Interaction of Metal Ions with Polynucleotides and Related Compounds. XIV. Nuclear Magnetic Resonance Studies of the Binding of Copper(II) to Adenine Nucleotides[*]

Nathan A. Berger and Gunther L. Eichhorn

ABSTRACT: Paramagnetic broadening of the proton magnetic resonance peaks of adenine nucleotides is used to determine Cu(II) binding sites, and their relation to molecular conformation. The resonance peaks of adenine protons are broadened by Cu(II) ions as follows: H-8 is broadened preferentially to H-2 in 3'-AMP, 5'-AMP, and poly(A); H-8 and H-2 are broadened equally in adenosine, 2'-AMP, cyclic 3':5'-AMP, and cyclic 2':3'-AMP. In tubercidin (7-deazaadenosine), H-2 is broadened preferentially to H-8. These data indicate that proton magnetic resonance broadening is useful in determining the binding site of metal ions on the adenine moiety; electronic changes produced in regions of the ring system remote from the metal binding site do not obfuscate the re-

sults, nor does copper binding to phosphate account for broadening of the base peaks. In general, Cu(II) ions can bind to multiple sites on the adenine base, with preference for a given site influenced by molecular associations which in the different AMP isomers is governed by the position of the phosphate on the ribose. The results obtained with 3'- and 5'-AMP can be interpreted by a binuclear 2:2 Cu-AMP complex in which the two bases are stacked with each Cu(II) bound to a phosphate of one AMP and N-7 of the other. The results obtained with 2'-AMP can be interpreted by a chelate involving N-3 and a phosphate group of the same molecule. N-7 is the preferred binding site of Cu(II) in poly(A). Broadening of ribose peaks is in line with these structures.

The biological reactions of adenine nucleotides are generally mediated by metal ions, and numerous previous investigations have been concerned with the elucidation of the structure of the metal complexes of these compounds. A very useful technique for studying complexation by metal ions is the broadening of the peaks of a nuclear magnetic resonance spectrum by paramagnetic metal ions. The degree of broadening of a particular resonance peak is related to the distance of the bound paramagnetic ion from the group exhibiting that resonance (Li et al., 1962; Eaton and Phillips, 1965). This technique has been applied previously in studies of nucleotide complexes (Cohn and Hughes, 1962; Sternlicht et al., 1965; Eichhorn et al., 1966) demonstrating that binding occurs to both phosphate and heterocyclic bases in nucleotides. However, the site of attachment of metal to base is still uncertain.

It has occurred to us that two phenomena exist that challenge the interpretations of nuclear magnetic resonance broadening studies. The first is the possibility that metal ions binding at one site on the molecule distort the electronic configuration of the whole molecule (Carrabine and Sundaralingam, 1970) so that broadening could occur at sites far removed from the metal binding site. Secondly, metal ions bound to phosphate groups that approach a proton on the base can serve to broaden the resonance peak of that proton without being attached at a site in its vicinity.

In the present paper we have addressed ourselves to these problems of interpretation of proton magnetic resonance broadening of adenosine nucleotides, using Cu(II) as the

paramagnetic ion, with the result that the method appears vindicated. We have then demonstrated that copper ions are capable of binding to several positions on the nucleotide bases, but that frequently one position is favored by stereochemical considerations, which are greatly influenced by the position of phosphate on the ribose.

Materials and Methods

Adenosine and adenine nucleotides were obtained from Sigma Chemical Co., tubercidin was from Calbiochem, and polyriboadenylic acid from Miles Laboratories. Nucleosides were dissolved directly in Me_2SO-d_6, 99.5% Diaprep Inc. Deuterium replacement of rapidly exchanging protons was performed either by adding 0.1 ml of D_2O directly to a nuclear magnetic resonance tube containing 0.5-ml solutions of nucleoside dissolved in Me_2SO-d_6, or by first dissolving nucleoside in D_2O, lyophilizing, and then redissolving in Me_2SO-d_6. Adenine nucleotides and poly(A) were dissolved directly in D_2O, 99.75%, Brinkman Instruments Inc. Poly(A) was subjected to gentle rotary shaking at 4° to effect solution. The pH of aqueous solutions was measured with a pH meter 25, Radiometer, Copenhagen, and is reported directly as read. Unless indicated otherwise, all solutions were adjusted to pH 7.5 with NaOD or DCl, Diaprep Inc. No buffers were used. Concentrations of aqueous solutions prepared for nuclear magnetic resonance were determined by ultraviolet extinction of $1/1000$ dilutions of nucleotide in 0.1 N NaOH. Ultraviolet spectra were obtained with a Cary 14 recording spectrophotometer.

Nuclear magnetic resonance spectra were obtained with the Varian NMR spectrometer, A60D. All spectra presented were obtained at similar radiofrequency field and spectrum amplitudes. Unless otherwise specified, the sweep width is 500 Hz and the sweep time is 500 sec. Chemical shifts in D_2O were measured from an internal standard of sodium 2,2-di-

FIGURE 1 spectrum:

Shift in CPS from DSS Internal Standard

	515	505	495	485	475	465
5' AMP	H-8			H-2		
2' AMP		H-8		H-2		
3' AMP		H-8		H-2		
2'3' Cyc AMP			H-8	H-2		
3'5' Cyc AMP			H-8 & H-2			
poly A					H-8	H-2

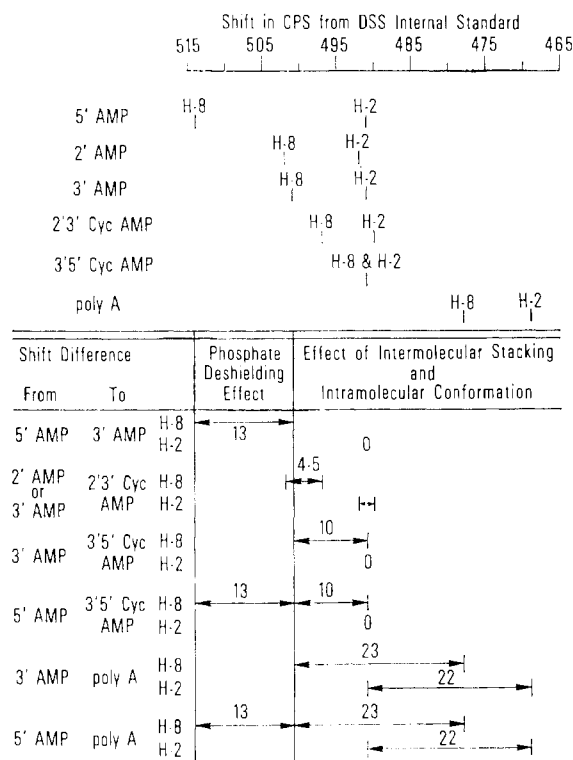| Shift Difference From	To		Phosphate Deshielding Effect	Effect of Intermolecular Stacking and Intramolecular Conformation
5' AMP	3' AMP	H-8 H-2	13	0
2' AMP or 3' AMP	2'3' Cyc AMP	H-8 H-2	4-5	⊢⊣
3' AMP	3'5' Cyc AMP	H-8 H-2		10 / 0
5' AMP	3'5' Cyc AMP	H-8 H-2	13	10 / 0
3' AMP	poly A	H-8 H-2		23 / 22
5' AMP	poly A	H-8 H-2	13	23 / 22

FIGURE 1: Components of proton magnetic resonance shift (60 MHz) of adenine nucleotides. Comparison of stacking and phosphate deshielding effects. Mononucleotides are 0.1 M and poly(A) is 0.067 M in D_2O (pH 7.5).

TABLE I: Proton Chemical Shifts (60 MHz).

		Purine			
	Concn (M)	H-8	H-2	6-NH$_2$	H-1'
Nucleosides in Me$_2$SO-d_6 at 37–40° a					
Adenosine	0.1	500	488	435	354
Tubercidin	0.1	440	484	(H-7) 419 397	361
Nucleotides in D$_2$O at 37–40°, pH 7.5 b					
5'-AMP	0.1	514	491		367
5'-dAMP	0.1	511	492		389
2'-AMP	0.1	502	492		370
3'-AMP	0.1	501	491		367
Cyclic 2':3'-AMP	0.1	497	490		376
Cyclic 3':5'-AMP	0.1	491	491		368
Poly(A)	0.038 0.067	478	469		340
	pH				
5'-AMP	6 0.1	508	490		367
	7.5 0.1	514	491		367
	9 0.1	514	491		367
	12 0.1	514	491		365

a Cycles per second from Me$_4$Si. b Cycles per second from sodium dimethylsilapentanesulfonate.

methyl-2-silapentanesulfonate,[1] NMR Specialties Inc. Tetramethylsilane (Me$_4$Si), Diaprep, Inc., was used as internal standard for solutions in Me$_2$SO-d_6.

