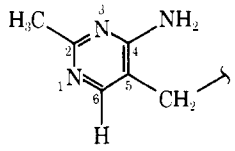
Table III. Predicted Chemical Shifts for N1 and N3



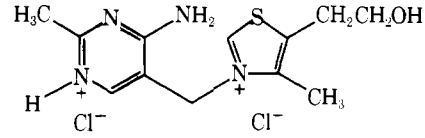
N1		N3	
Pyrimidine	82 ppm	Pyrimidine	82 ppm
2-CH_3	9	2-CH_3	9
4-NH_2	42	2-NH_2	65
3-CH_3	5	3-CH_3	5
Predicted N1 shift	138 ppm	Predicted N3 shift	161 ppm

Table IV. Half-Widths of ^{15}N Signals of **1** in Ethylene Glycol after Incomplete Neutralization

Proton decoupling target	Proton frequency decoupled, ppm	Half-widths in Hz of ^{15}N resonances		
		133 ppm	156 ppm	162 ppm
None		8.5	13.0	7.0
Pyrimidine proton	8.1	8.5	5.5	5.5
Thiazole proton	9.6	6.0	17-22	4.5

nance at 163 ppm (shifted from 165 ppm) was not. Selective proton decoupling of the pyrimidine C6 hydrogen sharpened the resonance at 156 ppm (see Table IV), which is in agreement with the assignments previously made using data from water and D_2O solutions of the hydrochloride.

The data in Table I show that the nitrogen signal which shifts the most on protonation of **1** is the signal at 209 ppm. With the assignment of this resonance to N1, thiamine hydrochloride, vitamin B_1, is properly represented as **3.**



3

Why thiamine protonates on N1 is not so clear. Adenine, with a somewhat similar arrangement of aminopyrimidine nitrogens, apparently protonates at its ring nitrogen (now N1) next to the $-NH_2$ group.[9] An important effect is possibly the C-N inductive effect and, in this connection, it is probably significant that 2-aminopyridine is a much weaker base than 4-aminopyridine.[10]

References and Notes

(1) Supported in part by the National Science Foundation and by the Public Health Service, Research Grant 11072 from the division of General Medical Science.
(2) For a general review see (a) *Annu. Rev. Biochem.*, **38**, 213 (1969); or for a review of enzyme action see (b) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms", Vol. 2, W. A. Benjamin, New York, N.Y., 1966, Chapter 8.
(3) See for example "The Merck Index", 8th ed, Merck and Co., Rahway, N.J., 1968.
(4) D. J. Brown, E. Hoerger, and S. F. Mason, *J. Chem. Soc.*, 4035 (1955).
(5) (a) R. Breslow, *J. Am. Chem. Soc.*, **79**, 1762 (1957); (b) *ibid.*, **80**, 3719 (1958).
(6) M. Witanowski, L. Stafaniak, and H. Januszewski in "Nitrogen NMR", M. Witanowski and G. A. Webb, Ed., Plenum Press, New York, N.Y., 1973, pp 222-223.
(7) Reference 6, p 220.
(8) R. L. Lichter and J. D. Roberts, *J. Am. Chem. Soc.*, **93**, 5218 (1971).
(9) See V. Markowski, G. R. Sullivan, and J. D. Roberts, *J. Am. Chem. Soc.*, **99**, 714 (1977), for references and discussion.
(10) A. Albert, R. Goldacre, and J. Phillips, *J. Chem. Soc.*, 2240 (1948).

Magnetic Resonance Studies of Copper(II) Interaction with Nucleosides and Nucleotides

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Abstract: Complexes of copper(II) with the four common nucleosides have been studied by ESR and NMR at different pHs in water-Me_2SO solutions. For comparison some nucleotide complexes of copper were also studied. These studies provide information about the different types of complexes which can be formed and the effect of pH on the mode of interaction of the bases with copper. In the pH range between 8 and 12 copper ions are able to discriminate between 5'-ribonucleotides and 5'-deoxynucleotides. The information derived from these studies may be useful in understanding the role which copper ions play in certain enzymatic reactions and in metal polynucleotide interactions. These studies also suggest that copper(II) may be able to stabilize some very unusual polynucleotide structures.

Divalent metal ions have pronounced effects on the properties of polynucelotides. Magnesium, the divalent metal ion usually found in relatively high concentrations in vivo, stabilizes both DNA and RNA against thermal denaturation and in many cases is required for these molecules to function properly.[1,2] Mn^{2+} also stabilizes DNA and RNA and may substitute for Mg^{2+},[3] although with certain enzymes (e.g., reverse transcriptase, RNA and DNA polymerases) the replacement of magnesium by manganese induces translational errors.[4] In contrast to Mg^{2+} and Mn^{2+}, which stabilize secondary structures in DNA and RNA, copper ions destabilize DNA and RNA double helices,[5-7] and this is attributed to the ability of copper to bind to the nucleic acid bases.[5,7] Because of this special property we were interested in exploring the use of copper(II) as a probe of certain structural features in both RNA and DNA molecules. For example, tRNA molecules contain looped out regions[8] which might be expected to bind copper rather strongly, and this might be exploited in electron and nuclear magnetic resonance studies of these molecules. Certain special regions of the DNA (e.g., operator regions)

may be looped out[9] and it is possible that copper(II) ions could be used to locate these sites. Binding of copper was also of interest to us because of Eichhorn's prior suggestion that Cu(II) might be able to differentiate between ribonucleosides and deoxynucleosides by serving as a template for ribonucleoside bridges, ribose 2′ and 3′-OH groups.[10] Although the original evidence for such a differentiation was subsequently shown to be invalid,[11] the idea appeared worthy of further exploration using different experimental conditions. As we show, there are conditions under which copper differentiates between ribo- and deoxynucleosides. Finally, we note that copper complexes with nucleic acids may be of interest in light of the reported antitumor and anti-viral activity of copper thiosemicarbazone complexes.[12,13] To prepare for studies of the polynucleotides we first studied the interaction of copper with the individual bases, nucleosides and nucleotides.

A variety of different experimental techniques (CD, ORD, potentiometric titrations, NMR)[2,3,7,9,14] have been used to investigate the interaction of copper with nucleosides, nucleotides, and polynucleotides. In most of these studies, formation of copper complexes was followed by monitoring the effect of copper binding on some property of the nucleic acid base (optical, NMR, potentiometric). With ESR, however, it is possible to study metal binding via the effect which the base or other ligand has on the electronic properties of the copper ion. In this way we obtain information that is complementary to that obtained by other techniques. ESR also has the potential advantage that it can be used to study metal binding in condensed phase systems (e.g., chromosomes, high molecular weight protein-DNA complexes) which are not so amenable to investigation by some of the solution state techniques mentioned above.

Some limited ESR studies on copper-nucleic acid complexes have already been reported. Ropars and Viovy[15] studied the room temperature ESR spectra of some copper-nucleic acid systems, but their studies were complicated by the fact that ESR line shapes at room temperature depend on both the rotational motion[16,17] and the ligand-metal bond properties[18,19] of the complex. Bemski et al.[14] studied the low-temperature (77 K) ESR spectra of copper-nucleic acids, but their data were limited to a fixed metal-to-ligand ratio at neutral pH, and no structural information was obtained from the data. Furthermore, some of their data show evidence for aggregation of the copper(II) they observed a single-line ESR spectrum with no resolved structure for $CuSO_4$ aqueous solution at 77 K, whereas the $CuSO_4$ aqueous solution should have resolved hyperfine structure at 77 K).

In the present paper, ESR is used to investigate the variety of complexes formed between Cu(II) and various nucleosides and nucleotides. To eliminate spectral complications arising from rotational motion of the complexes, most ESR measurements were carried out at 77 K using solvents which form good glasses. Some room temperature ESR and NMR studies were also carried out. Since the nature of the copper-nucleic acid interaction is very sensitive to pH, complex formation was examined over a pH range extending from 3.5 to over 12. As we shall demonstrate, different complexes are formed with each nucleoside at different pHs. In some cases, only one molecule is bound to a single copper, but under appropriate experimental conditions two and sometimes four nucleosides can simultaneously coordinate with a single copper ion. A new diamagnetic complex of the ribonucleosides is observed and conditions under which copper selectively distinguishes between ribo- and deoxynucleotides have been found.

Experimental Section

(a) **Magnetic Resonance.** A Varian E-3 spectrophotometer (X-band) was used to measure the ESR spectra, and diphenylpicryl hydrazil was used as a reference standard to calibrate the ESR parameters. Samples dissolved in glassing solvents were immersed in liquid nitrogen during the low-temperature measurements (77 K) and a constant stream of He gas was passed through the liquid nitrogen to eliminate bubbling. A Varian HR-300 spectrometer operated in the field sweep mode was used in the NMR studies. NMR experiments were carried out in Me_2SO to eliminate problems from the strong water peak and to permit resonances from the exchangeable protons to be observed. ESR experiments demonstrated that the behavior of the complexes in Me_2SO solutions parallels the behavior in aqueous solutions (same complexes formed in both solvents at the same pH). The apparent "pH" of Me_2SO solutions was measured using a Beckman 3500 pH meter with a silver-silver chloride electrode.

