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Cupric Ion–Adenosine Triphosphate System. Proton Magnetic Resonance Line-Broadening Studies†

Isaac Feldman* and Victorio Wee‡

ABSTRACT: An investigation of Cu^{2+}-induced broadening of the H_8 and H_2 signals in the proton magnetic resonance (pmr) spectra of the Cu(II)–ATP and Cu(II)–AMP systems, containing very low Cu^{2+} concentrations ($\leqq 10^{-4}$ M) and very low Cu:nucleotide ratios ($2–4 \times 10^{-4}$) with D_2O as solvent, has been carried out. The pD dependences and concentration dependences of these line broadenings were correlated with the changing distribution of the various species in hydrolytic equilibrium in the ranges pD 4.4–10.4 and 0.02–0.25 M. The results indicate that 1:1 complexation, with backbonding, predominates in the Cu(II)–ATP system at a pD $\leqq$ 5.4, where only unhydrolyzed complexes exist, when the ATP concentration is 0.02 M, but that at 0.25 M ATP a considerable fraction of the backbound unhydrolyzed complexes have 1:2 stoichiometry because of ring stacking of the two adenine groups. Only 1:1 stoichiometry seems to prevail for the hydrolyzed species, *i.e.*, $CuATP(OD)^{3-}$ and $CuATP(OD)_2^{4-}$, even at the higher nucleotide concentration. Although backbonding is evident for the monohydroxy complex, it is relatively insignificant for the dihydroxy monomer. Since there should be even less tendency for backbonding in the diol-dimer, $[CuATP(OH)]_2^{6-}$, than in $CuATP(OH)_2^{4-}$, because of the tendency for adenine-ring stacking in the dimer, we believe that these results satisfy one of the requirements of the recent theory proposed by Feldman (*Jerusalem Symp. Quan. Chem. Biochem. IV*, 528 (1972)) for the mechanism of metal ion catalysis of ATP dephosphorylation, namely, that Cu^{2+} is *not* bound to the adenine group of the active species.

Because of the very great catalytic effect of the cupric ion on the nonenzymatic dephosphorylation of adenosine 5′-triphosphate (ATP) (Tetas and Lowenstein, 1963), the Cu(II)–ATP system has been investigated in many laboratories. Spiro *et al.* (1968) concluded that the ground-state active species for this reaction is the diolated dimer, $ATPCu(OH)_2$-CuATP, which had previously been shown by Taqui Khan and Martell (1962a) to be coexistent in the pH 5–9 region with several monomeric complex species, $CuATP^{2-}$, $CuATP(OH)^{3-}$, and $CuATP(OH)_2^{4-}$. Claims that Cu^{2+} is attached to the adenine moiety in the active species (Schneider and Brintzinger, 1964; Miller and Westheimer, 1966) have been disputed by Feldman (1972). Previous demonstrations of the simple fact that Cu^{2+} changes the infrared (ir) spectrum (Schneider and Brintzinger, 1964) and the proton magnetic resonance (pmr) spectra (Eichhorn *et al.*, 1966) of adenine nucleotides do not relate to this particular question, because of the multispecies composition of the system.

We have carried out a study of the pH dependence of the Cu^{2+}-induced broadening of the pmr signals of the adenine moiety of ATP, since complexation of the paramagnetic Cu^{2+} ion by the adenine moiety should be detectable by such line broadening (Swift and Connick, 1962) and since this

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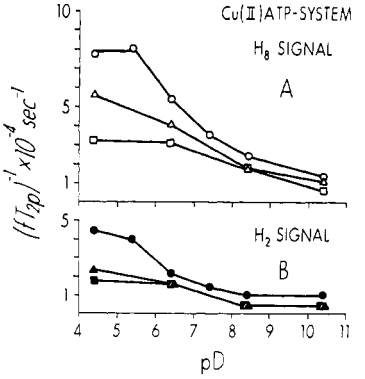

FIGURE 1: pD dependence of normalized Cu^{2+}-induced broadening of ATP H_8 and H_2 pmr signals. Circles, 0.25 M ATP ($f = 2 \times 10^{-4}$); triangles, 0.10 M ATP ($f = 4 \times 10^{-4}$); squares, 0.02 M ATP ($f = 4 \times 10^{-4}$); temperature, 27°.

broadening effect should be related to the pH-dependent distribution of the various ground-state species.

