The Interaction of H^+, Zn(II) and Cu(II) with Adenine and 9-Methyladenine

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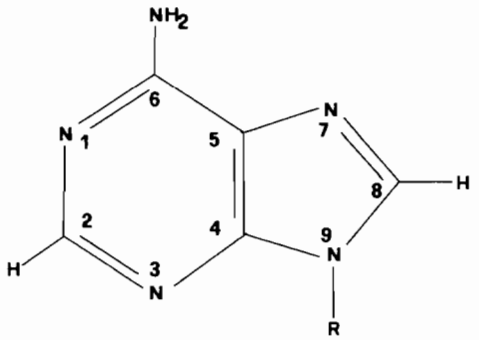
Abstract

The interaction of H^+, Zn(II) and Cu(II) with adenine (A), and 9-methyladenine (9-MeA) is examined by means of potentiometry, spectrophotometry, ^1H NMR and ESR spectroscopy. Quantitative evaluation of the protonation and of the stability constants of the 1:1 complexes with Cu(II) and Zn(II) is given for both adenine and 9-methyladenine ligands. Analysis of possible bonding sites are discussed based on ^1H NMR titration curves and on the stabilities of the considered species. Additionally, Cu(II) forms strong dimeric complexes with adenate (N9 deprotonated adenine), which acts as a bridging ligand via N9 and N3 atoms. The species formed and the values of their formation constants are given.

Introduction

Proton and metal ion interactions with purinic and pyrimidinic bases influence the structure and reactivity of nucleic acids [1]. Although there are many studies of this subject [2], owing to the biological relevance of the nucleic acids and their clinical implications [3], there are still some aspects which have not been completely clarified. Few studies about the stability constants of the metal complexes with the bases have been reported [4]. Most of the reports discuss the binding sites of the molecules and their relative affinity to the metal, but the results are controversial [5–7].

The aim of the present work is twofold: to determine the stability constants for the protonation, Zn(II) and Cu(II) complexation with adenine (A) and 9-methyladenine (9-MeA) at biological conditions of temperature (37 °C) and ionic strength (0.15 M $NaNO_3$ in H_2O), and to identify the binding sites of both compounds and the main influence of the N9-substitution.



Scheme 1. R = H adenine (A); R = CH_3 9-methyladenine (9-MeA).

Adenine (see Scheme 1) has four nitrogen atoms, labelled N1, N3, N7 and N9, suitable for metal binding. The NH_2 group is nearly coplanar with the ring [8], and the amino nitrogen-to-carbon bond has an appreciable double-bond character; delocalization of electron density to the rings can be expected. Therefore, the nitrogen atom of the amino group is not active as a binding site compared to the ring nitrogen atoms. 9-Methyladenine has only three ring nitrogen atoms (N1, N3 and N7) as possible binding sites, since N9 is blocked by the methyl group. As it has been pointed out [1, 8], 9-methyladenine is a compound quite suitable for the comparison of metal-ion interactions with the base in adenine, nucleosides and nucleotides, because their N9 atoms are involved in the glycosyl bonds and thus not available as binding sites. At weak acidic, neutral and basic pH, unsubstituted adenine interacts with metal ions via the N9 position, therefore giving completely different species in solution [1].

First protonation of 9-methyladenine and of neutral adenine is usually assigned to the N1 atom. This assignment has been proposed from X-ray crystallographic studies [9]. In solution, the possibility of simultaneous protonation of other sites cannot be excluded, although most authors assume that this only happens to a minor extent. A ^{15}N NMR spectroscopy study showed that the signal corresponding to the N1 atom is the most shifted upon protonation [10], and it was concluded that this is the main site for protonation. Small downfield shifts for the other nitrogen atoms could not be inter-

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preted in terms of protonation of the N atom only, and some partial protonation of N3 and N7 or their involvement in conjugation with the protonated N1 atom was suggested.