(b) **Chemicals.** Anhydrous reagent grade copper(II) chloride was used throughout this study. Nucleosides and nucleotides were purchased from Terra-Marine Bioresearch, La Jolla, Calif. In some ESR experiments we used a solvent consisting of a 1:1 mixture (by volume) of water and glycerol. However, since copper reacts with glycerol at high pH (>8) most experiments were carried out using a 1:1 mixture (volume) of water and Me_2SO. In the NMR experiments, Me_2SO was used as a solvent in order to monitor the behavior of the exchangeable protons of the nucleosides. It is, therefore, important to note that the pH dependence of the ESR and optical experiments demonstrate the complexes exhibit parallel behavior in the Me_2SO-water solutions and in neat Me_2SO, with the same spectra observed in the two different solvent systems at approximately the same measured pH. The only difference noted was in the case of uridine where a new species which formed at high pH (12) in water was not observed in neat Me_2SO. Thus, while the "apparent" pH measured in neat Me_2SO may not correspond to the true pH, it is clear that there is a very close correspondence with the pH measured in Me_2SO-water mixtures.

Results and Discussion

The ESR spectra of axially symmetric copper complexes in rigid glass solutions can be interpreted in terms, of the following spin Hamiltonian (second-order perturbation terms, quadrupolar and nuclear Zeeman terms, have been neglected):[19]

$$\mathcal{H} = \beta[g_{\parallel}H_zS_z + g_{\perp}(H_xS_x + H_yS_y)] + AS_zI_z + B(S_xI_x + S_yI_y) \quad (1)$$

where β is the Bohr magneton, $g_{\parallel}$ and A are the g-value and hyperfine components, respectively, parallel to the molecular symmetry axis, $g_{\perp}$ and B are the g-value and hyperfine components, respectively, perpendicular to the molecular symmetry axis, S is the electron spin, and I is the nuclear spin. Interaction of the unpaired copper electrons with ligand nitrogen nuclei can give extra hyperfine structure (superhyperfine coupling) and this adds another term to Hamiltonian:

$$A_{N\parallel}S_zI_{N_z} + A_{N\perp}(S_xI_{N_x} + S_yI_{N_y})$$

where $A_{N\parallel}$ and $A_{N\perp}$ are the superhyperfine splittings in the parallel and perpendicular hyperfine components, and I_N is the nitrogen nuclear spin. In favorable cases, the stoichiometry of the copper-nucleoside complexes can be determined from the number of superhyperfine lines and their intensity ratios.

In the low-temperature ESR measurements, only the parallel (along the molecular symmetry axis of the complex) ESR parameters $g_{\parallel}$ and $A_{\parallel}$ can be obtained directly from the spectra, and it is these parameters which are tabulated and used to interpret the spectra. We first consider the various copper complexes which are formed with adenosine over the pH range extending from 3.5 to 12. The results of this analysis facilitate the discussion of copper(II) complexes with the other nucleosides as these often show rather similar behavior.

(a) **Adenosine-Copper(II) Complexes.** The ESR spectra of copper-adenosine complexes at pHs ranging from 3.5 to 12 are shown in Figure 1. From comparison of these spectra, it is evident that, depending upon the pH, a number of different types of copper complexes are formed. At the lowest pH, 3.5, only

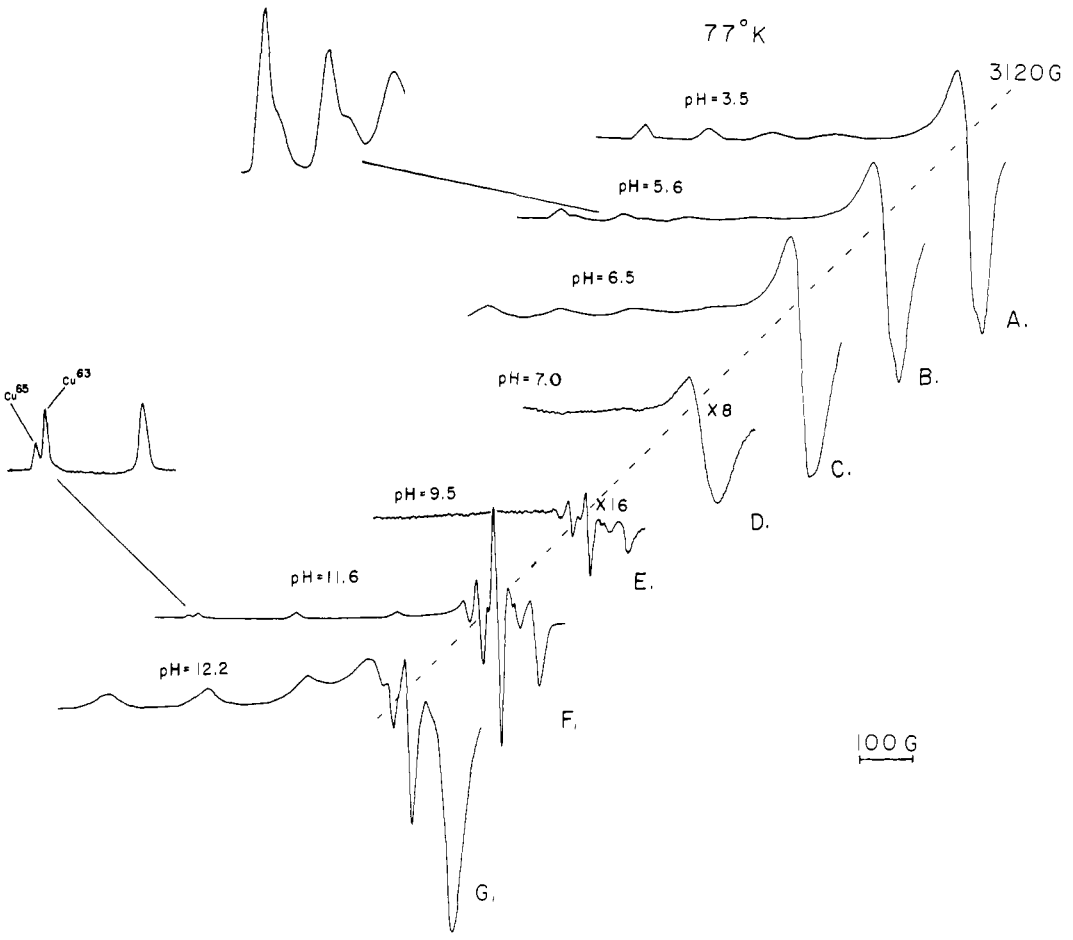


Figure 1. Effect of pH on the low-temperature (77 K) ESR spectra of Cu(II)–adenosine complexes in 50% H_2O–50% Me_2SO: $[Cu(II)^0 = 7$ mM; $[A]^0 = 37$ mM.

Table I. A Summary of the ESR Parameters of Copper(II)–Adenosine Complexes in 50% Me_2SO–50% H_2O at 77 K: $[Cu^{2+}] = 7$ mM, [A] = 37 mM

pH	$A_\parallel$, G	$g_\parallel$	Color[b]
3.5	119[c]	2.402[c]	Yellowish
5.6	119 (major) 135 (minor)	2.402 (major) 2.360 (minor)	Yellowish
6.5	135	2.360	Greenish-yellow
7–8	Ppt.	Ppt.	Ppt.
9.5	*a*	*a*	Green
11.6	185	2.235	Blue
>12	~185	~2.235	Blue