No broadening or shifts of DSS or Me$_4$Si were produced by Cu(II) from 10^{-5} to 10^{-3} M. After heating to 100° for 30 min or longer in the presence of Cu(II) and then recooling to 40° no change in shift or line characteristic was observed for DSS. No change was observed in a nucleoside or nucleotide on addition of DSS or Me$_4$Si, nor was any variation in metal binding observed in the presence or absence of these standards. During experiments performed at room temperature, the nuclear magnetic resonance probe is maintained at 37–40°. Heating of solutions in the nuclear magnetic resonance spectrometer was performed with the Varian variable-temperature accessory and all temperatures were calibrated by the splitting of the peaks of the Varian ethylene glycol temperature calibration sample. Temperature was elevated by 10° increments and samples were equilibrated at each level for 20 min before recording spectra.

A syringe was used to quantitatively load nuclear magnetic resonance tubes with 0.5 ml of ligand solution. Spectra were initially obtained in the absence of metal; then calibrated capillary pipets, Clay Adams Micropets, were used to introduce CuSO$_4$ in D$_2$O or Cu(CH$_3$COO)$_2$ in Me$_2$SO-d_6 to solutions in nuclear magnetic resonance tubes. The technique of incremental addition of Cu(II) to the same solution was used to study progressive broadening.

[1] Abbreviation used: DSS, sodium 2,2-dimethyl-2-silapentanesulfonate.

Results

Chemical Shifts in the Adenine Nucleotide Series. Effects of Phosphate Deshielding and Base Stacking. The chemical shifts of the proton nuclear magnetic resonance spectra of adenosine, tubercidin, the adenosine mononucleotides, and poly(A) are presented in Table I and Figure 1. Base peak assignments and shifts were those established for the adenine nucleotides (Jardetzky, 1960; Jardetzky and Jardetzky, 1960; Bullock and Jardetzky, 1964; Broom et al., 1967). Ribose peaks are those established by Feldman and Agarwal (1968). The identity of the protons associated with the amino group at position 6 of adenosine and of the ribose hydroxyl protons are as indicated by Gatlin and Davis (1962) and were confirmed by deuterium replacement of these rapidly exchanging protons in Me$_2$SO-d_6 to which D$_2$O was added (Kokko et al., 1961). Peak assignments in tubercidin are those of Schweizer et al. (1968) and, as also indicated by these investigators, the specific deshielding effect of the 5'-phosphate on the H-8 of the adenine ring is clearly demonstrated by its downfield shift in 5'-AMP compared to the shift of the H-2 resonance. When the phosphate is located elsewhere on the ribose ring, as in 2'- or 3'-AMP, no deshielding of the H-8 resonance occurs. The downfield shift of the H-8 resonance of 5'-AMP demonstrates that the deshielding increases as the pH increases from 6 to 7.5 but not past this point. This is in agreement with the demonstration (Schweizer et al., 1968) that the form of the 5'-phosphate with two negative charges exerts the maximum deshielding effect on the H-8 proton. Increasing the pH to 9 and 12 produces no further ionization of the phosphate and no further deshielding effect.

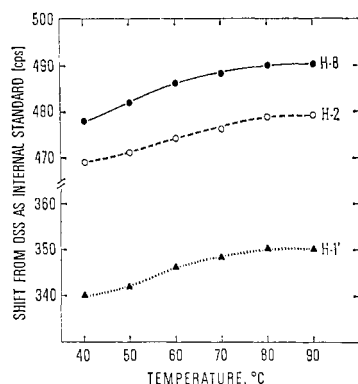In contrast to the downfield shift of the base proton resonances produced by the deshielding effect of the phosphate

FIGURE 2: Temperature dependence of proton magnetic resonance shifts (60 MHz) of 0.038 M poly(A) in D_2O (pH 7.5). Shifts are downfield from DSS as the internal standard.

FIGURE 3: Proton magnetic resonance shifts (60 MHz) of polymer at 40 and 90° compared to monomer at 40°. Solutions are 0.038 M poly (A) and 0.1 M 3'-AMP in D_2O (pH 7.5).

group, an upfield shift of the peaks results from the shielding effects of neighboring heterocyclic bases in stacked association (Broom et al., 1967; Schweizer et al., 1968). The shift of the base protons is also sensitive to the rotation around the glycosidic linkage and to the conformation of the ribose ring (Chan and Nelson, 1969; Prestegard and Chan, 1969). This sensitivity to intramolecular rotation was demonstrated by the adenine H-8, while the H-2 responds principally to stacking effects. A 4- to 5-cps upfield shift occurs for the H-8 resonance of cyclic 2':3'-AMP when compared to the H-8 of either 2'- or 3'-AMP (Figure 1). This increased shielding of the H-8 resonance in the cyclic 2':3' compound may be attributed to either conformational changes in the ribose or to an increased tendency of the five-membered component of the purine ring to participate in stacked association. The small accompanying shift of the H-2 suggests that both effects occur. The H-8 of cyclic 3':5'-AMP shifts 23 cps upfield from 5'-AMP and 10 cps from the H-8 of 3'-AMP. This marked upfield shift can be explained as the result of two major components as demonstrated in Figure 1. Formation of the cyclic 3':5'-phosphate constrains the phosphate to a side of the ribose away from the base. This results in removing the deshielding effect of the 5'-phosphate on the H-8 proton and accounts for the portion of the H-8 upfield shift of 13 cps, characteristic of removing the phosphate deshielding in going from 5'-AMP to 3'-AMP. The further upfield shift of 10 cps from 3'-AMP to cyclic 3':5'-AMP reflects the marked change in the ribose conformation due to formation of the cyclic 3': 5'-phosphate. Also, constraining the phosphates in the cyclic form places them at a distance from the purine ring in which they are less likely to interfere with the stacking tendency of the bases.

Joining of the adenine mononucleotides into the polymer produces an upfield shift of the proton resonance signals due to the increase in base stacking, designated as the polymerization shift (Jardetzky, 1964). The degree of base stacking in poly(A) is demonstrated by the large upfield shift of 22 cps for the H-2 resonance when compared to either the 3'- or 5'-AMP. The upfield shift of the H-8 resonance is 36 cps from the H-8 of 5'-AMP and 23 cps from the H-8 resonance of 3'-AMP. This 23-cps difference from the H-8 resonance of 3'-AMP to the H-8 of poly(A) is similar to the H-2 shift and is due to polymerization. The additional shift of 13 cps from 5'-AMP is due to removal of the 5'-phosphate effect. No shift was observed in poly(A) resonances over a twofold concentration range, 0.038–0.067 M. This is apparently due to
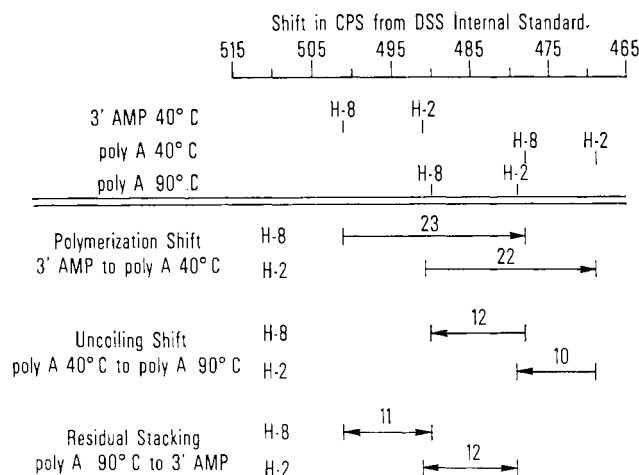
the already stacked nature of the polymer as compared to the monomer, which demonstrates a great increase in stacking over this concentration range (Broom et al., 1967; Schweizer et al., 1968). Similar to previous observations (McDonald et al., 1964; McTague et al., 1964), when poly(A) is heated from 40 to 90° a continuous and equal downfield shift of the H-8 and H-2 resonance occurs (Figure 2), which levels off by 90°. This downfield shift accompanies the noncooperative helix to random coil transition of poly(A) (Van Holde et al., 1965; Holcomb and Tinoco, 1965) and reflects a continuous decrease in stacking interactions over this temperature range. The final positions of the base proton resonances of poly(A) at 90° are still 11 to 12 cps upfield from their corresponding peaks in 3'-AMP, indicating that even in the random coil, the adenine bases are 50% more stacked than in the monomer (Figure 3). This proton magnetic resonance demonstration of the residual base stacking in poly(A) during denaturing conditions is similar to the difference in the ultraviolet extinction between the denatured poly(A) and its component monomers (Leng and Felsenfeld, 1966; Brahms et al., 1966).

