Stability of Some Metal-ion Complexes of Tubercidin (= 7-Deazaadenosine) in Aqueous Solution. An *o*-Amino Group inhibits Complexation at N^1 of Purines!

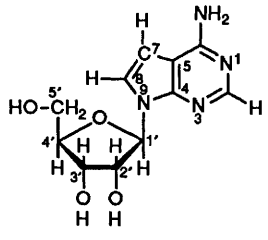
Liang-nian Ji,† Nicolas A. Corfù and Helmut Sigel *
Institute of Inorganic Chemistry, University of Basel, Spitalstrasse 51, CH-4056 Basel, Switzerland

The stability constants of the 1:1 complexes formed between Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} or Cd^{2+} and tubercidin (Tu) were determined by potentiometric pH titration in aqueous solution (I = 0.5 mol dm^{-3}, $NaNO_3$; 25 °C); some of the equilibrium constants were also independently measured by spectrophotometry. The self-association tendency of tubercidin was approximately quantified by ^1H NMR shift measurements; these experiments were hampered by solubility problems, but it could be assured that self-stacking is negligible under the conditions used for the determination of the stability constants of the $[M(Tu)]^{2+}$ complexes. Tubercidin is protonated at N^1 and at this site also metal-ion binding occurs. The fact that tubercidin has an *o*-amino group next to the N^1 site allows a quantification of the steric inhibitory effect of this *o*-amino group on the complexation tendency of the N^1 site. This effect is rather pronounced for Cu^{2+} and Ni^{2+} (the log stability constant is about 1.5 log units smaller than the constant expected on the basis of the basicity of N^1), and practically non-existent for Mn^{2+}; for Co^{2+}, Zn^{2+} and Cd^{2+} the effect is in between. These observations may be explained by assuming that a varying fraction of the $[M(Tu)]^{2+}$ complexes is formed *via* outer-sphere binding of a co-ordinated water molecule, *i.e.* by a hydrogen bond to N^1. Owing to the structural similarity of tubercidin with adenosine (Ado) (in Tu the N^7 of Ado is replaced by a CH unit) the reported results allow a more detailed evaluation of the N^1 and N^7 dichotomy for metal-ion binding present in adenosine complexes. Furthermore, for $[Cu(Cyd)]^{2+}$ (Cyd = cytidine) it is definitely shown, confirming an earlier suggestion, that the simultaneous presence of an *o*-amino *and* an *o*-carbonyl group next to the N binding site leads only to a somewhat reduced stability of the complex, indicating that a metal ion–carbonyl interaction compensates in part for the steric effect of the *o*-amino group.

Tubercidin is synthesised by molds and fungi.[1] It is a nucleoside with antibiotic properties and was first isolated in 1957 from the culture broth of *Streptomyces tubercidicus*;[2] its structure was described[3] in 1961 and later proven by total synthesis[4] and X-ray crystal structure analysis.[5,6] Tubercidin and its derivatives are antibacterial and antiviral agents, which are also active against some forms of cancer;[7] hence, it is not surprising that they are widely studied in enzymatic reactions,[8] and therefore a detailed study of the metal-ion affinity of tubercidin is justified in its own right, next to a further reason outlined below.

Tubercidin differs from the more common adenosine in the replacement of N^7 by a CH unit (see Fig. 1) and is therefore also known as 7-deazaadenosine.‡ Adenosine offers metal ions two binding sites, N^1 and N^7; this leads to a dichotomy of metal-ion binding to N^1 *versus* that to N^7, a problem which was first addressed in detail by Martin.[9] He showed that both metal-ion binding sites of purines must be considered; in addition, he provided ratios for the distribution of Ni^{2+}, Cu^{2+} and Zn^{2+} between the N^1 and N^7 sites of adenosine.

A re-evaluation[10] of the indicated analysis for the mentioned complexes of adenosine, including also an extension to the corresponding complexes with Mn^{2+}, Co^{2+} and Cd^{2+}, made it likely that the metal-ion affinity of N^1 in adenosine is smaller than originally assumed[9] due to steric hindrance of the amino group at C^6 which is in the *ortho* position to N^1 (see Fig. 1). Indeed, the present results obtained for tubercidin complexes confirm this; it is now definitely clear that an *ortho*-amino group inhibits metal-ion co-ordination at pyridine nitrogens



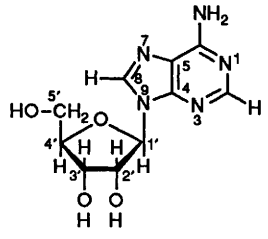


Fig. 1 Chemical structures of tubercidin (Tu) and adenosine (Ado)

† Work done during a leave from Zhongshan University, Guangzhou, People's Republic of China.

‡ Abbreviations: *A* = optical absorption; Ado = adenosine; AMP^{2-} = adenosine 5′-monophosphate; Cyd = cytidine; L = general ligand; M^{2+} = divalent metal ion; P = purine derivative; pic, α-picoline = 2-methylpyridine; py = pyridine; Tris = 2-amino-2-(hydroxymethyl)-propane-1,3-diol = tris(hydroxymethyl)aminomethane; Tu = tubercidin (= 7-deazaadenosine = 7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine); $TuMP^{2-}$ = tubercidin 5′-monophosphate (= 7-deaza-AMP^{2-}).

including N^1 of adenosine, and therefore a further and more sophisticated evaluation of the dichotomy of metal-ion binding in adenosine complexes is becoming possible.[11]

In addition it may be pointed out that the present results are meaningful also for considerations on the metal-ion binding properties of other nucleosides, *e.g.* cytidine [see section 3(*b*)], as well as for those of single-stranded nucleic acids.

Experimental

1(*a*) *Materials.*—Tubercidin (from *Streptomyces tubercidicus ca.* 95%) was from Sigma, St. Louis, MO, USA and used as obtained. However, from the potentiometric pH titrations and the curve-fit procedure for the determination of $pK^{H}_{H(Tu)}$ [see section 1(*c*)] the presence of any acid–base impurity could be excluded. The exact concentration of Tu was determined as given below. The disodium salt of ethylenediamine-*N,N,N',N'*-tetraacetic acid (Na_2H_2edta), potassium hydrogenphthalate, HNO_3, NaOH (Titrisol), a 10% tetramethylammonium hydroxide solution (which we converted into the nitrate), DNO_3 (99% D) and the nitrate salts of Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} (all pro analysi) were from Merck, Darmstadt, FRG. The perchlorate salt of Cd^{2+} was purchased from Ventron, Karlsruhe, FRG; all the other metal-ion perchlorates were obtained from Fluka, Buchs, Switzerland. The D_2O (99.8% D) and NaOD (98% D) were from Ciba-Geigy, Basel, Switzerland. All solutions were prepared with distilled CO_2-free water.

The exact concentrations of the tubercidin solutions were measured by titrations with NaOH. Its titre was determined with potassium hydrogenphthalate, and the concentrations of the stock solutions of the metal ions were established with edta.

1(*b*) *Proton NMR Spectroscopy.*—The ^1H NMR signals of tubercidin were assigned as previously.[12] The spectra were recorded with a Varian VXR 400 spectrometer (399.96 MHz) in D_2O at I = 0.1 mol dm^{-3} ($NaNO_3$) and 25 °C, using the centre peak of the tetramethylammonium ion triplet as internal reference. However, all measured chemical shifts were converted into the sodium 3-(trimethylsilyl)propane-1-sulphonate reference by adding 3.174 ppm.[13–15]

The pD of the D_2O solutions was measured with a Metrohm 6.0204.100 glass electrode connected with a Metrohm 605 digital pH meter (Metrohm, Herisau, Switzerland); the final pD of the D_2O solutions was obtained by adding 0.40 to the pH-meter reading.[16] The other parts of the experiments were done as before,[12] and the data were analysed as described[12] for the self-association constant of tubercidin [see also the Results section 2(*a*)] with a Hewlett-Packard Vectra 60PC MSDOS desk computer.

