

Co-ordination of copper(II) by amikacin. Complexation equilibria in solution and oxygen activation by the resulting complexes†

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Protonation and copper(II) co-ordination properties of amikacin (A) were studied in solution by potentiometry, and NMR, UV/VIS, CD and EPR spectroscopies. Mononuclear, tetragonal and five-co-ordinate complexes of stoichiometries ranging from $\text{Cu}(\text{H}_3\text{A})$ to CuH_{-2}A were found. The effects of amikacin on copper(II) binding by physiological copper(II) carriers, histidine and albumin, and facilitation of oxidation of 2'-deoxyguanosine by copper(II)–amikacin complexes were also investigated. The results indicate that complexation of Cu^{II} by amikacin should not be expected to affect copper(II) homeostasis in blood, but may contribute to the intracellular activity of the drug.

Amikacin is a semisynthetic aminoglycosidic antibiotic (a derivative of kanamycin A) active against Gram-negative bacteria, developed to combat gentamycin-resistant strains.¹ It kills bacteria by inhibiting the translation step in microbial protein synthesis and subsequently damaging cytoplasmic membrane.^{2,3} Amikacin has a broad spectrum of activity and is also widely used to prevent bacterial infections in cell cultures. A major disadvantage of amikacin, common to aminoglycosides, is its auditory-⁴ and nephro-toxicity.⁵ These result in a narrow therapeutic window of blood plasma concentrations, thereby reducing amikacin's use in therapy to life-threatening situations and requiring monitoring of the drug's plasma level.^{6,7}

Simple aminohexoses can bind copper(II) ions in the weakly acidic to neutral pH range. Primary binding occurs through the amino nitrogen. The effectiveness of the binding depends, however, on whether a five- or six-membered chelate ring can be formed with an appropriately located hydroxyl oxygen. If this is not possible a monodentate complex cannot withstand hydrolysis above pH 7.^{8,9} Otherwise, a range of chelate complexes is formed, with Cu^{II} bound through nitrogens and protonated or deprotonated sugar oxygens.^{8–15}

Binding of Cu^{II} to aminoglycosides has been the subject of several studies,^{16–21} with kanamycin being the one most widely studied.^{16–19} Binding modes similar to those discovered for simple aminosugars, but not involving hydroxyl oxygen deprotonations, were proposed. The main interest of the authors was to selectively block particular functional groups for synthetic purposes. Complexation phenomena were therefore not studied systematically beyond the assumed acylation reaction conditions.

Amikacin differs from kanamycin A by having the B1 amino group of the 2-deoxystreptamine moiety modified by acylation with 4-amino-2-hydroxybutyric acid. This modification, designed to prevent inactivation of the antibiotic by bacterial enzymes, adds to the molecule a peptide bond, a hydroxyl group and a terminal amino group. These potential donor groups are arranged so that they can all participate in a metal binding site alternative to, and possibly stronger than, the aminohydroxyl binding characteristic of aminosugars. The first objective of this study was therefore quantitatively and structurally to describe the interaction of Cu^{II} with amikacin in order to find whether such interaction might be possible *in vivo*. The

copper(II)–histidine 1:1 complex is probably a major component of the low-molecular-mass fraction of copper in blood plasma.²² It has a potential to form ternary complexes with aminosugar analogues, as was recently demonstrated,²³ and such complexes ought to be taken into account in the analysis of speciation *in vivo*. Therefore we also studied the formation of ternary complexes between Cu^{II} , amikacin, and histidine. Copper(II) was reported to enhance antibacterial activity of two aminoglycosides, gentamycin and streptomycin, in an *in vitro* test.²⁴ This finding does not seem to have been followed by detailed studies, but these preliminary results nevertheless serve another good reason for our study.

Acidic pH and anaerobic conditions^{25,26} suppressed the bactericidal activity of amikacin. These facts are believed to reflect diminished active transport of the antibiotic through the microbial cytoplasmic membrane, which results from the inhibition of bacterial metabolism under such conditions. However, interestingly, low pH is a factor discouraging copper(II) complexation with aminosugars and hypoxia would inhibit oxidative activity of such complexes. Hypoxia partially inhibited killing of the bacteria by amikacin even after transport had been restored.²⁵ This might indicate the existence of an oxygen-dependent component in the mechanism of amikacin bactericidal action.

We have recently discovered that copper(II) complexes of an iminosugar, 1-deoxynojirimycin, activate oxygen and hydrogen peroxide very efficiently, oxidising 2'-deoxyguanosine (dG) to its 8-oxo derivative.²³ It therefore seemed important to investigate whether copper(II)–amikacin complexes can catalyse oxidative processes that might contribute to their biological activity.

Materials and Methods

Amikacin was from Fluka, CuCl_2 , D_2O , NaOD, DCl, KNO_3 , ethane-1,2-diol, methanol and 3-(trimethylsilyl)propionic acid sodium salt (tsp) from Aldrich. L-Histidine, bovine serum albumin (BSA, essentially fatty acid free), dG and H_2O_2 , chelex 100 resin, sodium and potassium phosphates were from Sigma. The reference sample of 8-oxo-dG was a gift of Dr. K. S. Kasprzak (National Cancer Institute–Frederick Cancer Research and Development Center).

