(Hist)(HisLeu)]* (**2**). The term r_{EB} is the concentration ratio of bound EB to total DNA, and c_{EB} is the concentration of free EB (see *Exper. Part*).

After confirming the DNA-binding of these complexes, we performed cleavage experiments. When DNA was incubated with increasing the concentrations of **1** and **2**, supercoiled DNA (form I) was degraded to form II (relaxed circular), and then, in the case of **2** only, even slowly to form III (linear). The increased efficiency of DNA cleavage by [Cu^{II}(Hist)(HisLeu)] (**2**) is due to the presence of the two imidazole rings.

Fig. 8 shows the agarose-gel-electrophoresis patterns for the cleavage of DNA after being treated with **1** and **2**, respectively. The initial concentration of DNA was set at 0.156 μg/μl, and the concentration of the complexes was varied from 0 to 0.50 mM. At 1 mM concentration of the cleavage reagents, band broadening and slower migration of the plasmid DNA were observed, indicating DNA binding. Higher concentrations of the complexes (2 and 3 mM) led to the precipitation of the plasmid DNA as a white solid due to charge neutralization caused by extensive binding of the complexes. Generally, a single cut on a strand of supercoiled DNA leads to form II. A second cut on the complementary strand of the original cut site then linearizes the DNA to form III. Therefore, [Cu^{II}(Hist)(HisLeu)] (**2**) must have cut the DNA at least twice to convert it from form I to form III, and [Cu^{II}(EN)(HisLeu)] (**1**) must have cut the DNA once.

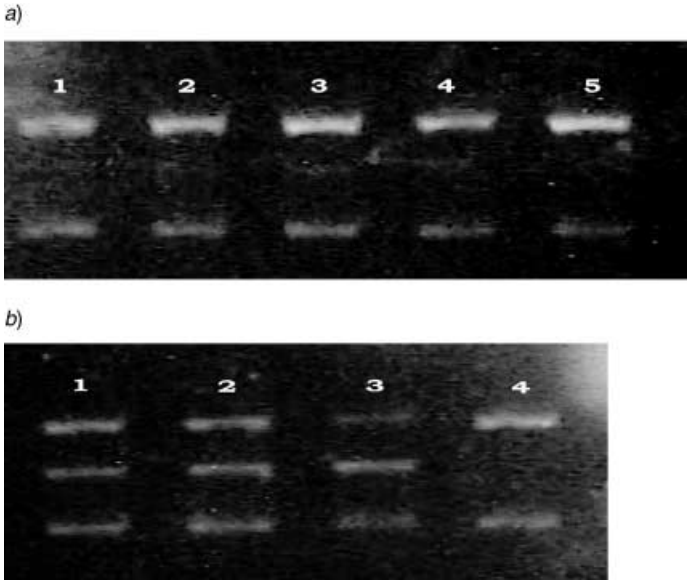


Fig. 8. *Agarose-gel-electrophoresis patterns for the cleavage of pUC19-DNA at various concentrations of* a) *[Cu^{II}(EN)(HisLeu)]* (**1**) *and* b) *[Cu^{II}(Hist)(HisLeu)]* (**2**). Conditions: 0.156 μg/μl pUC19-DNA, 5 mM aqueous *Tris*-HCl (buffer, 50 mM NaCl, 4 h at 37°. Assignments for experiments with **1**: *lane 1*, DNA control; *lanes 2–5*, DNA plus increasing concentrations of **1** (0.31, 0.89, 2.01, and 3.01 mM, resp.). Assignments for experiments with **2**: *lane 4*, DNA control; *lanes 1–3*, DNA plus increasing concentrations of **2** (0.125, 0.250, and 0.375 mM, resp.).

The extent of DNA cleavage was quantified *via* fluoroimaging (*Table 4*). Individual Cu^{2+} ions and free ligands showed no DNA cleavage at concentrations below 1.08 mM (*Fig. 9*). These results confirm that the complexes **1** and **2** are solely responsible for the degradation of DNA. One possible explanation for G/T recognition is the formation of

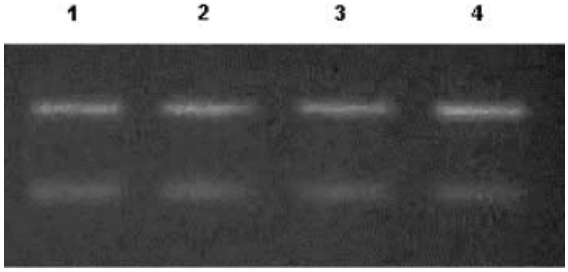


Fig. 9. *Agarose-gel-electrophoresis patterns for the cleavage of pUC19-DNA by free Cu^{2+}, (EN)(HisLeu), and (Hist)(HisLeu), respectively. Lane 1*, DNA control; *lane 2*, DNA plus Cu^{2+}; *lane 3*, DNA plus (EN)(HisLeu); *lane 4*, DNA plus (Hist)(HisLeu). The concentration(s) of both Cu^{2+} and of the free ligands were 0.375 mM, that of DNA 0.156 μg/μl.