Metal complexation sites of 9-substituted adenines have been studied by paramagnetic relaxation [11] and NMR diamagnetic shift techniques [12]. From these studies, it has been concluded that Zn(II) and Cu(II) bind to both N1 and N7 atoms, and that the N3 position is usually not involved in metal complexation because of the steric hindrance produced by the ribose sugar in nucleosides and nucleotides, or by the methyl group in 9-methyladenine. This conclusion obviously cannot be applied to adenine. In fact, the calculated order of electrophilic attack obtained by theoretical methods [13] is N3, N1 and N7. Experimental solution studies could not directly exclude the N3 position as a possible coordination site, since most of the spectroscopic techniques (^{1}H NMR, ^{13}C NMR) do not allow the distinction between the two N atoms, N1 and N3, in the 6-member ring. Very recent studies tried to clarify the roles of N1 *vs.* N7, by relating the values of the complex formation constants with the acidity constants of different compounds [5], or by examining the stabilities of metal complexes with adenines and purines substituted at the C2, C6, C8 or C9 atoms [6]. From these studies, different conclusions about the relative importance of the N1 and N7 as coordination sites were obtained.

Species formed in weak, neutral or basic Cu(II) solutions which also contain adenine have been scarcely studied [14–21]. These studies revealed that Cu(II) complexation with adenate (deprotonated adenine) is rather strong; Reiner and Weiss [19] pointed out that these complexes should involve N3 and N9 simultaneously as binding sites. Additionally, X-ray crystallographic studies showed that this is true for solid complexes, which have also been described as dimers or other polymeric species [22].

Experimental

Materials

$Zn(NO_3)_2 \cdot 4H_2O$ (analytical grade) and $ZnCl_2$ (analytical grade) and $Cu(NO_3)_2 \cdot 3H_2O$ (puriss.) were obtained from Merck. Adenine (puriss.) was obtained from Fluka. 9-methyladenine was prepared following the method proposed in ref. 23. Benzimidazol (puriss.) was obtained from Aldrich Co.

All aqueous solutions were prepared using CO_2-free deionized water; the ionic strength was kept constant up to 0.15 M $NaNO_3$ in the potentiometric measurements, and up to 0.5 M $NaNO_3$ in the ^{1}H NMR measurements. Concentrations of the ligands in these solutions were directly obtained from the weighted amounts of the substances. Stock solutions of the metal ions were standardized previously. Acid and base, HNO_3 and NaOH, for potentiometric and spectrophotometric experiments were Merck Titrisol products. D_2O (100%) was obtained from Aldrich-Chemie, and the pD in the ^{1}H NMR titrations was adjusted with concentrated NaOD (Na dissolved in D_2O) and DCl (99.8%, Stohler Isotope Chemicals).

Apparatus

For the pH and pD measurements a Schott pH meter CG 803 and a combined Ross pH electrode (ORION 81-02) were used. The ^{1}H NMR spectra were recorded on a Varian EM-360-L 60 MHz spectrometer with a sweep width of 10 ppm and a sweep time of 5 min. Spectrophotometric measurements were carried out with a Unicam 1800 ultraviolet spectrophotometer linked to a Unicam 1805 program controller and a Unicam AR 25 linear recorder. ESR measurements were made with a Varian E 104 spectrometer (calibrated microwave frequency 9.097 GHz) in tubes of 1 mm diameter (Willmad Cat. Nr. 800). Potentiometric and spectrophotometric measurements were carried out in a thermostatized cell (37 °C) compartment. ^{1}H NMR spectra were recorded at 32 °C.

Calculations

All calculations were carried out at the CDC Cyber 830 computer of the University of Innsbruck. For the evaluation of formation constants and of the spectra of the pure species present in solution from the ^{1}H NMR data, the Fortran program NMROPT [24] was used. For the evaluation of the stability constants from the potentiometric data, the MINIQUAD program [25] was used. For the spectrophotometric evaluation of the stability constants and the species absorption spectra, the SQUAD [26] program was used.

^{1}H NMR Determinations

Protonation of 9-methyladenine and its complexation with Zn(II) were studied with the ^{1}H NMR titration method described previously [24]. The concentration of the ligand in the titrations (5 titrations with 43 recorded spectra at different pD values) was varied between 0.028 M and 0.040 M, and the Zn(II) concentration was varied between 0.040 and 0.083 M. Protonation and Zn(II) complexation of adenine were also investigated by the ^{1}H NMR titration method. In this case, a total of 3 titrations with 23 spectra (adenine concentration from 0.018 M to 0.050 M and Zn(II) concentration from 0.025 M to 0.056 M) were analysed. Only solutions in which no precipitation of the ligand and no hydrolysis of the metal ion occurred were taken. In spite of the low solubility of the substances at neutral pD, we succeeded in our experiments by taking

advantage of the higher solubility of the ligands near the physiological temperature, and avoiding the use of solvents other than water (e.g. dimethyl sulfoxide) so often used in similar studies.

