ESR and Titrimetric Investigation of ATP*–Copper Solutions

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Aqueous ATP–copper solutions were investigated by ESR spectroscopy and potentiometric titrations in the millimolar concentration range at ATP:Cu ratios from 1:1 to 8:1. It could be proved that from neutral to basic pH $Cu_2(ATP)_2(OH)(5-)$ and $Cu_2(ATP)_2(OH)_2(6-)$ are formed, instead of the commonly assumed $Cu(ATP)(OH)(3-)$ species. Further, a $Cu(ATP)_2(6-)$ complex was found and its stability constant determined. At pH > 10.5 and ATP:Cu ratio greater than 1:1, a stable complex is formed by one Cu(2+) and two ATP molecules. This complex shows an ESR spectrum similar to that of adenosine-copper solutions at pH = 11.3 and can be described formally as $Cu(ATP)_2(OH)(9-)$ or $Cu(ATP)_2(10-)$. The stability constants calculated by both titration and the ESR method show good agreement.

Introduction

Intending to investigate ternary complexes containing Cu(2+) and ATP in dilute aqueous solutions by a recently developed ESR titration method [1], we first had to reinvestigate the complex formation of ATP and Cu(2+) in solution.

Several potentiometric titration studies of ATP and Cu(2+) [2–6] have been performed. Some more recent work deals with ternary complexes of [7–13, 15], partly with those of biogenic amines, ATP and Cu(2+) [7, 11, 12], which have been found in synaptosomes [7].

Further, spectrophotometric titrations [10, 11, 14, 15], NMR [18, 19], IR [20, 27], RAMAN [22], optical rotation [14, 21] and calorimetric [13] measurements have been performed to investigate ATP–Cu solutions. The hydrolysis of ATP catalysed by Cu(2+) has also been thoroughly investigated [15–17].

There remain, however, some important questions: almost all the investigations have been performed with an equivalent molar amount of ATP compared to Cu(2+) or less, and an attempt to evaluate the stability constants for complexes containing two ATP molecules and one metal ion proved unsuccessful by the potentiometric titration method [12]. In addition, the question of whether the hydroxylated species Cu(ATP)(OH)(3–) is a monomer or not has not yet been answered [15]. The ESR titration method used in our work proved useful to answer both questions and a special advantage of this method is that it permits discrimination between complexes containing one or two metal ions [1]. Thus, all stability constants were calculated from the ESR titrations and these results were compared with potentiometric titration data.

Experimental

Materials

Copper was used as $CuCl_2 \cdot 2H_2O$ analytical grade (Mallinckrodt), ATP was obtained from Serva as $Na_2H_2ATP \cdot 3H_2O$ 'puriss'. All solutions were prepared using CO_2 free distilled water. The acid and base used for titration were 'Titrisol' products (Merck).

Potentiometric Titrations

Stock solutions of ATP(4–) were always freshly prepared by rapidly titrating the dissolved $Na_2H_2ATP \cdot 3H_2O$ to the equivalence point. Then an appropriate amount of HCl was added and after the addition of the metal solution the titration was carried out immediately, in order to minimize errors due to dephosphorylation [15]. 50 cm^3 solutions containing $1–2 \cdot 10^{-3}$ *M* Cu(2+) and ATP in a ratio to Cu(2+) of 1:1 to 5:1 were titrated at 20 ± 0.3 °C with 0.05 *N* NaOH at an ionic strength of 0.1 *M* $NaNO_3$ from pH = 4 to 9.

ESR Experiments

The titrations were carried out like the potentiometric titrations but at a concentration of Cu(2+) of $4.9 \cdot 10^{-3}$ *M* in all cases and base concentrations of

*'ATP' denotes adenosine 5'-triphosphate.

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0.2 *N* and 0.5 *N*. Seven titrations with five different ATP:Cu ratios (1:1 to 8:1) were performed. For the ESR measurements, 8 to 15 samples of 0.20 cm^3 per titration were taken from the titration vessel by a syringe. To prevent dephosphorylation the samples were immediately frozen in liquid N_2 and rapidly thawed just before the ESR measurement. The spectra were recorded at 20 °C within two minutes.