Broadening of Proton Resonance Peaks by Copper(II) Ions. Paramagnetic broadening effects due to Cu(II) were observed at base to Cu(II) ratios of 10^2–10^4; no shifts are produced in the presence of this large excess of ligand to paramagnetic metal ion. The broadening produced when increments of Cu(II) are added to adenosine in Me_2SO-d_6 (Figure 4) demonstrates that Cu(II) coordinates to base in preference to ribose whose peaks remain narrow while several base protons broaden. The maintenance of the sharp resonance peak of the 6-amino group of the adenine ring, while the other two base protons are broadened, clearly indicates that the amino group is not involved in binding, as previously demonstrated (Eichhorn et al., 1966). Due to differences in hydrogen bonding and polarity in Me_2SO and D_2O, ligand binding is not necessarily expected to be identical in these solvents. Solvent-dependent variations in base association and hydrogen bonding of the amino group to Me_2SO (Katz and Penman, 1966) could result in alteration of metal binding sites. The H-8 and H-2 protons of the adenine ring broaden simultaneously and no distinction can be made between binding near these positions on opposite sides of the base. Coordination of Cu(II) to nitrogen atoms neighboring both these positions would account for the simultaneous broadening of their proton resonances. These results
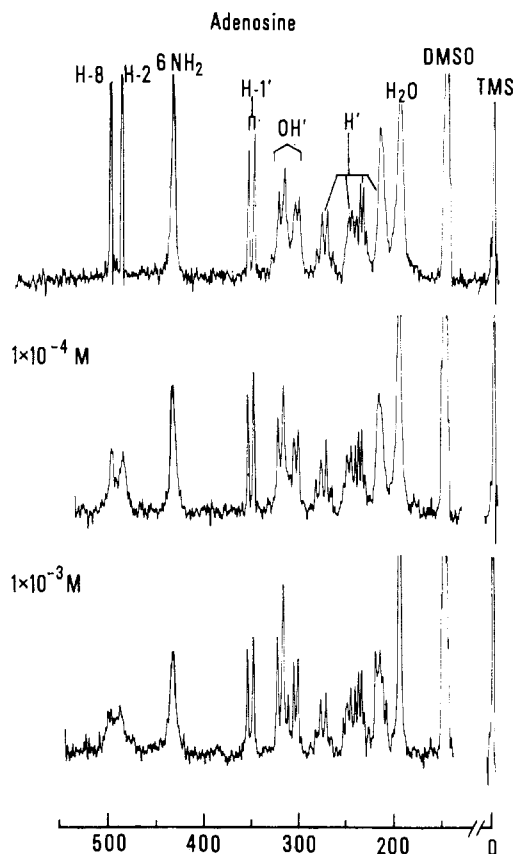
Adenosine

Tubercidin

FIGURE 4: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M adenosine in Me₂SO-d_6. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Protons on the base and ribose H-1′ are specifically labeled. The ribose hydroxyl protons are designated collectively as OH′ and the remaining ribose protons as H′. Abscissa is in cycles per second downfield from Me₄Si (TMS) internal standard.
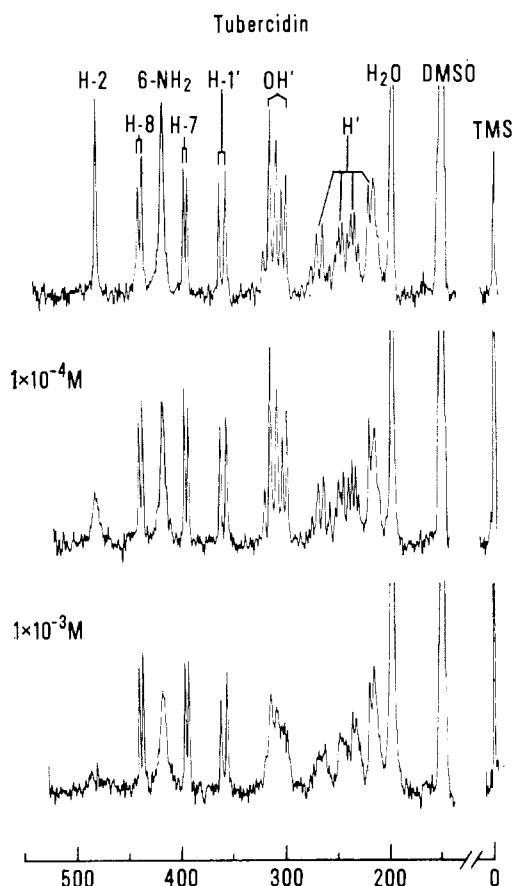
FIGURE 5: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M tubercidin in Me₂SO-d_6. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Ribose protons are designated collectively as in Figure 4. Abscissa is in cycles per second downfield from Me₄Si (TMS) internal standard.

might also be explained by binding of Cu(II) to one unique position on the base with the paramagnetic broadening effect being transmitted through both rings.

Is the Paramagnetic Effect of Copper(II) Transmitted from One Ring to the Other? Recent crystallographic studies on the copper(II) complex of guanine have shown that binding of copper at one position, N-9, on the purine brings about changes in the bond lengths elsewhere in the molecule (Carrabine and Sundaralingam, 1970). As a result the possibility must be considered that the copper induces electron changes throughout both rings of the purine molecule that could result in the broadening of proton resonances other than those close to the binding site. To determine whether the paramagnetic broadening effect could be transmitted to another ring of the purine molecule, the interaction of Cu(II) and tubercidin was examined. Tubercidin, which is 7-deazaadenosine, has the N-7 atom replaced by carbon, leaving no free nitrogen ligand on the five-membered ring to coordinate Cu(II), whereas two such nitrogen atoms remain on the six-membered ring. Cu(II) would induce broadening of the nearby H-2 resonance if coordinated to the N-1 or N-3 atoms, but broadening of H-7 or H-8 would have to be due to long-range transmittance of paramagnetic effects. On addition of Cu(II) to tubercidin (Figure 5) the H-2 resonance is completely broadened while the other base protons, 6-NH₂, H-8, and H-7, retain their narrow line widths. Broadening of the H-2 resonances in tubercidin occurs at a similar base:Cu(II) ratio as does base

proton broadening in adenosine. At higher concentration of Cu(II) the ribose hydroxyl protons of tubercidin also broaden.

This selective broadening clearly demonstrates the binding of Cu(II) in the H-2 region, to either the N-1 or N-3 or possibly both sites. Broadening of the tubercidin ribose hydroxyl protons demonstrates that Cu(II) also binds to these sites in this analog. Although Cu(II) produces drastic broadening of the H-2 resonance, the absence of such effects on the H-7 or H-8 resonances indicates that the broadening effect of the paramagnetic ion bound to the six-membered ring is not transmitted through this modified purine to the five-membered ring. Since tubercidin differs from adenosine only in the substitution of a carbon for a nitrogen at position 7, these results confirm that the broadening observed at H-8 in adenosine is due to Cu(II) coordinating directly to N-7.

Effect of the Position of Phosphate in AMP Isomers. In 5′-AMP addition of Cu(II) broadens the H-8 proton resonance in preference to the H-2 (Figures 6 and 7). This is in contrast to adenosine where Cu(II) broadens both base peaks simultaneously. The preferential broadening of the H-8 proton in the nucleotide indicates that the addition of phosphate at the 5′-ribose position favors coordination of the Cu(II) to the N-7 position of the base.