Potentiometry

Potentiometric titrations of binary and ternary complexes of Cu^{2+} with amikacin and histidine in the presence of 0.1 M KNO_3 were performed at 25 °C using pH-metric titrations over

† Supplementary data available: chemical shifts and coupling constants. For direct electronic access see <http://www.rsc.org/suppdata/dt/1998/153/>, otherwise available from BLDSC (No. SUP 57312, 5 pp.) or the RSC Library. See Instructions for Authors, 1998, Issue 1 (<http://www.rsc.org/dalton>).

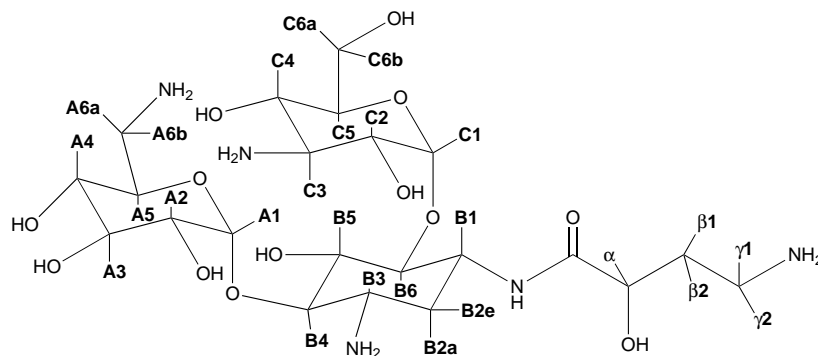


Fig 1 The molecule of amikacin in the fully deprotonated form (A). Non-exchangeable protons are marked in bold

the range pH 3–11.5 (Molspin automatic titrator) with NaOH as titrant. Changes in pH were monitored with a combined glass–calomel electrode (Russell) calibrated daily in hydrogen concentrations by HNO₃ titrations.²⁷ Sample volumes were 1.5 cm³. Concentrations of ligands were 10⁻³ mol dm⁻³. Ligand : Cu²⁺ molar ratios between 2:1 and 1:1 (binary system) and 1:1:1 (ternary system) were used. The data were analysed using the SUPERQUAD program.²⁸ Standard deviations computed thereby refer to random errors only. They give, however, a good measure of the importance of a given species in solution.

Spectroscopy

The CD spectra were recorded at 25 °C on a JASCO J-600 spectropolarimeter over the range 190–750 nm, using 1 and 0.1 cm cuvettes. For ligand spectra the amikacin concentration was 10⁻³ mol dm⁻³. For complexation experiments, samples with 2:1 and 1:1:1 ligand-to-metal ratios were used, with a Cu²⁺ concentration of 10⁻³ mol dm⁻³. Additionally, the binary system was checked for aggregation/oligomerisation by recording the spectra at pH 7, 9 and 11 at 0.003 and 0.01 mol dm⁻³. In the BSA competition experiment samples containing 7 × 10⁻⁴ mol dm⁻³ BSA and Cu^{II} in 0.15 mol dm⁻³ NaCl were titrated with 0.06 mol dm⁻³ histidine or 0.06 mol dm⁻³ histidine + 0.06 mol dm⁻³ amikacin. These samples were adjusted to pH 7.4 with small amounts of concentrated HCl or NaOH. The removal of Cu^{II} from BSA by histidine + amikacin was followed by recording the CD spectra after each increment at titrant. The spectra are expressed in terms of Δε = ε_l - ε_r, where ε_l and ε_r are the molar absorption coefficients for left and right circularly polarised light, respectively.

Electronic absorption (UV/VIS) spectra were recorded on a Beckman DU-650 spectrophotometer over the spectral range 190–1100 nm in 1 and 0.1 cm cuvettes, using the same samples as in the CD measurements. The EPR spectra were recorded at 120 K on a Bruker ESP 300E spectrometer at the X-band frequency (9.3 GHz). Ethane-1,2-diol–water (1:2) was used as a solvent in order to obtain homogeneity of frozen samples. Sample concentrations were similar to those used in CD measurements. Proton NMR spectra of 0.005 mol dm⁻³ amikacin were recorded at 27 °C with a Varian VXR500S spectrometer at 499.84 MHz; tsp was used as internal standard. The pH* (pH reading of the electrode not corrected for isotope effects) of the samples was adjusted by adding small volumes of concentrated DCl or NaOD. The correlation (COSY) spectrum was run in the absolute value mode and processed using standard Varian software.