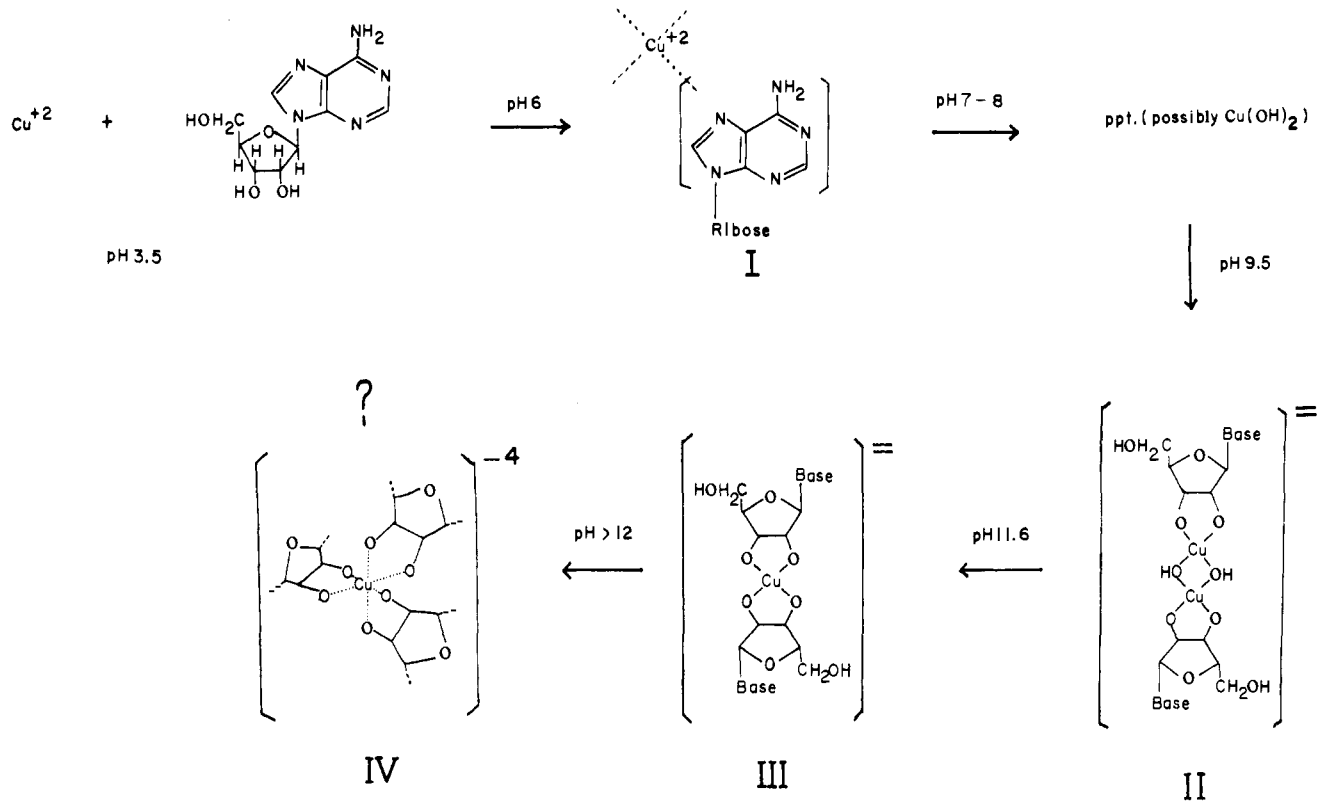
[a] Clear solution with very small ESR signal. [b] There is no apparent change in the color of the solution between room temperature and liquid nitrogen temperature. [c] The corresponding ESR parameters for free copper in aqueous solution are 120 G and 2.402, respectively.

one type of complex is present (Figure 1A), and this species has $A_\parallel$ and $g_\parallel$ values which are identical with those observed for free copper in an aqueous solution ($A_\parallel = 120$ G, $g_\parallel = 2.402$; see Table I). When the pH is increased to 5.6, a new species with $A_\parallel$ and $g_\parallel$ values of 135 G and 2.360, respectively, is produced (Figure 1B) and by pH 6.5 this latter species is the only copper species detected (Figure 1C). The ribose OH groups (pK_a values for ribose OH groups are ~12) are not the binding site at pH 6.5 since at the same pH copper solutions in polyols such as ethylene glycol and glycerol exhibited an ESR spectrum characteristic of free copper(II).[20]

To account for these observations and the magnitudes of $A_\parallel$ and $g_\parallel$, we attribute the spectrum at pH 6.5 to a complex (I) in which the copper is bound to ring nitrogen atoms of adenine (N_7, or possibly N_3 or N_1), and this is consistent with the NMR observations.[21] (Since the ESR spectrum at pH 6.5 is independent of the adenosine concentration to copper ratio over a range extending from 1:1 to 10:1, we conclude that only a single adenosine is bound to each Cu(II).) At pH 7–8, the solution becomes cloudy, presumably due to formation of copper hydroxide precipitate. At pH 9.5, the solution became clear and green (the precipitate completely dissolves), but the ESR signal (Figure 1E) remained almost undetectable (reduced by a factor of over 50 compared with the pH 6.5 solution). The great loss of intensity with no evidence for precipitation, if the adenosine-to-copper ratio is greater than 1, clearly indicates the formation of some soluble diamagnetic complex with a stoichiometry of one copper per adenosine.

NMR experiments, carried out in Me_2SO to eliminate problems arising from a strong water peak and rapid exchange of the N–H protons, confirm the formation of a diamagnetic copper complex with adenosine around pH 9.5 and these data are shown in Figure 2. Addition of copper chloride to a "pH" 6.5 Me_2SO solution of the nucleoside severely broadens the H_2, H_8, and N–H_2 resonances but only slightly broadens and shifts the $H_{1'}$ resonances. This indicates that the copper is binding to the base (N_3, N_7, or N_1, step 1 in Scheme I).[5,7] Around "pH" 8, there is evidence for formation of a precipitate and the H_2, H_8, and N–H_2 resonances begin to sharpen. At "pH" 9.5, the solution is again clear, the NMR spectrum is

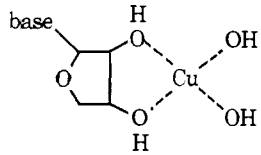
Scheme I



quite sharp, and well-resolved splittings are observed in the $H_{1'}$ resonance (see Figure 2D). There is no broadening of the resonances from the ribose OH protons. These observations confirm the fact that copper forms a *diamagnetic* complex with adenosine at "pH" 9.5.

To further demonstrate the diamagnetism of this copper-adenosine complex, we examined the broadening of the water signal in an aqueous solution containing 50 mM Cu^{2+} and 0.2 M 5′-AMP. In the pH 7-8 region where no ESR signal is detected, there was no observable broadening of the water resonance (within 10%). At pH 12, however, where a paramagnetic complex is observed, the excess water line width was >5 Hz. These experiments demonstrate that the solutions become diamagnetic when the ESR signal disappears.

The diamagnetic species is not a simple copper hydroxide complex since copper hydroxide is insoluble in the pH range from 8 to 11. Furthermore, the fact that copper-deoxynucleotide solutions have immense precipitation at this pH (9.5) indicates that the vicinal hydroxyl groups of the ribose are required for the formation of this soluble complex. To account for this set of observations, we propose structure II (Scheme I) for the diamagnetic copper complex. Ordinarily, ribose protons titrate around pH 11-12 so it is evident that chelation with copper has shifted their pK_a by ~2 pH units. The diamagnetism of II is attributed to spin pairing of the unpaired electrons of the two copper atoms in the complex. Glycerol and ethylene glycol form diamagnetic soluble complexes with copper(II) around pH ~10, consistent with the fact that they too possess vicinal hydroxyl groups which, when ionized, can chelate with the copper ion and form a complex analogous to the adenosine complex.[20] Reinert and Weiss[22] had proposed the following structure for the copper-ribose complex.



This structure cannot be correct, however, since a monomeric copper species would be paramagnetic, whereas we find that the complex is diamagnetic at this pH.

When the pH of the copper-adenosine system is raised to 11.6, the solution turns blue and a strong, well-defined ESR signal (Figure 1F, Table I) is detected. There is only a slight difference (0.004) in the values of g_x and g_y so the complex must have approximately square-planar geometry. The fact that the ESR spectrum of the paramagnetic copper(II)-adenosine complex exhibits resolved copper hyperfine components at room temperature (Figure 3) indicates that the complex is rather small and from the ESR line widths (m_I = $\frac{1}{2}$, $\frac{3}{2}$ hyperfine components) we calculate that the hydrodynamic radius of the complex is 7-10 Å (rotational correlation time $\tau_c \sim 4 \times 10^{-10}$ s).[23,24]

Copper complexes with 2′-, 3′-, and 5′-AMP were also examined. Aqueous solutions of Cu(II) and 5′-AMP (3′-AMP or 2′-AMP) are all cloudy at pH 7. At pH ~10, the Cu(II) 5′-AMP solution is clear green, but the 2′- and 3′-AMP Cu(II) solutions remain cloudy. From this, we conclude that the ribose OH groups in adenosine and 5′-AMP are essential to formation of the soluble diamagnetic complex. Upon further increase of pH, the ESR behavior of the Cu(II) 5′-AMP system is the same as the Cu(II)-adenosine system (Scheme I), but the Cu(II) 2′-AMP and Cu(II) 3′-AMP solutions still contain immense precipitate with no detectable ESR signal.