1(*c*) *Potentiometric* pH *Titrations.*—The pH titrations were carried out with a Metrohm E536 potentiograph, E655 dosimat and 6.0202.100 (JC) combined macro glass electrodes. The buffer solutions (pH 4.64, 7.00 and 9.00; based on the scale of the U.S. National Bureau of Standards)[17,18] used for calibration were also from Metrohm. The direct pH-meter readings were used in the calculations of the acidity constants; *i.e.* these constants are so-called practical, 'mixed' or Brønsted constants. The negative logarithms of these practical acidity constants given for aqueous solutions at I = 0.5 mol dm^{-3} and 25 °C may be converted into the corresponding concentration constants by subtracting 0.04 log unit;[19] this conversion term contains both the junction potential of the glass electrode and the hydrogen-ion activity coefficient (see, *e.g.*, ref. 20). It should be noted that for the stability constants of metal-ion complexes no conversion is necessary.

The acidity constant $K^{H}_{H(Tu)}$ of N^1-protonated tubercidin was determined by titrating 50 cm^3 of aqueous 1.54 × 10^{-3} mol dm^{-3} HNO_3 (I = 0.5 mol dm^{-3}, $NaNO_3$; 25 °C) in the presence and absence of 10^{-3} mol dm^{-3} tubercidin under N_2 with 1 cm^3 of 0.085 mol dm^{-3} NaOH and by using the differences in NaOH consumption between such a pair of titrations for the calculations. The value for $pK^{H}_{H(Tu)}$ was calculated from 13 independent pairs of such titrations by taking into account the species H^+, Tu and $H(Tu)^+$ and by using a curve-fit procedure with a Newton–Gauss non-linear least-squares program within the pH range determined by about 3 and 97% neutralization for the equilibrium $H(Tu)^+$/Tu. The calculation was carried out with a Hewlett-Packard Vectra 60PC MSDOS desk computer connected with a Brother M-1509 printer and a Graphtec 3100 plotter. The determination of $pK^{H}_{H(Tu)}$ was repeated at I = 0.1 mol dm^{-3} ($NaNO_3$) and 25 °C by four independent pairs of titrations (in this case 0.02 log unit should be subtracted to obtain the concentration constant of $pK^{H}_{H(Tu)}$;[21] see also above in the preceding paragraph).

It should be emphasized that in the described calculation procedure the ionic product of water (K_w) and the hydrogen-ion activity (γ) (to be more exact: the mentioned 'combined' term for converting the measured data into hydrogen-ion concentration) do *not* enter into the calculations, because we evaluate the *differences* in NaOH consumption between two corresponding solutions; *i.e.* always solutions with and without ligand are titrated (see above). The advantage of this procedure is (aside from not needing K_w and γ values) that impurities in the solvent or in the salts (see also below), as well as systematic errors, *etc.*, cancel.

The stability constants $K^{M}_{M(Tu)}$ of $[M(Tu)]^{2+}$ were measured under the same conditions used for most of the pairwise acidity-constant titrations, *i.e.* I = 0.5 mol dm^{-3} ($NaNO_3$), 25 °C and [Tu] = 10^{-3} mol dm^{-3}, but $NaNO_3$ was partially or fully replaced by $M(NO_3)_2$. For most M^{2+} systems (*i.e.* Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} or Cd^{2+}) titrations were made with $[M^{2+}]$ = 0.1667 mol dm^{-3} (*i.e.* Tu:M^{2+} = 1:167), and for Cu^{2+} with 0.1333 mol dm^{-3} (*i.e.* Tu:Cu^{2+} = 1:133); for Cu^{2+} and Cd^{2+} also $[M^{2+}]$ = 0.0833 mol dm^{-3} (*i.e.* Tu:M^{2+} = 1:83) were used. Each final value for $K^{M}_{M(Tu)}$ is the result of at least four independent pairs of titrations.

The stability constants $K^{M}_{M(Tu)}$ were calculated in two ways. (*i*) By taking into account the species H^+, $H(Tu)^+$, Tu, M^{2+} and $[M(Tu)]^{2+}$,[22] and by collecting the data every 0.1 pH unit from about 5% complex formation to a neutralization degree of about 85% or to the beginning of the hydrolysis of $M(aq)^{2+}$, which was evident from the titrations without tubercidin. However, even though the results obtained in this way agreed within the error limits with the following evaluation method, it turned out that due to the very small depression of the buffer region {the $[M(Tu)]^{2+}$ complexes are rather unstable} the agreement of the results between the independent titrations were more satisfactory if (*ii*) the curve-fitting procedure described above for $pK^{H}_{H(Tu)}$ was also used to determine an apparent acidity constant, K'_a, for the deprotonation of $H(Tu)^+$ in the presence of a large (and hence during the titration constant) excess of M^{2+}.[23] Values for $K^{M}_{M(Tu)}$ are then obtained from equation (1). In fact, for certain metal ions (especially

$$K^{M}_{M(Tu)} = (K'_a - K^{H}_{H(Tu)})/(K^{H}_{H(Tu)}[M^{2+}]_{tot}) \quad (1)$$

Mg^{2+} and Ca^{2+}) consistent and reasonable results could only be obtained with this evaluation procedure. Therefore, the stability constants given in the Results section 2(*c*) (Table 2, see below) are all based on calculations with equation (1).

1(*d*) *Spectrophotometric Measurements.*—The acidity constant $K^{H}_{H(Tu)}$ was also determined by spectrophotometry. The UV spectra ([Tu] = 3 × 10^{-5} and 7.5 × 10^{-5} mol dm^{-3}) were recorded on a Cary 219 instrument in aqueous solutions at 25 °C and I = 0.5 mol dm^{-3} ($NaClO_4$) with 1 cm quartz cells. The pH of the solutions was adjusted by dotting with relatively concentrated $HClO_4$ or NaOH and measured with a Metrohm 605 digital pH meter using a Metrohm 6.0202.100 (JC) glass

electrode. The spectrophotometric data were analysed with the mentioned Hewlett-Packard Vectra desk computer analogously as previously described for NMR data.[12] The spectra measured for solutions with [Tu] = 3×10^{-5} mol dm^{-3} (four experiments) were evaluated at 213 and 227 nm where H(Tu)$^+$ has a minimum and a maximum in its absorption spectrum, respectively, and also at 238 nm where Tu has a minimum in its spectrum; in addition the absorption differences were evaluated between 213 and 227 nm, as well as between 227 and 238 nm. Those spectra taken with [Tu] = 7.5×10^{-5} mol dm^{-3} (one experiment) were evaluated at 238 (minimum of the Tu spectrum), 270 (maximum) and 300 nm (shoulder), and also by the absorption differences between 238 and 270 nm, as well as between 270 and 300 nm. An example of an experimental series with some of its evaluated data is shown in Fig. 3 (see below). The final result given in Table 1 of section 2(*b*) is the average of five independent series of experiments with in total 25 evaluations at different wavelengths or absorption differences between different wavelengths.