Our preliminary experiments showed that, because of signal overlapping, line broadening small enough to be accurately measurable could be obtained only by using 10^{-4} M Cu^{2+} or less. Unfortunately, at such a low Cu^{2+} concentration the dimeric species is absent. Nevertheless, a pmr study of the Cu(II)–ATP system should be informative concerning interaction between Cu^{2+} and the adenine moiety in the ground state dimer, since each of the two Cu^{2+} atoms in the diol–dimer, at higher Cu^{2+} concentration, and the Cu^{2+} atom in the dihydroxy monomer, at lower Cu^{2+} concentration, should be bonded in a similar coplanar[1] fashion to the same four ligands, namely, β- and γ-phosphoryl oxygen atoms (Cohn and Hughes, 1962) and two hydroxyl groups (Taqui Khan and Martell, 1962a).

Experimental Section

Pmr spectra were recorded with a Jeol 4H-100 (100-MHz) spectrometer kept in a 24° constant-temperature room, using highest quality grade (Wilmad Glass Co.) nuclear magnetic resonance (nmr) tubes. Probe temperature was 27°. A Jeol RA-1 spectrum accumulater was used to increase signal-to-noise ratio when desired.

Aqueous adenine nucleotide solutions at pH 7 were freed of paramagnetic impurities by passing them through a Chelex-100 resin bed, *which had previously been washed with* 10^{-5} M *HCl until the eluate reached pH 5.* This procedure prevented alkaline hydrolysis of ATP during the treatment! As described previously (Wee *et al.*, 1974), these solutions were then mixed with aqueous $Cu(NO_3)_2$, pH was adjusted, and the mixture was alternately lyophilized and dissolved in D_2O three times, with 100% D_2O being used for the final solution. Immediately after each spectrum was taken, the pD (*i.e.*, Beckman Research pH meter reading + 0.4 (Glasoe and Long, 1960)) was measured and the 260-nm absorbance was obtained to check the final nucleotide concentration.

Resolution of the spectra in the competition studies into their overlapping ATP and AMP components was accomplished by computer using the assumption that the component bands are Lorentzian line shapes and additive.

[1] Because of severe Jahn-Teller distortion, four coplanar bonds are so much shorter and stronger than the other two trans bonds in six-coordinated Cu(II) complexes that they resemble square-planar complexes much more closely than octahedral ones (Basolo and Pearson, 1968; Cotton and Wilkinson, 1972).

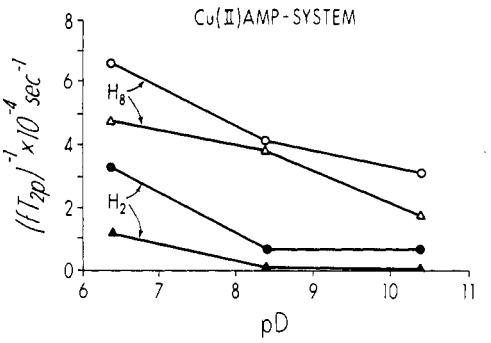

FIGURE 2: pD dependence of normalized Cu^{2+}-induced broadening of AMP H_8 and H_2 pmr signals. Circles, 0.25 M AMP; triangles, 0.1 M ATP; $f = 4 \times 10^{-4}$ in each case; temperature, 27°.

Results

The Cu^{2+} ion causes broadening of the signals of both adenine protons, H_8 and H_2, in the pmr spectra of the Cu(II)–ATP and Cu(II)–AMP systems containing 10^{-4} M, or less, Cu^{2+} in a pH- and concentration-dependent manner without producing detectable change in the chemical shifts.