Zn(II) and H interactions are subject to fast exchange between coordinated and free species, so that the population of each individual species cannot directly be measured. Each observed peak corresponds to the average signal of each proton for the mixture of species. The chemical shifts of the protons were measured with and without the addition of the Zn(II) salt as a function of pD. They were related to the resonance signal of trimethylammonium (TMA) salt as internal standard and the assignment of signals was made according to previous studies [5, 13]. ^{1}H NMR chemical shifts of solutions containing the substance benzimidazol were also measured at pD values where it is either completely protonated or not protonated.

Electrode calibration for the ^{1}H NMR experiments was made externally in terms of acid concentration and without correction for pD. For measurements made at pD below 1, junction potentials of the electrode were considered [27]. The ionic strength was kept in excess in all solutions (0.5 M $NaNO_3$) so that ^{1}H NMR shifts were not affected by concentration changes of the components [5]. The shifts of the solutions at very low values of pD (<1) display a small influence due to changes in the ionic strength, but in this case no quantitative evaluation was intended.

Potentiometric Determinations

Potentiometric determinations of adenine and 9-methyladenine protonation and of Cu(II) and Zn(II) complexation of these ligands were carried out at 37 °C and 0.15 M $NaNO_3$ (biological conditions). Calibration of the cell was made using Gran plots [28].

Adenine and 9-methyladenine show a protonation step in the 3–5 pH range. Adenine also shows deprotonation at basic pH (pH > 8). Further protonation of the ligands occurs at a pH below 1, and, therefore, was not studied potentiometrically. For the protonation of 9-methyladenine, 3 titration curves (76 experimental points) were recorded in which the ligand concentration was varied from 0.0078 M to 0.0242 M. For the study of the protonation and deprotonation of adenine, 6 titrations (118 experimental points) where the ligand concentration was varied from 0.0148 M to 0.0286 M were performed.

For the Zn(II) and Cu(II) complex formation with 9-methyladenine, 4 (74 experimental points) and 5 (93 experimental points) titrations were made, respectively. The concentrations of the ligand and of the metal ion varied from 0.0131 M and 0.0074 M to 0.0199 M and 0.0215 M for the Zn(II) complexation, and from 0.0081 M and 0.0076 M to 0.0184 M and 0.0189 M for the Cu(II) complexation, respectively.

For the Zn(II) and Cu(II) complexation of adenine 10 (152 experimental points) and 9 (200 experimental points) curves were recorded. The concentrations of the ligand and the metal varied from 0.0108 M and 0.0052 M to 0.0212 M and 0.0220 M for Zn(II) complex formation, and from 0.0147 M and 0.0018 M to 0.0270 M and 0.0193 M for the Cu(II) complex formation.

ESR Determinations

Some sample solutions (0.1 ml) were taken from the potentiometric vessel during the Cu(II) complexation studies with adenine and 9-methyladenine. The ESR spectra of these solutions were recorded and analysed with the same method as described previously [29].

Spectrophotometric Determinations

Cu(II) complexation of adenine was studied in detail using the spectrophotometric method [25, 30]. A total of 85 solutions at different ligand-to-metal ratios (1 to 10) and concentrations of Cu(II) from 0.0010 to 0.0200 M were prepared and their spectra recorded in the 430–690 nm absorption range. Absorbance readings were taken directly from the recorded spectra at intervals of 20 nm (14 points per spectra).

Results and Discussion

Protonation, Cu(II) and Zn(II) Complexation with Neutral Adenine and 9-Methyladenine

^{1}H NMR determinations

In Table I, the ^{1}H NMR shifts of all species studied are given. They correspond to the pure species shifts. In Fig. 1, ^{1}H NMR titration curves (for H8 and H2) obtained as in ref. 24, corresponding to the first protonation and Zn(II) complexation of 9-methyladenine, are given. Protonation of 9-methyladenine shifts both the H2 and H8 peaks to lower fields and, to a lesser extent, the signals corresponding to the methylene groups (not shown in Fig. 1). Proton H8 is somewhat more shifted than the H2 peak. Further addition of acid (pD < 1) causes a strong downfield shift of H2 below the H8 signal. This picture suggests that the first protonation of 9-MeA occurs not only in one site – N1 – as suggested by other authors [2, 8], but at two or even all three nitrogens, N1, N7 and N3.