The stability constants $K^{M}_{M(Tu)}$ of the $[Co(Tu)]^{2+}$, $[Ni(Tu)]^{2+}$ and $[Cd(Tu)]^{2+}$ complexes were also determined by spectrophotometry, *i.e.* by recording difference spectra of the UV region between 230 and 320 nm on the mentioned Cary instrument. The desired pH was adjusted to a value between 6 and 7, *i.e.* for each series of experiments to an exact value (mostly one close to pH 6.7), as described in the preceding paragraph. To achieve this the tubercidin solution was adjusted to such an alkaline pH that after mixing with the metal perchlorates a pH somewhat higher than the desired one was reached; then by using (only) $HClO_4$ the final pH was adjusted. One 1 mm quartz cell in the reference beam contained the metal(II) perchlorate (0.067–0.3, sometimes to 0.50 mol dm^{-3}) and the other tubercidin (10^{-3} mol dm^{-3}); one cell in the sample beam contained the mixed system and the other water. All four cells contained in addition $NaClO_4$ to maintain I at least at 0.5 mol dm^{-3} (25 °C); it is evident that in some few instances I reached 1.5 mol dm^{-3}. The metal(II) perchlorates contribute only little to the absorption (A) in the mentioned UV region. These difference spectra (for an example see Fig. 4) showed a rather broad maximum in the 270–280 nm region; they were evaluated at 270, 280 and 290 nm. The stability constants were calculated from these data by a Benesi–Hildebrand-like method,[24,25] *i.e.* with equation (2). Plots of

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max} K_{app}} \cdot \frac{1}{[M^{2+}]_{tot}} + \frac{1}{\Delta A_{max}} \qquad (2)$$

$1/\Delta A$ *versus* $1/[M^{2+}]_{tot}$ resulted in straight lines confirming that 1:1 complexes are formed. For the three mentioned metal ions representative examples are shown in Fig. 5 (see below), where also additional details are given in the legend. From the intercepts with the x and y axes one obtains the apparent stability constant for the considered complex, K_{app}/dm^3 mol^{-1}, at the pH of the experiment (for $y = 0$ holds $x_0 = -K_{app}$, hence $K_{app} = -x_0$), and ΔA_{max} (for $x = 0$ holds $y_0 = 1/\Delta A_{max}$, hence $\Delta A_{max} = 1/y_0$), from which the absorption coefficient, $\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1}, for the difference spectrum of the M^{2+} 1:1 complex may be calculated ($\Delta\varepsilon = \Delta A_{max}/d[L]_{tot}$, where d = length of cell in cm and $[L]_{tot} = [Tu]_{tot}$).

It must be noted that the constants determined with equation (2) are apparent constants, K_{app}, which are valid only at the pH of the experiment (see Fig. 5). They become independent of pH if one considers the protonation of N^1 of tubercidin, which is different for each set of experiments. The competition of the proton is taken into account[25,26] with equation (3) (by using

$$\log K^{M}_{M(Tu)} = \log K_{app} + \log(1 + [H^+]/K^{H}_{H(Tu)}) \qquad (3)$$

$pK^{H}_{H(Tu)} = 5.29$). The final results given in Table 3 of Section 2(*c*) are the average of three independent series of experiments with in total nine evaluations in the case of $[Ni(Tu)]^{2+}$ or $[Cd(Tu)]^{2+}$ and two series of experiments with in total four evaluations in the case of $[Co(Tu)]^{2+}$. It may be added that the corrections according to equation (3) were very small in the present instances because all experiments were done at pH $\geqslant$ 6; *i.e.* Tu exists to the largest part not in a protonated form.

These experiments for the mentioned M^{2+}/Tu systems were difficult to carry out, as the spectral alterations were small. In addition, there was a drift in the results obtained for K_{app} in the evaluations at 290, 280 and 270 nm (*i.e.* the constants increased somewhat within this order), though the results usually overlapped within the limits of one standard deviation (1σ); in the case of $[Co(Tu)]^{2+}$ reasonable results were obtained only for the evaluation at 290 and 280 nm. For the Mn^{2+}/Tu system difference spectra could be measured and these are similar to those shown in Fig. 4, but no reproducible stability constant was obtained, though a value for $\Delta\varepsilon$ could be estimated (see Table 3). The Tu systems with Cu^{2+} and Zn^{2+} could not be studied at all due to hydroxo-complex formation and precipitation at the pH needed for this type of experiment.

Results

2(*a*) *Self-association Properties of Tubercidin.*—Self-stacking is a well known phenomenon of purine (P) derivatives[27] and therefore any measurements designed for the quantification of monomeric species must be carried out under conditions where this effect has been considered.[28] Unfortunately, our aim to measure the self-stacking tendency of tubercidin by ^{1}H NMR shift experiments, a method previously demonstrated to be very useful,[12–14,29] and to quantify the effect according to the isodesmic model for an indefinite non-co-operative self association,[12,30] as expressed in equation (4), was considerably

$$(P)_n + P \rightleftharpoons (P)_{n+1} \qquad (4a)$$

$$K = [(P)_{n+1}]/[(P)_n][P] \qquad (4b)$$

hampered by the low solubility of tubercidin. Only solutions of tubercidin with concentrations of up to about 8×10^{-3} mol dm^{-3} could be prepared at 25 °C and $I = 0.1$ mol dm^{-3} ($NaNO_3$). As one would expect, under these latter conditions self association is not yet very pronounced and therefore the upfield shifts measured in ^{1}H NMR experiments between solutions of 2×10^{-3} and 7.6×10^{-3} mol dm^{-3} tubercidin are only about 0.016 ppm for H^2 and H^7 (see Fig. 1), and 0.009 and 0.003 ppm for H^8 and $H^{1'}$, respectively.

With these limitations in mind, it is not surprising that no curve-fitting procedure[12] could be carried out with the three variables K [equation (4b)], δ_0 (shift at infinite dilution, *i.e.* for monomeric P) and δ_∞ (shift of P in an infinitely long stack) for the six data points measured for a given proton of tubercidin (see Fig. 2). However, the experimentally observed variation of the upfield shifts for H^2, H^7, H^8 and $H^{1'}$ as a function of the tubercidin concentration is satisfactorily explained by using $K = 15$ dm^3 mol^{-1}, as is nicely seen from the solid curves shown in Fig. 2. This value for the self-association constant K corresponds to that determined earlier for adenosine.[30] Considering the close relationship between tubercidin and adenosine (see Fig. 1) a similar self-stacking tendency is expected for these two nucleosides. Indeed, this expectation is further supported by the identity of the self-association constants ($K = 2.1 \pm 0.3$ dm^3 mol^{-1}, each)[12] measured for tubercidin 5′-monophosphate ($TuMP^{2-}$) and adenosine 5′-monophosphate (AMP^{2-}).

Calculations for the experimental data pairs shown in Fig. 2 with $K = 15 \pm 3$ dm^3 mol^{-1} (value for Ado $\pm 2\sigma$)[30] give for the chemical shifts of H^2, H^7, H^8 and $H^{1'}$ for δ_0 8.159 ± 0.004 (error limits always 2σ), 6.675 ± 0.004, 7.374 ± 0.003 and 6.136 ± 0.002 ppm, and for δ_∞ 7.90 ± 0.09, 6.43 ± 0.09, 7.23 ± 0.06 and 6.08 ± 0.03 ppm, respectively; the corresponding shift differences, $\Delta\delta = \delta_0 - \delta_\infty$, for H^2, H^7, H^8 and H^1

