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Complexes between Nucleic Acid Bases and Bivalent Metal Ions. 4: 1 Cytosinium Copper Chloride Complex[1)]

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A new cytosinium copper chloride complex was synthesized from diluted hydrochloric acid solution.

The site of copper ion being coordinated with cytosine was examined by means of infrared and selective broadening in proton magnetic resonance (PMR) spectrum. On the basis of these data, it was suggested that copper ion is coordinated with the $C_{(2)}$=O of cytosine, and that composition of the cytosinium copper chloride complex is 4: 1 ratio of ligand to metal.

On the basis of infrared and PMR spectral studies, it was suggested that the $N_{(3)}$ site of cytosine is strongly bound to proton rather than to copper ion under an acid condition.

Keywords——cytosine; cytosinium; metal complex; copper; coordination site; IR spectra; PMR spectra

Introduction

It is of great interest that nucleic acids which are indispensable for the phenomena of life require metal ions in functioning. Metal ions are bound directly or indirectly through the water molecule to the base or phosphate sites on nucleic acids, and stabilize or destabilize the double helix of nucleic acids, binding to phosphate[3)] or to bases,[4)] respectively. However, no detailed answer is yet given to a question at molecular level as to which site of the base, metal ions are bound in bringing about destabilization of nucleic acids. As the first step to elucidate this, the authors are investigating the interactions of nucleic acid base with metal ions.

The present study was undertaken to investigate the interaction of cytosine with copper (II) ion, and to examine the resulting crystal of the cytosinium copper chloride complex. The cytosinium copper chloride complex was a new metal complex in which the ratio of cytosine to copper was quite different from that of bis-(4-aminopyrimidine -2-one) copper (II) chloride (2: 1 cytosine copper chloride) complex, whose X-ray crystallographic analysis has been reported by Carrabine and Sundaralingam,[5)] and other cytosine copper complexes have been reported by Melzer.[6)]

Experimental

Materials——Cytosine (special-grade, purchased from Wako Pure Chemical Industries, Ltd.) was recrystallized from warm water to be used. Copper chloride was commercially available (from Wako Pure Chemical Industries, Ltd.). Deionized water was redistilled and used as water throughout the experiment. Hydrochloric acid (special-grade, Wako Pure Chemical Industries, Ltd.) was diluted with water, and a series of the dilution was used.

1) Presented in part at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April, 1974.
2) Location: *1–33, Yayoi-cho, Chiba-shi, 280, Japan.*
3) G.L. Eichhorn and Y.A. Shin, *J. Am. Chem. Soc.*, **90**, 7323 (1968).
4) Y.A. Shin and G.L. Eichhorn, *Biochemistry*, **7**, 1026 (1968).
5) J.A. Carrabine and M. Sundaralingam, *Chem. Commun.*, **1968**, 746.
6) M.S. Melzer, *Chem. Commun.*, **1967**, 1052.

Apparatus——Infrared spectra were obtained with Hitachi Models EPI-285 and 215 by KBr disk method, since the isolated crystals were insoluble in organic solvents such as chloroform. Ultraviolet (UV) spectra were measured on a Hitachi spectrophotometer, Model EPS-3T. Proton magnetic resonance (PMR) spectra were obtained with JEOL, Model NM4H-100 spectrometer operated at 100 MHz.

Synthesis of the Cytosinium Copper Chloride Complex——Cytosine was dissolved in 0.1 N hydrochloric acid till the concentration of 0.5 w/v %. Copper chloride was added to the solution till a final concentration of 1 w/v %. Then, the solution was stirred at 65° for 7 hours, and was kept in a desiccator at room temperature to concentrate slowly. After about a week, yellow, column crystal was obtained. This crystal was washed with 0.1 N hydrochloric acid, water, and ethanol in the order given. —2: 1 Cytosine copper chloride complex was isolated by Melzer's method.[6]