The extent of the protonation on each site cannot be described quantitatively since it is difficult to know exactly how the protonation of one site affects the other sites, and how the influence passes from one ring to the other. On the other hand, accord-

TABLE I. Chemical Shifts[a] (ppm to TMA)

	H2	H8	Others
9-MeA	4.72	4.80	0.50[b]
H(9-MeA)	5.08	5.26	0.72[b]
H_2(9-MeA)	6.30	5.60	1.06[b]
Zn(9-MeA)	5.04	5.22	0.69[b]
A	4.93	4.94	
HA	5.18	5.23	
$H_{-1}A$	4.92	4.77	
B		5.00	4.32[c]
HB		5.87	4.47[c]

[a]For the species which contain adenine (A) or 9-methyladenine (9-MeA), the shifts shown have been calculated from the analysis of the experimental data with NMRCON program [24]. The estimated errors in the given values are less than 0.02 ppm. For benzimidazole (B) the given values are directly obtained from the experimental spectra. [b]Shift corresponding to 9-methylene protons. [c]Shift corresponding to the benzene protons (approximate value).

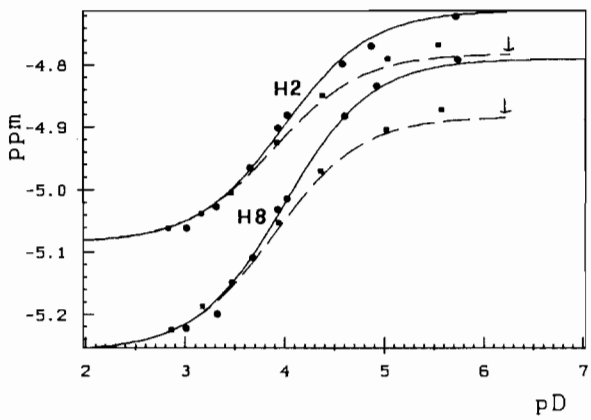

Fig. 1. Calculated and experimental ^{1}H NMR titration curves for the H8 and H2 protons of 9-methyladenine: 0.040 M 9-MeA —— calculated, • experimental; 0.04 M 9-MeA + 0.040 M Zn(II) – – – calculated, ▪ experimental; ↓ precipitation.

ing to our ^{1}H NMR results (Table I), the second protonation of 9-methyladenine will take place mainly over the 6 ring atoms.

Substitution of the methyl group in the 9 position of 9-methyl adenine by a proton in adenine causes a shift of both H2 and H8 peaks to lower fields, giving very close signals. Protonation of neutral denine shifts both H2 and H8 peaks to lower field, but to lesser extent than in the case of 9-methyladenine. Deprotonation of adenine, at basic pH, causes the signal corresponding to H8 to shift to higher field, while the H2 peak remains located at nearly the same position, therefore displaying a crossing of signals.

In Table I, the shifts produced by the protonation of benzimidazol are also given. The protonation site in this substance is related to the site in the 5-membered ring of purine. This substance was used here to study the magnitude of the shifts induced by the protonation of the nearby atoms, and to see how the proton signals in one ring are affected by the protonation of a N atom in the other ring. The results obtained showed that, on the one hand, the ^{1}H NMR peak of benzimidazol is considerably more shifted than H8 in adenine and 9-methyladenine. On the other hand, each ring system seems to be somewhat 'closed' to the influence of protonation of the N atom in the other ring, since the shifts corresponding to the aromatic protons are less changed (Table I). This result would confirm our proposal that protonation of neutral adenine and 9-methyladenine occurs simultaneously in both ring systems.

