Comparison of the Metal Ion Coordinating Properties of Tubercidin 5'-Monophosphate (7-Deaza-AMP) with Those of Adenosine 5'-Monophosphate (AMP) and 1,N^6-Ethenoadenosine 5'-Monophosphate (ε-AMP). Definite Evidence for Metal Ion–Base Backbinding to N-7 and Extent of Macrochelate Formation in M(AMP) and M(ε-AMP)

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Abstract: The stability constants of the 1:1 complexes formed between Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Cd^{2+} and tubercidin 5'-monophosphate ($TuMP^{2-}$ = 7-deaza-AMP^{2-}) or adenosine 5'-monophosphate (AMP^{2-}) were determined by potentiometric pH titration in aqueous solution (I = 0.1 M, $NaNO_3$; 25 °C). The corresponding stability data for some 1,N^6-ethenoadenosine 5'-monophosphate (ε-AMP^{2-}) complexes are taken from our earlier work. All the experimental conditions were carefully selected such that self-association of the nucleotides (NMP) and their complexes was negligibly small; i.e., it was made certain that the properties of the monomeric M(NMP) complexes were studied. On the basis of recent measurements with simple phosphate monoesters (R-MP^{2-}, R is a noncoordinating residue; Massoud, S. S.; Sigel, H. *Inorg. Chem.* **1988**, *27*, 1447–1453), it is shown that all M(TuMP) complexes show exactly the stability expected for a sole phosphate coordination of the metal ion. Hence, in all the M(AMP) complexes where an increased complex stability is measured (i.e., with Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Cd^{2+}), this can definitely be attributed to macrochelate formation with N-7 of the adenine residue; replacement of N-7 in AMP by a CH group is the only structural difference of TuMP. Introduction of the 1,N^6-etheno bridge into AMP (= ε-AMP) transforms the adenine moiety into a phenanthroline-like binding site with excellent coordinating properties (Sigel, H. *Chimia* **1987**, *41*, 11–16); consequently the stability of the M(ε-AMP) complexes (M^{2+} = Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}) is greatly enhanced due to strong backbinding of the phosphate-coordinated metal ion to the ε-adenine residue. By use of the mentioned results for simple M(R-MP) complexes as basis for the correlation between complex stability and phosphate group basicity, the extent of macrochelate formation was quantified for the M(AMP) and M(ε-AMP) complexes: the percentages are between about 15–70 and 60–100, respectively, for the 3d ions and Zn^{2+} or Cd^{2+}; the alkaline earth ions show no base backbinding with the possible exception of Mg(AMP) and Mg(ε-AMP) where traces of macrochelates might occur. The structures of these macrochelates are discussed, and the evidence for inner-sphere versus outer-sphere binding of the metal ions to N-7 and the phosphate group is summarized and evaluated.

Nucleotides play a key role in all kinds of metabolic pathways, and with the phosphoryl and nucleotidyl transfers being metal ion dependent, the corresponding complexes have found wide attention.[2-4] Much effort[5-8] was devoted to the complexes of ATP,[9] and the properties[10,11] and structures[12,13] of binary M(ATP)$^{2-}$ systems in aqueous solution are now relatively well described. Depending on the kind of metal ion involved, coordination occurs not only to the phosphate chain but also to N-7, leading thus to macrochelates.

Comparison of the available knowledge on adenine nucleotides shows that the fraction of macrochelates formed in aqueous solution with M(ATP)$^{2-}$ complexes is well defined, including information on inner-sphere and outer-sphere interactions with N-7.[12,13] For M(ADP)$^{-}$ the existence of macrochelates with N-7 has also been proven by ^{1}H NMR shift experiments and stability constant comparisons,[14] and the approximate fractions of the closed species are known. Spectroscopic studies of solid complexes containing Mn^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} and AMP suggest also an N-7 interaction,[15] and indeed by X-ray crystal structure analysis N-7 coordination has been repeatedly confirmed[16,17] with 9-substituted adenine derivatives and AMP. However, the information on macrochelation in aqueous M(AMP) complexes[7,18,19] is not comprehensive and is of a more preliminary nature.