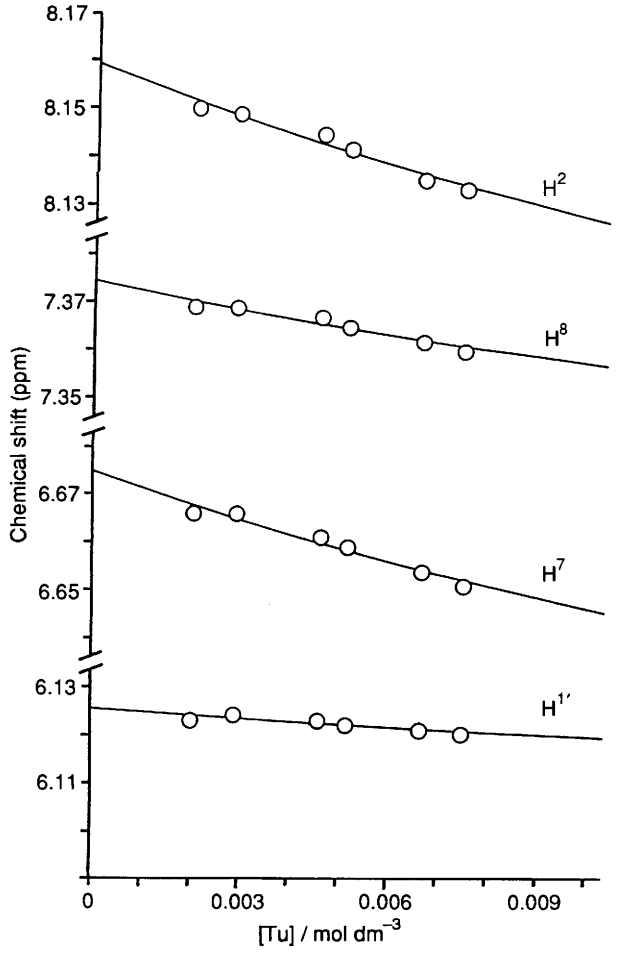


Fig. 2 Variation of the chemical shift of H^2, H^7, H^8 and $H^{1'}$ with increasing concentrations of tubercidin in D_2O solutions at pD 8.31, 25 °C and I = 0.1 mol dm^{-3} ($NaNO_3$). The spectra were measured on a Varian VXR 400 spectrometer at 399.96 MHz, relative to internal NMe_4NO_3 and converted into values relative to sodium 3-(trimethylsilyl)propane-1-sulphonate by adding 3.174 ppm. The curves shown here are the computer-calculated best fit of the experimental data (calculated with K = 15 dm^3 mol^{-1}), using the indefinite non-co-operative stacking model [equation (4)]; the resulting shifts are given in the text of section 2(*a*)

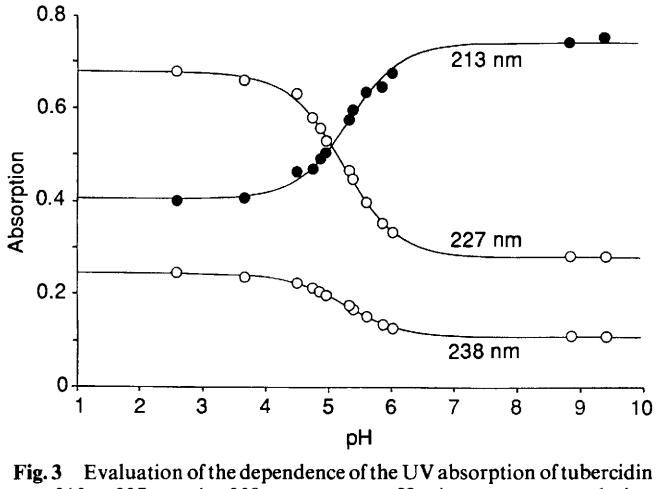


Fig. 3 Evaluation of the dependence of the UV absorption of tubercidin at 213, 227 and 238 nm on pH in aqueous solution (measured in 1 cm cells; [Tu] = 3 × 10^{-5} mol dm^{-3}; I = 0.5 mol dm^{-3}, $NaClO_4$; 25 °C) by plotting the absorption *versus* pH. The solid curves represent the computer-calculated best fits of the experimental data points at pH 2.60, 3.68, 4.50, 4.75, 4.87, 4.97, 5.34, 5.40, 5.62, 5.87, 6.03, 8.84 and 9.40 (from left to right), which leads for this experimental series to $pK^H_{H(Tu)}$ = 5.34 ± 0.13 (3σ), 5.25 ± 0.07 and 5.27 ± 0.08 for the evaluation at 213, 227 and 238 nm, respectively, for the deprotonation of $H(Tu)^+$ [see also sections 1(*d*) and 2(*b*)]

Table 1 Negative logarithms of the acidity constants [equation (5)] in aqueous solution for monoprotonated tubercidin (Tu),[a] pyridine (py),[b] and 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)[b] at 25 °C and various ionic strengths (I)

I/mol dm^{-3}	$pK^H_{H(Tu)}$	$pK^H_{H(py)}$	$pK^H_{H(Tris)}$
0.1	5.21 ± 0.03[a,c]	5.24 ± 0.02[b]	8.09[b]
0.5	5.29 ± 0.02[a,c]	5.31 ± 0.02[b]	8.15[b]
0.5	5.30 ± 0.04[a,d]		

[a] This work. So-called practical constants are given; see section 1(*c*). The errors given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] From ref. 33. [c] Determined by potentiometric pH titrations; $NaNO_3$ was used as background electrolyte. [d] Determined by UV spectrophotometry (see also Fig. 3); $NaClO_4$ was employed as background electrolyte.

are 0.26 ± 0.09, 0.25 ± 0.09, 0.14 ± 0.06 and 0.06 ± 0.03 ppm, respectively.

Clearly, in stacks of different purine derivatives the aromatic rings may be in somewhat different positions towards each other; hence, if the extents of upfield shifts are compared, this should be done with the average values. With this reasoning in mind it is comforting to note that the average upfield shift of H^2, H^8 and $H^{1'}$ for tubercidin (0.15 ppm) is about half of the corresponding value for adenosine (0.33 ppm)[30] and that this ratio is in excellent agreement with that observed for $TuMP^{2-}$ (0.30 ppm)[12] and AMP (0.62 ppm).[12] Consequently the estimation, K = 15 dm^3 mol^{-1}, for the self-association constant of tubercidin is in excellent agreement with all experimental facts. Moreover, calculations with K = 10 and 20 dm^3 mol^{-1} indicate that beyond these limits the average upfield shifts become unreasonably large or small, respectively, in the light of the above considerations and therefore we conclude that K = 15 ± 5 dm^3 mol^{-1} (25 °C, I = 0.1 mol dm^{-3}, $NaNO_3$) for the self-association of tubercidin. With increasing protonation at N^1 a decreasing self-stacking tendency is expected.[30]

2(*b*) *Conditions for the Potentiometric* pH *Titrations and Acidity Constant of* $H(Tu)^+$.—To obtain a large degree of formation of the complexes formed between metal ions and tubercidin it is desirable to work at high concentrations of the reactants. However, in the preceding section 2(*a*) we have seen that tubercidin is subject to self-stacking and therefore low concentrations are requested. As a compromise, all potentiometric pH titrations of this work have been carried out with 10^{-3} mol dm^{-3} tubercidin solutions. Calculations with the self-association constant K = 15 dm^3 mol^{-1} and [Tu] = 10^{-3} mol dm^{-3} show that under these conditions 2.8% of tubercidin exists as dimers and 0.06% as trimers; *i.e.* about 97% of tubercidin is present under the mentioned experimental conditions as monomeric species, and hence we conclude that the following results apply to monomeric tubercidin.