Properties——The cytosinium copper chloride complex was soluble in acids and water, decomposed in alkaline solution, and insoluble in many organic solvents. The measurement of its melting point was attempted by raising the temperature up to 300°, but neither crystal had a melting point despite the color change around 250°. The UV absorption spectrum of the cytosinium copper chloride complex in 0.1 N hydrochloric acid showed a maximum absorbance at 277 nm (cytosine, at 275 nm). The hygroscopicity and the color of the Cytosinium copper chloride complex were not changed by drying over P_2O_5 under reduced pressure. *Anal.* Calcd. for $(C_4H_5N_3O)_4\ CuCl_2 \cdot 4HCl$: C, 26.52; H, 3.35; N, 23.20; Cu, 7.99. Found: C, 26.69; H, 3.35; N, 23.30; Cu, 7.87.

Thin-Layer Chromatography (TLC)——Very few methods of TLC are available for metal complexes. For the detection of nucleic acid bases and nucleosides, TLC method have been reported by Randerath[7] and Sakaguchi, *et al.*[8] Its modification was used for the present investigation. Each of the metal complexes, cytosine, and cytosine hydrochloride was subjected to TLC by using cellulose as support and 0.01 N hydrochloric acid as developing solvent. All the spots were identified under UV light as dark spot.

Rf values	Cytosine:	0.61
	Cytosine hydrochloride:	0.70
	Cytosine copper chloride complex:	0.68
	Cytosinium copper chloride complex:	0.75

PMR Spectra——Cytosine and cytosine hydrochloride were dissolved in CF_3COOH (TFA) and in Me_2SO-d_6 (DMSO-d_6), respectively, to a concentration of 0.2 M. Then, copper chloride was added to each solution. The broadenings for the $C_{(5)}$-H, the $C_{(6)}$-H, the $C_{(4)}$-NH_2, the $N_{(1)}$-H, and the $N_{(3)}$-H resonances in each solution were observed at 24°. PMR solvents used were commercially available (from Wako Pure Chemical Industries, Ltd.). Internal references used were TMS (in DMSO-d_6) and DSS (in TFA).

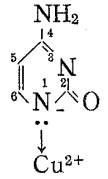
Results and Discussion

Infrared Spectra

The relevant infrared absorption bands are reported in Table I. Of the absorption bands in the spectrum of the cytosinium copper chloride complex, those at 784-(δC-H), 880-(δNH_2 out-of-plane), 1236-(δC–H in-plane), 1270-(νC–NH_2), 1545-(δNH in-plane), 1730-(νC=O), 2700—3000 cm^{-1} (νN–H), and 3350 cm^{-1} (νNH_2) are attributed to the cytosine structure,[9] indicating that the complex contains the cytosine skeleton.

The followings are the prominant characteristics in this infrared spectrum of the cytosinium copper chloride complex when compared with that of cytosine or cytosine derivatives. (I) New bands appear at 818-, 840-, 976-, 1005-, 1220-, 1405-, 1545-, 1572-, and 3140 cm^{-1}. (II) Three absorption bands occur at 1623-, 1679-, and 1730 cm^{-1} which in the spectrum of cytosine appear as a single, broad and strong band in the 1600—1700 cm^{-1} region.

The variations noticed in the 2500—3500 cm^{-1} region are that the $N_{(1)}$-H (stretching vibration) band which is the characteristic band of cytosine[9] is extremely weakened, and that a new band appears at 3140 cm^{-1}. It is presumed from the



Fgi. 1. Cytosine Anion Form

7) K. Randerath, *Biochem. Biophys. Res. Commun.*, **6**, 452 (1961).
8) T. Sakaguchi and S. Yoshimitsu, *Bunseki Kagaku*, **19**, 1549 (1970).
9) C.L. Angell, *J. Chem. Soc.*, **1961**, 504.