In a comprehensive effort to study the stability and structure of purine nucleoside monophosphate complexes formed with Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Cd^{2+}, we are first comparing the coordinating properties of AMP with those of tubercidin 5'-monophosphate ($TuMP^{2-}$) (cf. Figure 1). TuMP differs from AMP in the replacement of N-7 by a CH unit (Figure 1)[20-22] and is therefore also known as 7-deaza-AMP. This comparison should allow an evaluation of the influence of N-7 on the coordinating properties of AMP^{2-}, which is indeed, as shown now,

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(9) Abbreviations: AMP^{2-}, ADP^{3-} and ATP^{4-}, adenosine 5'-mono-, -di-, and -triphosphate; ε-AMP^{2-}, 1,N^6-ethenoadenosine 5'-monophsphate; GMP^{2-}, guanosine 5'-monophosphate; IMP^{2-}, inosine 5'-monophosphate; L, general ligand; M^{2+}, divalent metal ion; NMP^{2-}, nucleoside 5'-monophosphate; R-MP^{2-}, phosphate monoester (R may be any organic residue, e.g., phenyl or nucleosidyl); $TuMP^{2-}$, tubercidin 5'-monophosphate (= 7-deaza-AMP^{2-}).
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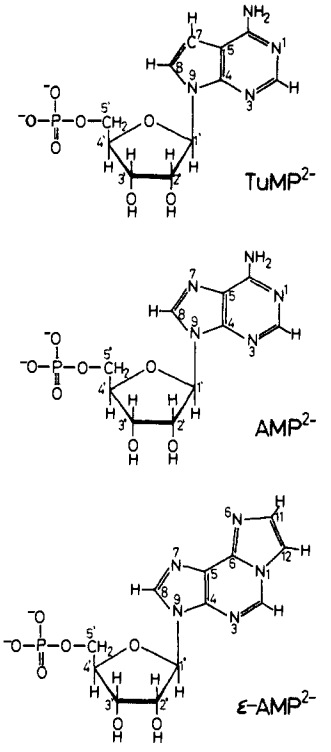

Figure 1. Chemical structure of the nucleoside 5'-monophosphates (NMPs) considered in this study.[9] AMP^{2-} and its derivatives are shown in their dominating anti conformation.[8,20,21] To facilitate comparisons between AMP and ε-AMP, the conventional atom numbering for adenines is also adopted for ε-AMP, a procedure which is common.[20,22]

considerable for several metal ions. This is even more true if a 1,N^6-etheno bridge is formed, which leads to a 1,10-phenanthroline-like binding site at the adenine residue[20] in the so-called ε-AMP^{2-} (Figure 1);[9] therefore some previous stability data[19] for M(ε-AMP) have now been reevaluated to allow a direct comparison with the present results. Our next effort is to compare the solution properties of complexes of 2'-AMP^{2-}, 3'-AMP^{2-}, and 5'-AMP^{2-}.[23]

Tubercidin is synthesized by molds and fungi,[24] and the nucleoside and its derivatives are antibacterial and antiviral agents; they are also active against some forms of cancer[25] and are widely studied in enzymic reactions.[26] Hence, a detailed study of the metal ion affinity of $TuMP^{2-}$ is justified in its own rights and not only due to the comparisons possible with AMP^{2-}. Certainly, one must expect that the structures of M(AMP) and M(TuMP) in solution differ considerably as the lack of N-7 in $TuMP^{2-}$ prevents any macrochelate formation. Indeed, the complexing properties of $TuMP^{2-}$ are those of a simple phosphate monoester.

One of the main obstacles in studying metal ion systems with AMP and its derivatives in solution is the known self-association of adenine nucleotides.[10] This means low concentrations of AMP must be employed in the experiments, a condition usually fulfilled with UV-spectroscopic studies[5,7] but not in NMR measurements, where high AMP concentrations, e.g., between 0.06 and 0.3 M,[27] render the data useless for any structural interpretation on monomeric M(AMP) complexes due to the rather large self-association. Moreover, UV studies of AMP complexes are also not easy to carry out as their formation degree is low compared with that of $M(ADP)^-$ or $M(ATP)^{2-}$ species.