To account for this set of observations, we propose structure III shown in Scheme I for the complex which is obtained at pH 11.6 in the presence of an excess of adenosine. This fits with our observation that other diols such as glycerol, ribose, and ethylene glycol also form paramagnetic complexes at pH ~12 with ESR parameters (A = 183 G, g = 2.235), which are nearly identical with those of the adenosine complex. Fructose and copper(II) also form a 2:1 complex which exhibits an identical (but less resolved) ESR spectrum.[25] When the adenosine-to-copper ratio is reduced from about 6:1 to 1:1, there are changes in the distribution of intensity in the ESR spectrum which we attribute to the formation of a 1:1 cop-

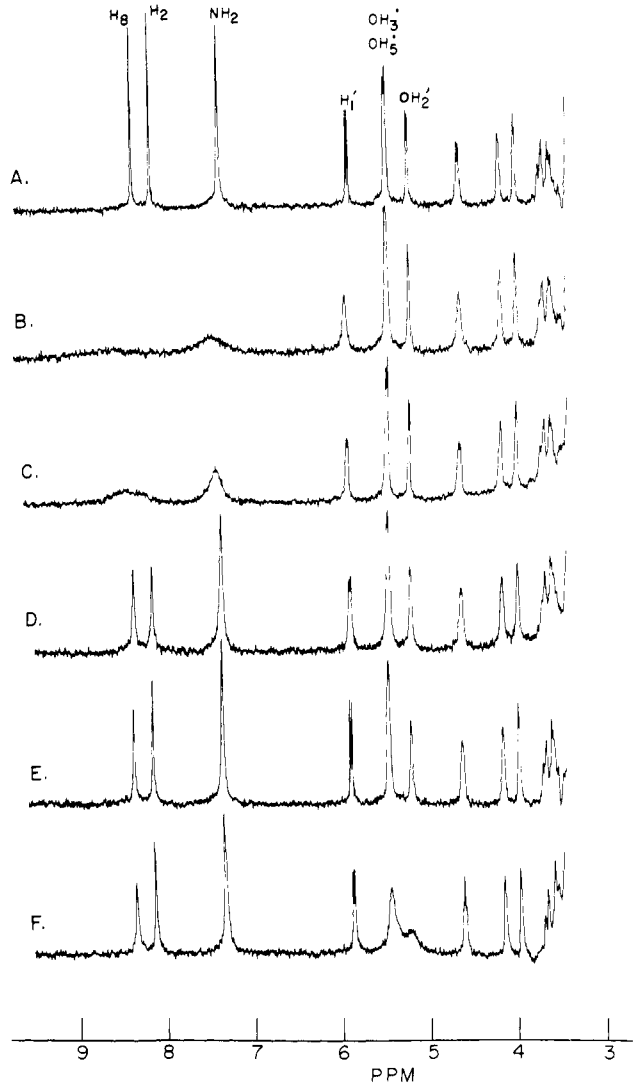


Figure 2. The "pH" dependence of the room-temperature NMR spectrum of Cu(II)-adenosine complexes in Me_2SO. In A there is no copper, but all other solutions contained 1.5 mM Cu(II). The adenosine concentration in all solutions was 0.1 M. The apparent pH values are: A and B ~6.5, C ~9, D ~9.5, E ~10.4, F ~11.

per-ribose complex in which one ribose and two OH groups are bound to a single copper. When the adenosine-to-copper ratio is decreased below 1:1, precipitation (presumably $Cu(OH)_2$) is observed.

At pH 12.2, there is a slight redistribution of intensity in the perpendicular hyperfine components of the ESR spectrum, but no change in the superhyperfine splittings. Since free ribose hydroxyl protons are also ionized at pH ~12, the minor changes observed in the ESR spectrum could be due to formation of the tris complex (structure IV, Scheme I) or addition of OH groups in the axial positions. A summary of the pH dependence of the interaction of copper and adenosine is given in Scheme I.

(b) Guanosine–Copper(II) Complexes. NMR studies have shown that at pH 6–7, copper(II) binds strongly to the N_7 position of guanosine.[11,26] The ESR work described below indicates that, depending upon the pH of the solution, other sites (N_1 of base, 2′- and 3′-hydroxyl groups of ribose) can compete effectively with the N_7 site for binding copper(II).

The effects of pH on the ESR spectra of copper complexes of guanosine in Me_2SO-water are summarized in Table II. At pH 2.8, the ESR data (Table II) indicate two different types of copper species are present. The major species is clearly due to free copper ion, and the minor species is attributed to copper

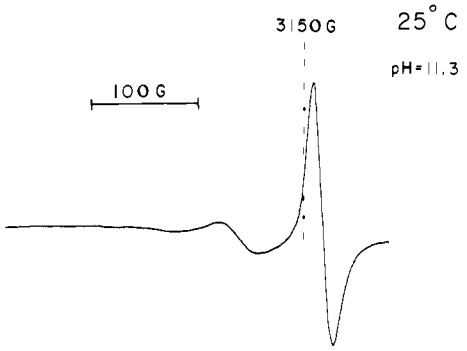


Figure 3. The room-temperature ESR spectrum of a Cu(II)-adenosine complex at pH 11.3: $[Cu(II)]^0 = 5$ mM; $[A]^0 = 40$ mM.

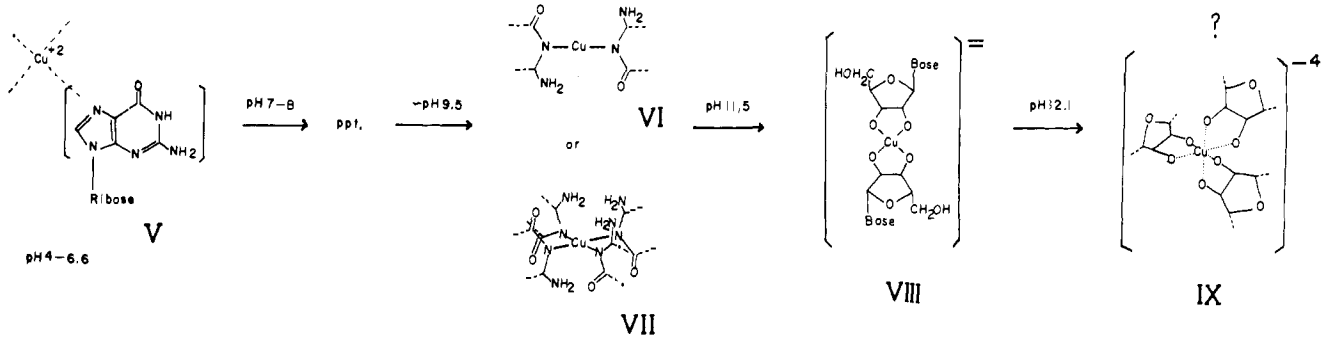
Table II. ESR Parameters of Copper(II)-Guanosine in 50% Me_2SO-50% Water (77 K): $[Cu^{2+}] = 15$ mM, [G] = 80 mM

pH	$A_\parallel$, G	$g_\parallel$	Color
2.8	120[c] (major); 150 (minor)	2.402[c] (major); 2.321 (minor)	Yellow
6.6	150	2.321	Green
8–9	Ppt.	Ppt.	Ppt.
9.6	175[a]	2.296[a]	Green
11.5	185	2.235	Blue
12.1	185[b]	2.235[b]	Blue

[a] With superhyperfine structure (Figure 5). [b] The peak intensity distribution is different from that at pH 11.5 (refer to the analogous case in the copper-adenosine system for additional details). [c] The corresponding ESR parameters for free copper in aqueous solution are 120 G and 2.402, respectively.

bound to N_7 and/or N_3 of guanosine. At pH 6.6, only the latter species is observed. Between pH 8 and 9, the solution is cloudy and a small ESR signal with superhyperfine structure appears (Figure 4A). By pH 9.9, the solution is clear and green and a strong ESR signal with considerable superhyperfine structure is seen (Figure 4B). This ESR behavior is quite different from that observed in the copper-adenosine system which forms a soluble *diamagnetic* copper dimer species in the pH range 9 to 10. The difference between adenosine and guanosine can be attributed to ionization of the N_1H proton of G[10,27] leading to strong binding of copper to N_1 which suppresses formation of the diamagnetic copper-ribose species. The superhyperfine structure in the spectrum in Figure 4B provides additional information about the structure of the complex. We find that the spectrum can be fit using two sets of resonances each with five lines, peak intensity ratios of 1:2:3:2:1, peak-to-peak separations (within each set) of 12.2 G, and absorption line half-widths of 5.2 G. (The choice of the line width and separation between the two sets of lines are crucial (±1 G) in obtaining a good fit to the experimental spectrum.) The centers of the two sets of lines are separated by 22 G. The experimental spectrum (Figure 5A) has the line intensity ratio 1:2.1:4.2:3.6:4.2:2.2:1, in reasonably good agreement with the simulated spectrum (Figure 5B) which has line intensity ratios 1:1.9:3.6:3.2:3.5:1.9:1. Each set has five lines and this implies $(2I + 1) = 5$, or $I = 2$. Since nitrogen has nuclear spin of 1, this indicates that in the complex each copper is chelated to at least two equivalent nitrogens (structure VI in Scheme II). It is possible each copper is actually bound to four guanosines through their N_1 positions in a manner such that pairs of guanosine groups are equivalent. This might involve a complex in which the molecular plane containing two guanosines is perpendicular to the plane containing the other two guanosines

