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THE INTERACTION OF METAL IONS WITH NUCLEIC ACIDS. A NUCLEAR MAGNETIC RESONANCE RELAXATION TIME STUDY OF THE COPPER(II)-INOSINE 5'-MONOPHOSPHATE SYSTEM IN SOLUTION

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The nature of binding between copper ions and inosine 5'-monophosphate was studied using $^{13}$C, $^1$H, and $^{31}$P nuclear magnetic resonance techniques. The results imply that at least two species are present in the solution with the metal to ligand ratio of 1:1 and 1:2 (termed, respectively, ML and ML$_2$); however, the ML complex predominates in the Cu(II)-IMP system. The results indicate that the copper ions bind directly to two different sites on the inosine ring i.e., to N$_7$ and to N$_1$. The distribution of the species ML$_1$ and ML$_2$ is nearly equal. The relative dipolar and scalar contributions to the proton and carbon-13 transverse relaxation have been assessed.

Numerous studies used the selective NMR line broadening by a copper(II) ion to locate the metal ion binding sites in inosine and its derivatives (Maskos, 1978a; and references therein). All these studies were essentially qualitative. A systematic study of copper(II) nucleotide complexes has now been undertaken on a more quantitative basis in order to elucidate structures in solution.

The values of the transverse relaxation times ($T_{2M}$) of nuclei on coordinated ligands can be obtained from line broadening measurements only if the fast exchange condition is satisfied. This condition has seldom been verified experimentally in the earlier NMR selective broadening experiments. Furthermore, even if the values of $T_{2M}$ for the various relaxing species could be measured, no geometrical information about the ligand bound can be obtained unless it is shown that the dipolar interaction is dominant.

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The relative contributions of hyperfine and dipolar interactions to the relaxation time of each nucleus have not been determined in previous line broadening studies. We have, therefore, reinvestigated the Cu(II)-IMP system. $T_1$ and $T_2$ measurements were used to determine the relative contributions of the dipolar and hyperfine interactions to the measured relaxation times.

Furthermore, the $T_{1p}$ measurements can be used to estimate the conformation of the Cu(II)-IMP complex and yield further insight into the binding of Cu(II) to the hypoxanthine ring.

In view of the doubts presented by Espersen & Martin (1976a) some binding site assignments in IMP were checked by using longitudinal relaxation. The present study has shown that the binding assignments based on line-broadening studies (Berger & Eichhorn, 1971b; Maskos, 1978a) are correct. The results imply that at least two species are present in the solution with the metal to ligand ratio of 1:1 and 1:2 (termed, respectively, ML and ML$_2$); and in the Cu(II)-IMP system the ML complex predominates. The system is found to be in "fast exchange", the distances, however, between the metal ion and the carbon and proton nuclei can not be estimated from $T_1$ measurements because the dipolar interaction contributing to relaxation is not only between the paramagnetic ion and the affected nucleus but there is also a significant contribution of interactions from unpaired spin density on the ligand.

MATERIALS AND METHODS

Materials

IMP (disodium salt) was obtained from Sigma Chemical Co.; to remove paramagnetic impurities the aqueous solutions (pH 7.2) were shaken with Chelex-100 cation exchange resin (Bio-Rad Laboratories) or passed through a Dowex chelating resin (Sigma Chemical Co.). Preliminary proton relaxation results obtained on IMP showed that this procedure was not sufficient to remove all paramagnetic impurities. To sequester all paramagnetic ions, 5 µl of 1 mm-EDTA was added to each of the solutions. The effect of paramagnetic ion impurities on the relaxation rates was practically eliminated by these treatments and the relaxation times remained constant.

Stock solutions of copper(II) ions in $^2$H$_2$O were prepared from CuCl$_2$·$^3$H$_2$O crystals.

Measurements

The proton NMR spectra were measured in Fourier transform$^1$ (FT) mode with a Varian HFT-80 (80 MHz for protons) instrument or with

$^1$ Abbreviations used: FT, Fourier transform; PD, pulse delay; DRR, differential relaxation rate.
a Varian XL-100-12 NMR spectrometer (100.1 MHz for protons) equipped with a Varian 620-L computer. Some spectra were also measured with a Varian A-60 continuous-wave spectrometer. The field-frequency lock was obtained from the deuterium resonance of the solvent.

The $^{13}$C spectra were recorded in FT mode with proton noise decoupling with a Varian $^{13}$CFT (20 MHz for $^{13}$C) or with a Varian XL-100-12 NMR spectrometers (25.2 MHz for $^{13}$C). The free induction decays of transient signals induced by rf pulses were accumulated as 16 k data points in the time domain when observing a 4000 Hz width for 25° MHz instrument and 8 k for 20 MHz spectrometer.

The $^{31}$P NMR spectra were obtained on a Varian XL-100-12 NMR spectrometer (40.5 MHz for $^{31}$P) in the FT mode.

The sample temperature was controlled by a calibrated Varian-4343 variable-temperature unit. Samples were in the probe for at least 10 min before the measurements were begun.

The $T_2$ results were obtained from the line widths at half-height from spectra that were results of free induction decay (FID) values of 16 or 8 k data points.