The proximity of the 5′-phosphate to the H-8 proton in 5′-AMP is demonstrated by the deshielding effect observed in the nuclear magnetic resonance spectrum. The possibility
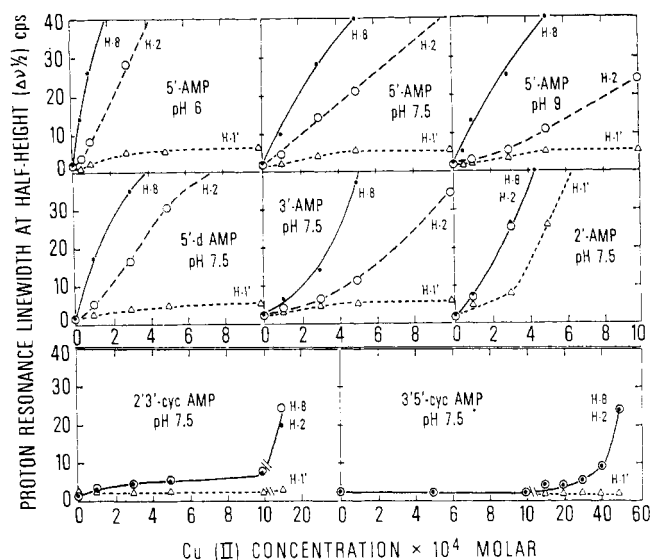
FIGURE 6: Effect of Cu(II) on (60 MHz) proton magnetic resonance line width at half-height of adenine nucleotides. Solutions are 0.1 M in D$_2$O.

exists that the preferential broadening of H-8 is due to the presence of Cu(II) ions on the phosphate being brought into juxtaposition to the H-8. This problem is resolved by examining alterations in Cu(II) binding to base when the nucleotide phosphate is varied among the 5', 2', 3', and the cyclic 2':3' positions.

Comparing the chemical shifts of 2'-AMP and 3'-AMP to 5'-AMP demonstrates that no deshielding of the adenine H-8 protons is produced by the 2'- or 3'-phosphates, indicating that in solution these phosphates do not approach this site on the base. It can be demonstrated by molecular models that in the case of 3'-AMP the phosphate is constrained to the side of the ribose opposite that of the base and cannot be manipulated into close proximity with the base no matter how the base–sugar or sugar–phosphate bonds are rotated. Cu(II) preferentially broadens the H-8 of 3'-AMP (Figure 8). The possibility of copper bound to phosphate approaching the H-8 is eliminated by the configuration of this molecule, confirming coordination to N-7 in 3'-AMP as in 5'-AMP.

Cu(II) broadens the base peaks of 2'-AMP in the same range as the other AMPs but H-8 and H-2 are broadened simultaneously (Figure 9). Both base peaks are also broadened simultaneously in cyclic 2':3'-AMP and cyclic 3':5'-AMP, but higher concentrations of Cu(II) are required with the cyclic nucleotides to produce broadening. It is apparent that the presence and location of the phosphate drastically alters the nature of copper binding to base.

In addition to the broadening of the base proton resonances, the ribose proton resonances are also selectively broadened. In 3'- and 5'-AMP the H-1' proton resonance demonstrates the least Cu(II) induced broadening of all ribose protons. The 2' and 3' proton resonances broaden simultaneously and along with the base protons of 3'-AMP, while in 5'-AMP the 2' is broadened slightly before the 3' but also along with the base. In contrast, the H-1' resonance of 2'-AMP broadens along with the base protons, while the 3', 4', and 5' protons exhibit little broadening effects. In the cyclic AMPs (both 2':3' and 3':5') and in adenosine in Me$_2$SO, base proton broadening occurs well in advance of that of any ribose peaks.

Cu(II) binding to 5'-AMP and 2'-AMP was also studied at
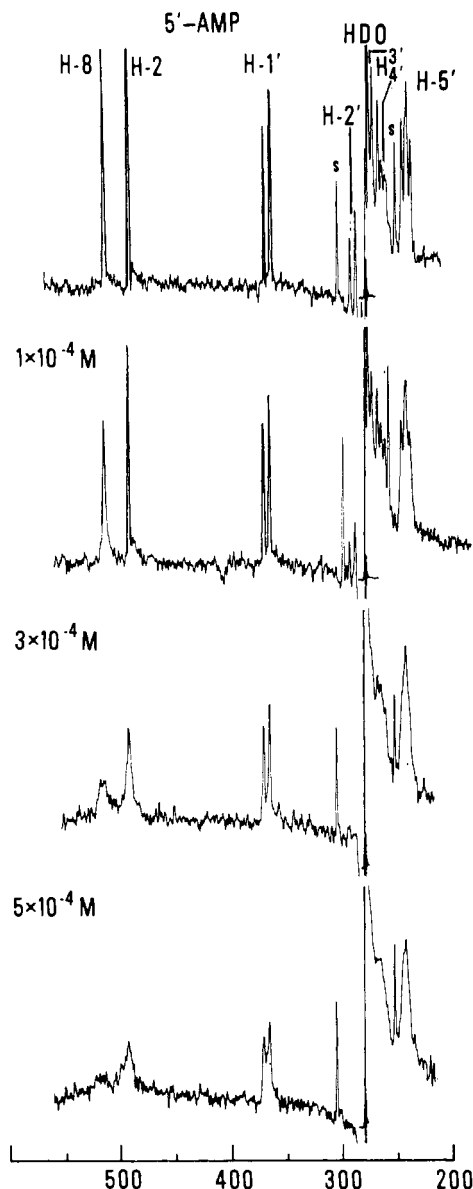

FIGURE 7: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M 5'-AMP (pH 7.5) in D$_2$O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands of water are labeled s. The two protons at the 5'-ribose position are labeled together as H-5'. All other base and ribose peaks are indicated. Abscissa is in cycles per second downfield from DSS as the internal standard.

lower than 0.1 M nucleotide concentrations, as follows: 0.07, 0.035, and 0.015 M. The contrasting effects noted for the base and ribose protons of 5'-AMP and 2'-AMP at 0.1 M also occurred at these lower concentrations. In particular, the simultaneous broadening of H-2 and H-8 by Cu(II) in 2'-AMP, as contrasted with the preferential broadening of H-8 in 5'-AMP, is observed throughout this concentration range.

Figure 6 demonstrates that equal quantities of the paramagnetic Cu(II) ion cause more selective broadening of H-8 compared to H-2 when the pH is increased. The decrease in total binding with increasing pH is apparently due to an increasing proportion of the Cu(II) ions involved in copper hydroxide formation until at pH 12 most of the copper enters into this complex and no base broadening is observed. The observed increase in phosphate deshielding occurred between
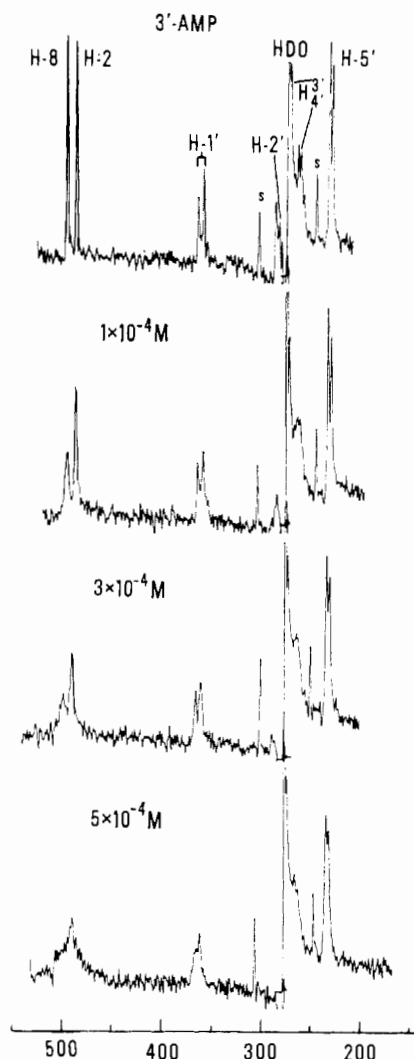
FIGURE 8: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M 3'-AMP (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Peaks are labeled as in Figure 7. Abscissa is in cycles per second downfield from DSS as the internal standard.

FIGURE 9: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.1 M 2'-AMP (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Peaks are labeled as in Figure 7. Abscissa is in cycles per second downfield from DSS as the internal standard.

pH 6 and 7.5 (Table I), but preferential broadening of H-8 by Cu(II) continues to increase until pH 9, demonstrating again that Cu(II) is coordinated directly to N-7 and indirectly influenced by the status of the phosphate.

