Base Stacking. An important question regarding dihydrouridine is whether the saturated base can participate in parallel stacking of the bases. In fact, the base stacking configuration observed here is in many respects similar to that observed in the known planar pyrimidine systems[18] with the carbonyl oxygen atoms O(2) of the two molecules lying either over or close to the rings of adjacent bases. Figure 6 shows some intermolecular contacts between atoms of stacked bases in dihydrouridine and related saturated bases in views normal and parallel to the bases.

(18) C. E. Bugg, J. Thomas, M. Sundaralingam, and S. T. Rao, *Biopolymers*, **10**, 175 (1971).

Acknowledgments. It is a pleasure to thank Dr. Douglas Rohrer for preparing the crystals. We gratefully acknowledge the United States National Institutes of Health for generous support of this research through Grant No. GM 17378. Further thanks are due to the University Computing Center for use of their facilities and supported by grants from the Wisconsin Alumni Research Foundation with funds administered by the University Research Committee.

Interaction of Metal Ions with Polynucleotides and Related Compounds. XVIII. The Multiplicity of Reactions of Copper(II) with Inosine and Its Derivatives

N. A. Berger and G. L. Eichhorn

Contribution from the Laboratory of Molecular Aging, Gerontology Research Center, National Institutes of Health, National Institute of Child Health and Human Development, Baltimore City Hospitals, Baltimore, Maryland 21224. *Received March 18, 1971*

Abstract: Inosine displays remarkable versatility in its coordination to copper ions under various conditions. Proton magnetic resonance studies in D_2O indicate that copper(II) binds to both the N-7 and N-1, O-6 areas of inosine at intermediate pH, preferring N-7 at pH 3, but N-1 at pH 7 and above. The N-1 (O-6) site of inosine is also favored by heating to 80°. Blocking with methyl groups limits Cu(II) binding to N-7 in 1-methylinosine and N-1 in 7-methylinosine. In dimethyl sulfoxide Cu(II) preferentially binds to the hydroxyl groups of the ribose portion of inosine and its 1-methyl and 7-methyl derivatives, as indicated by pmr broadening and continuous variation studies in the visible, demonstrating 2:1 copper–nucleoside binding. The visible spectra are compatible with the occurrence of copper–copper bonds. These characteristics of the spectra of Cu(II)–ribose complexes are absent in deoxyinosine and triacetylinosine.

Copper has profound effects on the structures of polynucleotides and DNA. Under appropriate conditions Cu(II) is capable of bringing about both the unwinding and rewinding of the double helix.[1-4] Unwinding is aided by coordination of Cu(II) ions to electron-donor sites on the purine and pyrimidine bases resulting in withdrawal of electrons and weakening of interstrand hydrogen bonds.[1] Rewinding and renaturation require that some of the bases on opposite strands be held together, or in register, until conditions favor the return of the double helix.[2] The bases may be held in register by copper ions coordinated between bases of the opposing strands.

In earlier studies in which nmr was used to determine the nature of the Cu(II) binding sites in polynucleotides and their constituents several of these coordination sites were identified.[5-8] When paramagnetic copper ions bind to a ligand, the protons in the vicinity of the paramagnetic ion are rapidly relaxed resulting in the broadening of their characteristic proton magnetic resonance peak.[9,10] Using this technique, Cu(II) ions were demonstrated to bind to multiple sites on the adenine base with preference for a given site influenced by molecular conformation and association, which in the AMP isomers varies with the position of the phosphate on the ribose.[7] Cu(II)-induced broadening of the H-8 and H-2 resonances of 5′-IMP was observed and attributed to the possibility of a chelate involving N-7 and O-6 as proposed earlier[11,12] or also coordination at N-1.[8] Both the H-8 and H-2 of poly(I) were also broadened by Cu(II), although the broadening of the H-8 in advance of H-2 indicates preferential coordination at N-7 on the polymer.[8] The present study was undertaken to delineate more clearly under what conditions the various Cu(II) binding sites on inosine (see formula) are favored. The nucleosides are used to eliminate the complication of metal ion bound to

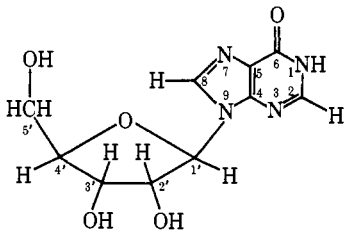
(1) G. L. Eichhorn, *Nature (London)*, **194**, 474 (1962).
(2) G. L. Eichhorn and P. Clark, *Proc. Nat. Acad. Sci. U. S.*, **53**, 586 (1965).
(3) G. L. Eichhorn and E. Tarien, *Biopolymers*, **5**, 273 (1967).
(4) G. L. Eichhorn and Y. A. Shin, *J. Amer. Chem. Soc.*, **90**, 7323 (1968).
(5) M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 176 (1962).
(6) G. L. Eichhorn, P. Clark, and E. D. Becker, *Biochemistry*, **5**, 245 (1966).
(7) N. A. Berger and G. L. Eichhorn, *ibid.*, **10**, 1847 (1971).
(8) N. A. Berger and G. L. Eichhorn, *ibid.*, **10**, 1857 (1971).
(9) N. C. Li, R. L. Scruggs, and E. D. Becker, *J. Amer. Chem. Soc.*, **84**, 4650 (1962).
(10) D. R. Eaton and W. D. Phillips, *Advan. Magn. Resonance*, **1**, 103 (1965).
(11) E. Frieden and J. Alles, *J. Biol. Chem.*, **230**, 797 (1957).
(12) A. T. Tu and C. G. Friedrich, *Biochemistry*, 7, 4367 (1968).

Table I. Proton Chemical Shifts at 60 MHz

	H-1	H-8	H-2	H-1′	3′-OH	2′-OH, 5′-OH	H-2′	H-3′	H-4′	H-5′	CH_3
Nucleosides in D_2O at 40° [d]											
Inosine		500	493	366			286	267	257	235	
Deoxyinosine		498	491	388			165	278[a]	248	231	
1-Methylinosine		499	501	363			285	267	256	235	219
7-Methylinosine		(555)[b]	495	370			284	265	252[c]	236	252
Nucleosides in DMSO-d_6 at 40° [e]											
Inosine	740	499	483	353	323	303	269	249	236	216	
Deoxyinosine	742	497	483	379	319	298	150	263	232	216	
1-Methylinosine		499	504	352	324	305	271	249	238	216	211
7-Methylinosine		560	482	356			275	249		220	231

[a] Under water peak. [b] Measured in H_2O. [c] Under CH_3 peak. [d] Cps from DSS internal standard. [e] Cps from TMS internal standard.

phosphate and thus to reconfirm specific base binding of Cu(II) in the absence of phosphate.