The $T_1$ values were measured in partially relaxed FT experiments according to Freeman & Hill (1969). The pulse sequence used was (-PD-180°-t-90°)$_n$, where PD is a long waiting period to ensure complete relaxation between individual (180°-t-90°) sequences. Usually 14 different $t$'s for proton and 8 $t$'s for carbon were taken with the longest $t$ not more than the null point. Since it is practically impossible to obtain the very initial relaxation rates in the FT experiments it was considered to be more practical to take relaxation data up to 50% recovery of magnetization and use it as an approximation of the initial relaxation rate rather than to try to obtain the latter directly from experiment.

The experimental data reported for protons are the mean of at least two measurements done on the same sample.

Deuterium substitution at $H_8$ was performed on the solutions of a normal compound for which relaxation data had been taken, simply by heating it directly in an NMR tube at about 90°C for 3 h. Then, relaxation data were taken for the deuterated compound.

The concept and equations of the theory of effects of paramagnetic ions on the NMR spectra, essential for the present work, are given in Appendix.

RESULTS AND DISCUSSION

At very small concentrations of Cu(II) in IMP solutions both the $H_8$ and $H_2$ resonance signals are broadened to an extent dependent on the IMP concentration. This concentration dependence at $p^2H = 7.4$ and $p = 2.5 \times 10^{-5}$ is demonstrated in Fig. 1. After an initial increase, the slope of $T_{2p}^{-1}$
versus [IMP] shows a decrease reaching finally a plateau. It is significant that a similar type of curves describe the transverse relaxation rate of these protons. These latter, $T_2^{-1}$, curves roughly describe the extent of association of IMP in solution.

Similarly to $T_{2p}^{-1}$, also $T_{1p}^{-1}$ values (Fig. 2) are strongly dependent on the IMP concentration. The steep slope at the beginning of $T_{1p}^{-1}$ curves of Fig. 2 cannot be attributed to an increasing concentration of the Cu(II)-IMP 1:1 complex since the stability constant, log $K = 3.26$ reported (Naumann & Sigel, 1974) for this complex indicates almost complete...
complexation of the metal at the lowest IMP concentrations studied. The results of the proton relaxation time studies are consistent with an equilibrium between the 1:1 and 1:2 complexes.

Consideration of the types of complexes leads to the conclusion that the following species should be taken into account: ML complexes involving metal ion binding to the phosphate group (MLₚ), to the ring (MLₘ), or to both simultaneously (MLₚₘ). Moreover, there are two binding sites on the ring: the nitrogen N₇, and nitrogen N₁ atoms (Berger & Eichhorn, 1971b; Maskos, 1978a).

If the nitrogen N₇ of the IMP molecule participates in complex formation, one would expect the N₁-H proton to dissociate at a lower pH than in the free ligand (Sigel, 1968). For example, from the data given for the guanosine-Cu(II) system one may calculate (Sigel, 1968) the shift of the acidity constant for the proton at N₁ under the influence of copper(II) coordinated to N₇, and obtain ΔpKₐ = pKₐ[guo] - pKₐ[ML₇guo] = 9.24 - 7.05 = 2.2. Similar calculations can be performed for other systems if the appropriate values are known (e.g., for Cu(II)-ITP system this difference is 1.7 (Sigel, 1968).

Also theoretical CNDO/2 calculations (Maskos, unpublished results) indicate that the electric charge of the hydrogen atom at the N₁ position of 9-methylhypoxanthine with copper(II) bonded to N₇, is more positive (+0.156) than that of uncomplexed 9-methylhypoxanthine (+0.120), suggesting and confirming the easier release of proton from the nitrogen atom. Certainly, as soon as N₁ is deprotonated this atom shows an increased tendency to form complexes. Hence, under the experimental conditions (p_{\text{H}} 7.4) the nitrogen N₁, as well as the nitrogen N₇ are potential binding sites for copper(II) ions. For this reason two possibilities should be considered i.e., MLₚₘ and MLₚₘₚ.

In addition, the situation is complicated by the fact that IMP molecules themselves self-associate at sufficiently high concentrations and the higher order complexes of the type ML₂, M₂L₂ or M₂L₃ can be formed (Berger & Eichhorn, 1971a; Frey & Stuehr, 1974; See er al., 1974). However, the binuclear structures of the type M₂L₂ or M₂L₃ with two or three stacked nucleotides and two Cu(II) are not considered in this paper because the experiments were performed with a large excess of ligand over Cu(II), 10 000/1 or even greater. Even if all the nucleotides were stacked in dimers or trimers, the probability of two Cu(II) occurring simultaneously on one pair would be one part or less in 10⁶ to 10⁷. Thus the binuclear structures can be neglected.

It is possible to estimate approximately the fraction of complexes in 1:2 form since this fraction should approximate the fraction of IMP in the dimer form.