Scheme II



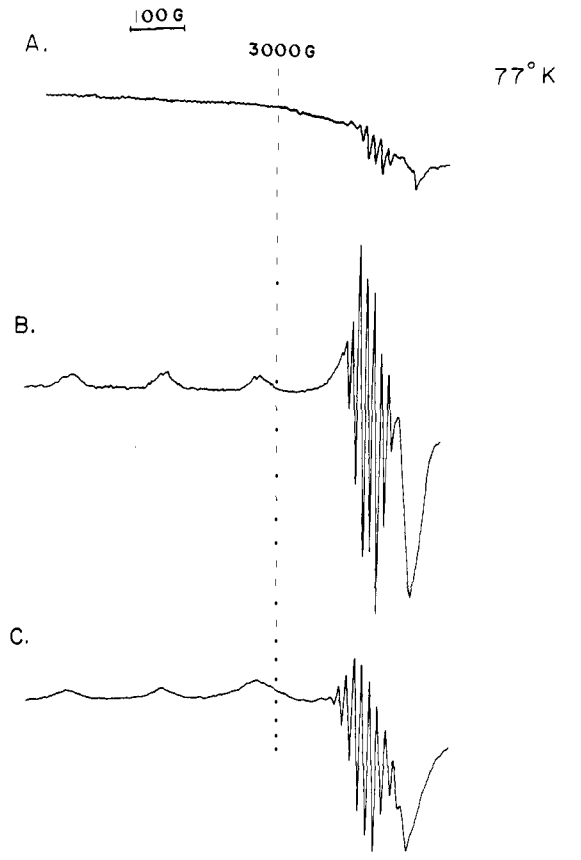


Figure 4. The low-temperature (77 K) ESR spectra of Cu(II)-guanosine complexes in 50% H_2O and 50% Me_2SO: (A) $[Cu(II)]^0 \simeq 7$ mM, $[G] \simeq 70$ mM, pH ~9.1; (B) $[Cu(II)]^0 \simeq 7$ mM, $[G] \simeq 70$ mM, pH ~9.9; (C) $[Cu(II)]^0 \simeq 23$ mM, $[G] \simeq 88$ mM, pH ~9.6.

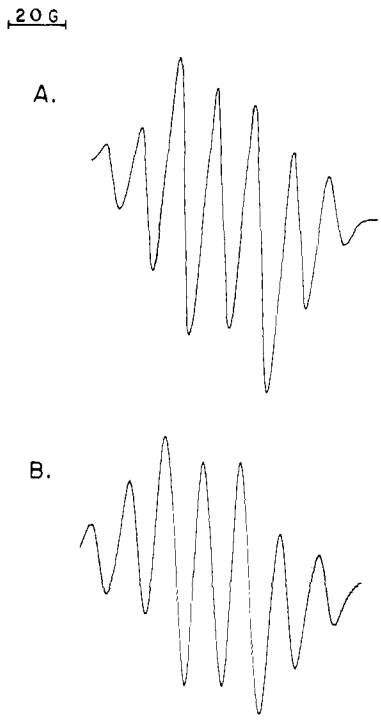


Figure 5. The ESR superhyperfine structure (perpendicular component) of Cu(II)-guanosine complex in 50% H_2O-50% Me_2SO: $[Cu(II)]^0 \sim 7$ mM, $[G]^0 \sim 70$ mM, pH ~9.9; (A) experimental spectrum; (B) computer-simulated spectrum.

and the copper ion. The exact nature of the complex formed in this pH range is sensitive to the precise experimental conditions since other measurements carried out at pH 9.6 and slightly different guanosine-to-copper ratios gave an ESR spectrum with a nine-line superhyperfine pattern (splitting constant 14.1 G, peak intensity ratio of 1:4:9:15:19:19:12:7:5; see Figure 4C). For $I = 4$ (four equivalent nitrogens attached to one copper), a nine-line superhyperfine pattern with peak intensity ratios of 1:4:9:16:19:16:9:4:1 is expected. Except for the last four lines, the calculated and experimental values are the same. We, therefore, attribute the ESR spectrum shown in Figure 4C to a complex in which four equivalent nitrogen atoms are attached to one copper (structure VII in Scheme II). The small discrepancy between the experimental and calculated intensity ratios for the four highest field peaks may be due to the following. In the computer simulation of the spectra (with the consideration of only first-order terms in the spin Hamiltonian), it was found that the $m_I = -1/2$ perpendicular ESR component in most intense[28] so the nine-line pattern observed (Figure 4C) is likely to have the main contribution from the $m_I = -1/2$ perpendicular component of the copper complex. However, the last four superhyperfine lines in the experimental spectrum (Figure 4C) may have a small intensity contribution from the $m_I = 3/2$ hyperfine component of copper. Since this effect was not taken into consideration in the calculation, some differences between the experimental and calculated intensity ratios in the last few superhyperfine lines can be expected.

At pH 11.5 a paramagnetic species with ESR parameters identical with those observed for the adenosine complex (Figure 1F) is observed and therefore attributed to a complex in which copper is bound to the ribose groups of two guanosines (structure VIII, Scheme II). Slight changes in the ESR pattern, which are observed when the pH is raised to 12.1, may be due to formation of a tris complex (structure IX in Scheme II) or addition of OH^- groups as axial ligands. A complete summary of the pH dependence of copper(II)-guanosine complexes in aqueous solution is given in Scheme II.

The behavior of the copper-guanosine complexes is quite similar in Me_2SO and in Me_2SO-water mixtures (see Figure 6). At pH 6.5, a complex with the same ESR parameters is observed in both solvents. Between 7.5 and 8.5, a new species

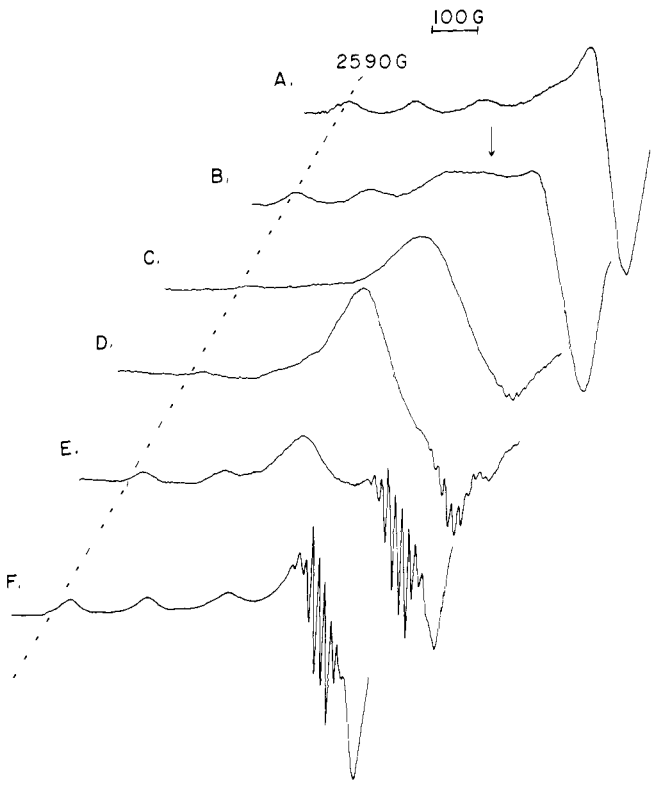


Figure 6. The "pH" dependence of the low-temperature (77 K) ESR spectra of Cu(II)-guanosine complexes in Me_2SO containing 5 mM Cu(II) and 100 mM guanosine: (A) "pH" ~6.5; (B) "pH" ~7.5; (C) "pH" ~8.5; (D) "pH" ~9.5; (E) "pH" ~10.2; (F) "pH" ~11.

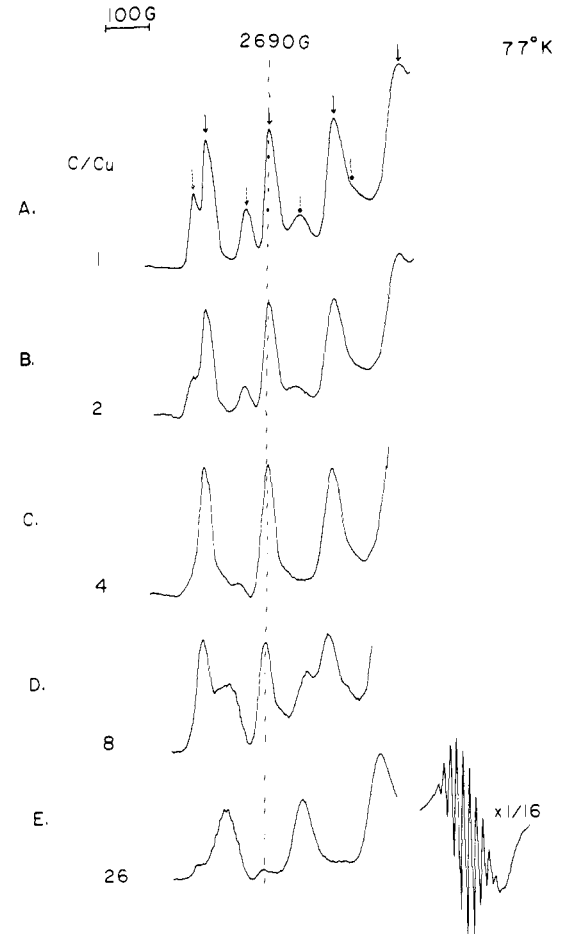


Figure 7. The low-temperature (77 K) ESR spectra of Cu(II)-cytidine complexes with different Cu:cytidine ratios. A–D show only the parallel hyperfine components of the ESR spectra while E shows the complete spectrum. The cytidine to copper ratios are indicated to the left side of each spectrum. Resonances from free copper are indicated by the dashed arrows; resonances from copper bound to the base are indicated by the solid arrows; solvent, 1:1 H_2O-glycerol.