Experimental Section

Materials and Methods. Inosine, 2′-deoxyinosine, 7-methylinosine, and 2′,3′,5′-triacetylinosine were obtained from Sigma Chemical Co., 1-methylinosine from Cyclo Chemical Co. Spectroquality DMSO, Matheson Coleman and Bell, was used for visible spectrophotometric studies which were carried out with a Cary 14 recording spectrophotometer and a Beckman DU.

Nmr studies were performed on the nucleosides in 99.5% DMSO-d_6, Diaprep Inc., and in 99.75% D_2O, Brinkman Instruments. 7-Methylinosine was also measured in distilled H_2O. The pH of aqueous solutions was measured with a pH meter 25, Radiometer, Copenhagen, and is reported directly as read. Nmr studies of the nucleosides in D_2O were performed at the pH at which they dissolved: 5.6 for inosine, 7.8 for 1-methylinosine, 7.9 for 7-methylinosine, and 6.6 for deoxyinosine. For pH variation studies solutions were adjusted with NaOD and DCl, Diaprep Inc.

Nmr tubes were quantitatively loaded and the technique of incremental addition of Cu(II) was used as previously described.[7] Reagent grade $CuSO_4 \cdot 5H_2O$, Fisher Scientific Co., was used in aqueous solution and $Cu(CH_3COO)_2 \cdot H_2O$, J. T. Baker Co., was used in DMSO. Spectra were obtained with the Varian nmr spectrometer A60D at the normal operating probe temperature of 37–40° and studies at elevated temperatures were performed with the Varian variable temperature accessory calibrated by the splitting of the peaks of Varian ethylene glycol temperature calibration sample. Chemical shifts were measured from internal standards of sodium 2,2-dimethyl-2-silapentane sulfonate (DSS), NMR Specialties Inc., in D_2O, and tetramethylsilane (TMS), Diaprep Inc., in DMSO-d_6.

Results and Discussion

Characteristics of the Nmr Spectra. The chemical shifts of inosine and deoxyinosine and the 1- and 7-methyl analogs in D_2O and DMSO-d_6 are presented in Table I. The peak assignments are those previously published for the base,[13,14] ribose,[15–17] and deoxyribose protons.[16,18] The nmr and uv properties of these compounds have been extensively investigated by Ts'o, *et al.*[13] The present investigations are in agreement with these earlier nmr studies and only those characteristics pertinent to the Cu(II) binding studies are discussed. Selective deuterium exchange of the purine H-8 proton[19] at 80° in D_2O was used to confirm the identity of the H-8 resonance of inosine, deoxyinosine, and 1-methylinosine. Partially deuterated nucleosides were lyophilized from D_2O and redissolved in DMSO-d_6; the partially deuterated peaks then confirmed the identity of the H-8 in DMSO. Since the protons on the ribose hydroxyls and N-1 position are also exchanged rapidly in D_2O, the identity of these protons was also confirmed by comparing a spectrum of the nucleoside dissolved directly in DMSO-d_6 to one obtained from a deuterated sample redissolved in DMSO-d_6.

As with most 6-substituted purines the H-8 resonance of inosine and deoxyinosine is downfield from the H-2 resonance.[20] The present study is in agreement with the earlier demonstration[13,14] that in 1-methylinosine the positions are reversed, with the H-2 resonance being downfield from the H-8. The reversal is due to the H-2 proton being shifted to lower field, having increased in acidity due to the 1-methyl substitution, while the H-8 proton is not affected.

The nmr spectrum of 7-methylinosine has several unusual features described by Ts'o, *et al.*,[13] and confirmed in these studies. Deuterium exchange of the H-8 proton of 7-methylinosine is complete within minutes of dissolving in D_2O at room temperature and therefore cannot be identified in this solvent although the H-2 is identified as a sharp peak at 495 cps, similar to the H-2 shift in inosine and deoxyinosine. In the nmr spectrum of 7-methylinosine in H_2O the H-8 is clearly apparent as a peak at 555-cps (Figure 4), of comparable line width to the H-2 which retains a similar shift to that in D_2O. This rapid exchange of the H-8 proton of 7-methylinosine and the 55-cps shift to low field when compared to inosine H-8 has been attributed to polarization of the molecule with a positive charge localized in the five-membered ring.[13]

In DMSO the H-1 proton of inosine is identified as a broad peak centered at 740 cps and a similar peak is observed at 742 cps for the H-1 proton of deoxyinosine. No such peak occurs in 1-methylinosine which has the proton replaced by a methyl group, nor can a resonance characteristic of the H-1 proton be identified in the nmr spectrum of 7-methylinosine. This observa-

(13) P. O. P. Ts'o, N. S. Kondo, R. K. Robins, and A. D. Broom, *J. Amer. Chem. Soc.*, **91**, 5625 (1969).
(14) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *ibid.*, **89**, 3612 (1967).
(15) C. D. Jardetzky, *ibid.*, **84**, 62 (1962).
(16) L. Gatlin and J. C. Davis, Jr., *ibid.*, **84**, 4464 (1962).
(17) I. Feldman and R. P. Agarwal, *ibid.*, **90**, 7329 (1968).
(18) C. D. Jardetzky, *ibid.*, **83**, 2919 (1961).
(19) F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, **29**, 1988 (1964).
(20) P. O. P. Ts'o, M. P. Schweizer, and D. P. Hollis, *Ann. N. Y. Acad. Sci.*, **158**, 256 (1969).