Apparatus

For the pH measurements a Schott pH meter CG 803 and an Ingold electrode 104051393 calibrated with standard buffer solutions (Merck) were used. The ESR spectra were recorded on a Varian E 104 spectrometer in tubes with a much smaller diameter (1 mm, Wilmad cat nr 800) than standard ESR tubes in order to reduce the dielectric losses caused by water.

Calculations

All calculations were carried out on the CDC Cyber 74 computer of the University of Innsbruck. 24 to 35 points per potentiometric titration curve and 400 points per ESR spectrum (digitized with a Summagraphics ID 2000, resolution 0.1 mm) were included.

Method

The ESR titration method was used as described in ref. [1] with minor extensions (integration procedure). The main differences from most other ESR investigations in this field are that the spectrum is recorded at room temperature and that the evaluation is focused on quantitative data (peak heights and stability constants calculated therefrom). The basic assumption used in the interpretation is, that the amplitude of the ESR signal is proportional to the concentration of a complex in solution. The spectrum recorded for a metal ligand solution is assumed to be the direct sum of the spectra of all species present. The experiments that justify these assumptions have been described in ref. [1] and were repeated at the beginning of this work. The reproducibility of the ESR spectra of such solutions was found to be in the range of ±1% as in ref. [1].

When the spectra of a series of ESR titrations had been obtained, the calculations of the single species spectra and of the stability constants were performed in the following way. Some (n) spectra are chosen where only m_1 complexes (having different and non-zero ESR spectra) are assumed to occur (certain pH range, only one ligand–metal ratio, *e.g.* 1:1). Then an initial set of pK values is assumed for these m_1 species (the pK values for the ligand itself are known from potentiometric titrations). From the pK values the concentrations of the species are evaluated and finally, a spectrum for each species can be calculated from m_1 of the n spectra by m_1 linear equations for every magnetic field or g value. As the spectra of the assumed single species and their pK values are known, spectra can be calculated for the other $(n - m_1)$ ESR titration points and compared with the experimental spectra. This procedure is repeated with a new set of pK values leading to other spectra, and the comparison of calculated and measured spectra will lead to a new fitting error. The pK values are varied iteratively to obtain the smallest possible fitting error. If a satisfactory fit of calculated to experimental spectra is not obtained, other or further species must be taken into account.

The whole procedure can be expanded to include a wider pH range and more metal–ligand ratios and, if still necessary, additional m_1 complexes can be taken into account and the spectra of these further species calculated until a good fit of calculated to experimental spectra is obtained for all ESR titrations.

In this work m_1 never exceeded a value of 2, indicating that in all cases sets of spectra could be chosen where only one or two further species having non-zero ESR spectra were present.

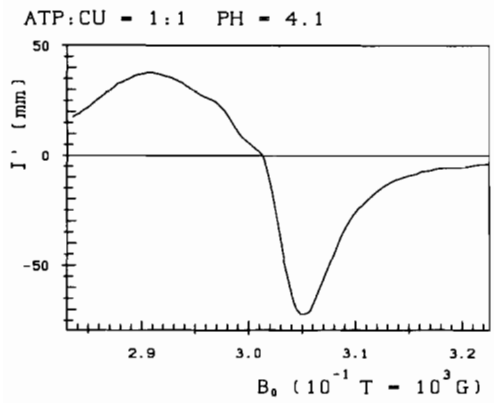
Small complex molecules containing two copper ions show a zero spectrum under the experimental conditions used here, due to the copper–copper interaction [*e.g.* 1, 23, 24]. Therefore a loss of intensity indicates the formation of complex molecules with two copper atoms. Consequently, a twofold integration of the ESR signals should give a direct means of measuring the amount of ESR detectable complex molecules containing one metal ion. Since many digital points per spectrum are available, this double integration can be performed easily and accurately by numerical methods. Unfortunately the error in the recorded spectra leads to a multiple error of the double integral (factor 6 for gaussian functions) and this error increases with the square of the spectral width. Since the spectra of ATP–Cu solutions are comparatively broad, the double integral can be used, therefore, only to examine whether the integrals of the calculated spectra are within the expected range, but not for the calculation of stability constants.