Table 4. *Cleavage of pUC19-DNA by Complexes* **1** *and* **2** *at Different Concentrations.* Cleavage, giving rise to a distribution of different DNA forms, was monitored by fluoroimaging technique. For details, see *Fig. 8* and the *Exper. Part*.

Concentration of reagent [mM]	DNA form [%][a])		
	Supercoiled	Nicked	Linear
[Cu^{II}(EN)(HisLeu)] (**1**):			
0	51.34	48.65	0
0.125	41.31	58.68	0
0.250	37.99	62.01	0
0.375	35.50	64.44	0
0.500	29.91	70.08	0
[Cu^{II}(Hist)(HisLeu)] (**2**):			
0	51.34	48.65	0
0.125	40.54	43.45[b])	15.27
0.250	38.61	34.20	27.16[c])
0.375	28.52	27.50	43.96[d])

[a]) Abs. Error (unless specified): ± 2. [b]) Error: ± 3. [c]) Error: ± 4. [d]) Error: ± 5.

a three-center H-bond involving the NH_2 group of guanine, the imidazole N(3) lone pairs, and the C-terminal C=O group of HisLeu.

Our results indicate that the C-terminal carboxy C=O group of HisLeu plays a key role in the cleavage of the phosphodiester backbone of DNA. *Bruice et al.* [48][49], in their study of bis(2-carboxyphenyl) phosphate, found that carboxy groups can participate in phosphodiester hydrolysis. Our findings support this, and detailed mechanisms will be investigated. This is also consistent with the observation that metals are much more efficient at promoting hydrolysis than are organic buffers of similar pK_a values [50]. The improved DNA-cleavage efficiency of [Cu^{II}(Hist)(HisLeu)] (**2**) compared to that of [Cu^{II}(EN)(HisLeu)] (**1**) may be due to the presence of *two* imidazole groups in the former. Similar observations were made earlier [51].

Experimental Part

1. *General*. Ethylenediamine (EN), histamine (Hist), histidylglycine (HisGly), histidylalanine (HisAla), histidylleucine (HisLeu), and ethidiumbromide[3]) (EB) were obtained from *Sigma* (USA). $Cu(NO_3)_2$ (anal. grade) was purchased from *Merck*. CT-(calf-thymus) and pUC19-DNA were obtained from *Fluka* (Switzerland) and from *Stratagene* (USA), resp. Agarose gel and *Tris*-HCl buffer were obtained from *Bangalore Genei* (India), and were used as supplied.

2. *Stock Solutions*. Conc. CT-DNA stock solns. were prepared in doubly distilled H_2O (pH 7.5) containing *Tris*-HCl (5 mM) and NaCl (50 mM). DNA Concentrations were determined by UV, based on an extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm [52]. Solns. of CT-DNA (pH 7.5; buffer as above) gave rise to a UV-absorbance (A_λ) ratio of A_{260}/A_{280} 1.8 to 1.9, indicating that the DNA was sufficiently free of protein [53]. All stock solns. were stored at 4° and used within one week. Concentrations of EB were determined spectrophotometrically [54], using an extinction coefficient of 5680 M^{-1} cm^{-1} at 480 nm. $Cu(NO_3)_2$ stock solns. were standardized volumetrically by titration with the disodium salt of EDTA (ethylenediaminetetraacetate) in the presence of a suitable indicator, as outlined by *Schwarzenbach* [55].

3. *Potentiometric Titrations*. Potentiometric titrations of ligands in the absence and presence of Cu^{2+} ($Cu(NO_3)_2$ stock solns.) were performed at a temp. of 35°. For every titration, fresh solid ligand was weighed directly into the reaction cell to avoid possible hydrolysis. Carbonate-free NaOH was prepared according to [56] and standardized by titration with potassium hydrogen phthalate. The ionic strength was kept constant, using 0.1M KNO_3 as supporting electrolyte, and relatively low concentrations (*ca*. 1 mM) of both ligand and metal ion. During the titration, a stream of N_2-free molecular O_2 was passed through the reaction cell to avoid adverse effects of CO_2. A *Digison DI-707* digital pH meter, fitted with a combined microglass electrode, was used to determine $[H^+]$ concentrations. The apparatus was calibrated for pH values below 3.5 and above 10.5 by means of standardized HCl and NaOH solns., resp. Each experiment was repeated at least twice. Further exper. details can be found elsewhere [57].