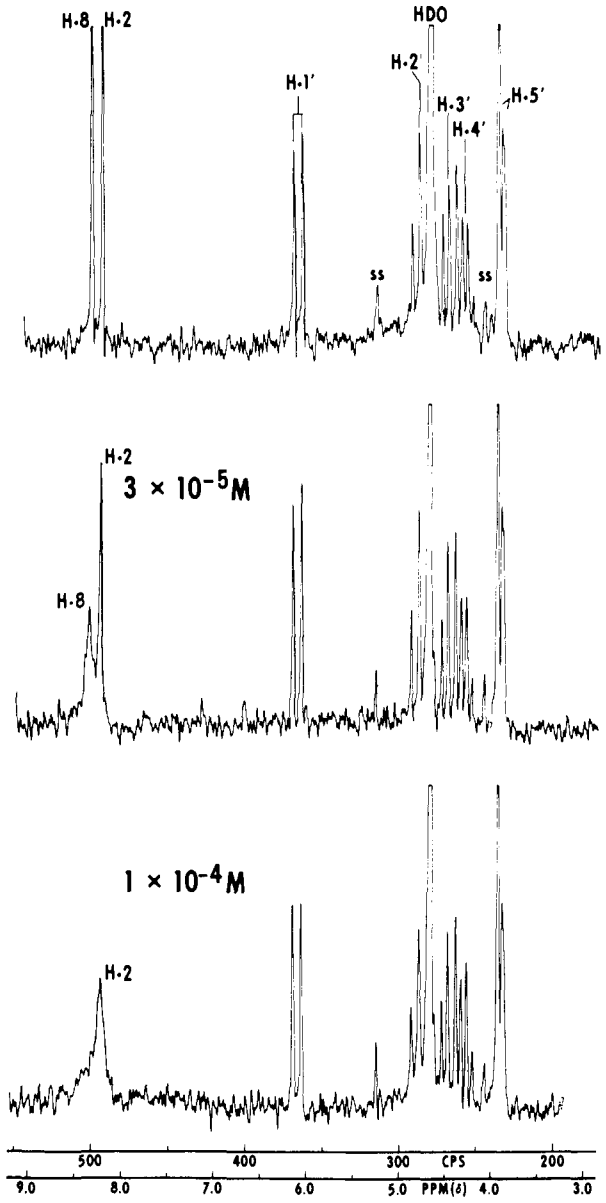


Figure 1. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* inosine in D_2O at pH 5.6 and 40°. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the others. Base and ribose peaks are specifically labeled. The two protons at the 5′-ribose position are labeled together as H-5′. Spinning side bands of water are labeled SS. Abscissa is in cycles per second and parts per million downfield from DSS internal standard.

tion has also been interpreted[13] to indicate that the 7-methylinosine molecule is in the polarized betaine form, with a negative charge located in the six-membered ring.

After several hours at room temperature the H-2 peak of 7-methylinosine in aqueous solution begins to decrease in intensity and a new peak appears 10 cps upfield. However, in DMSO the spectrum was stable for at least 24 hr at room temperature. Heating of 7-methylinosine to 80° in D_2O or DMSO-d_6 produced three new peaks in the H-2 region and split the single methyl resonance into a total of three nearby peaks. These new peaks in both the H-2 and methyl regions persist when the solutions are returned to room temperature. The nature of these altered products was not further explored. Since the apparent molecular rearrangement might obfuscate interpretation of the nmr effects, metal binding studies to 7-methylinosine were carried out only at 40° and immediately after preparation of solutions.

Nmr Determination of Copper(II) Binding to Inosine. The paramagnetic broadening effects produced by Cu(II) on the nmr spectrum of inosine in aqueous solution are demonstrated in Figure 1. Cu(II)-induced broadening is apparent at a metal concentration of 10^{-5} *M* (base to metal ratio of 10^4) which is similar to the previously observed Cu(II) concentrations required to broaden the base peaks of 5′-IMP, and poly-(I),[8] but an order of magnitude lower than the 10^{-4} *M* metal concentration (base to metal ratio of 10^3) required to broaden the base peaks of AMP.[7]

Selective broadening of the inosine H-2 and H-8 resonances while ribose proton resonances retain their narrow line width demonstrates coordination of Cu(II) to the purine portion of this nucleoside.

Since no phosphate is present in the nucleoside, these results confirm that Cu(II)-induced broadening of base protons in aqueous solutions is due to specific binding of Cu(II) to base and is not dependent on Cu(II) bound to phosphate. This is in agreement with the previous demonstration that broadening of base protons in the phosphate isomers of AMP is also due to coordination of Cu(II) to specific sites on the purine ring.[7] Both Mn(II) and Cu(II) coordinate to the phosphate groups of nucleotides; however, in contrast to the results obtained with Cu(II), recent nmr studies have demonstrated that with Mn(II) the base protons which are broadened are primarily those near the metal bound to phosphate.[21,22] These contrasting results are in line with the demonstration that Mn(II) has a relatively stronger tendency to bind phosphate rather than base, when compared to copper.[4]

As previously noted in IMP, both of the base proton resonances are broadened although in inosine the H-8 is clearly broadened more than and before the H-2. There are several alternatives to explain the observation that Cu(II) broadens both base resonance peaks. Previous studies have been interpreted as indicating chelation of metal ions between the N-7 and 6-oxy ligand atoms.[11,12] Either chelation in this position or simple coordination to the N-7 atom accounts for broadening of the nearby H-8 proton resonance. Binding at this site could also explain broadening of the H-2 resonance, if the paramagnetic effect were transmitted either from the N-7 through the purine system or in the case of the chelate from the 6-oxy group into the six-membered ring.

Another explanation is that Cu(II) can coordinate to multiple sites on the base and that at least two species of complexes exist in solution. A predominant species with Cu(II) coordinated at N-7 or chelated between N-7 and O-6 would account for the greater broadening of H-8, and the other species with Cu(II) coordinated to N-1 or N-3 would account for the broadening of H-2.

Coordination of Cu(II) to multiple sites on the purine ring has been demonstrated for adenosine and the phosphate isomers of AMP.[7] In tubercidin, an adenosine analog with carbon substituted for nitrogen at the 7

(21) M. J. Heller, A. J. Jones, and A. T. Tu, *Biochemistry*, **9**, 4981 (1970).
(22) S. I. Chan and J. H. Nelson, *J. Amer. Chem. Soc.*, **91**, 168 (1969).