8-Oxo-dG formation

Solutions of dG (10⁻⁴ mol dm⁻³) in 0.1 mol dm⁻³ sodium phosphate buffer, pH 7.4, were incubated in triplicate for 24 h at 37 °C in the presence of combinations of amikacin, histidine, Cu^{II} (0 or 10⁻⁴ mol dm⁻³), and H₂O₂ (0 or 10⁻³ mol dm⁻³). All stock solutions, except for Cu^{II} and H₂O₂, were purified with Chelex 100 prior to use. After incubation, the dG samples were

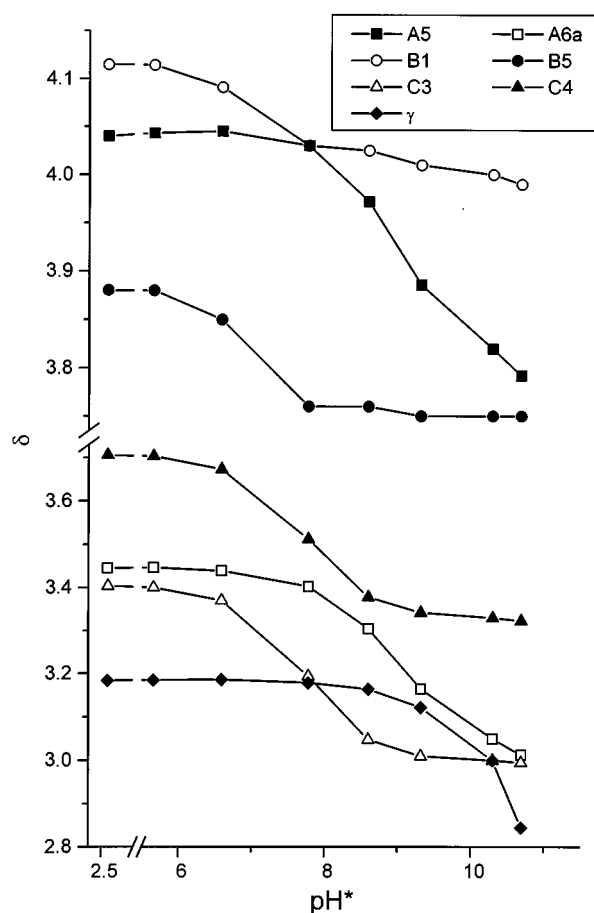


Fig. 2 Dependence of chemical shift (from tsp) of selected amikacin protons on pH*

analysed by HPLC without any additional pretreatment on a Beckman Gold system using UV detection (254 nm). A reversed-phase Beckman Ultrasphere ODS C₁₈ column (4.6 mm × 25 cm) was used. The mobile phase was 0.05 mol dm⁻³ KH₂PO₄ solution in 12% aqueous methanol. Results were quantified with the use of standard solutions containing known amounts of dG and 8-oxo-dG.

Results and Discussion

Protonation and conformation of the ligand

The amikacin molecule (Fig. 1) contains four amino groups. The protonation macroconstants were obtained from potentiometric titrations and are presented in Table 1. In order to correlate these values with particular protonation sites, one-dimensional NMR spectra were recorded in unbuffered D₂O solutions at various pH* (pH meter reading calibrated in water, uncorrected for D₂O isotopic effect) between 2.6 and 10.69 and

Table 1 Protonation of amikacin

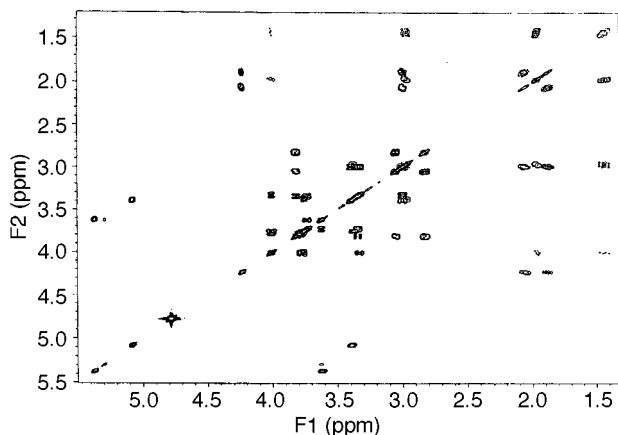
Species	log β^a	pK	pK*	Location of deprotonating amino group
H ₄ A	33.433(2)	6.831	6.98(7) ^b	Ring B
H ₃ A	26.602(2)	7.811	7.75(2) ^c	Ring C
H ₂ A	18.791(2)	8.890	9.08(6) ^d	Ring A
HA	9.901(2)	9.901		Aglycon chain

^a $\beta(H_xA) = [H_xA]/[A][H]^x$; standard deviations on the last digit are given in parentheses. ^b Calculated from B2a, B2e and B5/B6 signals. ^c Calculated from C2, C3 and C4 signals. ^d Calculated from A1, A5 and A6a signals.

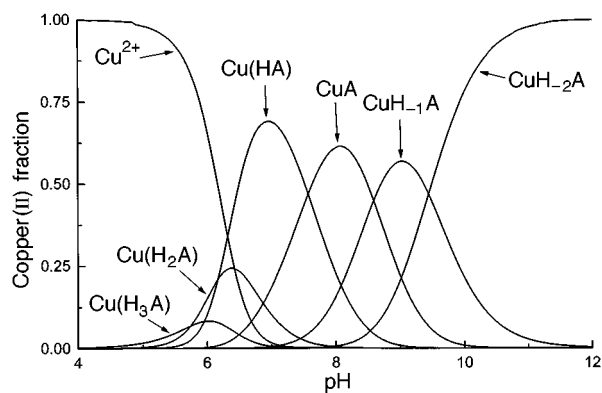
Table 2 Chemical shifts (δ , tsp) of amikacin protons at pH* 7.78. For notation, see Fig. 1

Ring A		Ring B		Ring C		Aglycon chain	
A1	5.517	B1	4.030	C1	5.125	α	4.271
A2	3.647	B2a	1.518	C2	3.575	$\beta 1$	1.977
A3	3.79 ^a	B2e	2.001	C3	3.194	$\beta 2$	2.178
A4	3.380	B3	3.094	C4	3.512	γ	3.179
A5	4.030	B4	3.500	C5	4.048		
A6a	3.403	B5	3.76*	C6	3.803		
A6b	3.165	B6	3.76*				

* Overlapping multiplet.