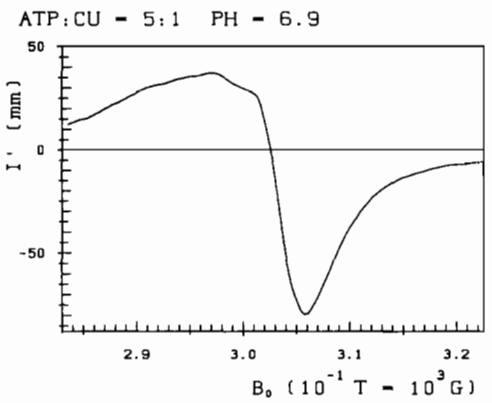
Results and Discussion

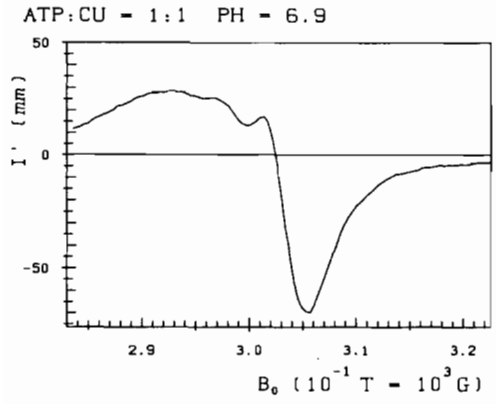
Part A: pH = 4 to 9.5

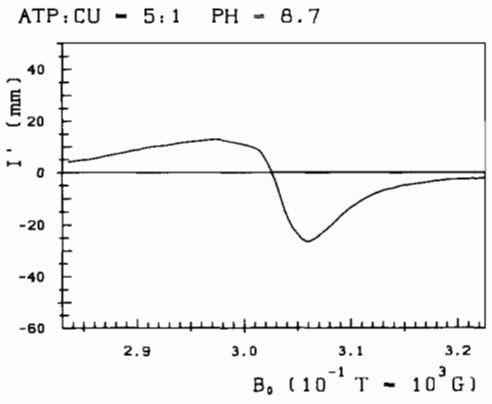
Figure 1 shows the most representative spectra of ATP–Cu solutions during titration.

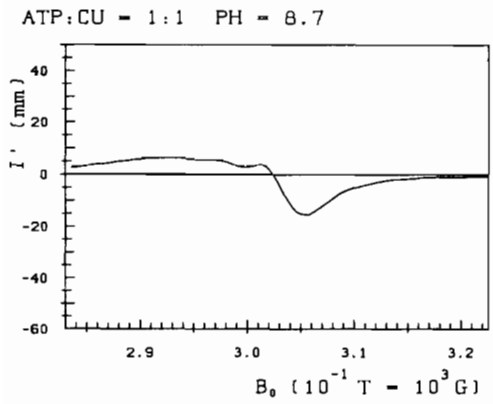
The spectra at acidic and neutral pH seem to be very similar. The experiments showed, however, that the differences between them are reproducible and significant. The spectra at an ATP:Cu ration of 5:1 have a different shape from the spectra at the 1:1 ratio, and the whole spectrum shifts to higher field

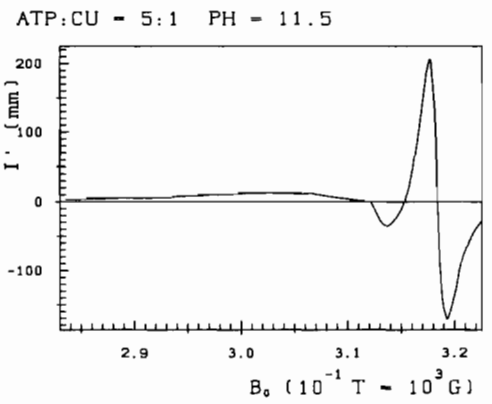












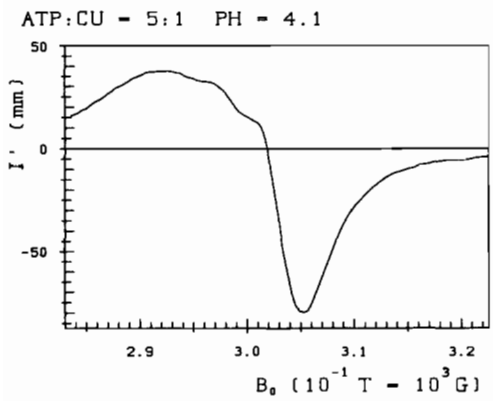


Fig. 1 Most representative spectra of ATP–Cu solutions during titration, concentration of Cu = 0.00490 *M* in all spectra, concentration of ATP is as indicated by the ratio.