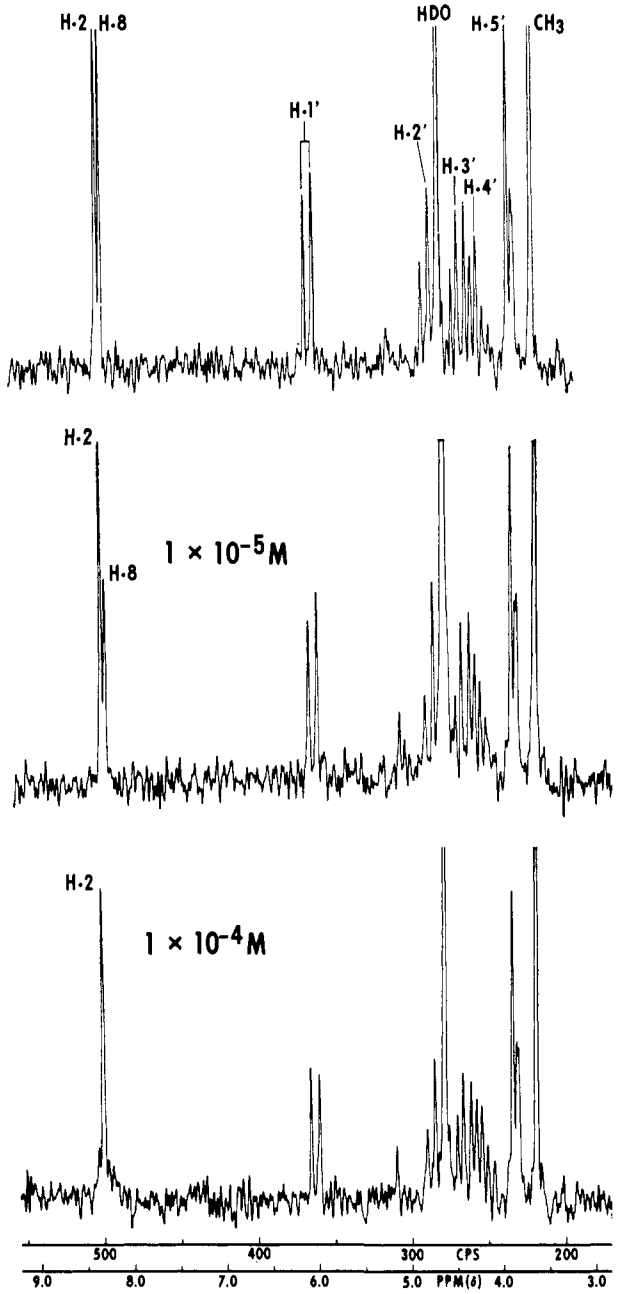


Figure 2. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* 1-methylinosine in D_2O at pH 7.8 and 40°. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the others. Peaks are labeled as in Figure 1. Abscissa is measured downfield from DSS internal standard.

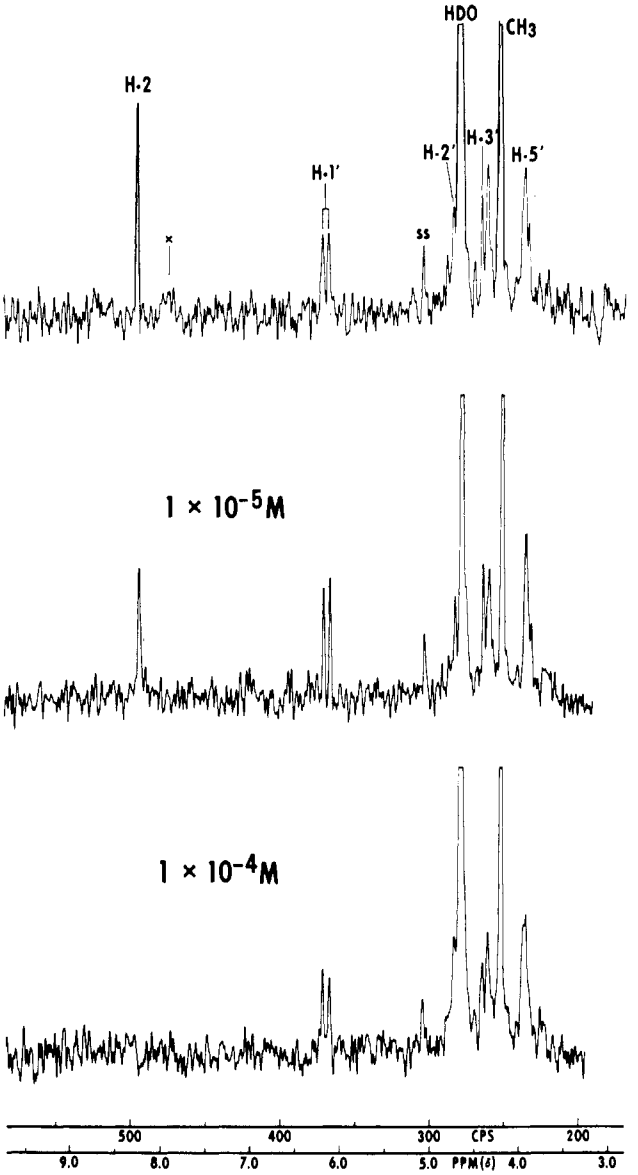


Figure 3. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* 7-methylinosine in D_2O at pH 7.9 and 40°. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the others. Peaks are labeled as in Figure 1. × designates the new peak which appears upfield from H-2 on standing. Abscissa is in cycles per second downfield from DSS internal standard.

position, Cu(II) coordinates to the nitrogen atoms of the six-membered ring, producing broadening of the neighboring H-2 resonance. No binding site is available for Cu(II) on the five-membered ring of tubercidin and no broadening was observed on the H-7 and H-8 resonances, clearly demonstrating that the paramagnetic broadening effect is not transmitted from ring to ring.[7] On the basis of this demonstration it is also unlikely that the paramagnetic effect is transmitted from ring to ring of inosine; however, if Cu(II) is chelated to the N-7 and O-6 positions, it is directly bound to a substituent of the six-membered ring and still might affect the H-2 resonance through this bond. To explore these alternatives, the binding of Cu(II) was studied with derivatives of inosine blocked by methyl groups at the 1- and 7-nitrogen atoms.