Table I. IR Data of Cytosine and Its Metal Complexes

Cytosine·H_2O	Tentative assignment	$(Cyt.)_4$ $CuCl_2$·4HCl	$(Cyt.)_2$ $CuCl_2$
3375 s	ν NH_2	3350 s	3370 s
3180 s	ν NH_2	3210 s	3320 s
3100sh	ν NH	3140 s	3170 s
2920m	ν NH	2925 s	2960sh
2850sh		2860m	2880m
2800m	ν NH	2800w	2800m
2700m	ν NH	2750w	
1665 s	δ NH_2 bending	1679 s	1680 s
1645 s	ν C=O	1730 s	1655 s
	ν $C=N^+$	1623 s	1628 s
1600sh	ν C=N+C=C	1590sh	
	δ NH in-plane	1572m	
1540m	δ NH in-plane	1545m	1550m
			1520m
1505m	ring vib.	1495m	1500m
1460 s		1470m	1470m
		1414m	1445w
		1405m	
1371 s	ν C–N (ring)	1365m	1367m
1290 s	ν C–NH_2	1270w	1295w
1235 s	δ CH, δ-ring	1236m	1238m
		1220 s	1225m
1138m			1140w
1105w	ring vib.	1105w	1105w
		1095w	
		1005m	1000w
978w		976m	985w
880m	δ NH_2 out-plane	880w	860w
	ν NH out-plane	840w	
814m	δ NH out-plane	818m	815w
787m	δ CH out-plane	784m	778m
		763sh	765m
		748m	730w

Cyt.: cytosine, vib.: vibration.

decrement of the $N_{(1)}$-H band that the cytosinium copper chloride complex contains the cytosine anion form, accordingly leading to a possibility of copper binding to the $N_{(1)}$ site of cytosine (in Fig. 1 and 2).

However, this assumption is denied because the 818 cm^{-1} and 1545 cm^{-1} bands assigned to the $N_{(1)}$-H out-of-plane and the $N_{(1)}$-H in-plane deformation vibrations,[9] respectively, are observed.

A new band at 3140 cm^{-1} is considered to be assignable to the $N_{(3)}$-H stretching vibration. In the spectrum of cytosine hydrochloride, like this complex's spectrum, the bands considered to be due to the $N_{(3)}$-H occur newly at 840-, 1570-, and 3145 cm^{-1}, when compared with the bands in the spectrum of cytosine itself. And, it was determined from a series of nuclear magnetic resonance (NMR) spectral studies on cytosine derivatives by Becker, *et al.*[10,11] that the $N_{(3)}$ site was protonated in cytosine hydrochloride. Accordingly, the bands at 840-, 1570-, and 3145 cm^{-1} in cytosine hydrochloride are assigned to the $N_{(3)}$-H out-of-plane deformation, the $N_{(3)}$-H in-plane deformation, and the $N_{(3)}$-H stretching vibrations, respectively, as cytidine derivatives.[12] Therefore, the new bands at 840-, 1572-, and 3140 cm^{-1} in the

10) E.D. Becker, H.T. Miles, and R.B. Bradley, *J. Am. Chem. Soc.*, **87**, 5575 (1965).
11) R.R. Shoup, H.T. Miles, and E.D. Becker, *J. Am. Chem. Soc.*, **89**, 6200 (1967).
12) M. Tsuboi, Y. Kyogoku, and T. Shimanouchi, *Biochim. Biophys. Acta*, **55**, 1 (1962).

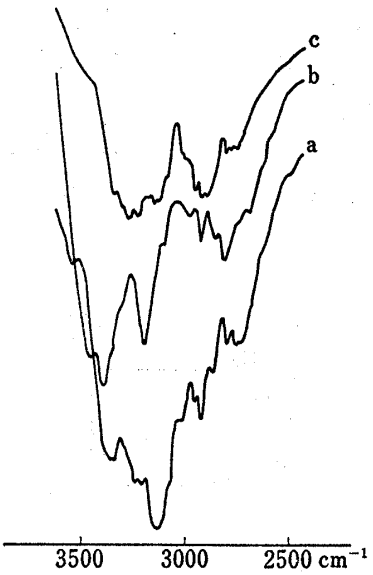


Fig. 2. Infrared Absorption Spectra from 2500 to 3500 cm^{-1} in KBr Disk

Abscissa, frequency of absorption in cm^{-1}; ordinate, per cent transmission on arbitrary scale. a: cytosinium copper chloride; b: cytosine; c: cytosine hydrochloride.