TABLE I. Equilibria Discussed in this Work.[a]

1	ATP(4−) + H(+)	→ HATP(3−)
2	HATP(3−) + H(+)	→ H_2ATP(2−)
3	Cu(2+) + HATP(3−)	→ Cu(HATP)(−)
4	Cu(2+) + ATP(4−)	→ Cu(ATP)(2−)
5	Cu(2+) + ATP(4−) + H_2O	→ Cu(ATP)(OH)(3−) + H(+)
6	2Cu(2+) + 2ATP(4−) + H_2O	→ Cu_2(ATP)$_2$(OH)(5−) + H(+)
7	2Cu(2+) + 2ATP(4−) + 2H_2O	→ Cu_2(ATP)$_2$(OH)$_2$(6−) + 2H(+)
8	Cu(2+) + 2ATP(4−)	→ Cu(ATP)$_2$(6−)
9	ATP(4−)	→ ATP(5−) + H(+)
10	Cu(2+) + 2ATP(4−) + H_2O	→ Cu(ATP)$_2$(OH)(9−) + 3H(+)
11	Cu(2+) + 2ATP(4−)	→ Cu(ATP)$_2$(10−) + 4H(+)
12	2Cu(2+) + 2ATP(4−) + nH_2O	→ Cu_2(ATP)$_2$(OH)$_n$(7−) + 3H(+)
13	2Cu(2+) + 2ATP(4−) + nH_2O	→ Cu_2(ATP)$_2$(OH)$_n$(8−) + 4H(+)

[a]n denotes that it was not determined whether H(+) came from metal bound water or from deprotonation of the ATP(4−) molecule.

from acidic to neutral pH. From neutral to basic pH the spectrum decreases with increasing pH and disappears at pH > 9. This is interpreted as the formation of a stable complex with two copper ions (see Method).

The equilibria discussed in this work are shown in Table I. The species commonly assumed to exist at an ATP:Cu ratio of 1:1 in the range of millimolar concentrations are given by eqns. 1–5, Table I (given by representing terms model A, Table II [9, 13, 15, 16]). A comparison of the spectra (Fig. 1) with species distribution diagrams for the ATP:Cu ratio 1:1 from the literature (*e.g.* [9]) shows that the decrease in the spectral intensity from pH = 7 to 9.5 is in agreement with the species concentration of Cu(ATP)(OH)(3−). Consequently, this complex