The most basic nitrogen in the tubercidin molecule (see Fig. 1) is N^1,[12] which undergoes protonation by forming $H(Tu)^+$. No further proton reactions occur in the pH range down to 2.[12] Neutral tubercidin may also release a proton, *i.e.* from the ribose residue,[31,32] but this reaction occurs only at pH > 12 and does not play a role in the physiological pH range. Hence, in the present context and in the pH range 2–12 only the equilibrium (5) needs to be considered. The acidity constant for

$$H(Tu)^+ \rightleftharpoons H^+ + Tu \qquad (5a)$$

$$K^H_{H(Tu)} = [H^+][Tu]/[H(Tu)^+] \qquad (5b)$$

$H(Tu)^+$ has been determined by potentiometric pH titrations and by UV spectrophotometry; an example of an experimental series with some of its evaluations for the latter method is shown in Fig. 3. The results are summarized in Table 1, together with some related data.[33]

Table 2 Logarithms of the stability constants, log $K^{M}_{M(Tu)}$, of some $[M(Tu)]^{2+}$ complexes [equation (6)][a] as determined by potentiometric pH titrations in aqueous solutions at 25 °C and I = 0.5 mol dm^{-3} ($NaNO_3$), together with the stability constants log K_{M/N^1} calculated for a non-inhibited co-ordination of the metal ions to an N^1-type ligand with the same basicity as N^1 in tubercidin but without an *ortho*-amino group.[b,c] The extent of the steric inhibition by the *o*-amino group is expressed by the difference log K_{M/N^1} − log $K^{M}_{M(Tu)}$

M^{2+}	log $K^{M}_{M(Tu)}$	log K_{M/N^1}[b]	log K_{M/N^1} − log $K^{M}_{M(Tu)}$
Ca^{2+}	0.09 ± 0.14		
Mg^{2+}	−0.01 ± 0.22		
Mn^{2+}	0.23 ± 0.16	0.20 ± 0.09	−0.03 ± 0.18
Co^{2+}	0.22 ± 0.12	1.32 ± 0.14	1.10 ± 0.18
Ni^{2+}	0.33 ± 0.07	1.88 ± 0.11	1.55 ± 0.13
Cu^{2+}	1.06 ± 0.08	2.49 ± 0.04	1.43 ± 0.09
Zn^{2+}	0.33 ± 0.10	1.02 ± 0.10	0.69 ± 0.14
Cd^{2+}	0.70 ± 0.14	1.31 ± 0.05	0.61 ± 0.15

[a] The error limits given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] The values for log K_{M/N^1} were calculated with the straight-line equations listed in Table IV of ref. 10 and $pK^{H}_{H(Tu)}$ = 5.29 from Table 1 of this work. [c] See also text in sections 3(*a*), (*c*) and (*d*).

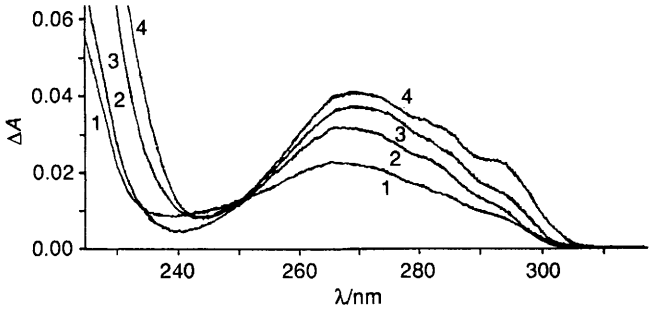


Fig. 4 UV difference spectra of aqueous solutions of tubercidin (10^{-3} mol dm^{-3}) at 25 °C in the presence of increasing concentrations of $Ni(ClO_4)_2$: 0.10 (1), 0.133 (2), 0.20 (3) and 0.30 mol dm^{-3} (4). One 1 mm quartz cell in the reference beam contained the corresponding concentration of $Ni(ClO_4)_2$ and another cell Tu (10^{-3} mol dm^{-3}), while the sample beam contained one cell with the mixed system and one with water. All four cells contained, where appropriate, in addition $NaClO_4$ to maintain I at 0.5 mol dm^{-3}. The pH was adjusted to 6.72 in the two cells which contained Tu. Evaluation of these spectra at 280 nm leads to the four points marked as ◇ in the plot for Ni^{2+} of Fig. 5

It is satisfying to see (Table 1) that the agreement between the constants obtained *via* potentiometric pH titrations and spectrophotometric measurements is excellent. Here it should be noted that the concentration of tubercidin for the spectophotometric experiments was 3 × 10^{-5} and 7.5 × 10^{-5} mol dm^{-3}, and therefore practically only monomeric tubercidin is present; hence, the agreement of the results confirms the above-mentioned conclusion that also for 10^{-3} mol dm^{-3} tubercidin solutions, as used in the potentiometric titration experiments, the properties of the monomeric species determine the result of the measurements.

From a comparison of the results collected in Table 1 it is further evident that the deprotonation of $H(Tu)^+$ is affected by a change in the ionic strength in the way common for nitrogen sites: a change from I = 0.1 to 0.5 mol dm^{-3} increases the basicity for the three examples in average by about 0.07 log unit. It is further evident that the basicity of N^1 in tubercidin is close to that of the nitrogen in pyridine. A comparison with the situation of N^1 in adenosine ($pK^{H}_{H(Ado)}$ = 3.61 ± 0.03; I = 0.1 mol dm^{-3}, $NaNO_3$; 25 °C)[12] reveals further that removal of the electron-withdrawing N^7 in adenosine and its replacement by a CH unit makes the nucleic base moiety of tubercidin more basic by about 1.6 log units. The difference of about 0.17 log unit between the micro acidity constant of H·TuMP·H$^{\pm}$ ($pk^{TuMP\cdot H}_{H\cdot TuMP\cdot H}$ = 5.38; I = 0.1 mol dm^{-3}, $NaNO_3$; 25 °C)[34,*] for deprotonation at the $H^+(N^1)$ site and the value for $H(Tu)^+$ ($pK^{H}_{H(Tu)}$ = 5.21 ± 0.03, *cf.* Table 1) is in the order expected for the inhibitory effect of a relatively far distant negatively charged 5′-$PO_2(OH)^-$ group as present in $H_2(TuMP)^{\pm}$.

2(*c*) *Stabilities of Some* $[M(Tu)]^{2+}$ *Complexes.*—The experimental data from the potentiometric pH titrations may be completely described by considering equilibria (5) and (6), provided the evaluation of the data is not carried into the pH range where hydroxo complexes form. The stability constants

$$M^{2+} + Tu \rightleftharpoons [M(Tu)]^{2+} \quad (6a)$$

$$K^{M}_{M(Tu)} = [M(Tu)^{2+}]/[M^{2+}][Tu] \quad (6b)$$

determined according to equilibrium (6a) are collected in column 2 of Table 2. It is evident that in general the error limits are rather large. However, this is not surprising as the stability of the $[M(Tu)]^{2+}$ complexes is low and therefore the depression of the buffer region of $H^+(Tu)$/Tu upon complex formation is very small: *e.g.* under the employed experimental conditions with Mg^{2+} the buffer depression corresponds only to about 0.07 log unit and even with Cu^{2+}, which is the most favourable case, only 0.40 log unit is reached. From equation (1) it is clear that any experimental error will become the more serious the more similar are the acidity constants K'_a and $K^{H}_{H(Tu)}$ [see section 1(*c*)].

For three of the $[M(Tu)]^{2+}$ complexes considered in Table 2, *i.e.* for $[Co(Tu)]^{2+}$, $[Ni(Tu)]^{2+}$ and $[Cd(Tu)]^{2+}$, the stability has also been determined by another independent method: the UV absorption spectrum of tubercidin is altered by the presence of divalent metal ions; this alteration can best be quantified by recording difference spectra [see Fig. 4 and section 1(*d*)], and from such spectra the stability constants of the mentioned three $[M(Tu)]^{2+}$ complexes were also determined as shown in Fig. 5 [regarding Mn^{2+}, Cu^{2+} and Zn^{2+} see section 1(*d*)]. The pH-dependent apparent stability constants, K_{app}, are obtained according to equation (2), and, by taking into account the competition of the proton using equation (3), constant values result which are independent of pH within experimental error. The average results of several experiments [see section 1(*d*)] are listed in column 2 of Table 3; the same table contains also the absorption coefficients, Δε, for the difference spectra of the corresponding $[M(Tu)]^{2+}$ complexes at three wavelengths. The error limits of the values given for log $K^{M}_{M(Tu)}$ are large, but this is not surprising as the spectral alterations resulting from the co-ordination of M^{2+} to N^1 of tubercidin are small (see Fig. 4). It is evident that this type of spectrophotometric measurements is rather prone to errors. However, comparison of the spectrophotometric results (Table 3) with those obtained from the potentiometric pH titrations (Table 2) shows that the stability constants for the three $[M(Tu)]^{2+}$ complexes, which were studied by both independent procedures, agree well within their error limits.