Table III. ESR Parameters of Copper(II)-Cytidine Complexes in a Rigid Glass (1:1 Water-Glycerol Mixture at 77 K), pH ~6.5

Complex	$A_\parallel$, G	$g_\parallel$
$[Cu(H_2O)_3(C)]^{2+}$	144	2.346
$[Cu(C)_4]^{2+}$	170	2.249

with an isotropic ESR spectrum is observed in Me_2SO, although a species with superhyperfine structure is beginning to form at pH 8.5. The species responsible for the isotropic ESR spectrum has not been identified. It has no counterpart in the aqueous solutions since copper hydroxide precipitates in the latter solutions. At pH 9.5 a species with superhyperfine structure is quite evident and by pH 10.2-11 it is the dominant species present in solution.

The ESR spectrum shown in Figure 6F was analyzed using the same method applied to the copper-guanosine aqueous system. From this analysis, we find the spectrum can be accounted for in terms of a complex in which the copper interacts with the N_1 nitrogens from two equivalent guanosines.

(c) **Cytidine-Copper(II) System.** In the ESR studies of the cytidine-copper(II) complexes, both the pH and the copper-to-cytidine ratios were varied because the nature of the copper-cytidine complex is very dependent on the cytidine-to-copper ratio. At neutral pH and a copper-to-cytidine ratio of 1:1, the parallel copper hyperfine components exhibit an ESR pattern characteristic of free copper (indicated by dotted arrow in Figure 7A) and base (N_3) bound copper (indicated by solid arrow in Figure 7A). From the area ratio of these two patterns ($m_I = \frac{3}{2}$, parallel components were chosen for comparison) the ratio of free copper to base bound copper is 1:2.16. (The estimation of the individual area of the two overlapping peaks in terms of Gaussian or Lorentzian line-shape analysis is not satisfactory so a simple smooth curve fitting method was used instead.) Since the initial copper-cytidine ratio is 1:1, and the resulting [free copper(II)]:[base bound copper(II)] ratio is 1:2.16, a complex involving more than one cytidine per copper seems unlikely. (The equilibrium constant for formation of the one-to-one cytidine to copper(II) complex is estimated from our data to be ~10^3 L/mol at about 0 °C.) When the cytidine-to-copper ratio is increased above 26/1, a new ESR signal with a nine-line superhyperfine pattern in both the parallel and perpendicular hyperfine components appears, and the signal from the free copper disappears (Figure 7E). Since no free

Table IV. ESR Parameters of Copper(II)-Cytidine Complexes in 50% Me_2SO and 50% H_2O at 77 K: $[Cu^{2+}]$ = 7 mM, [C] = 56 mM

pH	$A_\parallel$, G	$g_\parallel$	Color
2.7	119	2.404	Yellow
5.9	170 (major) 144 (minor)	2.249 (major) 2.344 (minor)	Green
8-9	Ppt.	Ppt.	Ppt.
10.2	185	2.237	Blue
12.0	185[a]	2.237[a]	Blue

[a] The ESR line intensity distribution at pH 12.0 is different from that at pH 10.2.

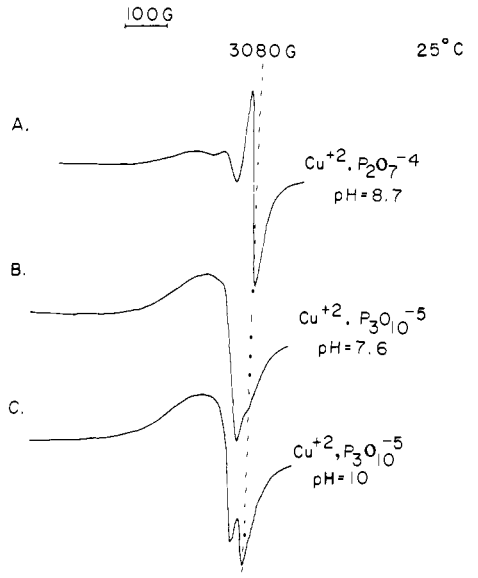


Figure 8. The room-temperature ESR spectra of Cu(II)-pyrophosphate and Cu(II)-triphosphate complexes in basic solutions (H_2O).

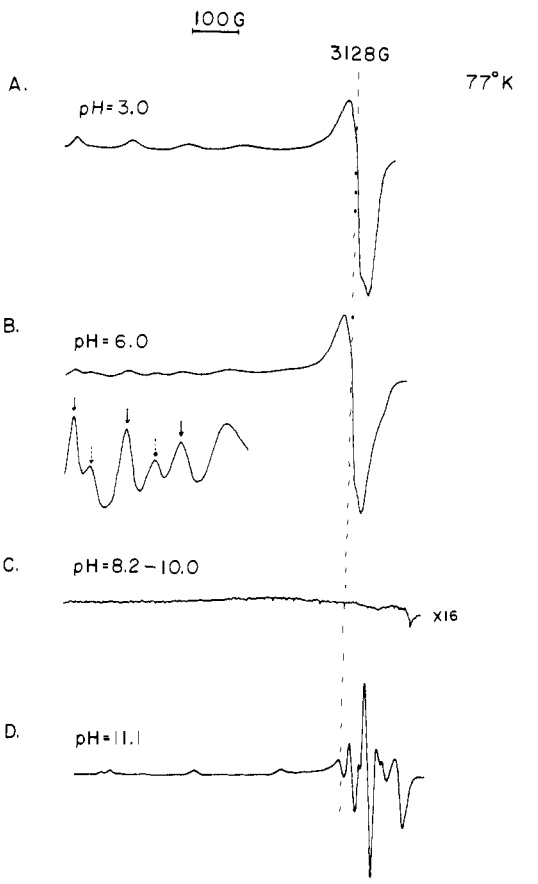


Figure 9. The pH dependence of low-temperature (77 K) ESR spectra of Cu(II)-uridine complexes in 50% H_2O-50% Me_2SO.

copper signal is observed, the nine-line pattern indicates the formation of a $[Cu(C)_4]^{2+}$ complex. The equilibrium constant for formation of the four-to-one complex from cytidine is estimated from the ESR data to be on the order of 10^8 L^4/mol^4. ESR parameters for the 1:1 and 1:4 copper(II)-cytidine complexes are listed in Table III.

The effect of pH on the ESR spectra of the copper-cytidine complexes in Me_2SO-water has been studied at a copper-to-cytidine ratio of 1:8, and the ESR parameters observed for the various complexes are summarized in Table IV. At pH 5.9, two different copper-cytidine complexes are present in the solution (Table IV). By referring to the data in Table III we see that the major species is $[Cu(C)_4]^{2+}$, and the minor species is $[Cu(C)(H_2O)_3]^{2+}$. At pH 10.2, the spectrum is characteristic of the bis(ribose-copper) complex which is also observed with adenosine (Figure 1F). The change in the ESR pattern at pH 12 (analogous to the one seen with the adenosine-copper complex) may be due to formation of a tris complex (see discussion of the copper-adenosine complex).

The pH dependence of the ESR spectrum of the copper-5′-CMP system is essentially the same as that of copper-cytidine in H_2O, indicating that the 5′-phosphate group has little effect on the nature of the complexes formed. At low pH (pH 3.8), the ESR spectrum indicates free copper(II) and the $[Cu(CMP)(H_2O)_3]^{2+}$ species are present. At neutral pH, the $[Cu(CMP)_4]^{2+}$ species with well-resolved superhyperfine structure starts to form. At high pH, the copper interaction with the ribose group is the same with or without the 5′-phosphate group.