The ^{1}H NMR analysis of Zn(II) complexes with 9-methyladenine allowed the calculation of the chemical shifts corresponding to the H2, H8 and methyl protons of the species formed, simultaneously with the calculation of their formation constant (Table II). As is seen from the values in Table I, in this case

TABLE II. Protonation and Metal Cu(II) and Zn(II) Complex Formation with Neutral Adenine and 9-Methyl Adenine[a]

	H	Zn(II)	Cu(II)
9-MeA	3.98(1)	1.2(1)	1.5(1)
	4.0(1)[b]	0.9(3)[b]	
A	4.02(2)	0.6(3)	2.25(6)
	4.0(2)[b]		

[a]Values of the log of the stability constants for the protonation equilibria and 1:1 complex formation equilibria obtained from the MINIQUAD [25] treatment of the potentiometric data. Values in parenthesis refer to three times the standard deviations in the last figure calculated by the program. Conditions: 37 °C and 0.15 M $NaNO_3$. [b]Values of the log of the stability constants obtained in the NMRCON [24] treatment of the ^{1}H NMR data. Values in parenthesis are the deviations in the last figure of the log of the constants, which cause a double value of the error-square function. Conditions: 37 °C and 0.5 M $NaNO_3$.

the three signals corresponding to H2, H8 and methyl protons are also shifted, although again the H2 and H8 peaks are considerably more influenced than the methyl signal. We had observed that protonation affects H8 somewhat more than H2; this is indeed more pronounced in the Zn(II) complex, showing that the contribution of the N7 site is probably more important in this case. The shifts to lower fields in the case of the Zn(II) complex are a little smaller than for protonation, as has usually been observed in other compounds [24], because of the lower

deshielding effect of Zn(II) ion compared to H on the protons near the bonding site. Addition of Zn(II) to adenine solutions produces very small changes on the observed shifts, showing that Zn(II) complexation with adenine is so weak that it is difficult to obtain any reliable value from the ^{1}H NMR data (see potentiometric results).

Values of the protonation constants of 9-methyladenine and adenine obtained from the analysis of ^{1}H NMR spectra (Table II) agree well with the obtained values from potentiometric data, showing that the method and the results obtained from the ^{1}H NMR analysis are reliable. The same can be said for the complexation of Zn(II) with 9-MeA in the expected precision range, but in this case this observation is even more remarkable, due to the difficulties involved in the determination of this small constant even with the well established potentiometric method (see below). It is interesting to point out here the possibility offered by the ^{1}H NMR method to obtain the NMR spectra of the pure species in solution, which give direct information about their structure.

Potentiometric determinations

In Table II the logarithmic values of the protonation constants of the ligands and of their 1:1 metal complexes with Zn(II) and Cu(II) obtained from the analysis of the potentiometric data are given. Values for the protonation constants of neutral adenine and 9-methyladenine are similar. Protonation only at N7 should be enhanced by N9 methylation, following the analogy of the protonation of imidazol (log K_H = 7.18 [19]) compared with the protonation of 1-methylimidazole (log K_H = 7.39, [15]). However, the protonation constant of 9-methyladenine is somewhat smaller than the one for adenine.

Cu(II) complexes of neutral adenine and 9-methyladenine display a different stability. The values obtained suggest that these two compounds differ not only in their complexes at neutral and basic pH (see later), where N9 of adenine deprotonates and determines complex formation, but also in their cationic complexes (formed at acid pH). From previous *ab initio* calculations [13] it was shown that the N3 atom should be the more favourable binding site in adenine. According to our results adenine acts as a monodentate ligand in acidic media, giving a 1:1 complex with Cu(II) where the contribution of N3 should be important, while in 9-methyladenine this site is sterically more hindered by the methyl group at N9, lowering the corresponding stability constant for the same 1:1 complex.

In the case of Zn(II), the complex with 9-methyl adenine has a higher stability than the corresponding complex with adenine. Moreover, at neutral pH, Zn(II) hydrolyses even in excess of adenine, in contrast to Cu(II), without forming any other complex species at this pH; this was also observed from the nearly unchanged ^{1}H NMR spectra of solutions of adenine containing Zn(II) in excess. The Zn(II) complex with adenine is rather weak, and only an approximate value for its formation constant can be obtained from potentiometric data and not at all from the NMR data. These facts can be interpreted by assuming that Zn(II) complexation via N7 is encouraged by methylation on the N9, and that in this case complexation via N3 is not as important as in the Cu(II) complexation case. The strengthening of complexation by methylation of the imidazol ring is also observed when the stabilities of the complexes with imidazol and methyl substituted imidazol are compared [5]. From the present study, complexation via N1 cannot be analysed, but it is supposed to have a similar contribution in adenine and 9-methyladenine.