Effect of Cu(II) on Proton Magnetic Resonance of Poly(A). Greater concentrations of Cu(II) were required to produce broadening of the nuclear magnetic resonance spectrum of poly(A) than of AMP (Figure 10). Since broadening of the nuclear magnetic resonance spectrum occurs when exchange of the paramagnetic ion between complexed and uncomplexed ligands is sufficiently rapid to produce enough complexed molecules at any instant to reflect the perturbing effect (Eaton and Phillips, 1965), the greater concentration of Cu(II) required to broaden the spectrum of poly(A) could be due to a restriction of the rate and freedom of exchange of Cu(II) ions by the more ordered structure of the polymer. Although greater concentrations were required in the polymer, H-8 was preferentially broadened, demonstrating that copper binds to the N-7 position in poly(A) as in 5'- and 3'-AMP. NaCl at a final concentration of 0.1, 0.2, and 0.4 M was added to poly(A) containing Cu(II) to see if a competing cation to dis-
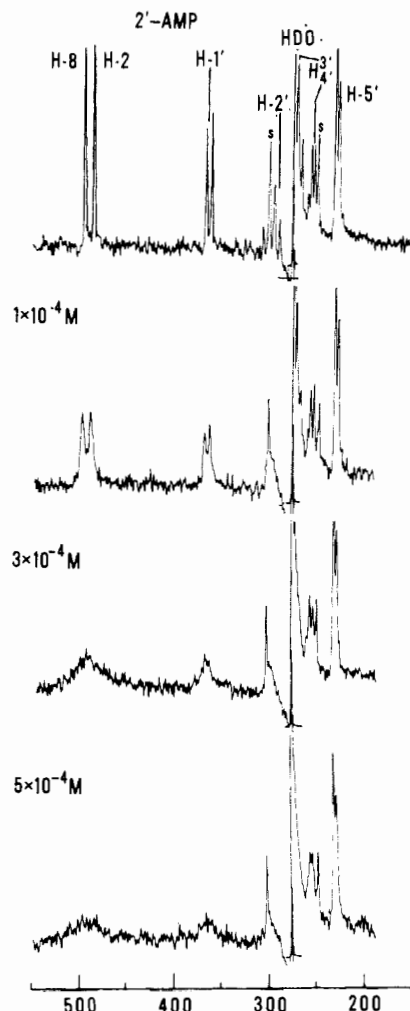
place Cu(II) from phosphate would alter the preferential base broadening observed in the nuclear magnetic resonance spectrum. In the presence of NaCl concentrations equal to or fourfold greater than polymer phosphate, the broadening effects of Cu(II) on poly(A) were the same as in the absence of salt, supporting the direct binding of Cu(II) to base in poly(A).

Discussion

Nuclear magnetic resonance has been used in these experiments to demonstrate that coordination of copper by adenine nucleosides and nucleotides involves direct binding to ligands on the purine ring and that coordination can occur with ligands on either the six-membered or five-membered ring. The simultaneous broadening of H-2 and H-8 would suggest that adenosine binds copper equally at sites on both the six-membered and five-membered rings. In tubercidin binding occurs to the only available nitrogen atoms which are confined to the six-membered ring. The copper-induced broadening of the nuclear magnetic resonance spectrum of tubercidin is observed for the proton resonance of the six-membered ring alone, and is not transmitted through the substituted purine system to affect the resonances of the five-membered

ring. Direct observation of the amino group protons at the 6 position in adenosine and tubercidin demonstrates that Cu(II) does not react at this site. In 2'-AMP, cyclic 2':3'-AMP, and cyclic 3':5'-AMP, as in adenosine, Cu(II) appears to bind sites on either the six- or the five-membered rings equally. In 3'- and 5'-AMP as well as 5'-dAMP, Cu(II) may still bind to either ring but in these isomers binding is preferentially to the N-7 ligand of the five-membered ring. In poly(A), as in 3'- and 5'-AMP, N-7 is the preferred ligand for Cu(II).

Effect of Phosphate-Bound Cu(II) on the Proton Magnetic Resonance of Bases. These experiments involve the direct observation of metal binding to the base and sugar moieties. Binding of metal to phosphate was not examined in this study but has previously been studied by nuclear magnetic resonance. Using ^{31}P nuclear magnetic resonance, Cohn and Hughes (1962) demonstrated the binding of Cu(II) and other divalent metal ions to the phosphates of AMP, ADP, and ATP. Shulman *et al.* (1965) also observed binding of paramagnetic metal ions to phosphate of ATP, and Sternlicht *et al.* (1968) to phosphate of AMP. This same technique was used by Eichhorn *et al.* (1966) to demonstrate binding of copper to the phosphates of dAMP and dTMP. Although not directly observed, it is probable that copper binds the phosphate of all nucleotides and polynucleotides studied.

The conformation of the purine and pyrimidine bases with respect to the furanose ring of the ribose has been extensively studied. The anti conformation was designated by Donohue and Trueblood (1960) as the rotation about the glycosidic bond which extends the 2-carbon of the base in the opposite direction of the 5'-ribose position. X-Ray diffraction studies have shown most of the β-pyrimidine and β-purine nucleosides and nucleotides to be anti (Sundaralingam, 1969). Using nuclear magnetic resonance, Schweizer *et al.* (1968) demonstrated that the preferred conformation of the nucleoside 5'-phosphates in solution is also anti. In this conformation the 8-purine position and the 6-pyrimidine position extend over the plane of the ribose and are the closest base atoms to the 5'-phosphate. The marked deshielding effects produced by the 5'-phosphate on the 8-purine and 6-pyrimidine position indicate the proximity of the phosphate to these base atoms. The possibility has been considered that the preferential base proton broadening of nucleotides, induced by paramagnetic ions, is due to the effects of a metal bound to phosphate, in proximity of the base. This type of broadening effect transmitted from a paramagnetic manganese(II) ion bound to a 5'-phosphate to its nearest base has been used (Chan and Nelson, 1969) in an nuclear magnetic resonance study of ApA as one of the methods to identify the 5' base and distinguish it from the nucleoside esterified at the 3'-ribose in this dinucleoside monophosphate.

The earlier work from this laboratory clearly demonstrated that base proton broadening was produced by paramagnetic metal ions, even in the absence of phosphate (Eichhorn *et al.*, 1966). Cu(II) caused base proton broadening when added to the nucleosides adenosine, cytidine, and guanosine, but not thymidine. Conversely, no base broadening was observed in the nucleotide thymidylic acid where phosphate binding was definitively demonstrated. Base broadening was also demonstrated in the absence of phosphate in the current study, where the nucleosides adenosine and tubercidin were both found to bind copper. These nuclear magnetic resonance studies with the nucleosides demonstrate that base proton broadening is due to direct binding of Cu(II) ions to the base.

Cu(II) binding studies to the phosphate isomers of AMP demonstrate that even the preferential broadening of the H-8
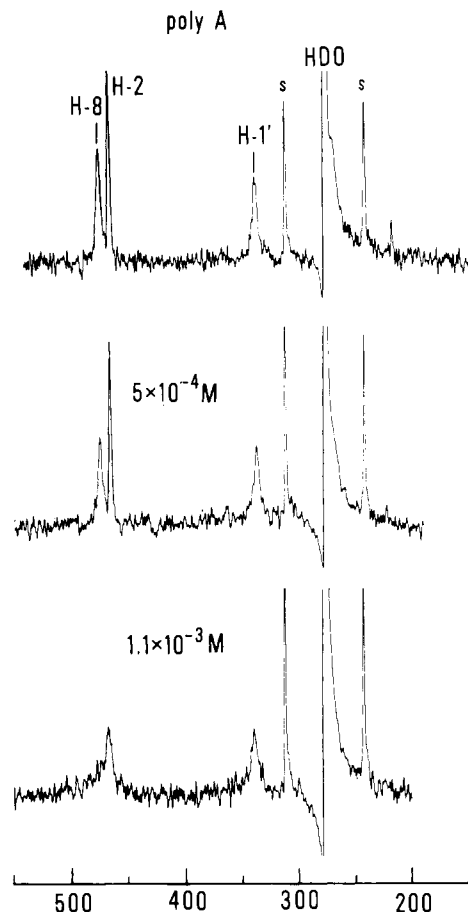

FIGURE 10: Effect of Cu(II) on proton magnetic resonance spectra (60 MHz) of 0.038 M poly(A) (pH 7.5) in D_2O. The top spectrum is the metal-free solution; the Cu(II) concentration is indicated for the others. Spinning side bands of water are labeled s. Abscissa is in cycles per second downfield from DSS as the internal standard.

proton in 5'-AMP is not due to the proximity of H-8 and copper bound to phosphate. In 3'-AMP the phosphate is constrained to one side of the ribose, the adenine is on the other side, and no matter how they rotate they are unable to approach each other. This conformation prevents copper bound to the 3'-phosphate from approaching the base, but the nuclear magnetic resonance demonstrates the same selective broadening of H-8 as in 5'-AMP, indicating that binding is similar, directly to the base and near H-8 in both 5'- and 3'-AMP. This selective base broadening of the H-8 resonance in 3'- and 5'-AMP demonstrates the preferential coordination of copper to the N-7 ligand of these isomers. In contrast, adenosine, cyclic 2':3'-, and cyclic 3':5'-AMP show no preferential binding site and binding probably occurs near both H-8 and H-2. It is apparent then that in the absence of phosphate, copper binds directly to the nucleoside base; however, the presence and position of phosphate influence the site where copper binds to base. These effects demonstrate that base proton resonance broadening is not due to Cu(II) ions bound to phosphate and simply held in proximity to the base of the same nucleotide.[1]

---

[1] While these studies demonstrate conclusively that broadening of H-8 in these compounds is due to binding of copper(II) ions at N-7, they do not necessarily reflect on the significance of H-8 broadening of the same substances by manganese(II) or other metal ions. Manganese(II) has a relatively stronger tendency to bind phosphate rather than base, when compared to copper (Eichhorn and Shin, 1968).