Metal-induced line broadenings are frequently expressed as normalized values, $\pi W_p/f$, or its equivalent $(fT_{2p})^{-1}$, where W_p is the difference between the line widths in hertz in presence and in absence of metal ion, f is the ratio of the total metal concentration, $[M]_t$, to total ATP concentration, $[ATP]_t$, and $T_{2p}^{-1} = T_2^{-1} - T_{2a}^{-1}$. T_2 and T_{2a} are the transverse relaxation times in presence and in absence of metal, respectively.

The pD dependences of the normalized Cu^{2+}-induced broadening of the ATP H_8 and H_2 signals are shown in Figure 1 for three ATP concentrations, 0.25 M ($f = 2 \times 10^{-4}$), 0.10 M ($f = 4 \times 10^{-4}$), and 0.02 M ($f = 4 \times 10^{-4}$). The pD dependences of the Cu^{2+}-induced broadenings of AMP H_8 and H_2 signals are shown in Figure 2. Like the Cu(II)–ATP curves, the Cu(II)–AMP curves decrease considerably above pD 6.4. It is also seen that the AMP H_8 signal is broadened about twice as much as the ATP H_8 signal at pD 8.4 and 10.4, but only about 20% more at pD 6.4. Because of solubilizing difficulty the Cu(II)–AMP system was not studied at pD 5.4 or below.

The results of an ATP–AMP competition study are presented in Figure 3. Figure 3A,B contains the normalized

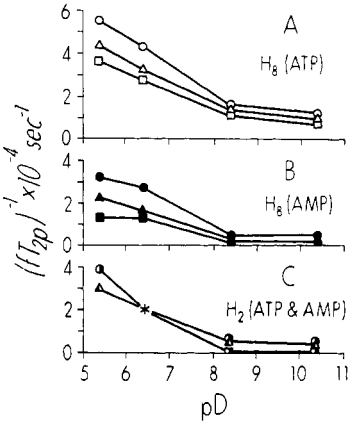

FIGURE 3: Equimolar ATP–AMP competition study of Cu^{2+}-induced broadening of H_8 and H_2 pmr signals; temperature, 27°. Concentration of *each* nucleotide: circles, 0.25 M; triangles, 0.1 M; squares, 0.02 M. Cu^{2+}/ATP molar ratio was 4×10^{-4} in each case. The asterisk represents six superimposed points, *i.e.*, for each nucleotide at each of three concentrations. Each of the other points in C represents two superimposed points, *i.e.*, for each nucleotide at the indicated pD's.

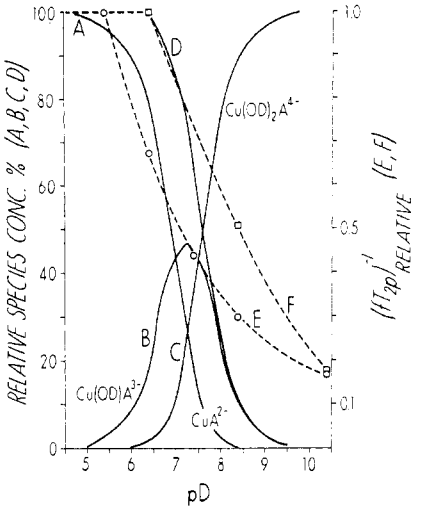

FIGURE 4: Comparison of relative normalized Cu^{2+}-induced broadening of ATP H_8 pmr signal with distribution of 1:1 Cu(II)–ATP complex species over pD range 5.4–10.4. Curves A, B, and C represent relative concentrations of $CuATP^{2-}$, $Cu(OD)ATP^{3-}$, and $Cu(OD)_2ATP^{4-}$, respectively, at $[Cu]_t = 10^{-4}$ M or less *if* only 1:1 complexes are considered. The species distribution was calculated after multiplying the aqueous stability constants of Taqui Khan and Martell (1962a) by 0.31, a deuterium isotope correction factor (Kakihana *et al.*, 1970). Curve D is summation of curves A and B. Curves E and F represent relative normalized broadening, $(fT_{2p})_{rel}^{-1}$, when $[ATP]_t$ is 0.25 and 0.02 M, respectively. $(fT_{2p})_{rel}^{-1}$ was obtained by dividing $(fT_{2p})^{-1}$ for a given pD by $(fT_{2p})^{-1}$ at pD 4.4, *at which point there is no hydrolysis of the copper.*