**Fig. 3** A COSY spectrum (500 MHz) of amikacin (0.005 mol dm⁻³) at pH* 10.3

titration curves for individual protons of amikacin molecule were generated (see Fig. 2 for examples). Data obtained from these spectra and from a COSY spectrum recorded at pH* 10.3 (Fig. 3) allowed us to assign the amikacin spin system. The assignments (Table 2) are in agreement with those reported previously for phosphate-buffered solutions and obtained with different two-dimensional NMR techniques.²⁹ The existence of parallel deprotonations was investigated by calculating pK* (pK values calculated with uncorrected pH-meter readings in D₂O) values from titration curves of individual protons of rings A, B and C with the use of the Hill equation. Protons of rings A and C yielded uniform titration curves, described by co-operativity coefficients of 0.8 ± 0.2 and 0.9 ± 0.1 , respectively. The pK* values obtained in these calculations are numerically very close to corresponding potentiometric macroconstants (see Table 1). The behaviour of ring B protons was somewhat more complicated. The pH* dependence of δ values for hydrogens B2a, B2e and B5/B6 can be very well described by a pK* of 6.98 and co-operativity coefficient of 0.9 ± 0.1 . On the other hand, signals for protons B3, B4, and, in particular, B1 are additionally sensitive to the deprotonation of the aglycon chain. The resulting pH* profiles appear to produce a higher pK value of ca. 7.5 for B3 and B4 (and very high errors of calculation), but in fact they are superpositions of B ring and aglycon deprotonations. Fig. 2

**Fig. 4** Species distribution for amikacin complexes for concentrations used in spectroscopic studies (0.001 mol dm⁻³ Cu^{II}, 0.002 mol dm⁻³ amikacin)

presents these profiles for B1 and B5, clearly showing this effect. The selective sensitivity of B1, B3 and B4 may indicate the location of the aglycon chain above the B ring, and an interaction between the B and aglycon amino group. However, our data indicate that the extent of parallel deprotonations for amino groups of these rings does not exceed 10%, and therefore protonation macroconstants can be assigned to particular amino groups, as presented in Table 1 [the pK and pK* values are numerically very close because deuterium isotopic effects on electrode readings (pH*) and on pK values tend mutually to cancel].³⁰

The conformation of the amikacin molecule remains essentially unchanged throughout the investigated pH* range, despite the charge change between +4 and 0. This is seen in the NMR spectra in the values of J_{HH} coupling constants, indicating chair conformations of all three rings. None of the J values varied by more than 0.5 Hz throughout the whole pH* range (see SUP 57312). Also, the magnitudes of the chemical shift changes of particular protons are determined solely by their distances from the deprotonating amino groups of their rings (SUP 57312). The CD spectrum of amikacin consists of two bands of opposite signs and similar magnitude ($\lambda = 216$, $\Delta\epsilon = +1.47$; 194 nm, $\Delta\epsilon = -2.0$ dm³ mol⁻¹ cm⁻¹), and remains virtually unchanged between pH 2 and 11.5. The only chromophore present that can absorb above 190 nm is the amide connecting the B ring with the aglycon chain. The lack of change in the CD spectra therefore confirms that the conformation of this part of the molecule is not affected by deprotonations.

The pK values found for the individual amino groups agree very well with those previously observed for similar moieties. The value for the aglycon chain is typical for aliphatic amines (around 10), the value for the C6-NH₂ group of ring A is almost identical to that obtained for 6-amino-6-deoxyglucopyranose, 8.94,¹⁵ and the value for the C3-NH₂ group of ring C is in the range typical for aminosugars, 7 to 8.²³ The relatively low value found for the amino group of ring B is due to the high overall charge of the fully protonated amikacin molecule, and possibly to the inductive effect of the neighbouring hydroxyamide moiety. An electrostatic interaction with the charged aglycon amine, emerging from the NMR data, is also likely.

The supplementary material contains lists of chemical shifts and J_{HH} constants vs. pH* and a list of cross-peaks observed in the COSY spectrum.

Copper(II) co-ordination

Stability constants of the copper(II)-amikacin complexes calculated from potentiometric titrations are presented in Table 3. A speciation diagram calculated with these constants for concentrations used in spectroscopic experiments is shown in Fig.