In general, the system is in equilibrium with respect to the monomeric and stacked dimeric forms of IMP, possibly including higher aggregates (although interphosphate repulsion should decrease formation of higher
polymers). In view of the lack of exact knowledge about the association equilibria of IMP in aqueous solution, we proceed to analyse our experimental results on the assumption that the IMP is in equilibrium between the monomeric form and a stacked dimetric form according to the following scheme, \( L + L = L_2 \). The fraction of the molecules in the dimeric form \( (f) \) may be obtained from the relation

\[
   f = \frac{4C + K_d - [(8C + K_d)K_d]^{1/2}}{4C}
\]

where \( C \) is the total concentration of IMP and \( K_d \) is the dissociation constant of the dimer. The dissociation constant \( K_d \) has been determined to be \( 3 \text{ M} \) from a separate measurement of the concentration dependence of the chemical shifts of \( H_2, H_8, \) and \( H_9 \), according to a treatment similar to that used by Wagner & Lawaczek (1972). From Eq. (1) \( f = 0.179 \) for \( 0.4 \text{ M}-IMP \) and only 0.059 for \( 0.1 \text{ M}-IMP \) solutions. In solution, Cu(II) is present probably in the ML as well as the ML_2 forms. However, even at the highest concentration of nucleotide applied in this study (\( 0.4 \text{ M} \)) the monomeric form predominates at equilibrium.

**The nature of the dimer in the ML_2 complex**

Two main orientations of the stacked IMP molecules in the dimer should be considered: cis and trans stacking orientations. The cis stacking orientation is defined as any parallel base stack in which the ribose moieties of adjacent molecules are near one another, and the trans stacking orientation is that in which the moieties are noticeably separated (Evans & Sarma, 1974). The cis stacking orientation is the same as that proposed by Broom et al. (1967) in which an alternate stack was further defined as a back to back (or face to face) arrangement of the bases, and a straight stack was defined as a face to back arrangement (the ribosyl substituents at \( N_9 \) are on the same side of the stack), (Broom et al., 1967).

In cis stacked IMP the \( H_8 \) proton is juxtaposed to the phosphate group on an adjacent molecule of IMP. However, in cis stacked orientation, \( H_2 \) is far removed from the phosphate group of the adjacent IMP.

To answer the question what is the orientation of stacked molecules which interact with Cu(II) we have used the same approach as Imoto (1977) [the NMR-DESERT method (Akasaka et al., 1975)], who studied the molecular orientation in the stacked dimer form of 5'-AMP. We have assumed that the fraction of Cu(II) is too small to have much effect on the intermolecular orientations of IMP. It is unlikely that the presence of Cu(II) ion may cause alteration of the stacking specificity and/or the stacking geometry.

From Eq. (8) in the paper of Imoto (1977) the distance \( r^{\text{inter}} \) between \( H_2 \) and \( H_8 \) of the adjacent hypoxanthine rings in the dimeric form can be obtained

\[
   \left( \frac{1}{r^{\text{inter}}} \right)^6 = \frac{1 + (k-1)f}{k \cdot f} \left\{ \left( \frac{1}{\rho} \right)^6 - \left( \frac{1}{\rho^{\text{intra}}} \right)^6 \right\}
\]

(2)
By introducing our data to the above equation, the value of $r_{\text{intra}}$ (intramolecular distance between $H_8$ and $H_2$ of the hypoxanthine ring) was assumed to be 6.5 Å. The factor $k$ was assumed to be 2.0 (Imoto, 1977). On the basis of the differential relaxation rate (DRR) of $H_2$ between before and after deuterium substitution at $H_8$ (DRR = 0.053 s$^{-1}$) the value of an average of the intra- and intermolecular $H_2$-$H_8$ distances in the monomeric and the dimeric stacked forms of IMP $r = [8 \times 10^{11} (\tau_e)/\text{DRR}]^{1/6} = 3.75$ Å ($\tau_e = 1.8 \times 10^{-10}$ s).

Hence, by introducing this value to Eq. (2), a value of 3.1 Å for $r_{\text{inter}}$ is estimated for 0.4 M-IMP solution. In the cis stacking arrangement, the distance between $H_2$ and $H_8$ of the adjacent hypoxanthine rings should be as large as 7 Å. However, the corresponding distance deduced from the present experiment is much smaller than expected. Thus the present result directly proves the existence of the stacking arrangement, in which $H_8$ of one hypoxanthine ring comes close to $H_2$ of its neighbour’s hypoxanthine ring. Trans stacking may be compatible with this close proximity of $H_8$ and $H_2$ of the adjacent hypoxanthine rings.

The present experiments give only the information on the distance between $H_2$ and $H_8$ of the adjacent hypoxanthine rings and therefore do not give any further details about the stacking geometry (for example, whether it is a straight stack or an alternate stack). However, it is possible to assume that adjacent bases are rotated by 180° with respect to each other, which favours dipole-dipole interactions. It is well known that this type of arrangement may be an important stabilizing force in the inosine and guanosine structures and it has been found in the structures of the 1:1 complexes of cobalt and nickel with IMP (Aoki, 1975).