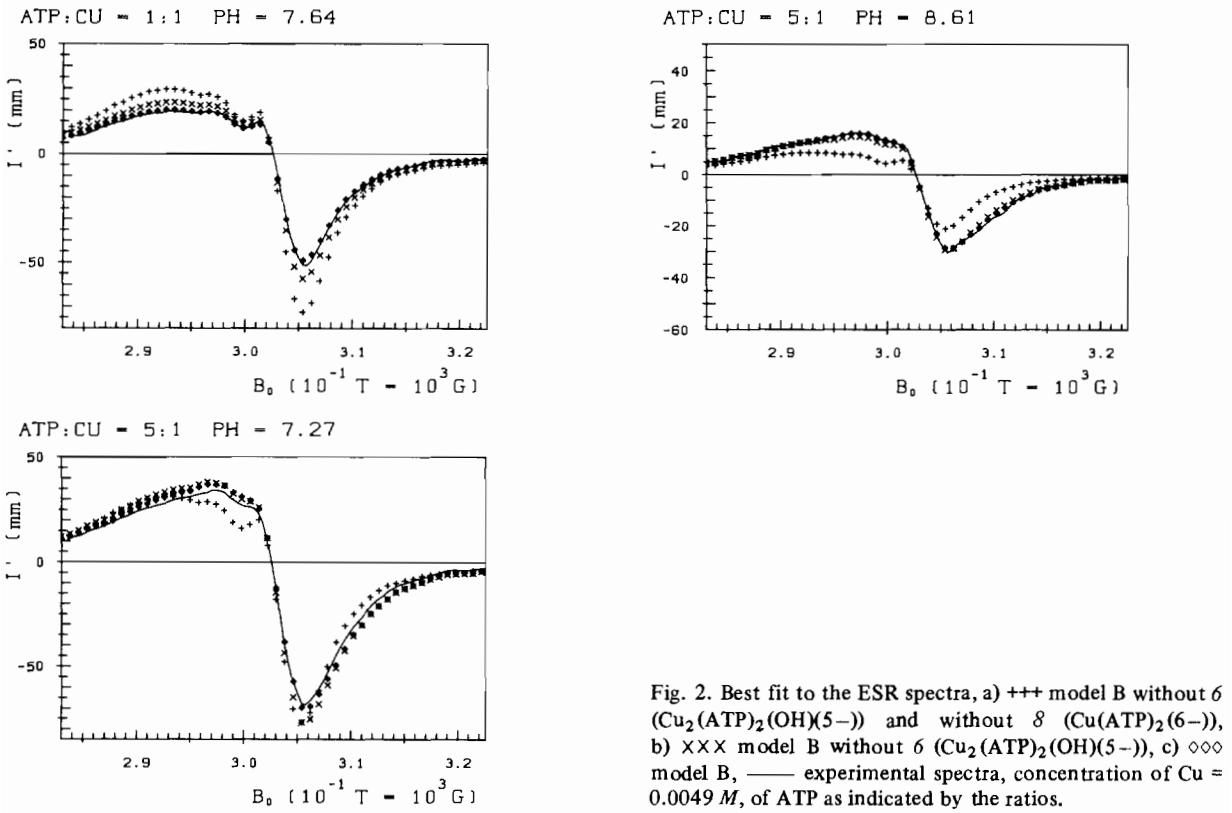


Fig. 2. Best fit to the ESR spectra, a) +++ model B without *6* (Cu_2(ATP)$_2$(OH)(5−)) and without *8* (Cu(ATP)$_2$(6−)), b) XXX model B without *6* (Cu_2(ATP)$_2$(OH)(5−)), c) ◇◇◇ model B, —— experimental spectra, concentration of Cu = 0.0049 *M*, of ATP as indicated by the ratios.

was assumed to be a dimer $Cu_2(ATP)_2(OH)_2(6-)$ as it shows no spectrum under the experimental conditions used here.

Thus we tried to fit our whole series of spectra (except the spectra recorded from very basic solutions which are discussed in part B) and the most satisfactory fit is shown in Fig. 2a in three examples. Two deficiencies can be noted:

i) The calculated spectra are too large for the 1:1 and too small for the 5:1 case from neutral to basic pH. This indicates that some species are missing, which induces an increase in the ESR signal from the 5:1 ratio to the 1:1 ratio.

ii) The spectra of the 1:1 case have a significantly different shape compared with the spectra of the 5:1 case. This indicates once more that a further species should be considered.

Consequently, we had to assume that a $Cu(ATP)_2(6-)$ species is being formed and indeed a much better fit of calculated and experimental data was then obtained (Fig. 2b). In addition, we tried to describe our data including many further species and their combinations: $Cu(HATP)_2(5-)$, $Cu(H_2ATP)_2(4-)$, $Cu(ATP)_2(OH)(7-)$ instead of $Cu(ATP)_2(OH)(6-)$, $Cu_2(ATP)_2(OH)(5-)$ and $Cu_2(ATP)_2(OH)_n(7-)$ instead of and/or in addition to $Cu_2(ATP)_2(OH)_2(6-)$.

Among these, only the additional assumption of $Cu_2(ATP)_2(OH)(5-)$ increased the quality of the fit significantly and thus we finally suggest model B (Table II), (Fig. 2c).