Table 3 Potentiometric and spectral data (CD, UV/VIS, EPR; λ/nm ; ϵ , $\Delta\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$; A_{\parallel}/G ; $G = 10^{-4} \text{ T}$) for the amikacin–copper(II) system

Species	$\log \beta^a$	UV/VIS λ (ϵ)	CD λ ($\Delta\epsilon$)	EPR	
				A_{\parallel}	g_{\parallel}
Cu(H ₃ A)	29.68(9)	Minor	Minor	130	2.35
Cu(H ₂ A)	23.94(1)	Minor	Minor	130	2.35
Cu(HA)	17.77(1)	628 (59)	649 (−0.41), ^b 362 (+0.10), ^c 297 (sh) (−0.81), ^d 267 (−1.34) ^e	187	2.24
CuA	10.20(1)	614 (70)	740 (+0.03), ^f 615 (−0.09), ^f 515 (+0.02), ^f 372 (+0.04), ^e 287 (−1.43), ^d 272 (sh) (−1.29) ^e	162	2.19
CuH _{−1} A	1.61(1)	670 (45) 607 (71)	>750 (−0.13), ^f 542 (+0.09), ^f 357 (+0.12), ^e 296 (sh) (−0.43), ^d 265 (−1.20) ^e	150	2.16
CuH _{−2} A	−7.83(1)	670 (45) 596 (67)	750 (−0.23), ^f 615(sh) (+0.25), ^f 547 (+0.40), ^f 273 (+2.38), ^g 229 (−2.14) ^h	Rhombic	

^a $\beta[M(\text{H}_x\text{A})] = [M(\text{H}_x\text{A})]/[M][\text{H}]^x[\text{A}]$; standard deviations on the last digit are given in parentheses. ^b d–d Electronic transition of Cu^{II} in a tetragonal complex. ^c O→Cu^{II} CT transition. ^d N→Cu^{II} CT transition. ^e NH₂→Cu^{II} CT transition. ^f d–d Electronic transition of Cu^{II} in a lower-symmetry complex. ^g Mixture of O, N[−] and NH₂→Cu^{II} CT transitions. ^h Intraligand transition.