From the results given in columns 2 of Tables 2 and 3 it is evident despite the relatively large error limits that the stability constants for the $[M(Tu)]^{2+}$ complexes show the usual trends: (*i*) complex stability with the alkaline-earth metal ions is lower than with the divalent 3d metal ions; (*ii*) for the divalent 3d metal ions a stability sequence corresponding to the Irving–Williams series[35] is observed; the relatively pronounced stability of the $[Cu(Tu)]^{2+}$ complex compared with that of the complexes of the other 3d ions and Zn^{2+} is characteristic[26] of nitrogen-donor ligands.

Finally, a comparison of the stability constants determined now for the $[M(Tu)]^{2+}$ complexes (Table 2) with those obtained previously[34] for the $[M(H\cdot TuMP)]^+$ species confirms

* Average of the two values (5.36 and 5.39) given in Fig. 2 of ref. 34.

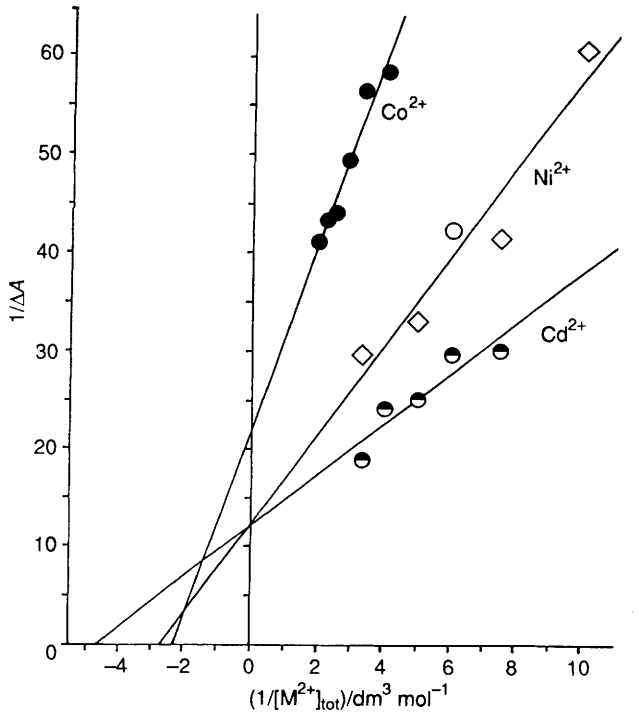


Fig. 5 Graphical determination of the apparent stability constant K_{app} for $[Co(Tu)]^{2+}$ (●), $[Ni(Tu)]^{2+}$ (○, ◇) and $[Cd(Tu)]^{2+}$ (◓) at pH 6.67, 6.72 and 6.67, respectively ([Tu] = 10^{-3} mol dm^{-3}; $I \geqslant 0.5$ mol dm^{-3}, $NaClO_4$; 25 °C). The straight lines were calculated by a least-squares procedure (regression). The difference spectra (all measured with 1 mm quartz cells) of the Co^{2+} systems were evaluated for the above plot at 290 nm, those for Ni^{2+} and Cd^{2+} at 280 nm; the four ◇ points for Ni^{2+} represent the evaluation of the UV difference spectra shown in Fig. 4. The intercept with the x axis [see equation (2) and section 1(*d*)] corresponds for Co^{2+}/Tu to $x_0 = -K_{app} = -2.296 \pm 0.425$ (1σ), *i.e.* $K_{app} = 2.296 \pm 0.425$ dm^3 mol^{-1}, hence log $K^{Co}_{Co(Tu)} = (0.361 \pm 0.080) + 0.018 = 0.38 \pm 0.08$ (1σ) [*cf.* equation (3)], for Ni^{2+}/Tu to $x_0 = -2.688 \pm 1.285$, *i.e.* $K_{app} = 2.688 \pm 1.285$ dm^3 mol^{-1}, hence log $K^{Ni}_{Ni(Tu)} = (0.429 \pm 0.208) + 0.016 = 0.45 \pm 0.21$, and for Cd^{2+}/Tu to $x_0 = -4.661 \pm 1.703$ *i.e.* $K_{app} = 4.661 \pm 1.703$ dm^3 mol^{-1}, hence log $K^{Cd}_{Cd(Tu)} = (0.668 \pm 0.159) + 0.018 = 0.69 \pm 0.16$ (1σ). The values given in Table 3 of section 2(*c*) for log $K^{M}_{M(Tu)}$ are the averages of several experimental series [see section 1(*d*)]

Table 3 Logarithms of the stability constants, log $K^{M}_{M(Tu)}$, of some $[M(Tu)]^{2+}$ complexes [equation (6)][a] as determined by UV difference spectrophotometry in aqueous solution at 25 °C and $I \geqslant 0.5$ mol dm^{-3} ($NaClO_4$, see also below), together with the difference absorption coefficients, $\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1},[b] for some $[M(Tu)]^{2+}$ complexes at the wavelengths of 270, 280 and 290 nm

M^{2+}	log $K^{M}_{M(Tu)}$	I/mol dm^{-3}	$\Delta\varepsilon_{270}$	$\Delta\varepsilon_{280}$	$\Delta\varepsilon_{290}$
Mn^{2+}	—	0.5–1.5	1000	900	500
Co^{2+}	0.24 ± 0.27	0.5–1.5	1050	920	600
Ni^{2+}	0.29 ± 0.24	0.5–0.9	1390	1130	890
Cd^{2+}	0.88 ± 0.29	0.5–0.9	750	820	670

[a] The error limits given are *three times* the standard error of the mean value. [b] The values for Δε were estimated by fixing x_0 on the x-axis with the aid of the $K^{M}_{M(Tu)}$ values determined by the potentiometric pH titrations (see Table 2) and by drawing a straight line through x_0 and the experimental data points measured by UV difference spectrophotometry (*cf.*, *e.g.*, Fig. 5); Δε was then calculated from the intercept with the y axis [see section 1(*d*)]. The error limits for the Δε values are estimated as approximately ±25%; in the case of $[Mn(Tu)]^{2+}$ [see section 1(*d*)] these limits may even be somewhat larger.

the earlier conclusion[34] that from the two isomers possible for $[M(H{\cdot}TuMP)]^{+}$ the one with the proton at N^1 and the M^{2+} at the phosphate group is dominating. By employing the present results also a detailed evaluation of the micro stability constants for the $[M(H{\cdot}TuMP)]^{+}$ complexes is possible, which again confirms the mentioned conclusion.

Discussion

3(*a*) *Steric Influence of an* ortho-*Amino Group on the Stability of Copper*(II) *Complexes formed with Purine* N^1 *Type or Pyridine-like Ligands: Construction of a Reference-line Plot.*— It has previously been indicated that an amino group in the *ortho* position to N^1, as present in adenosine, inhibits the complexation tendency of this pyridine-like nitrogen.[10] The equilibrium constants determined in the present study should allow a more quantitative evaluation of this effect. However, before this is attempted it is necessary to recall the already known correlations[9,10] between the stability of metal(II) complexes with imidazole-like (N^7) or pyridine-like (N^1) ligands and the basicity of the corresponding ligand binding sites. For the present we shall concentrate on the complexes formed with Cu^{2+}, because, as will become evident below, for this metal ion enough information on complexes formed with *ortho*-substituted pyridine-like ligands is available to allow a detailed evaluation.