Comparison with Potentiometric Data

The ESR results were compared with those of the potentiometric titrations and the literature information available for ATP–Cu complexes in solution (Table III). The analysis of the titration data was first focused on the 1:1 ATP:Cu ratio. With the above mentioned literature model (model A, Table II) a good fit was obtained. Assuming a dimeric com-

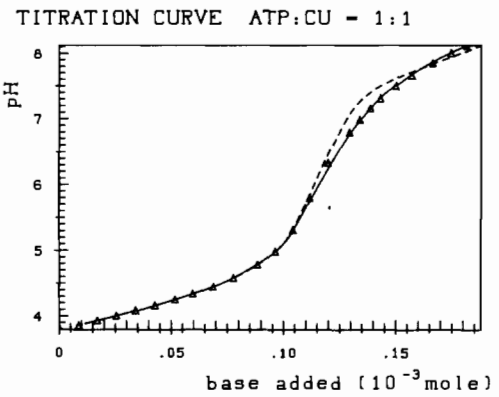


Fig. 3. Best fit to a titration of a ATP:Cu = 1:1 solution. Concentration of ATP = Cu = 0.002 *M*, —— experimental titration curve, a) – – – calculated using model B without species *6* and *8* (= without $Cu_2(ATP)_2(OH)(5-)$ and $Cu(ATP)_2(6-)$), b) ΔΔΔ calculated using model B without species *8* (= without $Cu(ATP)_2(6-)$).

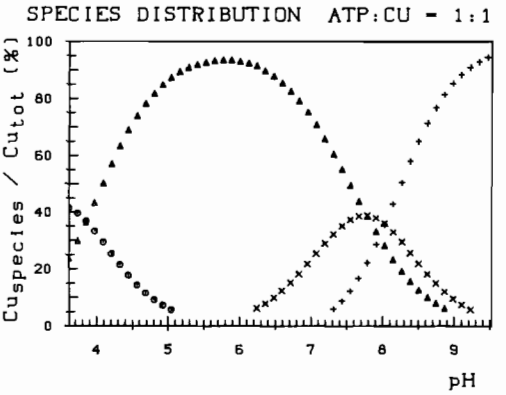


Fig. 4. Concentration ATP = Cu = 0.002 *M*, for pK values see Table II, model B. oo species *3* Cu(HATP)(–), ΔΔ species *4* Cu(ATP)(2–), XX species *6* $Cu_2(ATP)_2(OH)(5-)$, ++ species *7* $Cu_2(ATP)_2(OH)_2(6-)$.

TABLE II. Comparison of Species Combination and pK Values from Literature [15] to this Work.

Nr.[a]	Model A (literature)	pK lit	Model B (this work)	pK[b]
1	HATP(3–)	–6.42	HATP(3–)	–6.51
2	$H_2ATP(2-)$	–4.02	$H_2ATP(2-)$	–4.12
3	Cu(HATP)(–)	–3.12	Cu(HATP)(–)	–3.45
4	Cu(ATP)(2–)	–6.38	Cu(ATP)(2–)	–6.10
5	Cu(ATP)(OH)(3–)	1.79		
6			$Cu_2(ATP)_2(OH)(5-)$	–6.85
7			$Cu_2(ATP)_2(OH)_2(6-)$	1.19
8			$Cu(ATP)_2(6-)$	–7.96

[a]The numbers refer to Table I. [b]pK Values from potentiometric or ESR data (see Table III for details).

TABLE III. Comparison of pK Values from ESR Titrations, Potentiometric Titrations (tit) and Literature (lit), (for definition of the equilibria see Table I).