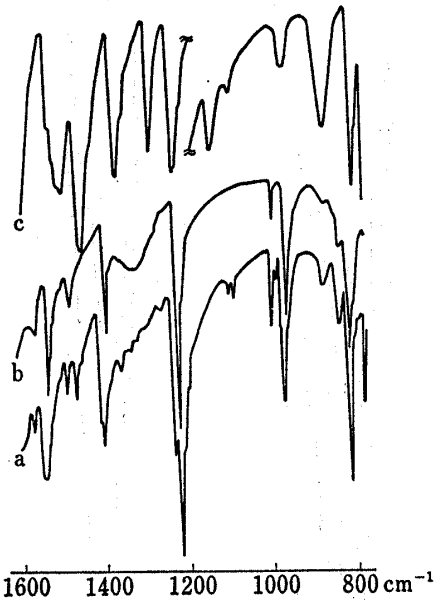


Fig. 3. Infrared Absorption Spectra from 800 to 1600 cm^{-1} in KBr Disk

Abscissa, frequency of absorption in cm^{-1}; ordinate, per cent transmission on arbitrary scale. a: cytosinium copper chloride; b: cytosine hydrochloride; c: cytosine.

spectrum of the cytosinium copper chloride complex are the absorption bands due to the $N_{(3)}$-H in that order as described above (in Figs. 2 and 3).

The variation observed in the 1600—1800 cm^{-1} region is that the single, broad, and strong band due to the C=N stretching, the C=O stretching, and the NH_2 bending vibrations found in the spectrum of cytosine, was split to three bands at 1623-, 1679-, and 1730 cm^{-1} in the spectrum of the cytosinium copper chloride complex. The absorption band at 1679 cm^{-1} is considered to be assignable to the NH_2 bending vibration, because, in the spectrum of cytosine hydrochloride, that strong band of cytosine was split into two bands at 1680- and 1720 cm^{-1}. The assignments of these bands are given to the $C_{(2)}=O$ stretching vibration at 1720 cm^{-1} and the NH_2 bending vibration at 1680 cm^{-1} from the infrared spectral studies on cytosine derivatives by Angell,[9] and on cytidine derivatives by Miles[13] and Tsuboi, *et al.*[12]

Moreover, in the spectrum of the cytosine copper chloride complex,[5] the bands assigned to the above-described vibrations are observed as a shoulder at 1655 cm^{-1} and as a strong band at 1680 cm^{-1} (in Fig. 4).

It is presumed in the same way that the band at 1730 cm^{-1} in the cytosinium copper chloride complex is assigned to the $C_{(2)}=O$ stretching vibration. This band is higher in frequencies by 10 cm^{-1} than that of cytosine hydrochloride. It is suggested from this result that copper ion coordinates with the $C_{(2)}=O$ in the cytosinium copper chloride complex, as in cytosine copper chloride complex of which the $C_{(2)}=O$ band is higher in frequencies by 10 cm^{-1} than that of cytosine itself (in Fig. 4).

The absorption band at 1623 cm^{-1} of the cytosinium copper chloride complex is considered to be assignable to the $C=N^+$ stretching vibration. In the spectrum of the cytosine copper chloride complex in which copper ion coordinates with both the $N_{(3)}$ site and the $C_{(2)}=O$ site, as in Fig. 5 structure (A), the $C=N^+$ (stretching vibration) band appears as a sharp and strong band at 1628 cm^{-1}. Then, in the spectrum of cytosine hydrochloride in which the $N_{(3)}$ site is protonated, the $C=N^+$ (stretching vibration) band is observed at 1625 cm^{-1}.[9,12,13]

13) H.T. Miles, *Proc. Natl. Acad. Sci. U.S.*, 47, 791 (1961).