It is expected that copper ions should not have a great influence on the intermolecular orientations of IMP i.e., that such dimeric trans species react with Cu(II) ions giving the ML$_2$ complex. If one assumes that the broadening effect has only an intermolecular origin, then the observed broadening of the $H_2$ proton signal is also consistent with the trans stacking orientations. However, as the $H_2$ and $H_8$ signals are broadened by Cu(II) nearly to the same extent even at the lowest IMP concentrations used in this study, i.e., the levels at which very little intermolecular stacking exists, this indicates that the observed broadening effects are not of an intermolecular but rather of an intramolecular origin. This means that copper ion broadening studies cannot be used to obtain reliable information concerning the type of orientation of stacked IMP molecules.

The most important conclusion arising from the present results is that even if all the stacked IMP molecules form ML$_2$ complex with copper ions, the amount of this species does not exceed 18% in 0.4 M solutions. For this reason the Cu-IMP system is analysed below assuming that the ML species dominates in the solution.
The ML complexes

For a system containing only 1:1 complex, $ML_p^{5+}$, and a large excess of free IMP, with magnetic coupling between copper ion and a proton $H_8$ (binding at $N_7$) Eq. (A1), (see Appendix), would be reduced to

$$(pT_{2p})^{-1} = \frac{1}{T_{2M} + \tau_M}$$

(i.e., a negligible concentration of free metal ion is assumed.

However, if there exists simultaneously a second 1:1 complex, $ML_p^{5+}$, differing from $ML_p^{5+}$ in that $H_8$ and the copper ion are too far apart to couple, Eq. (A1) would be reduced to

$$(pT_{2p})^{-1} = \frac{1-x}{T_{2M} + \tau_M}$$

where $x$ represents the mole fraction of Cu(II) present as $ML_p^{5+}$ form in solution.

If only 1:1 complexes are considered the ratio $[b]/[a]$ in Eq. (A1) is equal to $(1-x)[Cu]_{tot}/[IMP]_{eq}$, where $[Cu]_{tot}$ is the total copper concentration and $[IMP]_{eq}$ is the equilibrium concentration of IMP in the monomeric form. For a copper-IMP mixture having all the metal ions in a 1:1 complex, substitution of this expression into Eq. (A1) leads to

$$(pT_{2p})^{-1} = \frac{(1-x)[IMP]_{tot}/[IMP]_{eq}}{T_{2M} + \tau_M}$$

The following assumptions are made: (1) $[IMP]_{tot} \approx [IMP]_{eq}$; (2) The concentration of the species $ML_p^{5+}$ and $ML_p^{5+}$ is nearly equal i.e., $x = 0.5$. It will be shown further that the latter assumption is reasonable. Factor $x$ will be omitted in the equations presented below, it was, however, always used in the calculations.

Proton $T_{2p}$ values of the Cu(II)-IMP system are independent of the observation frequency (60, 80 or 100 MHz), and no shift in the resonance of the free ligand was observed even at the highest Cu(II) concentrations applied. The lack of any frequency dependence of the line width in the Cu(II)-containing solution suggests that the fast exchange limit is obtained in this case. The absence of chemical shift effects is consistent with observations on other Cu(II)-containing systems (Esperen & Martin, 1976a; Dillon & Rossotti, 1973; Beattie et al., 1976). Hence a $\Delta \omega_b$ mechanism in the fast exchange limit is not responsible for the observed line broadening. Consequently, Eq. (A4) can be applied to this system; this is justified because for Cu(II) and its complexes the relatively long electron spin relaxation time ($\tau_S$) make $T_{2M}$ contributions, in the full Swift & Connick (1962) equation for $T_{2p}$, considerably greater than those arising from $\tau_M$. 
Values of the $^1$H longitudinal and transverse relaxation rates obtained at 80 MHz are tabulated as $T_{1p}^{-1}$ and $T_{2p}^{-1}$ in Table 1.

Also carbon's $T_{2p}$ were measured at two different frequencies (20.1 and 25.2 MHz), and as for the protons, were found to be frequency independent within the experimental error. Again no shift in the $^{13}$C resonance of the ligand was observed in the presence of copper(II).

### Table 1

Summary of $^1$H $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values ($s^{-1}$) for Cu(II)-IMP solutions

Experiments were carried out in $^{2}$H$_2$O at p$^2$H of 7.4, at 26°C. [IMP] = 0.2 M; [Cu]/[IMP] = 2.5 x $10^{-5}$

<table>
<thead>
<tr>
<th>Proton:</th>
<th>$H_8$</th>
<th>$H_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1p}^{-1}$</td>
<td>1.20</td>
<td>1.041</td>
</tr>
<tr>
<td>$T_{2p}^{-1}$</td>
<td>2.50</td>
<td>3.50</td>
</tr>
<tr>
<td>$T_{1p}/T_{2p}$</td>
<td>2.08</td>
<td>3.36</td>
</tr>
</tbody>
</table>

The $^{31}$P linewidth increased with concentration of Cu(II), (Fig. 3), suggesting that Cu(II) ions definitely bind to the phosphate of IMP.