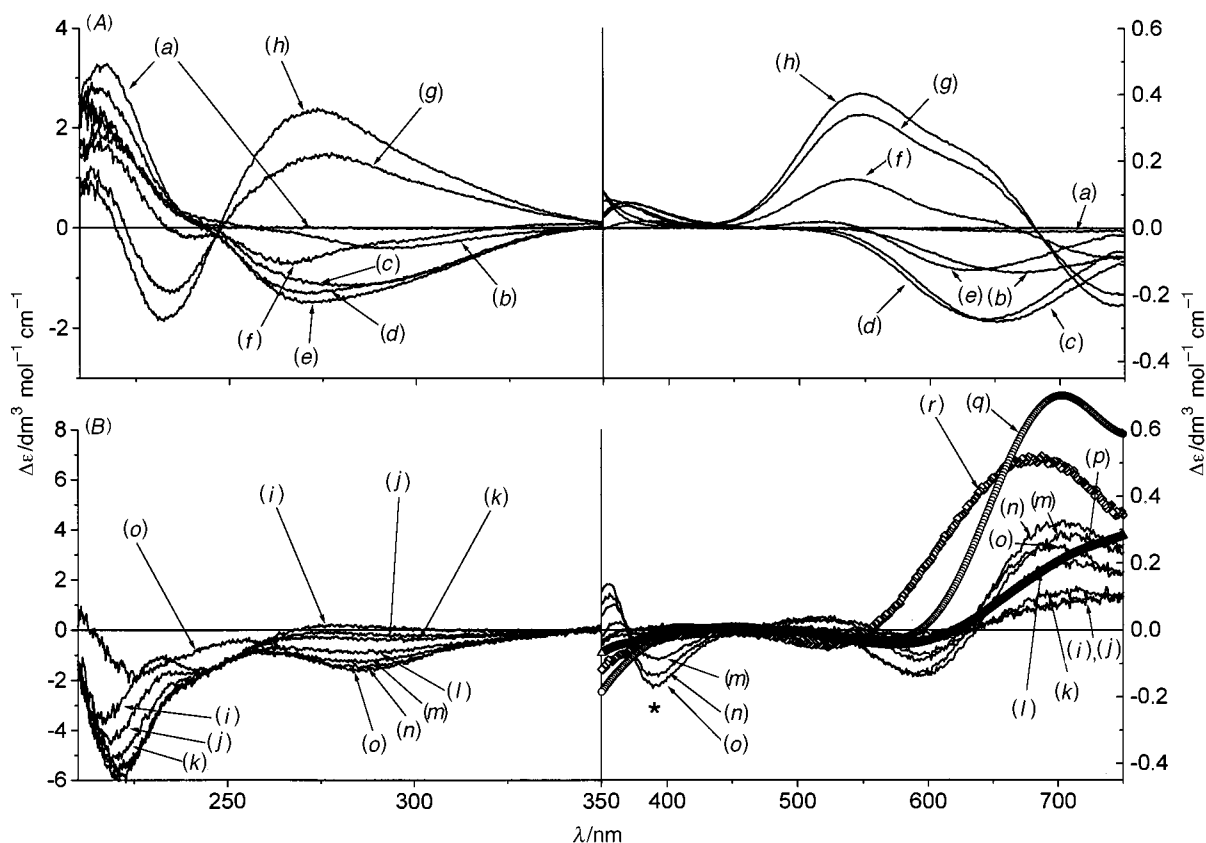


Fig. 5 The CD spectra of copper(II)–amikacin complexes. (A) Binary system, amikacin:Cu^{II} = 2:1 at various pH: (a) 5.51, (b) 6.02, (c) 7.02, (d) 7.41, (e) 8.11, (f) 8.88, (g) 10.00 and (h) 11.50. (B) Ternary system, amikacin:histidine:Cu^{II} = 1:1:1 at various pH: (i) 5.62, (j) 6.67, (k) 7.14, (l) 7.95, (m) 8.67, (n) 9.69 and (o) 11.2. For comparison, spectra of Copper–histidine complexes are overlaid: (p) CuL, (q) CuL₂ and (r) CuH_{−1}L. The asterisk marks the unique CT band in the ternary system (see text)

4. Only 1:1 complexes were detected by potentiometry and EPR spectroscopy gave no indication of the formation of copper(II) dimers. The pH dependence of the absorption and CD spectra exhibited excellent consistency with potentiometric speciation, and thus allowed us to calculate spectroscopic parameters of particular complex species (Table 3). All bands located above 250 nm could be assigned to particular d–d or charge transfer (CT) transitions on the basis of previous studies of copper(II) complexation to aminosugars^{8–11,23} and peptides.^{31–34} Examples of experimental CD spectra are presented in Fig. 5(A). No aggregation was found in the solutions studied.

The concentration of the initial complex at low pH, Cu(H₃A), is too low for this species to be characterised by absorption and CD spectra. However, a complex species could be seen in the parallel part of the EPR spectra beside the copper(II) aqua-ion at pH 5–6, where Cu(H₃A) and Cu(H₂A) are

present. This species has parameters characteristic of one nitrogen donor co-ordinated to copper(II) in aminosugar systems (1N complexes).^{14,23} The Cu(H₃A) complex releases two protons with pK values of 5.74 and 6.26, forming the Cu(HA) species. Spectroscopic parameters of this complex are consistent with two nitrogen (2N) co-ordination. In particular, its EPR parameters are very similar to those seen previously for CuL₂ complexes of aminosugars (A_{\parallel} of 170–190 G, g_{\parallel} 2.23–2.24).^{9,14–16} Three further deprotonations are seen, each one lowering the effective symmetry of the complex and introducing profound changes in spectroscopic parameters. The stoichiometry of the final high-pH complex, CuH_{−2}A, indicates that in addition to four amino groups, two other donor groups deprotonate. These additional groups, described by negative indices for the hydrogen in the formula, must be bound to Cu^{II} because they would not deprotonate otherwise. The amino nitrogen of ring B is the most likely anchoring site for Cu^{II} in the amikacin molecule,

due to its lowest pK . This binding results in the formation of the $\text{Cu}(\text{H}_3\text{A})$ complex. Inspection of molecular models indicates that such binding excludes a possibility of an involvement of the C ring amino group in co-ordination. Therefore the deprotonation pattern of the complex must include a proton dissociation from this amino group, without the participation of copper(II) binding. The pK for formation of the CuA complex, 7.57, is numerically closest to the protonation constant of the C-ring nitrogen in free amikacin. The formation of this complex, and of the further ones at higher pH, results, however, in significant rearrangements of the complex structure. It is difficult to see how a deprotonation of the amine group at least 6 Å away from the Cu^{II} might cause that. Therefore, the C-ring amino group must deprotonate at a lower pH. In our recent study of protonation of kanamycin B, an aminoglycoside with five amino groups, we found that the lowest pK is 5.74, identical to the pK for formation of the $\text{Cu}(\text{H}_2\text{A})$ complex of amikacin from $\text{Cu}(\text{H}_3\text{A})$.³⁵ Both deprotonating species have the same overall electrostatic charge of 5+ [in $\text{Cu}(\text{H}_3\text{A})$ it is due to monodentate Cu^{2+} co-ordination]. However, the exact reasons for such a low pK value remain unclear. As a result of the non-coordinative deprotonation in ring C, the $\text{Cu}(\text{H}_3\text{A})$ and $\text{Cu}(\text{H}_2\text{A})$ complexes have the same copper(II) binding site, and thus identical EPR spectra. This leads to the amplification of their spectral pattern, which becomes detectable by EPR spectroscopy. The molecular model indicates that both the amide nitrogen and the C5 oxygen of the B ring can easily complete six-membered chelate rings when Cu^{II} is bound to the B amino group. The pK value corresponding to the formation of $\text{Cu}(\text{HA})$, 6.17, is closer to those found for amide binding, usually 5–6,^{31,32} than for deprotonated sugar oxygen co-ordination, 6.5–7.^{8–15,23} The d–d band in the CD spectrum of $\text{Cu}(\text{HA})$ is negative, as is always the case for 2N complexes of simple peptides, while copper(II) complexes of aminosugars with deprotonated oxygens invariably have a positive band between 700 and 600 nm. The chelate ring formed by binding to both nitrogens at ring B is six-membered, as opposed to the five-membered rings in typical peptide complexes. The literature data discussed in ref. 23 indicate, however, that the five- and six-membered rings in aminosugar complexes do not differ much in stability, due to a fixed conformation of donor atoms offered by the rigid ligand. Deoxystreptamine amide is conformationally similar to aminosugars (*cf.* the NMR results), and so we can safely conclude that in the $\text{Cu}(\text{HA})$ complex Cu^{II} is co-ordinated to amikacin through both nitrogens of ring B. The oxygen of the C5–OH group of this ring can easily occupy the apical position in the copper(II) co-ordination sphere in $\text{Cu}(\text{HA})$ (as defined by the 2N co-ordination), thus completing a set of three fused chelate rings. Alcoholic oxygen co-ordination, well known for aminosugar complexes, is manifested by a characteristic $\text{O} \rightarrow \text{Cu}^{\text{II}}$ charge transfer (CT) band at *ca.* 360 nm (Table 3).³⁶ This oxygen deprotonates while bound to Cu^{II} , yielding CuA . The pK value for this process, 7.53, is typical for analogous processes of formation of equally charged $\text{CuH}_{-2}\text{L}_2$ complexes with aminosugars.^{8–15,23} Such strong apical co-ordination causes the decrease in complex symmetry, manifested by the appearance of three d–d bands in the CD spectrum of CuA and a slight rhombic distortion in its EPR spectrum (decrease of A_{\parallel}).