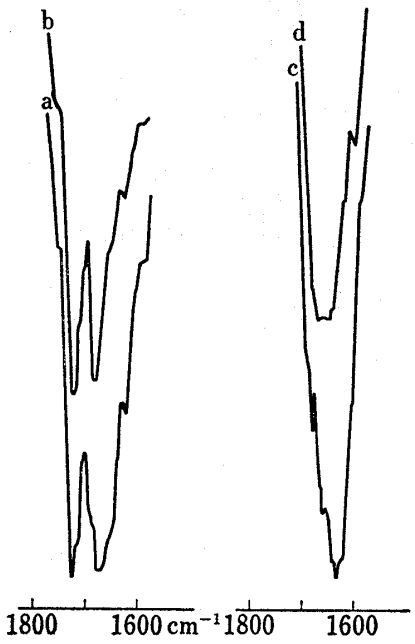
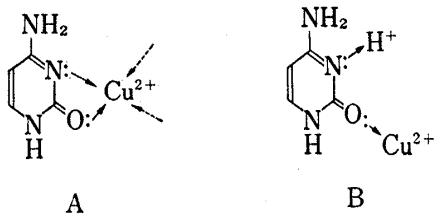


Fig. 4. Infrared Absorption Spectra from 1600 to 1800 cm^{-1} in KBr Disk

Abscissa, frequency of absorption in cm^{-1}; ordinate, per cent transmission on arbitrary scale. a: cytosinium copper chloride; b: cytosine hydrochloride; c: cytosine copper chloride; d: cytosine.



A B

Fig. 5

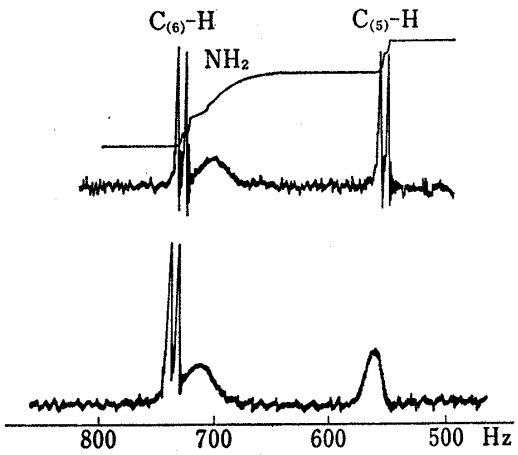


Fig. 6. Effect of Cu^{2+} on Proton Magnetic Resonance Spectra (100 MHz) 0.2 M Cytosine in DMSO-d_6

The upper spectrum is the metal-free solution; lower spectrum: Cu^{2+} was added to, till 2×10^{-4} M. Abscissa is in Hz downfield from TMS internal standard.

This may raise a question—whether the $N_{(3)}$ site is the site of metal binding or protonation. The above described facts indicate that the $N_{(3)}$ site is protonated in the cytosinium copper chloride complex (in Fig. 5).

In view of those together with Angell's, Miles's, and Tsuboi *et al.*'s studies on infrared spectra of cytosine derivatives, the structure (B) of the cytosinium copper chloride complex may be disclosed in Fig. 5 which shows the coordinated bond of copper ion to the $C_{(2)}=O$ of the cytosine hydrochloride.

Proton Magnetic Resonance Spectra

A selective broadening method in PMR spectral studies has been used to determine binding sites of paramagnetic metal ions such as copper ion to the nucleosides[14] and nucleotides.[15–17]

The $C_{(5)}$-H, $C_{(6)}$-H, and $C_{(4)}$-NH_2 resonances were measured by copper chloride being added to cytosine in DMSO-d_6 (in Fig. 6). Fig. 6 shows that the $C_{(5)}$-H resonance shifts by 4 Hz to downfield accompanying broadening, the $C_{(6)}$-H resonance shifts by 3 Hz to downfield without a broadening, and the $C_{(4)}$-NH_2 resonance shifts by 12 Hz to downfield.