Based on the log K^{Cu}_{CuL}/pK^{H}_{HL} data pairs for the copper(II) complexes of adenosine **1**, 1-methylinosine **2**, inosine **3**, guanosine **4**, imidazole **5** and 1-methylimidazole **6** the regression line (7) for plots of log K *versus* pK_a of imidazole-like or N^7-type ligands has previously been calculated.[10] The

$$\log K_{Cu/N^7} = (0.499 \pm 0.019)pK_a + (0.766 \pm 0.084) \quad (7)$$

identification numbers given above with the names of the ligands are the same as used previously[10] and these numbers are also employed now in Fig. 6. The error limits given in equation (7) with the slope (m) and the intercept with the y axis (b) correspond to one standard deviation (1σ). With this equation and the pK_a value of any N^7 ligand the corresponding value for log K^{Cu}_{CuL} may be calculated. Within the pK_a range of the experimental data employed (see Fig. 6) the standard deviation, s.d. = 0.053 [which resulted from the differences between the experimental and calculated (with the straight-line equation) log K values of the mentioned individual data pairs], times 2 or 3 is considered as a reasonable error limit for the calculated log K^{Cu}_{CuL} value.

The corresponding facts as outlined in the preceding paragraph hold also for the regression line for pyridine-like or purine N^1 type ligands; this straight-line equation (8) is based

$$\log K_{Cu/N^1} = (0.415 \pm 0.010)pK_a + (0.296 \pm 0.065) \quad (8)$$

on pyridine **7**, 4-(2-thienyl)pyridine **8**, 4-methylpyridine **9**, 7-methylinosine **10** (for inosine **11** and Cu^{2+} no value was used),[10] and ammonia (**12**, see ref. 10) with s.d. = 0.013. The slopes of both these base lines [equations (7) and (8)][10] are in excellent agreement with those published earlier by Kim and Martin.[9,36]

The two mentioned straight lines [equations (7) and (8)] with their corresponding data pairs are plotted in Fig. 6, together with the following data pairs of ligands with an *ortho* substituent next to the pyridine nitrogen. For each of the following five systems the first value in parentheses is the ligand-identification number used in Fig. 6, the second and third values are the pK_a of the binding site and the logarithms of the stability constant for binding of Cu^{2+} (log K^{Cu}_{CuL}), respectively: 2-phenylpyridine (**13**, 4.74, 0.7),[37,38] tubercidin (**14**, 5.29, 1.06; this work), 2-methylpyridine (**15**, 6.06, 1.3),[37,38] 2-aminopyridine (**16**, 6.96, 1.71)[39] and 2-amino-3-methylpyridine (**17**, 7.23, 1.91).[39] The result of the least-squares calculation for log K *versus* pK_a gives the reference line [equation (9)] for copper(II)

$$\log K_{Cu/N^1,ortho} = (0.456 \pm 0.029)pK_a - (1.428 \pm 0.175) \quad (9)$$

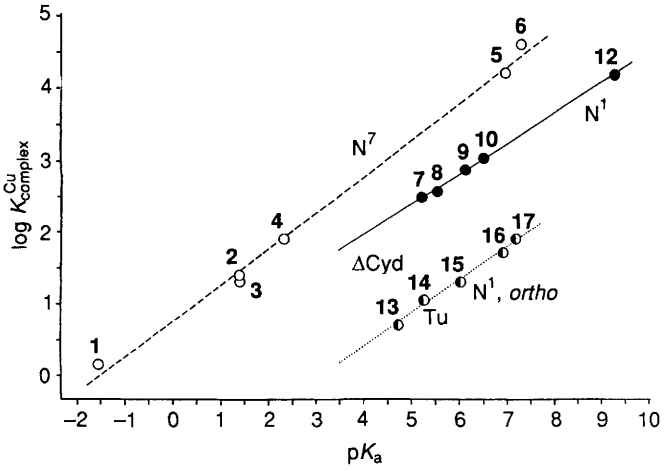


Fig. 6 Relationship between log K and pK_a for the 1:1 complexes of Cu^{2+} with imidazole-like or purine N^7-type ligands (○, broken line), pyridine-like or N^1-type ligands (●, full line), and pyridine-like or N^1-type ligands with an *ortho* substituent (◐, dotted line). The least-squares lines are drawn according to equations (7), (8) and (9); the inserted numbers correspond to the ligand numbers given in the text of section 3(*a*); only the point for $[Cu(Tu)]^{2+}$ is in addition identified (Tu). The point due to the complex formed between Cu^{2+} and cytidine (Cyd) is inserted for comparison (△); see also text in section 3(*b*)

complexes of pyridine-like or purine N^1 type ligands *with* an *ortho* amino group (s.d. = 0.024; correlation coefficient R = 0.994). The straight line identified in Fig. 6 as N^1,*ortho* is calculated with equation (9).

The data pairs for tubercidin **14**, 2-aminopyridine **16** and 2-amino-3-methylpyridine **17** in Fig. 6 fit on a straight line as is expected[40,41] because all three ligands are exactly of the same type with the *ortho* substituent, which is always an amino group, next to the nitrogen. The CH_3 and NH_2 groups are comparable in size and therefore it is not surprising that also the data for 2-methylpyridine **15** fit on this line; more surprising is that a phenyl substituent as present in 2-phenylpyridine **13** does not display a larger steric effect; the corresponding data pair also falls within the error limits on the mentioned straight line, which is in agreement with previous observations.[37] Hence, this reference line [equation (9)] quantifies the relation between log K and pK_a for purine N^1 or pyridine-like ligands with an *o*-amino group next to the nitrogen undergoing metal ion co-ordination. Needless to emphasize that this same reference line holds also for the N^3 of pyrimidines in the presence of an *o*-amino group.

3(*b*) *The Special Situation in Ligands with an* o-*Amino and an* o-*Carbonyl Group. The* $[Cu(Cyd)]^{2+}$ *Complex as an Example.*—The result described in the preceding section, *i.e.* the establishment of a reference line for copper(II) complexes with *o*-amino-substituted pyridines or purine N^1-type ligands, allows now the final proof for our earlier suspicion[10] that "it appears as highly likely that in N^1 or pyridine-like ligands with an *o*-amino *and* an *o*-carbonyl group the steric effect of the amino group is partially offset by a 'positive' oxo-metal ion interaction". A ligand of this type is cytidine (Cyd), and there are indications that metal ions not only co-ordinate to this ligand *via* the pyridine-like N but may also interact simultaneously with the carbonyl oxygen.[42,43] Indeed, the open triangle in Fig. 6 for $[Cu(Cyd)]^{2+}$, which is based on $pK^{H}_{H(Cyd)}$ = 4.14 and log $K^{Cu}_{Cu(Cyd)}$ = 1.56,[10] is clearly situated *between* the reference lines for purine N^1 or pyridine-like ligands and the same ligand type *with* an *o*-amino group.

It was concluded earlier[10,44] that the steric influence of an *o*-carbonyl oxygen is smaller than that of an amino group and indeed the data pairs for inosine and 7-methylinosine complexes fit well on N^1 reference lines[10] (see also **10** in Fig. 6); hence, if there is any steric influence on N^1 it is offset by an oxo-metal ion interaction.

With regard to $[M(Cyd)]^{2+}$ complexes one may suggest that part of the steric effect of the *o*-amino group is compensated for by hydrogen bonding of a metal ion-co-ordinated water molecule to the keto oxygen neighbouring to the nitrogen site at the other *ortho* position.

To conclude, from the results discussed in this and the preceding section 3(*a*) for copper(II) complexes it is evident that three different reference lines for pyridine-like or purine N^1-type binding sites (which also includes the N^3 site of pyrimidines) in nucleosides exist: (*i*) for metal-ion binding to freely accessible sites [equation (8)], (*ii*) for binding to sites which are sterically inhibited by an *o*-amino group [equation (9)], and (*iii*) for binding to sites with an *o*-amino *and* an *o*-carbonyl group. It is apparent that more work is needed, especially with metal ions other than Cu^{2+}, to provide a more general basis for this conclusion; in addition, also for Cu^{2+}, the detailed reference line for case (*iii*) still remains to be established.