The $^1$H NMR linewidths of heterocyclic protons increased considerably with concentration of Cu(II), (Fig. 4), suggesting that Cu(II) ions bound to two different sites on the ring, namely, nitrogen N$_7$ of imidazole ring, and nitrogen N$_1$ of pyrimidine ring. The differences observed in the $T_{2p}^{-1}$ values for $H_8$ and $H_2$ protons may indicate different distribution of the two species. However, these differences may originate from the different scalar contributions to the nuclear relaxation rates $T_{2M}^{-1}$ of these protons as the ratio $T_{1p}/T_{2p}$ is 2.08 and 3.36 for $H_8$ and $H_2$, respectively ($p = 2.5 x 10^{-5}$). These values are consistent with appreciable contributions of both the dipolar and scalar terms to $T_{2p}^{-1}$. Knowing that $T_{1p}^{-1}$ equals 1.041 s$^{-1}$ and 1.2 s$^{-1}$ for $H_2$ and $H_8$, respectively, one can calculate.

![Fig. 3](image_url)

Fig. 3. The effect of Cu(II) on the $^{31}$P transverse relaxation rate of the $^{31}$P nucleus of IMP. $p^2$H = 7.4 ± 0.2 at 29°C. The IMP concentration is 0.30 M.
the scalar contribution to $T_{2M}^{-1}$ of these protons by multiplying these values by 7/6. Hence, the dipolar term in the Solomon-Bloembergen Eq. (A6) is 1.22 s$^{-1}$ and 1.4 s$^{-1}$ for H$_2$ and H$_8$ protons, respectively. This means that scalar term contributes 65% to the nuclear relaxation rate $T_{2M}^{-1}$ of H$_2$ proton and 44% to $T_{2M}^{-1}$ of H$_8$, and explains the observed differences in the $T_{2p}^{-1}$ values for H$_2$ and H$_8$.

The effect of Cu(II) on the $^1$H longitudinal relaxation rates of the heterocyclic protons of IMP is shown in Fig. 5. The longitudinal relaxation
rates of $H_2$ and $H_8$ protons increased very similarly as concentration of Cu(II) was raised. Since the distance from metal ion to $H_8$ (binding at $N_7$) and the distance from metal ion to $H_2$ (binding at $N_1$) is nearly equal, the similar values of $T_{1p}^{-1}$ for these protons indicate that the distribution of the species is nearly equal (assuming the same dipolar correlation time for $H_2$ and $H_8$).

**Temperature-dependence studies**

The plot of $T_{2p}$ as a function of the reciprocal of the absolute temperature for $^{31}$P resonance of IMP is shown in Fig. 6. No shifts in the phosphorus resonance position could be observed.

As the temperature of the Cu(II)-IMP solution was raised, the line broadened at first but later the trend was reversed, and the increase in temperature resulted in further narrowing of the line.

![Graph showing temperature dependence of P/T2p](image)

**Fig. 6.** The temperature dependence of $pT_{2p}$ of the $^{31}$P nucleus of IMP. $p^2H = 7.4 \pm 0.2$. The IMP concentration is 0.30 M, $p = [Cu]/[IMP] = 5 \times 10^{-5}$.

The value of $\tau_M = 1 \times 10^{-6}$ s was obtained from the data (100% binding to the phosphate was assumed) at the lowest temperature used (302 K).

At low temperature i.e., below 320 K we are in the range where $npT_{2p} = \tau_M$ and, as would be expected, $\tau_M$ decreases with increasing temperature.

For the analysis of the NMR results to be discussed below, a value for the electron spin relaxation time ($\tau_S$) must be assumed (strictly speaking there is no single $\tau_S$ for Cu(II) but rather several $\tau_S$ values). To the extent that $\tau_S$ of Cu(II) in the Cu(II)-IMP complex is the same as in the [Cu(II)·(H$_2$O)$_6$] complex ($\tau_S = 1.43 \times 10^{-9}$ s; Poupko & Luz, 1972) we can predict $A/h$ for the $^{31}$P resonance in IMP with the same high accuracy.
At high temperatures the line width for the Cu(II)-IMP complex is determined by $T_{2M}$. From this and Eq. (A6) a value of $A/h = 8.7 \times 10^6$ Hz was derived, assuming 100% scalar contribution to the transverse relaxation rate of $^{31}$P. The $A/h$ value observed for the interaction of Cu(II) with $^{31}$P nucleus of IMP is of the same order of magnitude as those observed between Cu(II) with $^{17}$O of H$_2$O (Swift & Connick, 1962; Poupko & Luz, 1972) and $^{31}$P in ATP (Sternlicht et al., 1965).