broadening values of the H_8 signals of ATP and AMP, respectively, for spectra of several equimolar ATP–AMP mixtures. Each H_8 curve descends rapidly between pD 5.4 and 8.4, but little change, if any, is seen between pD 8.4 and 10.4. This observation is similar to the H_8 behavior for the individual nucleotide spectra. The broadening in this equimolar competition study was larger for the ATP H_8 signal than for the AMP H_8 signal at each concentration and pD studied, in contrast to the broadenings in the individual nucleotide spectra. From Figure 3C it appears that the H_2 signals of the two nucleotides were equally broadened in the equimolar competition experiment at each pD between 5.4 and 10.4.

Discussion

Any investigation of the Cu(II)–ATP system must take into account its great complexity. In addition to the pH-dependent and concentration-dependent hydrolytic equilibria (Taqui Khan and Martell, 1962a), there is a further complication from the possibility that, unlike Mn^{2+}, the Cu^{2+} ion might bind to an adenine nitrogen atom directly, or, like Mn^{2+} (Glassman *et al.*, 1971), indirectly *via* an intervening H_2O molecule which is hydrogen bonded to an adenine nitrogen atom, *i.e.*, a $Cu–OH_2\cdots N$ bond. In either case, broadening of an adenine proton signal would result. It should be noted, however, that all reports of *direct* bonding of Cu^{2+} to a nitrogen atom of an adenine group have involved nonaqueous solvents or the solid state (Harkins and Freiser, 1958; Sletten, 1972; Goodgame and Price, 1968; Berger and Eichhorn, 1971). There may also be an equilibrium between complexes with different metal:ligand ratios, *e.g.*, 1:1 and 1:2 as in the Mn(II)–ATP system (Sternlicht *et al.*, 1968; Wee *et al.*, 1974) when $[M]_t$ is very low ($<10^{-3}$ M) and $[ATP]_t$ is relatively high ($\sim$0.3 M). Such a system might, therefore, contain Cu^{2+} bound both directly and indirectly to the adenine.

These complications preclude any quantitative, or even semiquantitative calculations from the pmr data of this system, except in certain specific cases where it is probable that one complex overwhelmingly predominates, *e.g.*, $CuATP^{2-}$ at pH <5 and $CuATP(OH)_2^{4-}$ at pH >9 (Taqui Khan and Martell, 1962a). Nevertheless, one can draw inferences and make qualitative conclusions concerning the Cu(II)–ATP system by utilizing the more quantitative information obtained previously (Wee *et al.*, 1974) for the Mn(II)–ATP system, *i.e.*, by noting similarities and differences between the pmr results obtained for the two systems and correlating these with the hydrolytic reactions in the Cu(II)–ATP system.

For instance, whereas there is negligible pD dependence of previous broadenings for the Mn(II)–ATP system above pD 6.4 when $[ATP]_t$ is 0.25 M, and above pD 4.4 when $[ATP]_t$ is 0.02 or 0.10 M, a four- to fivefold decrease in the Cu(II)–ATP system between pD 4.4 and 10.4 at each of these concentrations is seen in Figure 1. Undoubtedly, this difference is related to the fact that Cu^{2+} hydrolyzes strongly, even when in a complex, whereas Mn^{2+} does not do so.

This relationship is brought out in Figure 4, in which the relative line broadenings $(fT_{2p})_{rel}^{-1}$, for each of two ATP concentrations, 0.02 and 0.25 M, are compared with the pD-dependent species distribution which would be expected *if* only 1:1 complexes were present. $(fT_{2p})_{rel}^{-1}$ is defined here as the $(fT_{2p})^{-1}$ value for a given pD divided by the $(fT_{2p})^{-1}$ value at the pD, 4.4, *where no hydrolysis of the complexed copper occurs.* The species distributions in D_2O were estimated by assuming the deuterium isotope effect for the monohydrolysis of $CuATP^{2-}$ and $CuATP(OH)^{3-}$ to be the same as that found by Kakihana *et al.* (1970) for the Cu^{2+} ion.