The behavior of the Cu(II)-CTP system is rather different from that observed with cytidine and 5′-CMP. Even with a 64-fold excess of CTP over copper, the low-temperature ESR spectrum at neutral pH still shows that only the $[Cu(CTP)(H_2O)_3]^{2+}$ and free copper species are present. No superhyperfine pattern characteristic of $[Cu(CTP)_4]^{2+}$ is detectable. Evidently, complexation of the copper with the triphosphate groups in CTP prevents chelation to more than one cytidine base. To learn more about the role of phosphate in the metal-nucleotide complex, we examined the interaction of copper(II) with phosphate, pyrophosphate, and triphosphate anions. At basic pH the copper-phosphate solution is cloudy, indicating the formation of copper hydroxide precipitate; however, the copper-pyrophosphate solution is green and clear even at high pH, and the room temperature ESR spectrum exhibits resolved hyperfine structure (Figure 8A). The same ESR spectrum has also been observed by Shapnik et al.,[29] in their electrodeposition studies of Cu-$K_4P_2O_7$ in H_2O. The pH dependence of the room-temperature ESR spectrum of copper-triphosphate solutions (Figures 8B,C) indicates that there are at least two different copper-triphosphate complexes in the solution. The fact that pyrophosphate and triphosphate form stable, soluble complexes at neutral and basic pH whereas copper hydroxide precipitates in the copper-phosphate systems further supports our interpretation of the different behavior of 5′-CMP and 5′-CTP toward copper(II).

In neutral rigid glass (50% water, 50% glycerol), the ESR of the copper-poly(C) system indicates that two copper species are present; one is the free copper ion and the other has ESR parameters $A_\parallel$ = 144 G and $g_\parallel$ = 2.344, which are the same as those of $[Cu(CMP)(H_2O)_3]^{2+}$ and $[Cu(C)(H_2O)_3]^{2+}$ (see previous section). This indicates that copper is bound to N_3 of the cytidine residue in poly(cytidylic acid), consistent with the NMR findings.

(d) Uridine-Copper(II) System. Uridine weakly binds copper(II); the site of binding has not been established.[21] The following ESR studies show copper(II) is directly bound to the uridine N_3 nitrogen and suggest a possible structure for the complex.

The effect of pH on the ESR spectra of copper-uridine complexes in water-Me_2SO is shown in Figure 9, and the relevant ESR parameters for the different species are tabulated in Table V. At low pH (pH $\leq$4), most of the copper is free in the solution (Figure 9A), but at pH 6 (Figure 9B), a base-bound copper species ($A_\parallel$ = 141 G, $g_\parallel$ = 2.344) is formed. Since uridine is not ionized at neutral pH in the presence of copper(II),[30] the direct interaction of copper with N_3, which is indicated by the ESR data (structure XI in Scheme III),

Scheme III

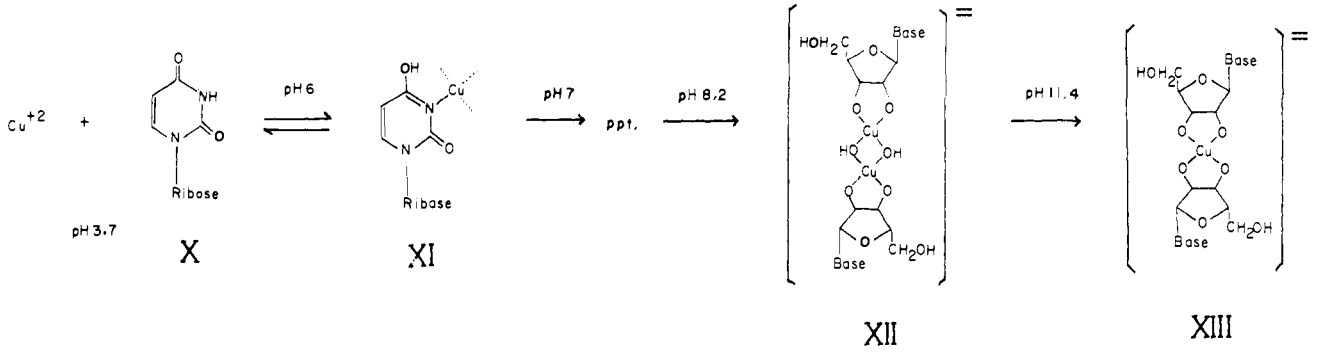


Table V. ESR Parameters of Copper(II)-Uridine Complexes in 50% Me_2SO-50% H_2O at 77 K: [Cu] = 6 mM, [U] = 50 mM

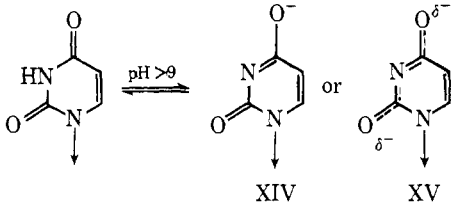
pH	$A_\parallel$, G	$g_\parallel$	Color
3.7	120	2.402	Yellow
6.0	120 (major) 141 (minor)	2.402 (major) 2.334 (*minor*)	Yellow
7-8	Ppt.	Ppt.	Ppt.
8.2-10	No ESR	No ESR	Green and clear
11.4	183	2.240	Blue

Table VI. ESR Parameters of Copper(II)-Uridine Complexes in Me_2SO at 77 K: [Cu^{2+}] = 11 mM, [U] = 100 mM

"pH"	$A_\parallel$, G	$g_\parallel$	Color
~6	120	2.402	Yellow
~8	Ppt.	Ppt.	Ppt.
~9.5	No ESR	No ESR	Green and clear
~10.5	175	2.247	Blue

suggests formation of a complex with the structure in which copper is bound to the N_3 of uridine in a tautomeric state.

In the pH range 7-8, a precipitate forms, but in the pH range 8.2-10, the solution is green and clear. No ESR signal can be detected from this clear solution indicating formation of the diamagnetic copper(II)-ribose complex formation (see structure II, Scheme I). It is interesting to contrast the behavior of guanosine and uridine since both have an imino proton (N_1 of guanosine and N_3 of uridine) which ionizes at pH ~9.3.[27] In the copper-guanosine system, copper binds to N_1 of guanosine in the pH range 8-10 (ESR spectrum with superhyperfine structure (section b) characteristic of base binding). However, in the same pH range (8.2-10) copper forms only the diamagnetic ribose complex with uridine instead of binding to N_3 of uridine. Perhaps this can be understood by the following analysis. At pH 9.3, the following ionization process for uridine has been suggested:[27,31]



From the structure of the anion (XIV) it can be seen that binding of more than one uridine anion to a single copper through the uridine N_3 nitrogen may be unfavorable because of oxygen anion repulsion, and the nonchelative binding through the negatively charged oxygen will not be as stable as the chelated diamagnetic copper-ribose complex (refer to structure XII below). Thus, at pH 8.2-10 structure XII is suggested for the complex formed between copper and uridine.

At high pH (pH 11.4) the aqueous solution of copper and uridine gave an ESR pattern characteristic of the paramagnetic copper-ribose complex (structure XIII) (Figure 9D). A summary of the pH studies in aqueous solution is presented in Scheme III.

(e) **Miscellaneous.** Inosine is structurally similar to guanosine, except that it lacks the amino group attached to C_2. From pH titration studies it was found[10] that the N_1 protons of G and I ionize at ~9.3 and 8.9, respectively. In the presence of copper(II), we found that copper-inosine aqueous solution gives a low-temperature ESR spectrum with nitrogen superhyperfine pattern characteristic of $[Cu(I)_4]^{2-}$ at pH 8.6, whereas formation of $Cu(G)_4^{2-}$ can be detected by ESR (vide supra) only when the pH is raised to 9.5. This slight difference in the effect of pH on complexes of G and I is in accord with the differences in their pK_a values. The ESR parameters for $Cu(G)_4^{2-}$ are A = 175 G, g = 2.296 and the corresponding values for $[Cu(I)_4]^{2-}$ are A = 188 G, g = 2.231. Using these ESR parameters and the approximate d-d transition energies (taken from visible spectral measurements), $\Delta E_{xy} = (E_{xy} - E_{x^2-y^2}) = \Delta E_{xz} = (E_{xz} - E_{x^2-y^2}) = 15\,000\ cm^{-1}$, the in-plane π-bonding parameter β_1^2 (Maki and McGarvey[18]) is calculated to be 0.77 for $[Cu(G)_4]^{2-}$ and 0.72 for $[Cu(I)_4]^{2-}$. The slightly larger in-plane π-bonding character (smaller β_1^2) of $[Cu(I)_4]^{2-}$ is probably related to the difference between guanosine and inosine in their ability to form coplanar complexes with copper(II). Thus, as expected on the basis of our analysis of guanosine complexes, the complexes with inosine are found to be essentially the same.

Conclusion

The ESR studies described here demonstrate that the nature of the Cu(II) complexes formed with nucleosides and nucleotides varies with the specific nucleic acid derivatives used, the pH, and in some cases the nucleic acid to copper ratio. In slightly basic solutions (pH ~8.5-10), copper specifically forms a water-soluble complex with the ribose OH groups of the ribonucleosides and 5′-ribonucleotides, but these complexes cannot form with any of the deoxynucleosides or the 2′- and 3′-ribonucleotides. Therefore, between 8.5 and 10 copper(II) is able to distinguish between the different compounds; this may have importance in the function of certain enzymatic systems such as the basic pH (pH 8.1) nucleic acid phosphorylation studies with different metals[32] and in translation errors in the RNA, DNA polymerase studies[4] which are induced by transition metals.