Species	pK ESR[a]	pK tit[a]	pK lit	Information available about structure in solution[b]
1 HATP(3−)		−6.51 ± 0.02	−6.51 [12] −6.42 [15]	protonation of γ-phosphate [27]
2 $H_2ATP(2-)$		−4.12 ± 0.02	−4.02 [11] −4.06 [15]	additional protonation of N(1) [25, 27]
3 Cu(HATP)(−)	−3.4 ± 0.4	−3.41 ± 0.14[d] −3.45 ± 0.04[c]	−3.12 [9, 16] −3.59 [12]	interaction with triphosphate, no interaction with adenine ring (Raman [22], NMR, [19]), phosphate is deprotonated before the adenine ring (IR, [27])
4 Cu(ATP)(2−)	−6.2 ± 0.3	−6.11 ± 0.07[d] −6.10 ± 0.02[c]	−5.95 [11] −6.38 [9] −6.03 [9] −6.34 [12]	complex formation at 6-NH_2, N(7) (separated by H_2O) and phosphate (Raman, [22]), at adenine moiety and phosphate (IR, [27]), macrochelate formed by Cu(2+), β + γ phosphate and N(7) (NMR, [19])
5 Cu(ATP)(OH)(3−)	no ESR signal	1.99 ± 0.03[c]	1.74 [15] 6.47 [4]	presumably hydroxyl ion bound to Cu (Raman, [22]) only weak interaction with adenine moiety (NMR, [19]), must contain more than one Cu (ESR, this work), Cu further away from adenine moiety than in Cu(ATP)(2−) (NMR, [19], Raman, [22]) phosphate groups still play a role in complex formation (see text)
6 $Cu_2(ATP)_2(OH)(5-)$	−6.9 ± 0.3	−6.86 ± 0.50[d] −6.85 ± 0.10[c]		
7 $Cu_2(ATP)_2(OH)_2(6-)$	1.7 ± 0.5	1.28 ± 0.30[d] 1.19 ± 0.05[c]	−1.91 [4]	
8 $Cu(ATP)_2(6-)$	−7.96 ± 0.25	−8.1 ± 0.8[d]	attempt proved unsuccessful [12]	The results from NMR [19], performed at ATP:Cu ratio = 2000) have to be attributed to this complex rather than to Cu(ATP)(2−)
9 ATP(5−)			adenosine: 12.5 [25] ~12 [26, 28]	deprotonation of sugar hydroxyls [25, 26, 28]
10 $Cu(ATP)_2(OH)(9-)$	22.9 ± 0.4			phosphate group has no more activity in complex formation (see text) binding to deprotonated ribose moiety proposed [26, 29]
11 $Cu(ATP)_2(10-)$	33.4 ± 0.5			
12 $Cu_2(ATP)_2(OH)_n(7-)$	~12 ± 1			ribose groups involved in complex formation (optical rotation, [21])
13 $Cu_2(ATP)_2(OH)_n(8-)$	~24 ± 1			

[a]The deviations indicate upon which change of pK value a doubling of the error is induced. Therefore these deviations do not describe only the reproducibility errors (which are smaller), but also take into account systematic inaccuracy. (For the ESR, a new spectrum for the thus changed pK has to be calculated for this error evaluation). [b]The comparison with the Raman [22] and with the IR [27] data as well as with the NMR data [19] might be incorrect because those methods were performed under rather different conditions (20 fold total concentration (0.1 *M*) for NMR, IR and Raman, ATP:Cu ratio 2000:1 for NMR). [c]Calculated with model A only from the 1:1 titration. [d]Calculated with model B. [e]Calculated only from 1:1 titration with model B without species *8* ($Cu(ATP)_2(6-)$).

plex $Cu_2(ATP)_2(OH)_2(6-)$ instead of Cu(ATP)(OH)(3−) the fit was insufficient (Fig. 3a) and could only be improved by the additional assumption of a $Cu_2(ATP)_2(OH)(5-)$ species (Fig. 3b) leading to the species distribution shown in Fig. 4. Then the analysis was expanded to titrations up to the ratio of ATP:Cu = 5:1. As in ref. 12, the analysis was found to be incapable of determining whether a $Cu(ATP)_2$ complex is formed or not. This is also reflected in the large deviation of the pK value for

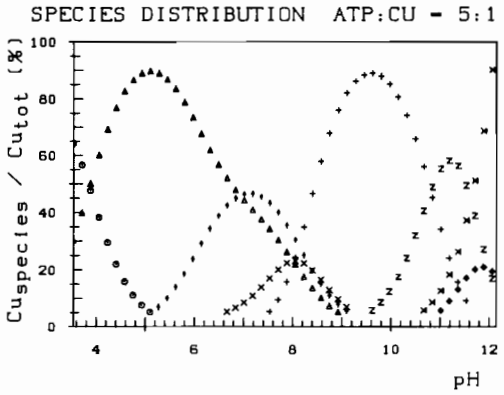


Fig. 5. Concentration of ATP = 0.025 *M*, Cu = 0.005 *M*. For pK values see Table II, model B and Table III. ⊙⊙ species *3* Cu(HATP)(–), △△ species *4* Cu(ATP)(2–), ×× species *6* $Cu_2(ATP)_2(OH)(5-)$, ++ species *7* $Cu_2(ATP)_2(OH)_2(6-)$, ↑↑ species *8* $Cu(ATP)_2(6-)$, ⋈⋈ species *10* $Cu(ATP)_2(OH)(9-)$, zz species *12* $Cu_2(ATP)_2(OH)_n(7-)$, ◇◇ species *13* $Cu_2(ATP)_2(OH)_n(8-)$.