Multiple Proton Resonance Broadening and Multiple Binding Sites. The nuclear magnetic resonance spectrum of adenine in nucleosides and nucleotides is composed of several base proton resonance peaks. Paramagnetic metal ion induced broadening of these peaks occurs in several fashions. In the first case, illustrated by tubercidin, the H-2 peak is broadened while the others, H-7 and H-8, remain essentially intact. In contrast, in the case of adenosine, both H-8 and H-2 broaden simultaneously. An intermediate case is produced in 5′-AMP where H-8 and H-2 both broaden, but H-8 is preferentially broadened. The degree of broadening of a proton's characteristic line width is proportional to the distance between the observed proton and the paramagnetic ion as well as the concentrations of ions at that point (Eaton and Phillips, 1965). It is necessary to determine, then, whether broadening of multiple base peaks reflects the distance of the protons corresponding to these peaks from a paramagnetic metal ion bound to one unique site on the base or whether multiple binding sites exist with metal ions near all broadened peaks.

Sternlicht *et al.* (1965) measured the paramagnetic metal-induced broadening of the H-8, H-2, and H-1′ peaks of ATP. Assuming 100% binding to a unique site, the distance was calculated from metal to these protons and binding was localized somewhere between the N-7 and 6-amino group. However, the distance calculated to the H-2 proton did not fit this location. They speculated on the possibility of two binding sites, N-7 and N-1. This implies the existence of two species of metal nucleotide complexes, one with metal bound to N-7 and another with metal bound to N-1. Binding at both positions with an equilibrium favoring the species with metal ions coordinated to the N-7 atom satisfied all their calculations, but the unique binding site was favored since the equilibrium was apparently not altered as a function of temperature variation or sensitive to shifting from pH 7 to 4.

The demonstration, in this study, of the variable fashion in which Cu(II) induces base proton resonance broadening in adenosine, tubercidin, and AMP and the alterations produced by pH variation favor a multiple base binding site explanation. In tubercidin, the only available nitrogens to act as ligands for Cu(II) are the N-1 and N-3 of the six-membered ring. The nuclear magnetic resonance spectrum of this complex demonstrates that copper coordinated to these ligands on the six-membered ring broadens the nearby H-2 proton resonance while having a negligible effect on the H-7 and H-8 protons on the opposite side of the purine ring. As in tubercidin, broadening of the H-2 resonance of adenosine can then be accounted for by coordination of Cu(II) to the N-1 and/or N-3 ligands of the six-membered ring. In contrast to tubercidin, when a nitrogen is present at the 7 position as in adenosine, Cu(II) can then also bind to this ligand on the five-membered ring and produce broadening of the nearby H-8 resonance. The simultaneous broadening of the H-2 and H-8 resonances of adenosine, 2′-AMP, and cyclic 2′:3′-AMP as well as cyclic 3′:5′-AMP indicate that multiple base binding sites exist and that in these isomers the concentration of complexes with copper bound to sites near H-2 approximately equals the concentratihn of species with Cu(II) bound to a site near H-8. Because of these results with tubercidin and some of the phosphate isomers of AMP, it is apparent that the preferential broadening of H-8 compared to H-2 in 3′- and 5′-AMP is also due to multiple-base binding sites with an equilibrium favoring the species with Cu(II) bound to the N-7 atom. Raising the pH shifts the equilibrium even further to favor coordination of Cu(II) to the N-7 ligand.

Simpson (1964) has previously postulated an equilibrium between methylmercury and two binding sites on both adenosine and inosine. Using proton and ^{13}C nuclear magnetic resonance, Read and Goldstein (1965) studied the protonation of the purine ring. Their results were consistent with protonation at all the basic sites with an equilibrium favoring N-1 > N-7 and N-9 > N-3. Yet another example of the similar order of reactivity of the adenine ring nitrogens was the demonstration by Singer and Fraenkel-Conrat (1969) that alkylation of 5′-AMP and poly(A) with dimethyl sulfate produces at least three isomers with the adenine methylated at either the N-1, N-3, or N-7 atoms.

The electron density calculations (Jordan and Pullman, 1968; Pullman, 1968, 1969), for adenine, indicate that the N-1, N-3, and N-7 ring nitrogens have a similar negative net atomic charge density, with N-3 being slightly less negative than N-7 and N-1, and therefore all are expected to be equally effective as bonding sites for Cu(II). The N-9 atom is eliminated as a possible site by formation of the glycosidic bond to ribose. Formation of the glycosidic linkage to ribose or esterification of phosphate at the 5′-ribose position produces no major charge redistribution among the adenine ring nitrogens (Boyd, 1969), leaving N-1, N-3, and N-7 as potential ligands. The amino group of adenine has a comparatively small negative charge. This nitrogen donates its lone pair of electrons to the π system of the purine ring and is not available to act as a donor for Cu(II) (Schneider *et al.*, 1964). Thus, when the amino group protons were observed in adenosine and tubercidin they were found not to be affected by Cu(II).

Preferential Coordination at One Site. Although it is apparent that Cu(II) ions can bind to various sites on adenosine or AMP, the proton magnetic resonance results indicate a definite preference for N-7 in 3′-AMP and 5′-AMP. A factor which could produce preferential coordination of a metal ion to one of several available sites is the geometry of the molecule. The complex associations which occur between the nucleic acid components result in numerous conformation changes which could either sterically hinder the accessibility of a potential ligand to a metal ion or bring distant ligands together to favor chelation.

The mode of intermolecular associations of nucleic acid components has been extensively studied. Katz and Penman (1966) and Shoup *et al.* (1966) demonstrated base pairing by hydrogen bond formation in nonpolar solvents. The extensive investigations by Ts'o and collaboraters (1969) demonstrate that in aqueous solution the principal method of association for nucleosides and nucleotides is by base stacking. The purine rings of 5′-AMP were shown to associate in these stacks in a face to face orientation, with the ribose phosphates of neighboring nucleotides extending on opposite sides of the stack. The 3′ isomer of AMP as well as 5′-dAMP also stack face to face. In these isomers the H-2 resonance showed greater shielding on stacking than the H-8, indicating that the H-2 spends more time being shielded by its stack neighbor than the H-8. Concomitantly, the six-membered rings containing the N-1 and N-3 atoms are more involved in the stack than the N-7 atom on the five-membered rings. This stacking interaction leaves the N-7 ligand more accessible than the N-1 or N-3 and so shifts the equilibrium to favor coordination of metal ions to N-7.

The molecular geometry of these associations and complexes was examined with Corey–Pauling–Koltun atomic models. When 5′-AMP associates, maintaining the preferred anti orientation of the adenine base to the ribose, and then is stacked in the face to face manner demonstrated by Ts'o *et al.* (1969), the N-7 ligand is the most exposed of the ring

nitrogens. It is also apparent that a metal bound to the phosphate of one nucleotide is in the proper location to coordinate to the N-7 of its stack neighbor. In fact, the conformation of this molecule allows for the easy formation of a symmetrical binuclear chelate ring composed of two metal ions and two 5'-AMPs in which each metal is bound to the phosphate of one AMP and the base of the other. This proposed arrangement is shown schematically in Figure 11.