Cu(II)-Adenate Complexes

In the following discussion, Table III and Figs. 2 and 3, β_{pqr} and pqr refer to the global formation constants and stoichiometric coefficients of the equilibrium $pL + qCu(II) + rH \rightleftharpoons Cu_qL_pH_r$, where L is adenate (N9-deprotonated adenine). Adenine is

TABLE III. Cu(II) adenate complexes at 37 °C and 0.15 N $NaNO_3$

Species $(pqr)^b$	log β_{pqr}pot[a,c]	log β_{pqr}spec.[a,d]	λ_{max}[e]	ϵ_{max}[f] (nm)
111	11.7(1)	–	–	–
220	17.9(1)	17.7(3)	580	413(15)
22 – 1	13.3(2)	12.5(3)	565	215(14)
420	–	27.2(3)	555	470(15)
42 – 2	10.4(9)[g]	8.9(3)	630	137(6)
101	9.47(1)	–	–	–
102	13.49(2)	–	–	–

[a]Values of the log of the formation constants for the equilibria $pL + q(Cu(II) + rH = Cu_qL_pH_r$, where L denotes adenate (N9 deprotonated adenine). [b]Stoichiometric coefficients of the detected species $Cu_qL_pH_r$. [c]Results obtained with the MINIQUAD treatment of the potentiometric data; the standard deviation of the least-square residuals in the total concentrations [25] was less than 0.0002 molar units; in parentheses, three times the standard deviations in the last figure given by the program. [d]Results obtained with the SQUAD [26] treatment of the spectrophotometric data; the standard deviation of the least-square residuals in the experimental absorbances was less than 0.01 units; in parentheses, three times the standard deviations in the last figure given by the program. [e]Wavelength values in nm for the maximum of the absorption bands of the considered species. [f]Molar absorbances at λ_{max} wavelengths of the considered species; in parentheses, values of the standard deviations given by SQUAD. [g]This value was obtained keeping the constant corresponding to the complex 420 unchanged in the refinement (see text).

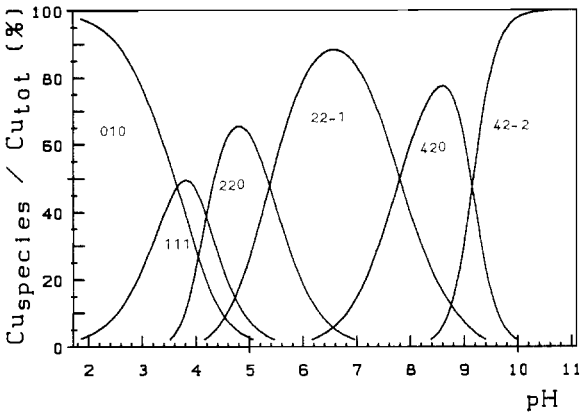

Fig. 2. Distribution of species as a function of pH; 0.0020 M Cu(II) + 0.0200 M adenine; same symbols as in Table III for the stoichiometric coefficients of the formed species; log β_{111} and log β_{220} taken from potentiometric results, log β_{22-1}, log β_{420} and log β_{42-2} taken from spectrophotometric results.

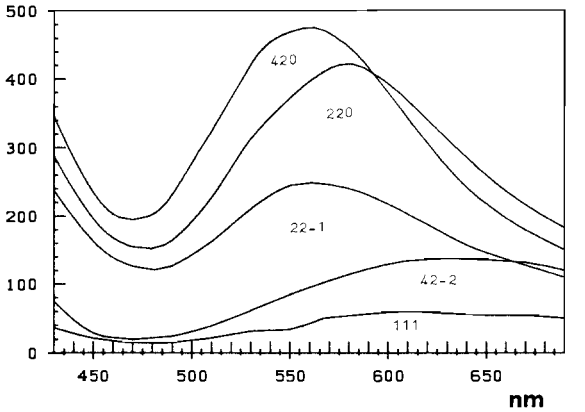

Fig. 3. Plot of the molar absorptivities of the Cu(II)–adenate complexes (14 points per spectra at each 20 nm) obtained with the SQUAD program [25]. See Table III.

noted as 101; therefore, negative values of r refer to the deprotonation of the water molecules attached to the central metal ion.