Under certain conditions, copper(II) is able to form complexes where as many as four bases are simultaneously bound to one metal. Conceivably this base binding ability could be

utilized in stabilizing unusual structures or interactions in certain polynucleotides. Quite independent of whether certain biological systems naturally make use of these interesting binding properties, the results presented here provide data which will be needed in ESR studies of the interaction of copper with polynucleotides. They also suggest ways in which pH may be used as an additional parameter to control the nature of the complexes formed.

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References and Notes

(1) A. Tissieres, J. D. Watson, D. Schlessinger, and B. R. Hollingworth, *J. Mol. Biol.*, **1**, 221 (1959).
(2) W. E. C. Wacker and B. L. Vallee, *J. Biol. Chem.*, **234**, 3257 (1959).
(3) B. M. Guirard and E. E. Snell, *Bacteria*, **4**, 26 (1962).
(4) D. K. Dube and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, **67**, 1041 (1975).
(5) G. L. Eichhorn, *Nature (London)*, **194**, 474 (1962).
(6) S. Hlai, *J. Mol. Biol.*, **11**, 672 (1965).
(7) C. Zimmer, *Z. Chem.*, **11**, 441 (1971).
(8) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, S. H. Marguisse, J. Merrill, R. Penwick, and A. Zamir, *Science*, **147**, 1462 (1965).
(9) H. Chan and R. Wells, *Nature (London)*, **252**, 205 (1974).
(10) N. A. Berger, E. Tarien, and G. L. Eichhorn, *Nature (London)*, **239**, 237 (1972).
(11) G. Brun, D. M. L. Goodgame, and A. C. Skapski, *Nature (London)*, **253**, 127 (1975).
(12) H. Petering, H. Buskirk, and J. Crim, *Cancer Res.*, **27**, 1115 (1967).
(13) J. Crim and H. Petering, *Cancer Res.*, **27**, 1278 (1967).
(14) G. Bemski, M. Rieber, and M. Wust, *FEBS Lett.*, **14**, 117 (1971).
(15) (a) C. Ropars and R. Vlovy, *C. R. Hebd. Seances Acad. Sci.*, **258**, 731 (1964); (b) C. Ropars and R. Vlovy, *J. Chim. Phys. Phys.-Chim. Biol.*, **62**, 408 (1965).
(16) H. M. McConnell, *J. Chem. Phys.*, **25**, 709 (1956).
(17) R. N. Rogers and G. E. Pake, *J. Chem. Phys.*, **33**, 1107 (1960).
(18) (a) A. H. Maki and B. R. McGarvey, *J. Chem. Phys.*, **29**, 31 (1958); (b) A. H. Maki and B. R. McGarvey, *ibid.*, **29**, 35 (1958).
(19) N. M. Atherton, "Electron Spin Resonance, Theory and Applications", Halsted Press, 1973.
(20) Y. Y. H. Chao and D. R. Kearns, *J. Phys. Chem.*, **81**, 666 (1977).
(21) N. A. Berger and G. L. Eichhorn, *Biochemistry*, **10**, 1857 (1971).
(22) H. Reinert and R. Weiss, *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 1321 (1969).
(23) N. Bloembergen, E. M. Purcell, and R. V. Pound, *Phys. Rev.*, **73**, 679 (1948).
(24) P. S. Hubbard, *Phys. Rev.*, **131**, 1155 (1963).
(25) R. Aasa, B. Malmstrom, P. Saltman, and T. Vanngard, *Biochim. Biophys. Acta*, **80**, 430 (1964).
(26) H. Sigel, C. F. Naumann, and B. Prijs, *Eur. J. Biochem.*, **46**, 589 (1974).
(27) J. J. Christensen, J. H. Rytting, and R. Izatt, *J. Chem. Soc. B*, 1643 (1970).
(28) R. Neiman and D. Kivelson, *J. Chem. Phys.*, **35**, 156 (1961).
(29) M. S. Shapnik, K. A. Zinkicheva, A. V. Il'yasov, and G. S. Vozdvizhenskii, *Russ. J. Inorg. Chem. (Engl. Transl.)*, **18**, 540 (1973).
(30) A. M. Fiskin and M. Beer, *Biochemistry*, **4**, 1289 (1965).
(31) R. C. Lord and G. J. Thomas, Jr., *Spectrochim. Acta, Part A*, **23**, 2551 (1967).
(32) C. Babinet, A. Roller, J. M. Dubert, M. N. Thang, and M. Grunberg-Manago, *Biochem. Biophys. Res. Commun.*, **19**, 95 (1965).

Communications to the Editor

Rate Constants for Spin Trapping. Primary Alkyl Radicals[1]

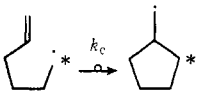
Sir:

The EPR spectroscopic technique of "spin trapping" has been used qualitatively to detect and identify transient free radicals for several years.[2] Its quantitative use in mechanistic studies has been hampered by the paucity of data on the rate constant, k_1, for the reactions of the radicals with the commonly employed spin traps.

$$\mathrm{R}\cdot + \mathrm{T} \xrightarrow{k_1} \mathrm{RT}\cdot \qquad (1)$$

What little rate data is available[3-9] rests on competition experiments with reactions having "known" rate constants. However, these "known" rate constants are themselves uncertain to varying degrees.[10] In view of the great potential of spin trapping, we have begun a program to determine accurate rate constants for the trapping of some commonly encountered radicals.

The 5-hexenyl radical, **H·**, isomerizes to cyclopentylmethyl, **C·**, at a rate which is reliably known.[11,12] Since both radicals are primary alkyls, the spin adducts that they form with a spin trap, T, will have very similar properties, i.e., similar EPR spectra and similar kinetic and thermodynamic stabilities. A nice distinction between the spectra of the two spin adducts, **HT·** and **CT·**, can be obtained by labeling the 5-hexenyl radical with C-13 in the 1 position, since hyperfine splitting (hfs) by this carbon will only be detectable in **HT·**. Any further reactions of **HT·** and **CT·** should have virtually equal rate constants and so the ratio of [**HT·**]/[**CT·**] should be independent of reaction time *unless* spin trapping is reversible, in which case this



$$\mathrm{H}\cdot + \mathrm{T} \xrightarrow{k^{\mathrm{T}}} \mathrm{HT}\cdot$$
$$\mathrm{C}\cdot + \mathrm{T} \longrightarrow \mathrm{CT}\cdot$$

ratio will decrease as the reaction proceeds. The rate constant for trapping, k^{T}, can be calculated from the relation

$$k^{\mathrm{T}} = k_c[\mathbf{HT}\cdot]/[\mathrm{T}][\mathbf{CT}\cdot]$$

provided the spin-adduct ratio is extrapolated to zero time if it shows any variation.

We have applied this procedure to phenyl-*N*-*tert*-butyl nitrone (PBN) and to 2-methyl-2-nitrosopropane (NtB)[13] using di([2-^{13}C]-6-heptenoyl) peroxide (90 at. % ^{13}C) as a *thermal* source of [1-^{13}C]-5-hexenyl in benzene at 40 °C. The ^{13}C hfs constants of the labeled **HT·** are 3.1 G, for T = PBN[14] and 5.0 G, for NtB;[15] so the lines due to **HT·** are resolved from those of **CT·** in both cases. For one trap, NtB, the [**HT·**]/[**CT·**] ratio decreased slightly with time (~20% between t = 5 and t = 30 min) suggesting that the addition is marginally reversible at 40 °C.[16] Using ~0.06 M peroxide and [PBN] = 0.44 and 0.30 M[18] and extrapolating to t = 0 gave $k^{\mathrm{T}}_{\mathrm{PBN}}/k_c$ = 0.75 and 0.75, respectively, and with [NtB] = 0.032 and 0.026 M,[17] $k^{\mathrm{T}}_{\mathrm{NtB}}/k_c$ = 50.9 and 50.4, respectively. At 40 °C, $k_c = 1.7_8 \times 10^5\ \mathrm{s}^{-1}$;[12] therefore $k^{\mathrm{T}}_{\mathrm{PBN}} = 1.3_4 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ and $k^{\mathrm{T}}_{\mathrm{NtB}} = 90.2 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. That the rate constant ratio, $k^{\mathrm{T}}_{\mathrm{NtB}}/k^{\mathrm{T}}_{\mathrm{PBN}} = 90.2/1.3_4 = 67.4$ is correct was confirmed by competitive experiments using the *n*-hexyl radical (from *n*-heptanoyl peroxide) in the presence of both spin traps. At