Curves A and E in this figure begin to decrease significantly near the same pD, 5.4, while curves D and F both start a sharp descent at pD 6.4. That is, at the higher $[ATP]_t$, 0.25 M, line broadening begins to decrease near the pD where the first OD^- group attaches to the metal of a 1:1 complex, whereas at a tenfold lower $[ATP]_t$ the broadening value does not fall until the metal binds a second OD^- group. As explained below, this comparison strongly suggests that when $[ATP]_t$ is only 0.02 M the complexation is primarily 1:1 up to pD 6.4, but that at the tenfold higher $[ATP]_t$ some 1:2 complexation occurs at this pD and below, although not necessarily to the exclusion of some coexisting 1:1 complex.

The Cu^{2+} ion has four coplanar strong-binding sites,[1] and only two of these are occupied by oxygen atoms of the triphosphate chain of ATP, these being β and γ oxygens (Cohn and Hughes, 1962; Sternlicht *et al.*, 1965). Consequently, the ligation of the Cu^{2+} atom of a 1:1 complex by one OD^- group would still leave one strong-binding site available for attachment of an adenine nitrogen atom, either directly or indirectly *via* an intervening D_2O molecule. Thus, monohydroxylation of a 1:1 complex need not lead to decreased broadening of the adenine signals. On the other hand, monohydroxylation of a 1:2 complex would necessarily cause decreased broadening, regardless of whether the adenine group is bound directly or indirectly in the unhydrolyzed complex. This would occur in the former case, because at least one of the magnetically coupled protons would, of necessity, move further away from the metal. In the latter case also, the effect of increased metal to proton distance should be seen, *unless* the water bridge $Cu–OD_2\cdots N$ were replaced by a hydroxyl bridge, $Cu–OD^-\cdots N$, in which case the metal to proton distance would probably be unchanged. In this case, decreased broadening would result from a decrease in the fraction of complexes in the 1:2 form, since the negative charge of the OD^- group

makes it a much poorer proton donor for hydrogen bonding, and therefore a much poorer bridging group, than D_2O (Hamilton and Ibers, 1968).

The broadening of the AMP signals at pD 5.4 and 6.4 in the equimolar competition study (Figure 3) is further evidence for the existence of some 1:2 complexations at these pD's since the relative values of the stability constants, 1.5×10^3 M^{-1} for $CuAMP^-$ and 1.4×10^6 M^{-1} for $CuATP^{2-}$ (Taqui Khan and Martell, 1962a,b) show that Cu(II)–AMP complexes would be negligible in the equimolar mixtures if only 1:1 complexes were present. For the same reason, the backbound[2] ligand of a 1:2 complex should be exclusively ATP even when AMP is present. The apparent equality of the ATP H_2 and AMP H_2 broadening values (Figure 3C) implies that in a 1:2 complex either nucleotide can function as the stackbound[2] ligand with about equal probability and also that the backbound ATP molecule is ligated *via* N_7. However, the ATP N_7 atom can also be the stackbound ligand atom, as can AMP N_7.

The $(fT_{2p})^{-1}$ values for pD 5.4 when each nucleotide is 0.25 M, shown in Figure 3, for the four signals—ATP H_8, AMP H_8, ATP H_2, and AMP H_2—are in the ratio 1.8:1.0:1.2:1.2. Such a small ATP H_8 value relative to the sum of the other three values would result if the exchange of the stackbound ligand of the 1:2 complex with bulk nucleotide is much more rapid than exchange of the backbound ligand. A full rationale for this conclusion was presented previously when we suggested this stepwise ligand exchange mechanism for the Mn(II)–ATP system at pD 5.4, in contrast to a simultaneous exchange of both ligands at higher pD (Wee *et al.*, 1974). True, there is no evidence that the adenine proton line broadenings are dipolar determined in the Cu(II)–ATP system, as has been shown for the Mn(II)–ATP system (Sternlicht *et al.*, 1965), but the following considerations make it seem highly unlikely that these results can be attributed to a scalar coupling.