ESR determinations

ESR spectra of solutions at acidic pH (pH < 3) containing Cu(II) and adenine display the same broad shape as solutions of Cu(II) ion in water. When the pH is increased, a small shift of the broad signal to higher fields is observed, as well as a decrease of its intensity until the signal disappears completely. Solutions which are strongly blue- and violet-coloured at weakly-acidic, neutral and basic pH (see spectrophotometric results) do not give any ESR spectra. All these facts are interpreted as the formation of only one ESR-detectable complex at weakly-acidic pH. When the pH is increased, species which contain more than one atom of Cu(II) ion per molecule (dimeric or other polynuclear species) are formed [31].

Analysis of solutions of Cu(II) ion which also contain 9-methyladenine gave the same patterns as the solutions with adenine in the first, acidic part. When the pH is increased, precipitation of Cu(II) is produced without further metal complexation by the ligand.

In this work, ESR was only used as a qualitative tool to indicate the presence of polynuclear species in the Cu(II) adenine system. In previous works [32], it was shown that ESR spectroscopy can be used satisfactorily to study Cu(II) complex equilibria in case of formation of monomer species or formation of monomer-dimer species simultaneously [33]. Unfortunately, in the present case only polynuclear species are detected at pH higher than 5, and therefore the ESR method cannot be used to study the equilibria of the species formed in the Cu(II) adenate system.

Potentiometric and spectrophotometric determinations

The proposed species together with the formation constants for the Cu(II) complexes with adenate (deprotonated adenine) are given in Table III, as obtained from the combined analysis of the potentiometric and spectrophotometric data. The species distribution at high ligand-to-metal ion ratio (10:1) is given in Fig. 2. In Fig. 3, the absorption spectra of all the species found in the Cu(II) adenate system are given in the 430–690 nm range.

The first species (111 in Table III) corresponds to the mononuclear species formed in acidic medium, where N9 is fully protonated and adenine acts as a monodentate ligand (see above). The value of its constant was obtained from the potentiometric analysis of the data in the acidic part of the titrations. Solutions which contain only this species display a weak blue colour with a broad absorption band at wavelengths over 600 nm.

The second proposed species is a dimer (see ESR results). From potentiometry it is seen that the number of protons released per Cu(II) atom is two. Some authors suggested, therefore [19], that this compound should be a monomer chelate with the stoichiometry 110, involving N3 and N9 atoms of the same adenine molecule as binding sites. However, this structure is not probable since it would involve a chelate ring of four members, which should be rather unstable, or at least less stable than the species considered here. On the other hand, previous studies of the Cu(II) adenate complexes in solid form revealed the formation of dimers [22] in which each atom of Cu(II) is simultaneously bonded to N9 and N3 atoms of different adenine molecules, with the ligand itself acting as a bridge between the

two central metal ions. This model agrees with the results obtained in the ESR analysis, since dimer species usually do not display an ESR spectrum [31]. The absorption spectrum of this species is shown in Fig. 3 (λ_{max} 580 nm, ϵ_{max} 413). The strong shift to lower wavelengths (of higher energy) of the maximum of the absorption band is larger than the one usually observed by the interaction or fwo N atoms [34], revealing that the structure of this complex is probably highly tetragonally distorted [35].

The complex 220 deprotonates giving the species 22–1 (Table III). This species is clearly obtained from the potentiometric data, since another proton is released per atom of Cu(II) at neutral pH, even for solutions where the ratio of ligand to metal ion concentration is 1. Other species proposed alternatively, such as 320 (three molecules of ligand and two of metal), give a worse fit of both potentiometric and spectrophotometric data. The precision for the stability constant obtained from the potentiometric data is still rather good, although its value differs a little from the spectrophotometric one. The absorption spectra of this species (see Fig. 3, λ_{max} 565 ϵ_{max} 215) is considerably less intense than the one corresponding to the previous species 220. This is in agreement with the fact that this species does not increase the number of N atoms per Cu(II) ion. The deprotonation should come therefore from one of the water molecules attached to the central metal ion. The inclusion of this species in the spectrophotometric analysis is very important in explaining the small changes in the experimental spectra (decreasing intensity) at neutral pH.