A temperature study of $T_{2p}$ of the base $^1$H resonances of IMP in the presence of Cu(II) ions was carried out at 80 MHz over the temperature range 299 - 358°C (Fig. 7). As seen from Eq. (A4), the temperature dependence of $T_{2p}$ is determined by the temperature dependence of the average lifetime of a molecule coordinated at any site ($\tau_M$) and the relaxation time of the nuclei when the molecule is coordinated to the metal ion ($T_{2M}$). For both protons, the data show a region of negative slope. This indicates that $T_{2p}^{-1}$ is controlled by the nuclear relaxation rate $T_{2M}^{-1}$. For these protons, lower temperatures would have to be attained to permit observation of the region where the relaxation is determined by the rate of the nucleotide-exchange process. From the value of $T_{2p}$ for H$_8$ at the lowest temperature shown in Fig. 7 an upper limit was calculated for $\tau_M$. This value is $\tau_M = np \cdot T_{2p} = 5 \times 10^{-6}$s. Temperature dependence of $T_{4p}$ for the H$_2$ and H$_8$ protons at 80 MHz were measured over the range 299 - 341°C. Figure 8 shows the temperature dependence of $T_{1p}^{-1}$ from which an activation energy of $\sim 8$ kcal/mol (a mean value from activation energy of 9 kcal/mol and 7 kcal/mol for H$_8$ and H$_2$, respectively) was obtained.

Bernheim et al. (1959) have calculated the activation energy (i.e. effective barriers to tumbling) of 2.6 kcal/mol for [Cu(H$_2$O)$_6$]$^{2+}$. Cox (1966) also found that the activation energy increased with the size of copper(II) complexes. The IMP-Cu-aquo species which is the predominant complex under the experimental conditions used, is larger than the hexa-aquo-copper(II) ion; the activation energy of 8 kcal/mol is, however, much too high for the tumbling of the complex. Presumably the dissociation constant of the
Cu-IMP complex may also be decreasing with increasing temperature to yield so large an effect.

No scalar shifts were observed in any of the IMP nuclei, under the conditions used ([IMP] ≫ [Cu(II)]). It is possible, however, to estimate the value of hyperfine interaction constant $A$ from the $T_{2p}^{-1}$ results knowing that the transverse relaxation rate of $H_8$ is in $44\%_o$ controlled by the scalar hyperfine interactions and under conditions of fast exchange is given by the equation:

$$T_{2p}^{-1} = np(T_{2M})^{-1} = \frac{np}{3} \frac{S(S+1)}{\hbar^2} A^2 \tau_e$$  \hspace{1cm} (6)

Hence, a value of $2.5 \times 10^6$ Hz for $A/h$ is estimated. This value should be compared with that of $3.6 \times 10^6$ Hz calculated in a similar way from the data for the $H_2$ proton. Since the transferred hyperfine interaction constant $A$ is a measure of covalency, the ratio of $A_{H_2}/A_{H_8}$ suggests that the Cu(II)-N$_1$ bond is more covalent than the Cu(II)-N$_7$ one.

The line widths of the $^{13}$C resonance of IMP in the presence of Cu(II) ions were measured only at some temperature values (above $37^\circ$C), hence no quantitative conclusions can be drawn from these data. It is important, however, that the temperature behaviour for the base carbon nuclei line widths is similar — $T_{2p}^{-1}$ values decrease with increasing temperature. This indicates that the fast exchange limit is obtained in these cases and the relaxation processes are dominated by the $T_{2M}^{-1}$ term in Eq. (A4).

Values of the $^{13}$C longitudinal and transverse relaxation rates obtained at 20 MHz are tabulated as $T_{1p}^{-1}$ and $T_{2p}^{-1}$ in Table 2. The high ratio of $T_{1p}/T_{2p}$ and the lack of any frequency dependence of the line widths indicates that a scalar term predominates in the equations for $T_{2p}^{-1}$ for carbons C$_4$, C$_5$, and C$_8$. The ratio $T_{1p}/T_{2p} = 4$ for carbon C$_6$ is consistent with $70\%_o$ contribution of the scalar mechanism to the relaxation of this carbon. The C$_4$ carbon atom is of special interest. It is located far from the copper(II) ion, however, as seen from Table 2, the ratio $T_{1p}/T_{2p}$ for
Table 2

Summary of $^{13}$C $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values (s$^{-1}$) for Cu(II)-IMP solutions

Experiments were carried out in $^2$H$_2$O at p$\text{H}$ 7.4 at 37 C. [IMP] = 0.4 M: $T_{1p}^{-1}$ values are normalized to a [Cu]/[IMP] ratio of $6.25 \times 10^{-3}$, however, $T_{1p}^{-1}$ values for C$_4$, C$_5$, and C$_6$ carbons were taken at [Cu]/[IMP] = $2.5 \times 10^{-5}$.

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<th>Carbon:</th>
<th>C$_3$</th>
<th>C$_4$</th>
<th>C$_5$</th>
<th>C$_6$</th>
<th>C$_7$</th>
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<td>$T_{10}^{-1}$</td>
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<td>0.077</td>
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<td>1.410</td>
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<td>$T_{20}^{-1}$</td>
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<td>4.06</td>
<td>5.94</td>
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<td>23.80</td>
<td>29.6</td>
<td>3.76</td>
<td>14.3</td>
</tr>
<tr>
<td>$T_{1p}/T_{2p}$</td>
<td>0.6</td>
<td>65</td>
<td>47</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

This carbon is the highest. This indicates that there is a significant amount of unpaired spin density that is transferred from the metal ion to this atom, and hence the copper(II) ion is directly bonded to the ring (Swift, 1973; Dwek, 1973). Indeed, theoretical CNDO/2 calculations (Maskos, unpublished results) indicate that there is 0.045 of the unpaired spin density of copper(II) ion located at the C$_4$ atom position.