The linkage of phosphate to N-7 *via* metal is in agreement with the demonstration by Sternlicht *et al.* (1968) that metal ions bind simultaneously to phosphate and base and that the length of time the metal remains bound to phosphate moiety is the same as the length of time metal is bound to the ring before chemical exchange occurs. These observations led them to propose a complex of two adenine nucleotides with a metal linking the phosphate of one molecule to the N-7 of another. It is now apparent that the preferred stacked conformation of 5'-AMP allows for both phosphates of neighboring nucleotides to be linked through metal to N-7 of the neighboring base. Such a complex differs from the 2-metal-2-nucleotide complex proposed by Agarwal and Feldman (1968) in which two uranyl ions are coordinated between ribose hydroxyls and phosphates of neighboring AMP molecules but not involved in base binding. In the structure suggested by the current study the metal ions linking phosphates to N-7's are held in such a manner that the paramagnetic ion is close to the H-2' and H-3', while the H-1' atom is held away from the metal on the opposite side of the ribose ring.

During the nuclear magnetic resonance observation of copper binding to 5'-AMP, the 2'- and 3'-ribose proton resonances were completely broadened, while the H-1' was only minimally affected. These copper-induced alterations of the ribose peaks in 5'-AMP fit well with the proposed conformation of two metals connecting the N-7 position to the phosphates of two stacked 5'-AMP molecules.

The proposed 2-metal-2-AMP complex linking each phosphate to N-7 of the neighboring base through copper forms just as easily with models of 3'-AMP as with 5'-AMP. In the 3'-isomer N-7 is again the preferred site for base binding since it is less hindered by stacking than the nitrogens on the six-membered ring. In addition to being more accessible to metal ions the N-7 position is also the most easily approached by metal bound to phosphate of stack neighbor. In this complex as in 5'-AMP the metal approaches the 2' and 3' hydrogens and is on the opposite side of the ribose from the H-1'. The nuclear magnetic resonance study of Cu(II) binding to 3'-AMP indicates that the preferential H-8 base proton broadening is accompanied by broadening of the H-2' and H-3' resonances while minimal effects occur at the H-1' resonance. As with the 5'-AMP the copper-induced broadening of the 2'- and 3'-ribose proton peaks of 3'-AMP support the 2-metal-2-AMP complex in which each metal ion linking phosphate to N-7 is close to H-2' and H-3'.

The 2-metal-2-AMP complex conforms with the simultaneous binding of metal to phosphate and base. It explains the preferential base binding to N-7 in 3'- and 5'-AMP and also the differential broadening effects observed for the ribose proton resonances. In forming these complexes all other preferential orientations of the molecule are maintained. The bases remain anti to the ribose, the six-membered rings are more stacked than the five-membered rings, and the five-membered rings of 3'-AMP are slightly more stacked than the five-membered ring of 5'-AMP. In addition to providing the geometry for the simultaneous binding of metal ions to phosphate and neighboring N-7, the stacking interactions
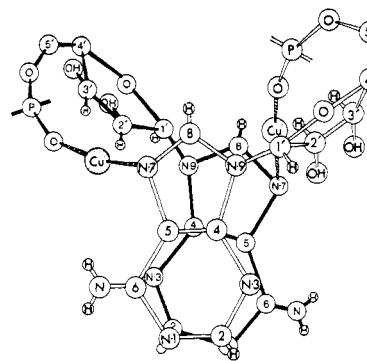

FIGURE 11: Schematic representation of binuclear chelate ring composed of two Cu(II) ions and two 5'-AMP molecules. The bonds of the AMP molecule in the foreground are white, and the atoms are numbered in boldface characters, while the bonds of the background molecule are black and the atoms are in fine characters. The bonds linking phosphate through copper(II) ions to N-7 are shaded. Cu, H, N, O, and P atoms are specifically indicated. The carbon atoms of the purine and ribose rings are designated only by position number. Hydrogens at the 4'- and 5'-ribose carbons and two of the oxygens at the phosphate atoms are omitted from the diagram. Structure proposed on basis of present experiments.

of the nucleotide bases also lend stabilization to this complex.

Moving the phosphate from the 5'- or 3'-ribose position to the 2' position produces interesting alterations in base stacking as well as Cu(II) binding. Schweizer *et al.* (1968) demonstrated that in 2'-AMP ionization of the phosphate produced a greater shift in the nuclear magnetic resonance spectrum of the H-2 resonance than of the H-8. This specific deshielding of the H-2 in 2'-AMP indicates the proximity of the phosphate to the six-membered ring of the adenine base. They found the presence of the 2'-phosphate reduced the stacking tendency of the six-membered ring by 30% and the five-membered ring by 50%, when compared to the association of 5'-AMP. This marked destacking tendency induced by the 2'-phosphate would remove steric hindrances and make all potential base ligands more equally accessible to metal ions. Also, this location of the phosphate is no longer favorable for metal bound to it to approach and bind the N-7 position.

The 2-metal-2-AMP complex linking phosphate to N-7, which is easily constructed for 3'- and 5'-AMP, can also be constructed for 2'-AMP. However, the complex with 2'-AMP forces the five-membered rings to overlap and the six-membered ring to diverge. This is exactly the opposite of the observed residual stacking tendency of 2'-AMP. It is apparent from the models that the 2-metal-2-AMP complex cannot be constructed for 2'-AMP while simultaneously preserving the stacking tendency of the bases. This distortion of the stacking in 2'-AMP minimizes the contribution of stacking interactions to the stability of the binuclear complex. In contrast, the proposed stacking geometry of 3'-AMP and 5'-AMP is identical with that which occurs in the 2-metal-2-AMP complex, allowing this binuclear chelate to be maximally stabilized by the stacking interactions of the bases.

Because of the alteration in stacking interaction, it appears that 2'-AMP is free to coordinate Cu(II) to either the N-1, N-7, or N-3 ligands and this accounts for the simultaneous broadening in the nuclear magnetic resonance spectrum of the H-8 and H-2 peaks. Cu(II)-induced broadening of the ribose proton resonances in 2'-AMP also occurs in a different fashion from the 3'- and 5'-nucleotides. In 2'-AMP the H-1' and
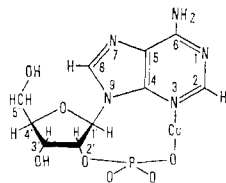
FIGURE 12: Structural formula of Cu(II) complex with 2 -AMP, proposed on basis of present experiments.

H-2' are broadened along with the base protons while the H-3' proton resonance retains its characteristic line width. The proximity of the phosphate to the six-membered ring and the broadening of H-1' and H-2' suggest that a Cu(II) ion coordinated to the N-3 atom could also be bound to the phosphate of the same molecule (Figure 12).

The purine rings do not stack in Me_2SO (Chan et al., 1964) and no stacking interaction was observed for adenosine in this solvent (Katz and Penman, 1966). This lack of self-association makes ligands on both the six-membered and five-membered rings equally accessible to metal ions. The simultaneous broadening of the H-8 and H-2 adenosine resonances, induced by Cu(II), indicate that as in 2'-AMP binding does occur with equal probability at the N-1 and N-3 atoms, on the one hand, and the N-7 atoms, on the other.

The broadening of base peaks in adenosine occurs at similar concentrations of Cu(II) as in 2'-, 3'-, and 5'-AMP. In the phosphate isomers the ribose protons were preferentially broadened in a manner which reflected the stacking geometry of the 2-metal–2-AMP complex or in the case of 2'-AMP the linking of 2'-phosphate with N-3 via metal. In contrast, adenosine, which has no phosphate, does not stack in Me_2SO and is not expected to form any type of self-associated complex, retains the sharp line width of all its ribose proton resonances.

Relationship of Molecular Association and Availability of Electron Donor Site for Coordination. The H-8 and H-2 resonances of cyclic 2':3'-AMP are equally broadened by copper, but at a slightly higher concentration than the resonances of other adenine nucleotides. The two base proton resonances of cyclic 3':5'-AMP which are overlapped in the nuclear magnetic resonance spectrum also appear to broaden simultaneously but require an even higher concentration of copper ions. The base proton resonances of the cyclic nucleotides demonstrated upfield shifts when compared to those of 2'-, 3'-, or 5'-AMP. These shifts were attributed to the removal of the phosphate deshielding effect, the subsequent increase in base stacking, and the alteration of ribose conformation due to formation of the cyclic phosphate. Due to these alterations in stacking and in the relation between adenine and the ribose, all of the potential base ligands apparently become equally less accessible to metal ions. This accounts for the simultaneous broadening of the H-8 and H-2 resonances in the cyclic compounds and also for the slightly higher concentration of Cu(II) required to induce this broadening.