Nmr Determination of Cu(II) Binding to Methylinosines. Addition of Cu(II) to 1-methylinosine (Figure 2) produces total broadening of the H-8 resonance while the H-2 retains its narrow line width. At the same Cu(II) concentration of 1×10^{-4} *M*, the H-2 resonances of inosine, as well as the H-8, are considerably broadened. The exclusive broadening of H-8 in 1-methylinosine clearly demonstrates binding of Cu(II) to the N-7 position, possibly involving the O-6 in a chelate. The maintenance of the sharp H-2 resonance demonstrates that metal bound at N-7 does not produce broadening at this position. This indicates that the broadening of H-2 in inosine is due to coordination of Cu(II) to the six-membered ring, and since it is eliminated by blocking the 1 position with a methyl group it is probable that N-1 is the coordination site on that ring.

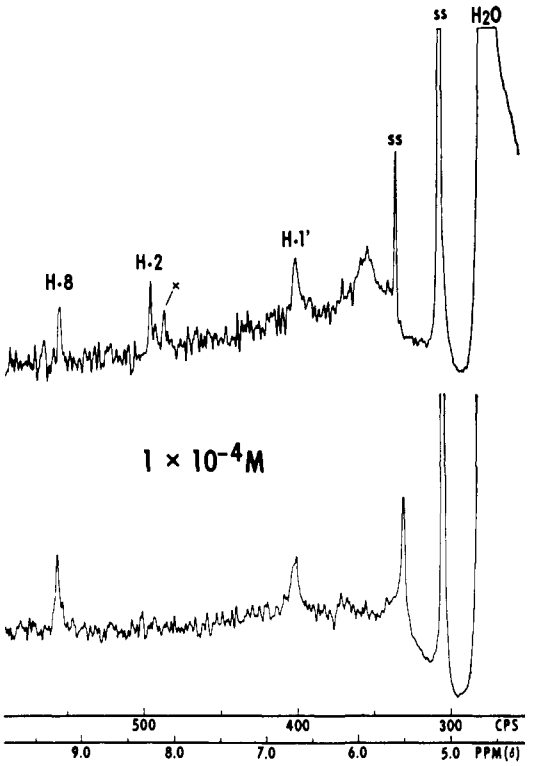


Figure 4. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* 7-methylinosine in H_2O at 40° The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the bottom spectrum. Peaks are labeled as in Figure 1. Abscissa is in cycles per second downfield from DSS internal standard.

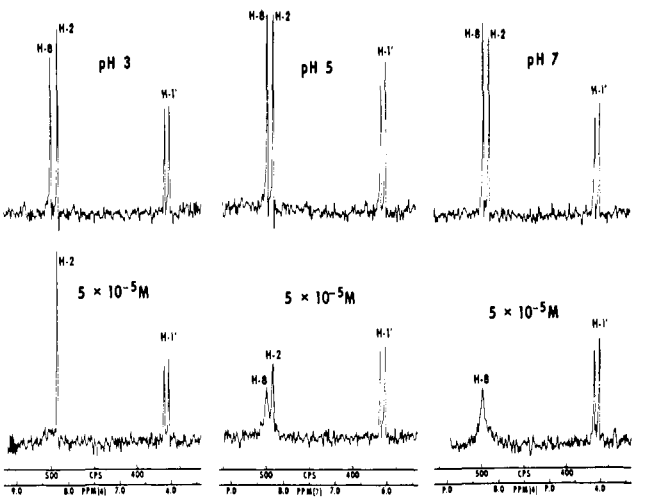


Figure 5. Effect of pH and Cu(II) on the low-field half of the pmr spectrum (60 MHz) of inosine in D_2O at 40°. The two spectra in each vertical column are at the pH indicated at the top of the column. In each case the top spectrum is the metal-free solution and the Cu(II) concentration is indicated for the bottom spectrum. Peaks are labeled as in Figure 1. Abscissa is measured downfield from DSS internal standard.

When Cu(II) is added to 7-methylinosine in D_2O, broadening of the only observable base peak, which is the H-2 resonance, is observed (Figure 3). It is apparent that the base proton is broadened before those of the ribose; however, since only one of the base protons is observable in D_2O, no indication of selective broadening of H-2 as compared to H-8 is obtained. To confirm selective broadening, a sample of 7-methylinosine in D_2O with sufficient Cu(II) present to broaden the H-2 was lyophilized and then redissolved in distilled H_2O, now revealing the same H-8 and H-1′ peaks that were observed in 7-methylinosine alone in H_2O. In Figure 4 it is apparent that the H-2 which was totally broadened by Cu(II) in D_2O remains totally broadened in preference to H-8 as well as to ribose. Although the peaks in H_2O are not as sharp as those in D_2O, it is apparent that 1×10^{-4} *M* Cu(II) totally broadens the H-2 resonance, while the H-8 is the same as in the absence of Cu(II). It is of interest to note that the peak which appears 10 cps upfield from H-2 does not occur in the presence of Cu(II), indicating that whatever structural alterations take place, copper remains in close proximity to the proton.

It should be recalled that 7-methylinosine is without a proton at the N-1 position and exists with a negative charge in the N-1,O-6 region. Selective broadening of the H-2 resonance demonstrates that Cu(II) binds in this region and again demonstrates that binding to a site on one ring does not produce broadening of the protons on the next. Although the results with 1-methylinosine implicate N-1 as a binding group, the negative charge in 7-methylinosine could reside on O-6 as well as N-1. The present studies do not generally distinguish between these two binding sites, and they are henceforth referred to as the N-1,O-6 region.

These studies demonstrate that coordination of Cu(II) to N-7 broadens the nearby H-8, coordination to N-1 broadens the nearby H-2, and in both cases the paramagnetic effect is observed only on the nearby proton. The Cu(II)-induced broadening of the inosine H-8 and H-2 resonances at pH 5.6 can then be best accounted for by multiple base binding sites with some coordination of Cu(II) to N-1 (O-6) and a predominant species of inosine coordinating Cu(II) at N-7.

Effect of pH Variation on Cu(II) Binding. With the existence of more than one complex having been established in solution, conditions were varied to determine those which favor formation of each complex. The effect of pH on Cu(II) binding was studied by incremental addition of Cu(II) to solutions of inosine ranging from pH 3 to 12. Figure 5 illustrates the drastic differences which result. At pH 3 Cu(II) totally broadens the H-8, having almost no effect on the H-2. In the pH range of 5 and 6, H-8 broadening approximates H-2, and at pH 7, H-2 is broadened more than H-8. As the pH continues to increase to 10, 11, and 12, the shifts of the base peaks are identical and no further distinction between H-8 and H-2 is possible. However, at pH 11 and 12 less total base broadening occurs.