this complex (Table III). Table III shows that the pK values calculated from ESR data are in good agreement with the pK values determined by potentiometric titration although both calculations are based on very different kinds of information (concentration of species in the ESR, number of protons released in the potentiometric titration).

Discussion of the Species $Cu_2(ATP)_2(OH)(5-)$, $Cu_2(ATP)_2(OH)_2(6-)$ and $Cu(ATP)_2(6-)$

The search for the first two of these species has been neglected since the publication of ref. 15, although the authors have not excluded the existence of dimeric species. This neglect was encouraged by an inaccurate citation of ref. 15 more recently [21]. We think that our experiments prove the existence of dimeric species. Furthermore, the combined titrimetric and ESR results prove the occurrence of both species ($Cu_2(ATP)_2(OH)(5-)$ and $Cu_2(ATP)_2(OH)_2(6-)$). As adenosine–Cu forms a precipitate in that pH range [26, 29], the triphosphate group can be assumed to play an essential role in the complex formation.

$Cu(ATP)_2(6-)$ was unambiguously detected only as a result of the use of the ESR spectroscopic method. It has a comparatively low stability constant (this species is only formed by about 50% of the Cu(2+) in the 5:1 case, Fig. 5), which is also the reason for the failure of the titration analysis in this case. Presumably copper is already coordinated strongly by one ATP molecule, preventing a similar strong binding of a second ATP molecule. Consequently it can be assumed that this complex is formed by means of some 'stacking' interaction [30] of the second ATP to the Cu–ATP complex.

Part B: pH = 9.5 to 12

Within this pH range a strong new ESR signal appears only at a ATP:Cu ratio larger than 1:1 (Fig. 1). The spectra recorded in this pH range can be explained and generated best by assuming a $Cu(ATP)_2(OH)(9-)$ species (ATP(4–) → ATP(5–) + H(+), pK = 12.5 was taken from ref. 25). Again we tried to fit our data with a variety of further species: $Cu_2(ATP)_2(OH)_n(7-)$; $Cu_2(ATP)_2(OH)_n(8-)$ in addition to $Cu_2(ATP)_2(OH)_2(6-)$, $Cu(ATP)(OH)_n(4-)$ to $Cu(ATP)(OH)_n(7-)$, $Cu(ATP)_2(OH)_n(8-)$ to $Cu(ATP)_2(OH)_n(12-)$, $Cu(ATP)_3(OH)_n(13-)$ instead of $Cu(ATP)_2(OH)(9-)$.

The complexes that lead to a good description are $Cu(ATP)_2(OH)(9-)$ or $Cu(ATP)_2(10-)$, $Cu_2(ATP)_2(OH)_n(7-)$ and $Cu_2(ATP)_2(OH)_n(8-)$.

$Cu(ATP)_2(OH)(9-)$ or $Cu(ATP)_2(10-)$

This complex shows an ESR spectrum very similar to that of copper adenosine solutions at pH = 11.3 [26]. Thus the triphosphate group can be assumed to play only a minor role in this complex. Chao and Kearns have made a proposal for the existence of such an adenosine copper complex assuming two ribose hydroxyls to be deprotonated in each adenosine ligand [26]. This corresponds to a $Cu(ATP)_2(10-)$ complex which can also fit our data but not as well as the $Cu(ATP)_2(OH)(9-)$ species.

In this high pH range it is difficult to decide which species is formed, because the number of protons set free upon complex formation does not influence very much its amount as a function of pH. Hence we cannot decide between these two species without doubt.

$Cu_2(ATP)_2(OH)_n(7-)$ and $Cu_2(ATP)_2(OH)_n(8-)$

Both species cannot be detected unequivocally as they do not display an ESR spectrum. If they exist, their pK value should be in the range indicated in Table III. Again our data do not allow a clear proof of the significance of these species.

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