4. *Determination of Complexation Constants*. The dissociation constants of the ligands EN, Hist, HisGly, HisAla, and HisLeu have been reported [30][31], but, for consistency, were remeasured under our experimental conditions. There was excellent agreement between our values and those reported. Stability constants of binary complexes of type ML^2) were determined by means of the computer program BEST [58]. The binary equilibria, omitting charges, are described below for the buffer region[1]) $m = 1-3$ for *a*) $M = Cu^{II}/Cu^{2+}$, L = EN or Hist (*Eqn. 1*); *b*) $M = Cu^{II}/Cu^{2+}$, A = HisGly, HisAla, HisLeu (*Eqn. 2*):

$$M + H_3L \rightleftharpoons ML + 3\ H^+$$

$$M + L \rightleftharpoons ML$$

$$K_{ML} = [ML] \cdot ([M] \cdot [L])^{-1} \quad (1)$$

and

$$M + H_2A \rightleftharpoons MA + 2\ H^+$$

$$M + A \rightleftharpoons MA$$

$$K_{MA} = [MA] \cdot ([M] \cdot [A])^{-1} \quad (2)$$

To determine the complexation constants for the ternary MLA systems[2]) (1:1:1 complexes) in the buffer region $m = 1-5$, the following equations were used:

$$M + H_2L + H_2A \rightleftharpoons MLA + 4\ H^+$$

$$M + L + A \rightleftharpoons MLA$$

$$K_{MLA} = [MLA] \cdot ([M] \cdot [L] \cdot [A])^{-1} \quad (3)$$

All complex-formation constants were subjected to computer refinement *via* the BEST software [58], considering possible species present in the soln., *i.e.*, H_2L^-, HL^{2-}, L^{3-}, HA, A^-, ML, ML_2, MA, HA, and MLA,

but excluding hydroxo and polynuclear species. The errors limits in K were minimized (σ fit -0.01 to $+0.001$). The BEST program was also used to generate complete species-distribution curves at various pH values.

5. *Spectral Experiments.* 5.1. *UV Spectra*. UV Spectra were recorded on a *Shimadzu 160A* spectrophotometer ($\lambda = 400-800$ nm), using 1-cm quartz microcuvettes. Spectra of **1** and **2** were recorded at 25° in buffered H_2O (pH 7.5; 5 mM *Tris*-HCl, 50 mM NaCl). For DNA-binding studies, an increasing amount of CT-DNA was added to the ligands, until the DNA/ligand ratio was *ca.* 1:1. All experiments were carried out at 25° (pH 7.5; buffer as above). After each addition, the mixture was shaken and equilibrated for 5 min, before the UV absorbance was recorded.

5.2. *^{1}H-NMR Spectra*. The NMR spectra of ligands, in the absence and presence of varying concentrations of Cu^{2+}, were recorded on a *Varian Gemini-200* (200 MHz) pulsed FT-NMR spectrometer in D_2O at a pD of 7.0 at 25°. Chemical shifts δ_H are expressed rel. to $SiMe_4$ (= 0 ppm) as an internal standard. The concentration of the free ligands was fixed at 30 mM.

5.3. *Fluorescence Spectra and Fluorescence Titrations.* Fluorescence spectra were recorded on a *SPEX-Fluorolog-0.22m* fluorimeter equipped with a 450-W Xe lamp. The slit widths were $2 \times 2 \times 2 \times 2$, and the emission range was 560–700 nm. Fluorescence titrations were carried out in H_2O (pH 7.5; *Tris*-HCl/NaCl buffer; see above) at 25°. Solns. containing DNA and EB were titrated against varying concentrations of **1** and **2**. The samples were shaken and equilibrated for 2–3 min before measurement. The DNA and EB concentrations were 112 and 110 μM, resp., those of the complexes were in the range of 0–140 μM. The samples were excited at 540 nm, and fluorescence emission was observed at 600 nm. For fluorimetric experiments concerning DNA cleavage, see *Sect. 6*.

Scatchard plots were obtained, under the above exper. conditions, by fluorescence titration of DNA against EB in the absence and presence of metal complexes. The initial DNA and EB concentrations were 53 and 100 μM. After each addition of EB to the solutions containing DNA and metal complexes, the emission spectrum was recorded (excitation at 540 nm) at 25°. The data were corrected for the volume changes during the course of the titrations, and analyzed by the method of *Lepec* and *Paoletti* [43] to obtain the concentrations of bound *vs.* free EB. Finally, *Scatchard* plots were obtained by plotting r_{EB}/c_{EB} against r_{EB}, where $r_{EB} = [EB]_{bound}/[DNA]$ and $c_{EB} = [EB]_{free}$ (see *Fig. 7*).

6. *DNA Cleavage.* Electrophoresis experiments were performed with PUC19-DNA in H_2O at pH 7.5. The DNA was exposed to the copper complexes **1** or **2** by adding to 16 μl of 5 mM *Tris*-HCl/NaCl buffer (pH 7.5) and 8 μl of DNA (0.2 μg/μl; in 10 mM *Tris*-HCl (pH 8.0) and 1 mM EDTA) varying amounts of **1** or **2**. The test solns. were incubated at 37° for 4 h, and the reactions were quenched by addition of bromophenol blue. The mixtures were then analyzed by agar-gel electrophoresis, using DMSO or glycerol as OH-radical scavengers. The 1%-agarose gels were run in *Tris*-acetate-EDTA (TAE) buffer at 50 V for 3 h, and the gels were stained with EB soln. to observe the cleaved DNA products. The extent of DNA cleavage was determined by means of the volume-quantification method (*UVI Doc Mw, v. 99.03*). The rel. amounts of the different forms of DNA (in %) were determined by dividing the fluorescence intensity I_F for a particular band by the sum of the I_F values for each band in that lane (see *Fig. 8* and *Table 4*).

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