The results at pH 3, which resemble those with 1-methylinosine, clearly demonstrate that Cu(II) is bound to the N-7 position of inosine. The fact that no broadening of the H-2 occurs confirms that, with inosine as with the methylated derivatives, the paramagnetic broadening effect is not transmitted from ring to ring. As the pH increases, the proton to N-1 bond is weakened, the p*K* being 8.96.[23] Dissociation of this proton leaves the N-1,O-6 region negatively charged,[23] and Cu(II) then binds to this region, preferentially broadening the H-2 resonance (Cu(II) binding can, of course, lower the p*K*). This is similar to the binding of

(23) J. J. Christensen, J. H. Rytting, and R. M. Izatt, *Biochemistry*, **9**, 4907 (1970).

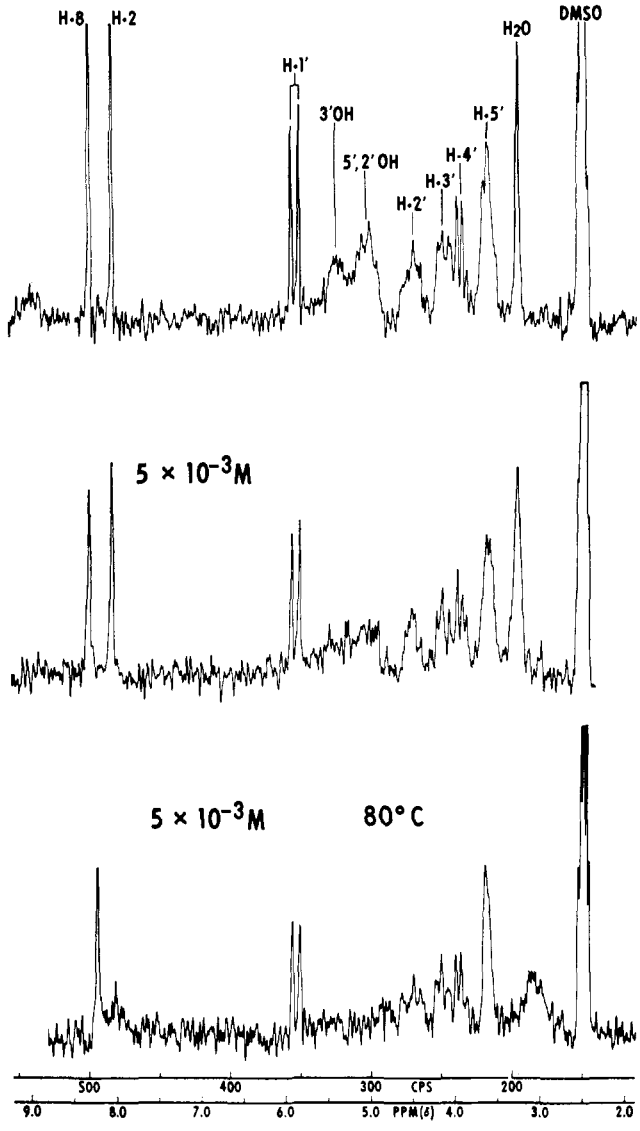


Figure 6. Effect of Cu(II) and temperature on pmr spectrum (60 MHz) of 0.1 *M* inosine in DMSO-d_6. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the others. The two upper spectra are at 40°, the bottom spectrum at 80°. Base and ribose protons are specifically labeled. The broad H-1 resonance appearing at low field is not included. Abscissa is in cycles per second and parts per million downfield from TMS internal standard.

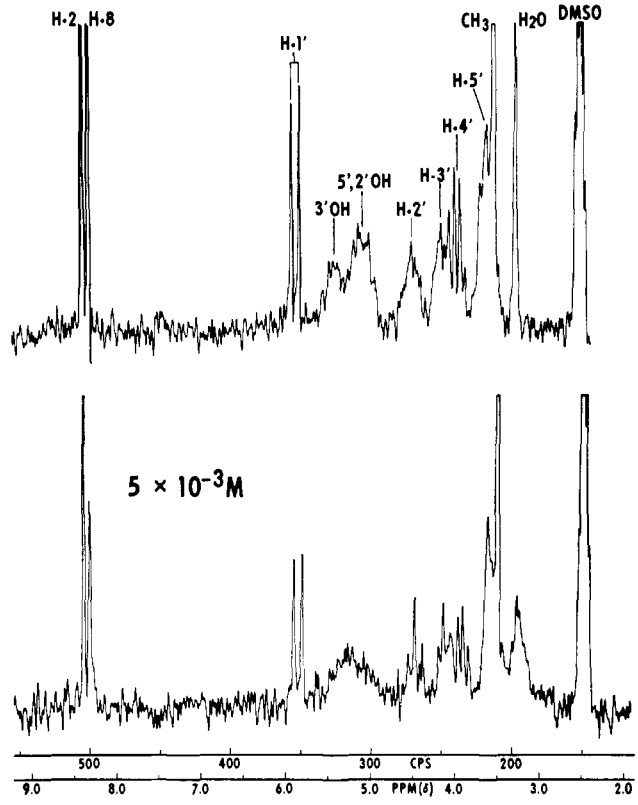


Figure 7. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* 1-methylinosine in DMSO-d_6 at 40°. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated on the bottom spectrum. Peaks are labeled as in Figures 1 and 6. Abscissa is measured downfield from TMS internal standard.

Cu(II) to the negatively charged N-1,O-6 region in 7-methylinosine. However, in contrast to 7-methylinosine, in which the 7 position is blocked, some binding still occurs to this position in inosine, producing some broadening of the H-8 resonance.