The Bloembergen–Solomon equation (Bloembergen, 1957; Solomon, 1955), which governs the effect of a paramagnetic metal ion on T_{2M}, the transverse relaxation time of a magnetic nucleus in its vicinity, may be simplified to

$$T_{2m}^{-1} = Cr^{-6}\tau_c + C'A^2\tau_e \qquad (1)$$

where C and C' are constants *for a given metal*, τ_c and τ_e are the correlation times for the anisotropic dipolar interaction and the isotropic hyperfine interaction, respectively, and A is the hyperfine coupling constant. If the unexpectedly small ATP H_8 value in the above ratio of the four broadening values were attributable to the scalar term, $C'A^2\tau_e$, in eq 1, this would imply a larger A value for the magnetic proton in the stackbound ligand than for the magnetic proton in the backbound ligand. This, in turn, would imply the *unlikely* situation where the stackbound ligand of the 1:2 complex is held by direct Cu(II)–N bonds, while the backbound ligand is bound *via* D_2O mediation.

These results and conclusions led to two predictions. First, decreasing the concentrations of the nucleotides in an equimolar competition experiment at pD 5.4 or 6.4 should increase the ratio of the ATP H_8 broadening to the AMP H_8 broadening, $(fT_{2p})_{ATP,H_8}^{-1}/(fT_{2p})_{AMP,H_8}^{-1}$, since the ratio of 1:1 to 1:2 complexes should then increase, and since AMP, in our picture, functions only as a stackbound ligand in the 1:2 complex when both nucleotides are present. In agreement, our results show a 42% increase in the $(fT_{2p})_{ATP,H_8}^{-1}/(fT_{2p})_{AMP,H_8}^{-1}$ ratio at both pD 5.4 and 6.4 when the two nucleotide concentrations were simultaneously decreased from 0.25 to 0.02 M. The smallness of the broadenings at higher pD, especially for the AMP signal, precluded meaningful calculations at higher pD.

Second, increasing the $[AMP]_t/[ATP]_t$ ratio above unity while $[ATP]_t$ and $[Cu]_t$ are kept constant, should increase the normalized broadening of both AMP signals and also the broadening ratios, $(fT_{2p})_{AMP,H_8}^{-1}/(fT_{2p})_{ATP,H_8}^{-1}$ and $(fT_{2p})_{AMP,H_2}^{-1}/(fT_{2p})_{ATP,H_2}^{-1}$. This prediction is made because $[ATP]_t$ governs not only the 1:1 concentration but also the total 1:2 concentration, while the ratio of stackbound AMP to stackbound ATP in 1:2 complexes should be equal to $[AMP]_t/[ATP]_t$, at least approximately. As predicted, when $[AMP]_t$ was raised from 0.1 to 0.4 M, while keeping $[ATP]_t$ at 0.1 M, the normalized broadening of each of the two AMP signals was increased 2.5–3 times at both pD 5.4 and 6.4, which is about midway between the predictable limits,[3] 1.6 and 4.0. Unfortunately, the overlapping of the two H_8 signals and of the two H_2 signals prevents accurate determination of the ATP broadenings when AMP is in excess. The AMP signals could be measured with fair accuracy, however, since the AMP signal is the major constituent in each band.

The very small, but nonzero, value of the broadening of the H_8 signal at pD 10.4, where dihydroxylation is complete, in Figures 1 and 4 is due to an experimental broadening value of only about 0.8 Hz. Since this value is only slightly higher than our experimental error, 0.5 Hz, one can conclude that most of the complexes at this pD are nonbackbound, *i.e.*, most of the metal binds only to the phosphate moiety of the nucleotide. If our high pD broadening value were indeed considered to be sufficiently significant to indicate a slight concentration of backbound dihydroxy complexes, OD^- bridging could be responsible.