Copper preferentially broadens the H-8 resonance of poly(A) as it does in 3'-AMP and 5'-AMP, but requires a concentration of copper ten times greater in the polymer than in the monomer. The preference of copper for N-7 in poly(A) indicates that the N-7 position of each base spends less time shielded by neighboring bases than do other potential base ligands. The requirement for higher concentrations of copper to produce broadening is a manifestation of the great in-

crease in base stacking when the monomeric units are joined in the polymer.

Acknowledgments

References

Agarwal, R. P., and Feldman, I. (1968), *J. Amer. Chem. Soc. 90*, 6635.

Boyd, D. B. (1969), *Theor. Chim. Acta 14*, 402.

Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.

Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Amer. Chem. Soc. 89*, 3612.

Bullock, F. J., and Jardetzky, O. (1964), *J. Org. Chem. 29*, 1988.

Carrabine, J. A., and Sundaralingam, M. (1970), *J. Amer. Chem. Soc. 92*, 369.

Chan, S. I., and Nelson, J. H. (1969), *J. Amer. Chem. Soc. 91*, 168.

Chan, S. I., Schweizer, M. P., Ts'o, P. O. P., and Helmkamp, G. K. (1964), *J. Amer. Chem. Soc. 86*, 4182.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237*, 176.

Donohue, J., and Trueblood, K. N. (1960), *J. Mol. Biol. 2*, 363.

Eaton, D. R., and Phillips, W. D. (1965), *Advan. Magn. Resonance 1*, 103.

Eichhorn, G. L., Clark, P., and Becker, E. D. (1966), *Biochemistry 5*, 245.

Eichhorn, G. L., and Shin, Y. A. (1968), *J. Amer. Chem. Soc. 90*, 7323.

Feldman, I., and Agarwal, R. P. (1968), *J. Amer. Chem. Soc. 90*, 7329.

Gatlin, L., and Davis, J. C., Jr. (1962), *J. Amer. Chem. Soc. 84*, 4464.

Holcomb, D. N., and Tinoco, I., Jr. (1965), *Biopolymers 3*, 121.

Jardetzky, C. D. (1960), *J. Amer. Chem. Soc. 82*, 229.

Jardetzky, C. D., and Jardetzky, O. (1960), *J. Amer. Chem. Soc. 82*, 222.

Jardetzky, O. (1964), *Biopolymers, Symp. No. 1*, 501.

Jordan, F., and Pullman, B. (1968), *Theor. Chim. Acta 9*, 242.

Katz, L., and Penman, S. (1966), *J. Mol. Biol. 15*, 220.

Kokko, J. P., Goldstein, J. H., and Mandell, L. (1961), *J. Amer. Chem. Soc. 83*, 2909.

Leng, M., and Felsenfeld, G. (1966), *J. Mol. Biol. 15*, 455.

Li, N. C., Scruggs, R. L., and Becker, E. D. (1962), *J. Amer. Chem. Soc. 84*, 4650.

McDonald, C. C., Phillips, W. D., and Penman, S. (1964), *Science 144*, 1234.

McTague, J. P., Ross, V., and Gibbs, J. H. (1964), *Biopolymers 2*, 163.

Prestegard, J. H., and Chan, S. I. (1969), *J. Amer. Chem. Soc. 91*, 2843.

Pullman, A. (1968), *Int. J. Quantum Chem. 2S*, 187.

Pullman, A. (1969), *Ann. N. Y. Acad. Sci. 158*, 65.

Read, J. M., Jr., and Goldstein, J. H. (1965), *J. Amer. Chem. Soc. 87*, 3440.

Schneider, P. W., Brintzinger, H., and Erlenmeyer, H. (1964), *Helv. Chim. Acta 47*, 992.

Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90*, 1042.

Shoup, R. R., Miles, H. T., and Becker, E. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 194.

Shulman, R. G., Sternlicht, H., and Wyluda, B. J. (1965), *J. Chem. Phys. 43*, 3116.

Simpson, R. B. (1964), *J. Amer. Chem. Soc. 86*, 2059.

Singer, B., and Fraenkel-Conrat, H. (1969), *Biochemistry 8*, 3261.

Sternlicht, H., Jones, D. E., and Kustin, K. (1968), *J. Amer.*

Chem. Soc. 90, 7110.

Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965), *J. Chem. Phys. 43*, 3133.

Sundaralingam, M. (1969), *Biopolymers 7*, 821.

Ts'o, P. O. P., Schweizer, M. P., and Hollis, D. P. (1969), *Ann. N. Y. Acad. Sci. 158*, 256.

Van Holde, K. E., Brahms, J., and Michelson, A. M. (1965), *J. Mol. Biol. 12*, 726.

Interaction of Metal Ions with Polynucleotides and Related Compounds. XV. Nuclear Magnetic Resonance Studies of the Binding of Copper(II) to Nucleotides and Polynucleotides[*]

Nathan A. Berger and Gunther L. Eichhorn

ABSTRACT: Proton magnetic resonance studies comparing the broadening effect of Cu(II) on the peaks of nucleotides and polyribonucleotides reveal that generally binding to monomer and polymer occurs preferentially at the same site. In poly(C), as in CMP, broadening of the H-5 resonance, but not the NH_2 proton peaks, indicates binding to N-3. Poly(U) and UMP, as well as uridine, also appear to bind Cu(II) at N-3; but the metal also exhibits an affinity for the hydroxyl groups of the ribose in uridine. Cu(II) broadens the H-2 and H-8 peaks of IMP simultaneously, while broadening H-8 in preference to H-2 in poly I; the former presumably involves chelation

to N-7 and O-6, whereas in poly(I) the O-6 atom is occupied in H bonding, thus restricting binding to N-7. The unwinding of the poly(A)·poly(U) double helix by heating can be followed by proton magnetic resonance, since the broadened peaks of the double helix are sharpened on unwinding.

In the presence of Cu(II) the broadening effects of the paramagnetic ion on the separate polymer strands are noted as soon as unwinding is complete, providing further evidence to support the possibility of bridging of polynucleotide strands by metal ions.

Metal ions have profound effects on the structure and function of nucleic acids and mono- and polynucleotides. They alter the coding specificity of polynucleotides acting as templates for protein synthesis (Szer and Ochoa, 1964), and are required for stabilizing the structure of tRNA (Fresco *et al.*, 1966). Their role in unwinding and rewinding of the double helix (Eichhorn and Clark, 1965; Shin and Eichhorn, 1968) and in degradation of polynucleotides (Butzow and Eichhorn, 1965) has been demonstrated. It is therefore of interest to determine the loci of interaction of metal ions with these substances.

Previously, nuclear magnetic resonance has revealed some of the binding sites of metal ions on the mononucleotides (Cohn and Hughes, 1962; Sternlicht *et al.*, 1965; Eichhorn *et al.*, 1966). The nuclear magnetic resonance technique of water proton relaxation enhancement has been used to study the binding of metal ions to DNA (Eisinger *et al.*, 1962) and to polyribonucleotides and tRNA (Cohn *et al.*, 1969). To determine if metal ions bind to the heterocyclic bases of the polynucleotides and to identify the binding sites, we investi-

gated the broadening effects of Cu(II) on the base proton resonances of the polymers as in the monomers.

This paper correlates the binding sites of Cu(II) on polynucleotides with those on the mononucleotides. Frequently the binding sites are the same in monomer and polymer; an interesting exception is the difference in binding observed for IMP and poly(I).

Because of the fact that proton magnetic resonance data can pinpoint reaction sites on the nucleotide bases, this technique is used to study the unwinding of the base-paired complex poly(A)·poly(U) in the presence of Cu(II) ions.

Methods

Uridine and 5'-UMP were from Sigma Chemical Co., 5'-IMP was from P-L Biochemicals, and homopolyribonucleotides were from Miles Laboratories. Nucleosides were dissolved directly in Me_2SO-d_6 and nucleotides in D_2O. Polynucleotides were dissolved in D_2O by gentle rotary shaking at 4°. Aqueous solutions were adjusted to pH 7.5 and molarity was determined as in the preceding paper. The base-paired complex poly(A)·poly(U) was prepared both by dissolving each homopolymer separately and then mixing or by dissolving them together. Similar spectra were obtained for complexes prepared by either method.

Nuclear magnetic resonance spectra were obtained with the Varian spectrometer, A60D, using the same calibrations and techniques indicated in the pre-