These nmr studies clearly demonstrate that at low pH Cu(II) coordinates almost exclusively to the N-7 atom of inosine. Between pH 5 and 6 Cu(II) is nearly equally distributed between coordination at the N-7 position and the N-1,O-6 region. By pH 7 the N-1,O-6 region is the preferential binding site. Further increases in pH decrease the amount of Cu(II) bound to base. This is similar to the demonstration by uv spectroscopy that methyl mercury binds to inosine N-7 at low pH, with increasing binding to N-1 occurring as the pH increases.[24]

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

Nmr Demonstration of Cu(II) Binding to Ribose Hydroxyls. The decrease in base broadening above pH 9 is due to copper hydroxide formation, and in the pH range of 11–12 it is probable that Cu(II) also binds to ribose hydroxyls. The pK for ionization of the inosine ribose hydroxyl protons is 11.84 at 40°,[23] making this a likely coordinating group at alkaline pH. Coordination of uranyl ions to ribose hydroxyls of adenine nucleotides has been demonstrated by nmr in this pH range[25] and potentiometric titrations have also demonstrated coordination of Cu(II) to ribose hydroxyls of adenosine and uridine at alkaline pH.[26]

Ribose hydroxyl protons are not observable in D_2O where they undergo rapid exchange with the solvent; however, they do not undergo exchange in DMSO where they usually can be observed. These protons were clearly demonstrated in the nmr spectra of inosine (Figure 6) and 1-methylinosine (Figure 7); they were difficult to resolve in deoxyinosine, and could not be identified in 7-methylinosine (Figure 8). Nmr studies of Cu(II) with the inosine nucleosides in DMSO (Figures 6 and 7) reveal major broadening of the ribose hydroxyl proton resonances which is then followed by broadening of base resonances as the Cu(II) concentration is increased. Broadening of the ribose and deoxyribose hydroxyl resonances in DMSO occurs at Cu(II) concentrations of 10^{-3} *M*, which contrasts with the 10^{-5} *M* Cu(II) concentration required to produce base broadening in aqueous solution. In DMSO, then, as

(25) R. P. Agarwal and I. Feldman, *ibid.*, 90, 6635 (1968).
(26) V. H. Reinert and R. Weiss, *Z. Physiol. Chem.*, 350, 1321 (1969).

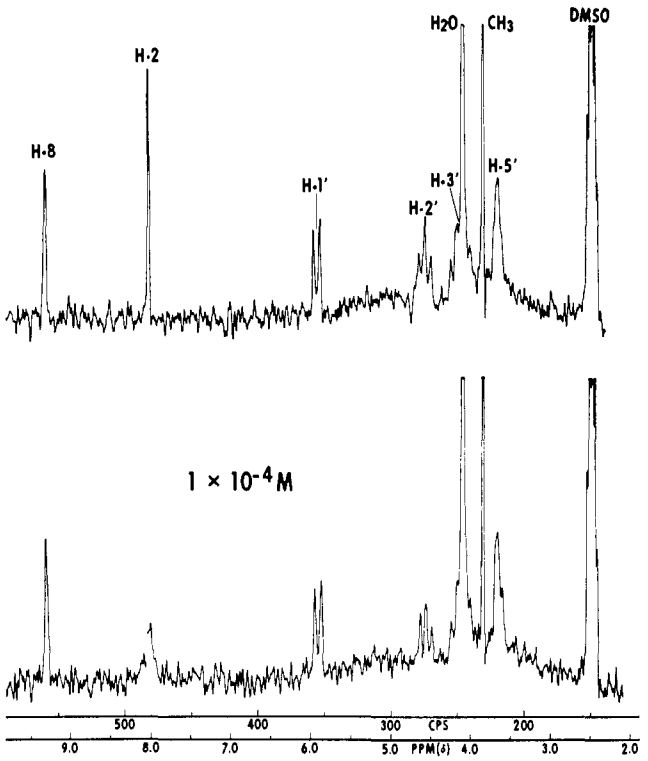


Figure 8. Effect of Cu(II) on pmr spectrum (60 MHz) of 0.1 *M* 7-methylinosine in DMSO-d_6 at 40°. The top spectrum is in the absence of metal; the Cu(II) concentration is indicated for the bottom spectrum. Peaks are labeled as in Figure 1. Abscissa is measured downfield from TMS internal standard.

in aqueous solution at alkaline pH, Cu(II) binds to hydroxyls in preference to sites on the inosine base. Coordination of Cu(II) to hydroxyl groups was previously noted with uridine in DMSO[8] and nmr studies now in progress clearly demonstrate this interaction with several other nucleosides.

Determination of Cu(II) Binding by Visible Spectrophotometry. Further revelations concerning the binding of Cu(II) resulted from an examination of the visible spectra of the complexes of Cu(II) acetate with inosine and its derivatives in DMSO. The spectra of equimolar concentrations of Cu(II) acetate with the ribonucleosides inosine, 7-methylinosine, and 1-methylinosine are contrasted in Figure 9 with that produced by the metal with deoxyinosine. All of the ribonucleosides produce a marked decrease in the visible absorption peak of Cu(II) acetate; they also produce a marked enhancement of the near uv absorption band of the Cu(II) acetate. The similarity of the spectral alterations produced by these analogs suggests that Cu(II) acetate is binding to similar ligand atoms in all three ribonucleosides. The relatively small increase in the near uv absorption produced by deoxyinosine suggests that the complex characterized by the high absorption does not form with deoxyinosine but only with inosine ribosides. Also, in contrast to the decreased spectral intensity produced in the visible peak of Cu(II) acetate by the ribonucleosides, binding of deoxyinosine results in an increase in absorption. This enhancement of the Cu(II) spectrum with deoxyinosine is the usual effect of Cu(II) binding to a ligand; a similar increase in the visible band of Cu(II) acetate was also produced by triacetylinosine.

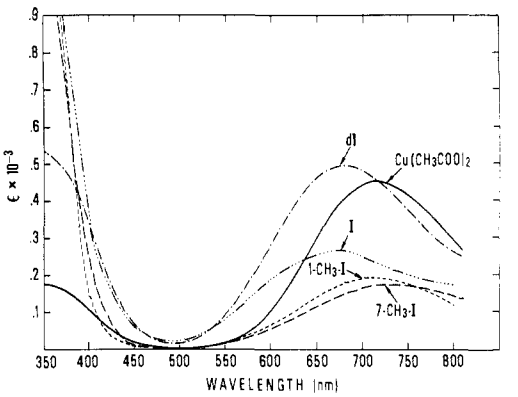


Figure 9. Effect of inosine nucleosides and deoxynucleosides on the visible spectrum of Cu(II) acetate in DMSO at 25°. The spectrum of 5 × 10^{-3} *M* Cu(II) acetate alone is labeled and indicated by the solid line. The spectra with the ligands are all at 5 × 10^{-3} *M* $Cu(CH_3COO)_2$ and 5 × 10^{-3} *M* ligand. The ligands are I, inosine; dI, deoxyinosine; 1-CH_3-I, 1-methylinosine; 7-CH_3-I, 7-methylinosine.