The following two deprotonations, to CuH_{-1}A and CuH_{-2}A , correspond to the binding of the two remaining amino groups to Cu^{II} . They are accompanied by the appearance of a two-band envelope in the absorption spectrum, characteristic for distorted square-pyramidal copper(II) complexes,^{37,38} as well as the increased rhombicity of the EPR spectrum. Each of these events results in extensive changes in the CD spectra. The centre of gravity of the band envelope shifts towards shorter wavelengths upon formation of CuH_{-1}A , indicating an increase of in-plane ligand field. The pK value, 8.59, corresponds to deprotonation at ring A in free amikacin (8.89). The molecular model

indicates that the A ring amine nitrogen binds *trans* to the amide nitrogen at Cu^{II} , creating a large (10-membered) macrochelate ring. Such binding redefines the co-ordination sphere by placing the B ring amine in the apical position. This explains the alterations of the signs of the d–d bands. The ligand-field effect of the A amine is stronger than from the B amine, as seen in the spectra [Fig. 5(A)]. There is little steric hindrance for the A ring binding, but also no additional effects that might enhance it, thus only a 0.3 log unit stability gain. The final remaining amino group, at the aglycon chain, can also bind only apically relative to the plane of CuA_{-1}H , again redefining the complex geometry. The absorption spectrum is insensitive to this rearrangement, but CD and EPR data clearly support it. The intensity of Cotton effects increases several-fold, indicating the increase of complex rigidity. The parallel EPR parameters cannot be extracted for the spectrum corresponding to this complex. This may be due to a heavy overlap of individual spectra in frozen samples. Another potential donor, the α -hydroxy group of the aglycon chain, is excluded from co-ordination by the rigidity of the amide bond, placing it away from Cu^{II} . A seven-membered chelate ring is formed. Such rings offer little entropic stabilisation, and thus a small stability gain of 0.5 log units.

Copper(II) competition experiments

The CD spectra of equimolar mixtures of amikacin, histidine and Cu^{II} feature a negative band at 390 nm, the intensity of which increases with increasing pH [Fig. 5(B)]. Such a band is absent from either binary system, thus indicating the formation of ternary complexes. On the other hand, EPR spectra of ternary mixtures could be easily deconvoluted into the spectra seen in binary complexes. However, the relative abundances of the binary components were different from those expected on the basis of their stability constants. These apparent discrepancies might indicate a dimeric or oligomeric and diamagnetic nature of the ternary complexes. Various stoichiometries are possible *a priori*: Cu_2AL , $\text{Cu}_2\text{A}_2\text{L}$ or Cu_2AL_2 being the simplest, but not necessarily the most likely (A stands for amikacin, L for histidine). The SUPERQUAD calculations accepted all these stoichiometries, as well as a set of monomeric ternary complexes, with similarly good fits. The nature of ternary copper(II)–amikacin–histidine complexes remains therefore open and will be a subject of our further studies.

Copper(II)–amikacin complexes are very stable: a simulation of the partition of Cu^{II} between amikacin and histidine in an equimolar system at pH 7.4, with binary complexes only, shows that amikacin binds as much as 35% of Cu^{II} at 10^{-3} mol dm^{-3} and 25% at 10^{-4} mol dm^{-3} of the components. The existence of ternary complexes should further increase the fraction of Cu^{II} in amikacin-containing species. We therefore tested whether amikacin could be capable of affecting copper(II) homeostasis in blood plasma by comparing the competitiveness of the 1:1 amikacin–histidine mixture *vs.* histidine alone for removal of Cu^{II} from the N-terminal binding site of BSA. Thus, 1 mol equivalent of Cu^{II} was added to BSA at pH 7.4 and the visible CD spectrum of the resulting complex was recorded. This $\text{BSA-Cu}^{\text{II}}$ complex was then titrated with histidine, with the pH maintained at 7.4. The competition for Cu^{II} between BSA and His was followed by recording the CD spectra after each addition of His. The removal of Cu^{II} from BSA was quantified with the use of ellipticity at 475 nm. At this wavelength, which is the maximum for the Cu^{II} –BSA complex, Cotton effects from histidine, amikacin and their complexes at pH 7.4 are negligible. A curve of partition of Cu^{II} between BSA and histidine was thus generated. The same procedure was then repeated, using a 1:1 mixture of histidine and amikacin. Fig. 6 presents both curves. One can clearly see that the presence of amikacin did not affect the speciation of Cu^{II} between albumin and histidine *in vitro*, and is thus unlikely to do so in blood plasma.