At basic pH the potentiometric analysis becomes more difficult. At low ratios of ligand to metal concentrations, hydrolysis and precipitation disturbs complex formation. At ratios higher than two, the precipitate is redissolved at basic pH, and at ratios higher than 5, there is no precipitation over the whole pH range. At basic pH (higher than pH = 10), the solutions have a clear blue colour, showing that the same species is obtained whether or not precipitation occured before.

Solutions with a high ratio of ligand to metal ion concentrations at weak basic pH (around pH = 8) display absorption spectra different from the ones described before. These solutions became strongly violet-coloured. The pH at which this occurs is close to the end point of the titrations, and it is very difficult under this condition to analyse the species formed using potentiometry. The changes observed in the spectrophotometric spectra are related to the formation of the next species which should have a higher number of N atoms per atom of Cu(II) ion (shift of the absorption to shorter wavelengths and increasing intensity). This proposed species 420 has four atoms of N attached to each atom of Cu(II). This complex should have a similar structure to the one described by Meester and Skapsky [35] for the binuclear solid complex $Cu_2(A)_4Cl_2$ (A = adenine). The two Cu(II) ions are held together by four bridging adenine ligands, which are coordinated through N9 and N3. The geometry at each copper atom is square pyramidal, the base plane being occupied by two *cis*-N9 atoms and two N3 atoms from four different adenine ligands, while the axial position is probably occupied in our case by a water molecule. This species fits the spectrophotometric data in the range of pH 7.5–9.5. Its spectrum is shown in Fig. 3 (λ_{max} 555 ϵ_{max} 470). The value of its formation constant could also be obtained from the spectrophotometric data. This species is responsible for the violet colour of solutions which contain an excess of ligand in the pH range 7.5–9.5. As was already mentioned, further addition of base changes completely the absorption spectra of the solutions. There is a strong shift to higher wavelengths, and the intensity of the absorption is also considerably decreased. Figure 3 shows the spectrum of the proposed species 42–2 (λ_{max} 630 ϵ_{max} 137). The inclusion of this species improves the fit of both potentiometric and spectrophotometric data in the high pH range (pH > 9); especially in the latter case, a more reliable value for the stability constant is obtained. The observed shift to higher wavelengths is related to the so called 'pentamine effect' [36] observed for the spectra of the species $Cu(NH_3)_5$. This effect is produced when there is increasing coordination in the axial positions of distorted tetragonal Cu(II) complexes, giving a decrease in the energy of the electronic transitions. In the species 42–2, this stronger axial interaction probably arises from the deprotonation of the two water molecules in the apical positions of the complex 420 (see before). Similar effects have been observed by other authors [34, 37], although there is little information about structures in solution.

The inclusion of other species instead of 42–2, such as 42–1, gives a worse fit of both potentiometric and spectrophotometric data. Another possibility, which was discarded, was the formation of species with higher nuclearity (higher number of central metal ions per complex molecule); such species could not explain the observed number of protons of the ligand released per Cu(II) ion in the complexation, nor could they fit the obtained spectrophotometric data.

The values finally proposed in this work for the stability constants of the formed species are (see Table III): $\log \beta_{101} = 9.47$, $\log \beta_{102} = 13.49$, $\log \beta_{111} = 11.7$, $\log \beta_{220} = 17.9$, $\log \beta_{22-1} = 12.5$, $\log \beta_{420} = 27.2$, $\log \beta_{42-2} = 8.9$. The first 5 values were obtained from the potentiometric data, and the last three from the spectrophotometric data.

Conclusions

N9 methylation of adenine blocks this atom as a binding site at neutral and basic pH, therefore preventing the formation of stable complexes in this pH region.

At acidic pH, N9 methylation displays a different influence depending on the metal ion. Cu(II) binds more strongly to adenine than to 9-methyladenine, reflecting a steric influence of the methyl group and, therefore, an important contribution of the N3 binding site. Zn(II) forms a more stable complex with 9-methyladenine than with adenine, showing that in this case the N9 methylation enhances the complexation via N7. This seems to be a highly interesting factor in the discussion of metal ion binding and the specificity of nucleosides and nucleotides.

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