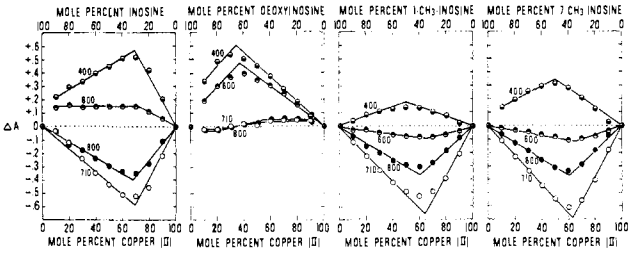


Figure 10. Continuous variation studies between $Cu(CH_3COO)_2$ and inosine, deoxyinosine, 1-methylinosine, and 7-methylinosine in DMSO at 25°. The total concentration of the two components at each experimental point is 1 × 10^{-2} *M*, but the molar ratio of the components changes from point to point as indicated on the abscissa. ΔA values plotted in the ordinates are obtained by subtracting from each observed absorbance at each ratio the absorbance calculated for a nonreacting mixture of the components. Each curve is labeled with the wavelength (400, 600, 710, and 800 nm) at which it was calculated. Several of the curves at the lower wavelengths do not continue linearly between 10 and 0% Cu(II).

It is apparent that all of the ribonucleosides bind Cu(II) in a similar fashion which requires the presence of a 2′-hydroxyl, as indicated by the fact that in deoxyinosine where the 2′-hydroxyl is absent and in triacetyl inosine where the 2′-hydroxyl is blocked, a different complex forms. Investigations in progress indicate that Cu(II) binding with similar spectral effects occurs with all ribonucleosides and with no deoxynucleoside.

Despite the similarity in the behavior of the ribonucleosides, suggestive of ribose hydroxyl binding, distinct differences exist in the spectrum produced by each analog, demonstrating that the bases play some role in determining the spectral alterations. Thus, while inosine decreases the visible absorbance and deoxyinosine increases it, the effect of the base is reflected in that both produce a shift in the absorption maximum of Cu(II) acetate from 715 to 680 nm, in contrast to the effects with the methylated bases.

Stoichiometry of Cu(II) Complex in DMSO. Figure 10 presents the results of continuous variation studies with Cu(II) and the inosine analogs in DMSO. The continuous variation curves at 710 and 800 nm for Cu(II) acetate with inosine, 1-methylinosine, and 7-

methylinosine all have minima in the region demonstrating the existence of a 2:1 metal–nucleoside complex. The curves at these wavelengths are almost identical for these three compounds, again suggesting the similarity of the complexes. In contrast, the 710- and 800-nm difference curve for deoxyinosine gives no indication of this complex formation.

Cu(II) acetate can exist as a dimer with a bond joining the copper atoms and four acetate molecules bridging the two metal atoms.[27,28] The formation of a similar complex with the three ribonucleosides with a ratio of 2:1 copper–nucleoside and its failure to form with the deoxynucleoside or with other Cu(II) salts, such as nitrate, coupled with the existence of Cu(II) acetate as a dimer, suggests that one of the bridging acetate ligands could be displaced by a nucleoside. One copper atom may be coordinated to the nucleoside 2′-hydroxyl and another to the 3′-hydroxyl with three acetate molecules still bridging the two metal atoms and a metal–metal bond between these copper atoms.

The continuous variation curves at other wavelengths give evidence for the probable existence of other complexes which may involve the bases.

Comparison of Visible and Nmr Spectroscopy. The visible and near-ultraviolet spectra of copper with inosine and analogs indicate formation of a complex with ribonucleosides which does not occur in the absence of the 2′-hydroxyl or when both 2′- and 3′-hydroxyls are blocked by acetylation. The nmr studies indicate binding of Cu(II) to ribose hydroxyls as well as to the bases. Broadening of the hydroxyls well in advance of the base proton resonances indicates them to be the preferred coordination sites in DMSO.

In contrast to inosine and 1-methylinosine, 7-methylinosine in DMSO exhibits broadening of the H-2 resonance by 1×10^{-4} *M* Cu(II) (Figure 8). Apparently the negatively charged N-1,O-6 region of this molecule is a strong coordinating site for Cu(II), as it is in aqueous solution. Binding of Cu(II) to the ribose hydroxyls of 7-methylinosine cannot be demonstrated by nmr since these protons are not evident. However, the absorbance decrease in the spectra of Cu(II) acetate and the continuous variation studies clearly demonstrate that 7-methylinosine does bind Cu(II) to the ribose hydroxyls as do the other nucleosides.

Effect of Temperature Variation on Cu(II) Binding. Upon heating inosine in DMSO from 40 to 80°, in the presence of a concentration of Cu(II) below that required to produce base broadening at 40°, the H-2 resonance becomes markedly broadened while the H-8 is only slightly broadened (Figure 6), demonstrating that temperature elevation favors Cu(II) binding to specific sites on the base. Similar effects are obtained with deoxyinosine, and in all cases the original spectrum returns on cooling.

In contrast to inosine, this effect in DMSO at elevated temperatures does not occur in 1-methylinosine where the 1 position is permanently blocked. Selective broadening of the inosine H-2 resonance suggests that heating increases dissociation of the proton from N-1, producing a negative charge center to which Cu(II) then coordinates. Evidence for proton dissociation under these conditions was obtained by observing a decrease in pH meter reading when a solution of inosine in DMSO was heated. This decrease is greater than the decrease in the pH meter reading when DMSO alone is heated.

Similar experiments with inosine in aqueous solution demonstrate that preferential Cu(II)-induced broadening of H-8 greater than H-2 could be partially reversed by heating to 80°. With 1-methylinosine heating results in narrowing of the H-8 resonance, but no increase in H-2 broadening occurs. Thus, in 1-methylinosine temperature elevation tends to dissociate Cu(II) from the N-7, and in inosine, where the H-1 proton is also dissociated, Cu(II) binding near the N-1,O-6 area is favored.

(27) T. Tsuchida and S. Yamada, *Nature (London)*, 176, 1171 (1955).
(28) R. L. Martin and A. Whitley, *J. Chem. Soc.*, 1394 (1958).