It is presumed from these results that copper ion mainly binds to the $N_{(3)}$ site and to the $C_{(2)}=O$ site. This interpretation is supported by the fact that copper ion is coordinated

14) G.L. Eichhorn, P. Clark, and E.D. Becker, *Biochemistry*, **5**, 245 (1966).
15) N.A. Berger and G.L. Eichhorn, *Biochemistry*, **10**, 1847 (1971).
16) W.G. Espersen, W.C. Hutton, S.T. Chow, and R.B. Martin, *J. Am. Chem. Soc.*, **96**, 8111 (1974).
17) W.G. Espersen and R.B. Martin, *J. Am. Chem. Soc.*, **98**, 40 (1976).

with both the $N_{(3)}$ and the $C_{(2)}=O$ sites in a 2:1 cytosine copper chloride complex of which the structure was reported by Carrabine and Sundaralingam[5] (refer to Fig. 5, structure A).

However, it is considered reasonably that the cytosinium copper chloride complex does not necessarily have the above described structure, since it was isolated from an acid solution in this experiment.

In the PMR spectrum of cytosine in TFA to which copper chloride was added, a broadening for the $C_{(6)}$-H resonance was observed contrary to the case of DMSO-d_6 (in Fig. 7). This indicates that copper ion is bound to the $N_{(1)}$ site or to the $C_{(2)}=O$ site of cytosine under an acid condition. In that situation, the $N_{(3)}$ site is considered to be protonated, as reported by Jardetzky, *et al.*[18] But the $N_{(1)}$-H and $N_{(3)}$-H resonances demonstrated by Jardetzky, *et al.* were not clearly observed in the present experiment (These bands may be overlapped by TFA side bands).

However, as it is presumed from the infrared spectrum of the cytosinium copper chloride complex that the $N_{(1)}$ site is protonated in cytosine under an acid condition, the $N_{(1)}$ and $N_{(3)}$ sites are considered to be protonated. Accordingly, the copper ion binding site may be the $C_{(2)}=O$ site of cytosine under a strong acid condition.

Furthermore, in the PMR spectrum of cytosine hydrochloride in DMSO-d_6 to which copper chloride was added (in Fig. 8), no variation was observed in the $C_{(4)}$-NH_2 resonance reported by Becker, *et al.*[10,11] and $N_{(1)}$-H and $N_{(3)}$-H resonances at 1210 Hz, but the $C_{(6)}$-H resonance shifted by 4 Hz to downfield and broadened, when compared with that of cytosine hydrochloride.

It is considered from these results that copper ion is bound to the $C_{(2)}=O$ site in the cytosinium copper chloride complex.

In the NMR spectral studies on copper complexation of cytidine by Eichhorn, *et al.*[14] and of cytosine by Li, *et al.*[19] when selective broadening of cytosine in DMSO-d_6 by the addition of copper chloride were measured, a broadening was observed only for the $C_{(5)}$-H reso-

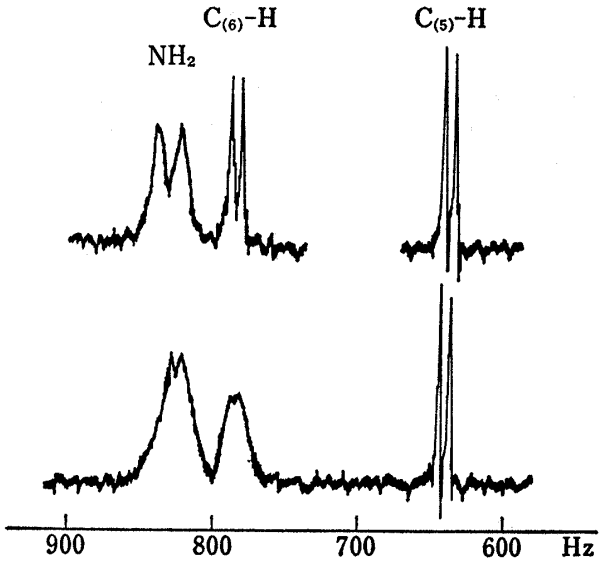


Fig. 7. Effect of Cu^{2+} on Proton Magnetic Resonance Spectra (100 MHz) 0.2 M Cytosine in CF_3COOH

The upper spectrum is the metal-free solution; lower spectrum: Cu^{2+} was added to, till 2×10^{-4} M. Abscissa is in Hz downfield from DSS internal standard.