The $(fT_{2p})^{-1}$ values for the AMP signals at the higher pD's *i.e.*, 8.4 and 10.4, in the equimolar competition experiment (Figure 3) are experimentally insignificant. This observation is in sharp contrast with the high pD broadening value of the AMP H_8 signals when no ATP is present (Figure 2), which not only is significantly large but is, in fact, larger than the high-pD ATP H_8 broadening value in absence of AMP (Figure 1). This is evidence that there is an insignificant concentration of 1:2 complexes *at the higher pD's*, which is not surprising in view of the weakness of OD^- bridging. That is, because of the primary strong metal–phosphate bonding OD^- bridging might be strong enough to cause a slight bit of backbonding in a 1:1 $CuATP(OH)_2^{4-}$ complex, but it is too weak to hold a stackbound ligand of a 1:2 complex.

[2] The 1:2 structure recommended in our previous paper (Wee *et al.*, 1974) for the Mn(II)–ATP system has the metal atom bound simultaneously to *both* of a pair of stacked adenine groups *via* water bridges, with one of these adenine groups, customarily described as "backbound," being part of a nucleotide molecule whose phosphate chelates the metal. The other, stacked but nonbackbound, adenine group will be designated the "stackbound" ligand in this paper.

[3] The (1:2)/(1:1) concentration ratio should equal $K_{1:2}$ [ATP + AMP] if, as we suggested above and in the previous Mn(II)–ATP paper (Wee *et al.*, 1974), stepwise dissociation occurs at pD's 5.4 and 6.4. Further, ATP and AMP should function with equal probability as the stackbound ligand. Thus, for an improbably large $K_{1:2}$, for which 1:2 complexation is complete, the AMP signal would increase 1.6 times when $[AMP]_t/([AMP]_t + [ATP]_t)$ is increased from 0.5 to 0.8. For an improbably small $K_{1:2}$, the 1:2 concentration would increase proportionally with the increase in *total* nucleotide concentration, *i.e.*, 2.5, and the AMP signal would increase fourfold.

Since the primary bonding structure of the Cu atom in the dihydroxy monomer should be similar to that for each of the two Cu atoms in the diol–dimer, and since the tendency for backbonding should be even less in the dimer than in the monomer, because of adenine-ring stacking in the dimer,[4] we believe that the results of this study satisfy one of the requirements of the recent theory of Feldman (1972) for the mechanism of metal ion catalysis of ATP dephosphorylation, namely, that Cu^{2+} is *not* bound to the adenine moiety of the active species.

Acknowledgment

We are grateful to Dr. William Simon and Miss Ruth Howard of the Biomathematics Department of the University of Rochester School of Medicine and Dentistry for putting a PDP8 computer at our disposal and for writing the SNAP computer program which enabled us to analyze the competition-study spectra.

Added in Proof

Apropos of footnote 1, since this paper was accepted Lewis *et al.* (1974a) have provided X-ray data for the diol dimer, $LCu(OH)_2CuL$, where L is the bidentate ligand, 2-(2-dimethylaminoethyl)pyridine. For each Cu atom, they found an equatorial plane consisting of two hydroxo oxygen atoms, 1.947 and 1.936 Å from the metal, and two nitrogen atoms, 2.017 and 2.056 Å from the metal, while the two axial sites were occupied by perchlorate oxygen atoms 2.716 and 2.782 Å from the metal. In a preceding paper, Lewis and Hodgson (1974b) found a very similar geometry for the monomeric bis complex, $CuL_2(ClO_4)_2$, having the same bidentate ligand.

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[4] Adenine-ring stacking is stronger in the UO_2(VI)–ATP diol–dimer, which is similar in structure to the Cu(II)–ATP diol–dimer, than in an uncomplexed ATP solution (Rich *et al.*, 1970, 1971). Also, like Cu^{2+}, the UO_2^{2+} ion catalyzes the nonenzymatic dephosphorylation of ATP very strongly and produces a sharp maximum in the rate–pH profile of this reaction near pH 5.5 in aqueous solution (Feldman *et al.*, 1967).