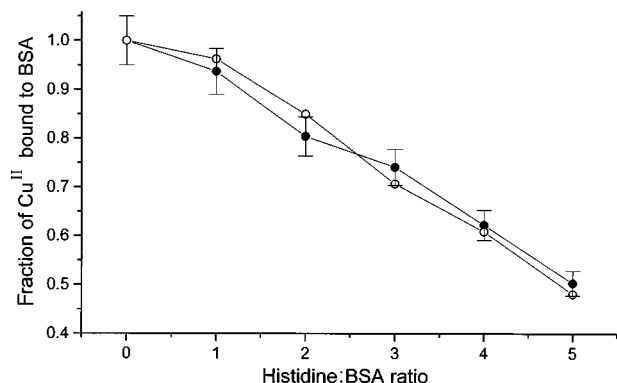


Fig. 6 Effect of amikacin on the partition of Cu^{II} between bovine serum albumin and histidine. Titration of $7 \times 10^{-4} \text{ mol dm}^{-3} \text{Cu}^{\text{II}}$ -BSA at pH 7.4 and 25°C with histidine (●), and 1:1 histidine + amikacin (○). The concentration of the complex was calculated from the ellipticity at 475 nm (see text for further details). Experimental error bars are shown on ● only for clarity

Table 4 Oxidation of dG by H_2O_2 facilitated by amikacin (A), histidine (His) and their copper(II) complexes. Concentrations (mol dm^{-3}): dG, Cu^{II} , His and A 10^{-4} ; H_2O_2 10^{-3} ; phosphate buffer (pH 7.4) 0.1; 24 h incubations at 37°C

Components	8-oxo-dG yield (%) ^a	dG decomposition (%) ^b
Control ^c	0.04 ± 0.01	0
Cu^{II}	0.17 ± 0.1	12 ± 1
His	0.03 ± 0.01	0
Cu^{II} + His	0.33 ± 0.06	31 ± 2
A	0.15 ± 0.03	0
Cu^{II} + A	3.5 ± 0.5	44 ± 4
Cu^{II} + A + His	9.4 ± 0.2	42 ± 3

^a Averages of five measurements \pm standard errors, normalised to initial dG concentrations. ^b Detection limit = 4%. ^c dG + H_2O_2 .

Oxidative properties of copper(II)-amikacin complexes

The compound 8-oxo-dG is an intermediate in the process of dG oxidation, important for its promutagenic properties. The reaction of its formation from dG also serves as a useful general indicator of the ability of a given system to exert oxidative damage. Results of the oxidation of dG by H_2O_2 in the presence of Cu^{II} , amikacin, or/and histidine, presented in Table 4, indicate that copper(II)-amikacin complexes are particularly specific in facilitating 8-oxo-dG formation by H_2O_2 . As much as 8% of reacted dG was converted into 8-oxo-dG in 24 h, as opposed to 1% generated by copper(II)-histidine, with a similar extent of dG decomposition. The ternary system, more likely to occur *in vivo* than the binary one, was even more specific, with 22%.

Oxygen metabolism generates appreciable amounts of H_2O_2 intracellularly. If even minute amounts of Cu^{II} are available, *e.g.* via degradation or damage of respiratory chain enzymes, then amikacin-induced oxidations may become an important element of cellular toxicity of the drug, by means of oxidative damage to DNA or other biomolecules. The results presented above also indicate a possibility that oxidative properties of copper(II) complexes of amikacin contribute to its bactericidal activity, because hypoxia was shown to help bacteria survive amikacin therapy.²⁵

Conclusion

Amikacin co-ordinates Cu^{II} at physiological pH through the amino and amide nitrogens and the C5 oxygen of the B (deoxystreptamine) ring [$\text{Cu}(\text{HA})$, CuA]. At higher pH also ring A and the aglycon chain amino groups are involved in the binding (CuH_{-1}A and CuH_{-2}A complexes, respectively).

The complexes are very strong, only slightly less stable than those of histidine. However, amikacin does not contribute to competition between histidine and serum albumin and thus should not be expected to affect copper homeostasis in blood plasma.

Copper(II)-amikacin complexes, both in the absence and presence of histidine, very efficiently facilitate hydroxylation of dG to promutagenic 8-oxo-dG by hydrogen peroxide, as opposed to copper(II)-histidine alone. We can therefore conclude that the interaction with Cu^{II} and resulting oxygen activation may become physiologically relevant intracellularly, especially in cells with active oxygen metabolism, thus contributing to both the therapeutic and toxic properties of this drug.

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