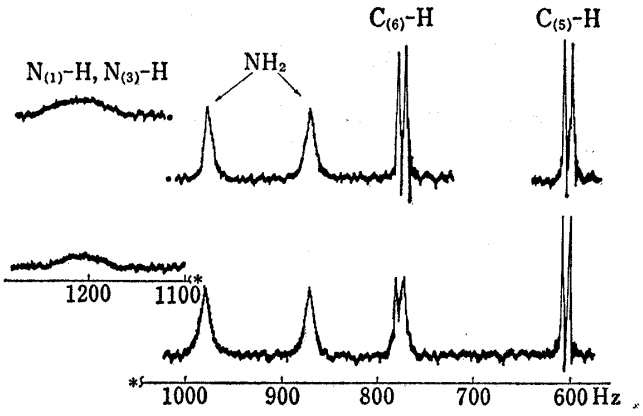


Fig. 8. Effect of Cu^{2+} on Proton Magnetic Resonance Spectra (100 MHz) 0.2 M Cytosine Hydrochloride in DMSO-d_6

The upper spectrum is the metal-free solution; lower spectrum: Cu^{2+} was added to, till 2×10^{-4} M. Abscissa is in Hz downfield from TMS internal standard.

18) O. Jardetzky, P. Pappas, and N.G. Wade, *J. Am. Chem. Soc.*, **85**, 1657 (1963).
19) S.M. Wang and N.C. Li, *J. Am. Chem. Soc.*, **88**, 4592 (1966).

nance. So, it has been concluded from their result that copper ion was bound to the $N_{(3)}$ site of cytosine.

However, in the PMR spectrum (in Fig. 6), a broadening for the $C_{(5)}$-H resonance was observed in agreement with their results, but the $C_{(6)}$-H resonance was observed to shift toward downfield. This shift is in disagreement with their results. In the spectrum of cytosine hydrochloride in DMSO-d_6, to which copper chloride was added, a broadening for the $C_{(6)}$-H resonance was observed.

It is difficult to conclude the metal binding site to a nucleic acid base only from the PMR spectral data, obtained by the addition of metal ions to nucleic acid solution. Furthermore, it is proposed that the use of several solvents is necessary when studying the metal binding site to the nucleic acid base.

Conclusion

The following are the results from the studies on a copper binding site to cytosine (in the cytosinium copper chloride complex).

The infrared spectral study; From the observation of the $N_{(1)}$-H, $N_{(3)}$-H, and $C_{(4)}$-NH_2 bands in the spectrum of this complex, it is considered that the complex does not have a cytosine anion form, but has a structure in which the $N_{(3)}$ site is protonated in cytosine. From the result that the $C_{(2)}=O$ band (stretching vibration) is shifted to higher frequencies by 10 cm^{-1} than that of cytosine hydrochloride, it is considered that copper ion coordinates with the $C_{(2)}=O$ in the cytosinium copper chloride complex.

The PMR spectral study; The PMR spectral studies on cytosine with copper ion in TFA and on cytosine hydrochloride with copper ion in DMSO-d_6, suggest the possibility that copper ion binds to the $C_{(2)}=O$ in cytosine under an acid condition.

In view of those data, it is suggested that copper is bound to the $C_{(2)}=O$ site of cytosine in the cytosinium copper chloride complex. The result of its elemental analysis has revealed the preparation of the cytosinium copper chloride complex in which the ratio of ligand to metal is 4: 1.

These infrared and PMR spectral results give us the interpretation that the $N_{(3)}$ site is strongly bound to proton rather than to copper ion under an acid condition. Accordingly, it can not be concluded that the $N_{(3)}$ is a site where cytosine base has the strongest affinity for metal ions at low pH.

Acknowledgement The authors are deeply indebted to Miss Ohida, Analytical center of this Faculty, who carried out elemental analysis and PMR measurements.