In terms of the Solomon-Bloembergen equations, these data indicate that $T_{1p}$ is dipolar in nature while the hyperfine term controls $T_{2p}$ in the fast exchange region.

The broadening of the C$_4$ line was observed also in all purine nucleosides and nucleotides previously studied (Maskos, 1978a-d) with the exception of 7-deazaadenosine, an adenosine analogue, where no broadening of the C$_4$ signal by copper was observed (Maskos, 1978d). These results support the direct copper(II) ion binding to the N$_7$ nitrogen of purine derivatives.

Since the dipolar term contributes insignificantly to the transverse relaxation rates of the carbon atoms one can neglect this contribution and estimate the values of hyperfine interaction constant $A/h$ from the $T_{2p}^{-1}$ results. These values, and $^1$H, $^{31}$P values are listed in Table 3.

Table 3

Scalar coupling constants for the nuclei of IMP

Value of $\tau_0 = \tau_s = 1.43 \times 10^{-9}$ s has been assumed for calculation.

<table>
<thead>
<tr>
<th>Nucleus:</th>
<th>$^{31}$P</th>
<th>H$_3$</th>
<th>H$_8$</th>
<th>C$_4$</th>
<th>C$_5$</th>
<th>C$_6$</th>
<th>C$_7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A/h$ (Hz) $\times 10^{-6}$</td>
<td>8.7</td>
<td>3.6</td>
<td>2.5</td>
<td>7.3</td>
<td>8.2</td>
<td>2.4</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The larger values of the coupling constant for $^{31}$P, and $^{13}$C, as compared to those of the protons, result in an enhancement of the scalar coupling relaxation.

APPENDIX

The theory of the effects of paramagnetic ions on the NMR spectra has been described elsewhere (Swift & Connick, 1962; Dwek, 1973; Swift,
1973; James, 1975). The following concepts and equations are essential for the present work.

For a two-component case, \( a \leftrightarrow b \), with \([a] \gg [b]\), and for the special case where the resonance frequency of the nucleus is not detectably shifted, the Swift & Connick’s (1962) equations are reduced to

\[
\frac{1}{T_2} = \frac{1}{T_{2a} + \frac{\tau_a^{-1} \cdot \tau_b}{T_{2b} + \tau_b}} = \frac{1}{T_{2a} + \frac{[b]/[a]}{T_{2b} + \tau_b}} = \frac{1}{T_{2a} + \frac{P_b}{T_{2b} + \tau_b}} \tag{A1}
\]

where \( T_{2a} \) is the transverse relaxation time for the free ligand nucleus; \( T_{2b} \) is the transverse relaxation time for the bound ligand nucleus; \( \tau_b \) is the lifetime of the bonded ligand; \( P_b \) is the amount of the complexed ligand; \( \tau_a \) is the lifetime of \( a \rightarrow b \) exchange.

The analogous equation for \( T_1 \) (Luz & Meiboom, 1964) is

\[
\frac{1}{T_1} = \frac{1}{T_{1a}} + \frac{P_b}{T_{1b} + \tau_b} \tag{A2}
\]

From Eq. (A2) we obtain the paramagnetic contribution to the longitudinal relaxation rate:

\[
\frac{1}{T_{1p}} = \left( \frac{1}{T_1} \right)_\text{obs} - \left( \frac{1}{T_1} \right)_0 = \frac{np}{T_{1M} + \tau_M} \tag{A3}
\]

where \((1/T_1)_\text{obs}\) and \((1/T_1)_0\) are the experimentally observed relaxation rates in analogous solutions in the presence and absence, respectively, of the paramagnetic metal ion. \( T_{1M} \) is the longitudinal relaxation time for a nucleus in the coordination sphere of the paramagnetic ion, \( \tau_M \) is the time the nucleus spends in the sphere of influence of the paramagnetic ion. If the nucleus is a part of a particular ligand coordinated to \( M \), \( n \) is the number of these ligands in one complex; \( p \) is the concentration ratio of paramagnetic ion to total ligand in solution.

For several paramagnetic ions having long electron spin relaxation times (\( \tau_s \), e.g., Cu(II) or Mn(II)), an equation for the paramagnetic contribution to the transverse relaxation rate, similar to Eq. (A3), may be obtained from Eq. (A1):

\[
\frac{1}{T_{2p}} = \left( \frac{1}{T_2} \right)_\text{obs} - \left( \frac{1}{T_2} \right)_0 = \frac{np}{T_{2M} + \tau_M} \tag{A4}
\]