3(*c*) *Size of the Steric Effect of the* o-*Amino Group on the Stability of Several* $[M(Tu)]^{2+}$ *Complexes.*—Comparison of the slopes of the reference lines shown in Fig. 6 for the co-ordination of Cu^{2+} to N^1 and N^1,*ortho* type ligands indicates that these slopes are very similar. Indeed, from equations (8) and (9) it is apparent that the slopes of the two straight lines nearly agree within a single standard deviation (1σ). Furthermore, the data pair for $[Cu(Tu)]^{2+}$ fits on the reference line identified as N^1,*ortho* in Fig. 6 and hence the vertical distance between the N^1 and N^1, *ortho* lines quantitatively represents the steric inhibition exercised by the *o*-amino group on the complexation of Cu^{2+} at N^1.

It is evident that the mentioned vertical distance is well represented by the difference between the value calculated for log K_{Cu/N^1} [from equation (8) with $pK^{H}_{H(Tu)}$] and the experimentally determined value for log $K^{Cu}_{Cu(Tu)}$. The same may also be surmised for other metal ions and therefore, by using $pK^{H}_{H(Tu)}$ = 5.29 (Table 1) and the straight-line equations published earlier for pyridine-like or purine N^1-type complex formations,[10] the values of log K_{M/N^1} for some other metal ions, as well as the corresponding differences, log K_{M/N^1} − log $K^{M}_{M(Tu)}$, have been calculated. These results are listed in columns 3 and 4 of Table 2.

As outlined above, the difference log K_{M/N^1} − log $K^{M}_{M(Tu)}$ quantifies for a given metal ion the extent of the steric inhibition exercised by an *o*-amino group on the co-ordination of this metal ion to a pyridine-like or N^1-type ligand. From the results listed in the fourth column of Table 2 it is apparent that the extent of inhibition depends on the metal ion; at least three categories are evident. (*i*) The first category encompasses the complexes of Cu^{2+} and Ni^{2+}, and possibly also of Co^{2+}; the average inhibition is in these cases close to 1.4 log units. (*ii*) The next category consists of the complexes of Zn^{2+} and Cd^{2+} where the inhibitory effect of the *o*-amino group is considerably smaller, though still quite significant; the metal-ion affinity of N^1 decreases here in the presence of an *o*-amino group by about 0.65 log unit. (*iii*) The third category contains Mn^{2+}, and probably also Mg^{2+} and Ca^{2+}; in the case of Mn^{2+} no inhibition by the *o*-amino group is observed, *i.e.* the data pair for Mn^{2+}/Tu falls within the error limits on the reference line given previously[10] for a non-inhibited N^1-type co-ordination.

3(*d*) *Further Evidence for the Steric Effect of an* ortho *Substituent on the Metal-ion Affinity of a Pyridine-like Binding Site in Complexes of* Ni^{2+} *and* Zn^{2+}.—It is unfortunate that at present not enough equilibrium constants are available to allow construction of N^1,*ortho* reference lines also for metal ions other than Cu^{2+} [for the latter see equation (9) and Fig. 6]. However, at least one more comparison is possible based on some experimental data available for the complexes of Ni^{2+} and Zn^{2+} with 2-methylpyridine (or α-picoline, pic).

In Table 2 of ref. 38 values are listed for $[M^{2+}]_{tot}$, pK'_a and

$pK^{H}_{H(pic)}$ (I = 0.1 mol dm^{-3}, $NaClO_4$; 25 °C); therefore with equation (1) and the mentioned data the following stability constants may be calculated, log $K^{Ni}_{Ni(pic)}$ = 0.78 ± 0.35 and log $K^{Zn}_{Zn(pic)}$ = 0.86 ± 0.30. With the straight-line equations given earlier[10] for a pyridine-like or N^1-type complex formation of Ni^{2+} and Zn^{2+}, and $pK^{H}_{H(pic)}$ = 6.06 ± (0.04),[38] one obtains log K_{Ni/N^1} = 2.06 ± 0.11 and log K_{Zn/N^1} = 1.30 ± 0.11; hence, the differences log K_{M/N^1} − log $K^{M}_{M(pic)}$ may be calculated; these correspond to 1.28 ± 0.37 and 0.44 ± 0.32 log units for the nickel and zinc systems, respectively.

It is very satisfying to observe that these differences agree well within their error limits with the corresponding (and more precise) data listed in the fourth column of Table 2. Moreover, the inhibitory effect of an *o*-methyl group corresponds also here to that of an *o*-amino group [as already discussed for Cu^{2+} in section 3(*a*)], and most important, the categories discussed above at the end of section 3(*c*) are confirmed; the value for Ni^{2+} clearly falls into category (*i*) and that for Zn^{2+} into category (*ii*). This means that different metal ions are definitely affected to a different extent in their affinity to N^1 sites by the presence of an *o*-amino or *o*-methyl group. Hence, a re-evaluation of this effect[10] on the metal-ion distribution[9,10] between the N^1 and N^7 sites in $[M(Ado)]^{2+}$ complexes is clearly necessary.[11]

3(*e*) *Why is the Steric Effect of an* o-*Amino Group on the* N^1-*Type Co-ordination Different for Different Metal Ions? Indirect Evidence for the Formation of Outer-sphere Complexes.*—The fact that inhibition by an *o*-amino group is strong for Ni^{2+} or Cu^{2+}, intermediate for Zn^{2+} or Cd^{2+}, and practically not existent for Mn^{2+} cannot be explained by differences in the geometry of the co-ordination spheres of these metal ions. An explanation for this observation may be given if one assumes that part of the M–N^1 interaction occurs in an outer-sphere manner; that the formation of such outer-sphere species is rather likely agrees with conclusions reached for the adenine residue in different connections.[11,45–47]

Indeed, if one assumes for the very unstable $[Mn(Tu)]^{2+}$ complex an outer-sphere interaction with N^1 *via* a co-ordinated water molecule by hydrogen bonding to N^1 the steric effect of the *o*-amino group is expected to be minor; the same type of interaction might be surmised for the complexes of Mg^{2+} and Ca^{2+}. In contrast, in $[Cu(Tu)]^{2+}$ or $[Ni(Tu)]^{2+}$ the M–N^1 interaction is expected to occur predominantly by an inner-sphere bond, and such an interaction should be sterically affected in a negative way by an *ortho* substituent, like an amino group.

Some support for the reasonings developed above may be obtained from a comparison of the absorption coefficients, Δε, of the UV difference spectra (Fig. 4). The electronic perturbation of the tubercidin ring system upon metal-ion co-ordination to N^1 is expected to be considerably more pronounced for an inner-sphere than for an outer-sphere co-ordination. With this in mind it is interesting to observe that the averages of the Δε values listed in Table 3 for some $[M(Tu)]^{2+}$ complexes decrease in the order $[Ni(Tu)]^{2+}$ > $[Co(Tu)]^{2+}$ > $[Cd(Tu)]^{2+}$; it is unfortunate that $[Mn(Tu)]^{2+}$ cannot definitely be placed in this series, but it may well be at the end (see $\Delta\varepsilon_{290}$ in Table 3). In any case, the given order corresponds to that found for the decreasing steric effect of an *o*-amino group [see section 3(*c*) and Table 2]. In other words, the described observations agree with the suggestion: the more the metal-ion co-ordination occurs in an outer-sphere manner, the lesser is the electronic perturbation of the aromatic tubercidin-ring system and also the smaller is the inhibiting steric effect of the *o*-amino group.

To conclude, it is more and more evident that the structural fine tuning of nucleotide–metal ion complexes[28,47] can only be understood if the detailed metal-ion binding properties of nucleosides are known. The same type of background information is also needed to predict the kind of binding of labile metal ions to single-stranded nucleic acids.

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