The paramagnetic contributions to the \( T_1 \) and \( T_2 \) relaxation times of a spin 1/2 nucleus with gyromagnetic ratio \( \gamma \) perturbed by a paramagnetic ion are given by Solomon & Bloembergen (1956):

\[
\frac{1}{T_{1M}} = \frac{2}{15} \frac{S (S+1) \gamma^2 g^2 \cdot \beta^2}{r^6} \cdot \left( \frac{3\tau_c}{1 + \omega_S^2 \cdot \tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2 \cdot \tau_c^2} \right) + \frac{2}{3} \frac{S (S+1) A^2}{\hbar^2} \left( \frac{\tau_e}{1 + \omega_S^2 \cdot \tau_e^2} \right) \tag{A5}
\]
\[
\frac{1}{T_{2M}} = \frac{1}{15} \frac{S(S+1) \gamma^2 g^2 \beta^2}{r^6} \left( 4\tau_e + \frac{3\tau_e}{1+\omega_J\tau_e^2} + \frac{13\tau_e}{1+\omega_S\tau_e^2} \right) + \frac{1}{3} \frac{S(S+1) A^2}{\hbar^2} \left( \tau_e + \frac{\tau_e}{1+\omega_S^2 \tau_e^2} \right)
\]

where \(S\) is the electron spin quantum number; \(g\) is the electronic \(g\) factor \((g = 2.111;\ \text{Maskos, 1974})\); \(\beta\) is the Bohr magneton; \(\omega_J\) and \(\omega_S\) are the Larmor angular precession frequencies for the nuclear spins and electron spins, respectively; \(r\) is the ion-nucleus distance; \(A\) is the hyperfine coupling constant; \(\tau_e\) and \(\tau_s\) are the correlation times for the dipolar and scalar interactions, respectively.

In both Eqs. (A5) and (A6), the first term represents the dipolar and the second the scalar contributions to the relaxation rates.

The correlation times are given by
\[
1/\tau_e = 1/\tau_R + 1/\tau_S + 1/\tau_M
\]
and
\[
1/\tau_s = 1/\tau_S + 1/\tau_M
\]
where \(\tau_R\) is the rotational correlation time; \(\tau_S\) is the electron spin relaxation time; \(\tau_M\) is the mean lifetime of a nucleus in the sphere of influence of the paramagnetic ion.

For small molecules coordinated to Cu(II), the rotational correlation time \(\tau_R\) is the shortest correlation time and is therefore the dominant correlation time in Eq. (A7). For complexes of Cu(II) with most ligands the inverse scalar correlation time, \(\tau_s^{-1}\), becomes nearly equal to \(\tau_s^{-1}\), rather than to \(\tau_M^{-1}\). As the \(\tau_s\) for Cu(II) is long, so for reasonably large frequency values, \(\omega_S^2 \tau_e^2 \gg 1\), and the hyperfine term \([i.e.,\ the\ second\ term\ in\ Eq.\ (A5)]\) will not contribute appreciably to \(1/T_{1M}\). Because of the form of Eq. (A6), however, the hyperfine term may contribute to \(1/T_{2M}\). The magnitude of the contribution of the hyperfine term depends on the hyperfine coupling constant \(A\).

Hence, the corresponding Solomon-Bloembergen equation for the inverse longitudinal relaxation time for the nuclei of ligands bound to copper ions is:
\[
1/T_{1M} = \frac{1}{15} S(S+1) \gamma^2 g^2 \beta^2 \cdot 6 \cdot \tau_e \cdot r^{-6} = a \cdot 6 \cdot \tau_e \cdot r^{-6}
\]

where
\[
a = \frac{1}{15} S(S+1) \gamma^2 g^2 \beta^2 = 1.375 \times 10^{-32} \text{ cm}^6 \text{ s}^{-2}
\]

REFERENCES


Berger, N. A. & Eichhorn, G. L. (1971a) Interaction of metal ions with polynucleotides and related compounds. XIV. Nuclear magnetic resonance studies of the binding of copper(II) to adenine nucleotides. Biochemistry, 10, 1847 - 1857.

Berger, N. A. & Eichhorn, G. L. (1971b) Interaction of metal ions with polynucleotides and related compounds. XV. Nuclear magnetic resonance studies of the binding of copper(II) to nucleotides and polynucleotides. Biochemistry, 10, 1857 - 1864.


**ODDZIAŁYWANIE JONÓW METALI Z KWASAMI NUKLEINOWYMI BADANIE UKŁADU MIEDZI(II)-5'-MONOFOSFORAN INOZYNY W ROZTWARZIE METODĄ POMIARU CZASÓW RELAKSACJI JĄDROWEGO REZONANSU MAGNETYCZNEGO**

**Streszczenie**

W roztworze wodnym istnieją w równowadze co najmniej dwa typy kompleksów miedzi (II) z IMP o stosunku metalu do liganda 1:1 (ML) i 1:2 (ML₂), jednakże dominującą formą są kompleksy ML. W kompleksach typu ML₂, których zawartość nie przekracza 18%, występuje trans orientacja cząsteczek nukleotydów. Tworzone są dwa kompleksy typu ML, tj. ML$^{5}$ i ML$^{8}$; stężenie obu kompleksów jest jednakowe. Zarówno dipolowe, jak i skalarnie oddziaływania są odpowiedzialne za relaksację poprzeczną heterocyklicznych protonów IMP w układzie Cu(II)-IMP. Relaksacja poprzeczna fosforu i atomów węgla jest zdominowana oddziaływaniami skalarnymi.

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