With the indicated problems in mind we decided to study the structure of M(AMP) and related complexes in solution by evaluating precise stability data from pH titrations. To be able to select correct experimental conditions, we have recently quantified by ^1H NMR shift experiments the stacking tendencies of AMP^{2-} and $TuMP^{2-}$ in D_2O according to the isodesmic model of indefinite noncooperative self-association;[21] for AMP also several protonation degrees were considered.[28] With regard to the present context, the following information is crucial. (i) The self-association varies within the series: AMP^{2-} (K = 2.1 M^{-1}) < $D(AMP)^-$ (K = 3.4 M^{-1}) < $D_2(AMP)^{\pm}/D(AMP)^-$ in a 1:1 ratio (K = 5.6 M^{-1}) > $D_2(AMP)^{\pm}$ ($K \approx 2$ M^{-1}) > $D_3(AMP)^+$ ($K \leq 1$ M^{-1}).[28] (ii) In a 5 mM AMP solution about 95% of the total AMP exists in the monomeric form (calculated with K = 5.6 M^{-1}).[21,28] However, as metal ion coordination is known to favor the self-association of adenine nucleotides,[10,13,14,20] the neutral adenosine is probably more representative for this special case. (iii) In a 1 mM adenosine solution about 97% of the total Ado exists in the monomeric form (calculated with K = 15 M^{-1}).[21,28] Hence, no experiments aiming for the properties of monomeric M(AMP) species should be carried out in concentrations higher than 10^{-3} M; in fact, to be on the safe side it is advised to use concentrations of 5×10^{-4} M only, or below. Consequently, in our potentiometric pH titrations mostly 3×10^{-4} M NMP solutions were employed.

Experimental Section

Materials. Tubercidin 5'-monophosphoric acid and the sodium salt of adenosine 5'-monophosphate (1.5 Na^+) were from Sigma Chemical Co., St. Louis, MO. $HClO_4$ and $NaClO_4$ were from Merck AG, Darmstadt, FRG, and $Zn(ClO_4)_2$ was from K & K Laboratories, Cleveland, OH. All the other reagents were the same as used recently.[29]

The preparation of the solutions and the determination of their exact concentrations was carried out as before.[29]

Potentiometric pH Titrations. The experiments were performed and evaluated exactly as described,[29] but some additional points warrant mentioning.

(1) The acidity constants $K^H_{H_2(NMP)}$ and $K^H_{H(NMP)}$ of $H_2(NMP)^{\pm}$ were determined by titrating 50 mL of aqueous 0.54 (or sometimes 1.08) mM HNO_3 and $NaNO_3$ (I = 0.1; 25 °C) in the presence and absence of 0.3 mM NMP^{2-} under N_2 with 1 (or 2) mL of 0.03 M NaOH and by using the differences in NaOH consumption between two such titrations for the calculations. For 5'-AMP another set of experiments (0.9 mM HNO_3, 0.57 mM 5'-AMP^{2-}, and 0.05 M NaOH) gave the same results. The stability constants of the complexes (I = 0.1; 25 °C) were determined under identical conditions, but $NaNO_3$ was partly or fully replaced by $M(NO_3)_2$ as described.[29] The stability constants $K^M_{M(AMP)}$ were computed for each pair of titrations by taking into account the species H^+, $H_2(AMP)^{\pm}$, $H(AMP)^-$, AMP^{2-}, M^{2+}, and M(AMP).[30]

(2) In case of the Zn^{2+}/5'-AMP system we faced an experimental problem. The titration curves showed the normal shape up to a "critical" pH at which the system "collapsed"; i.e., suddenly much more OH^- was consumed and then a precipitation appeared. This observation was not made with D-ribose 5'-monophosphate or any of the other phosphate monoesters studied previously,[29] and must therefore depend on the adenine residue. Due to this "collapse" the pH range that could be evaluated was small and therefore not only the usual[29] NMP:Zn^{2+} ratios of 1:56 and 1:28 were employed but also those of 1:14 and 1:7. If ClO_4^- was used as counterion instead of NO_3^-, the effect was somewhat smaller. Nevertheless, reliable results were obtained from 13 independent titrations of aqueous solutions with NO_3^- and six titrations in the presence of ClO_4^- (see Table VI; vide infra).

(3) With TuMP the formation of $M(H{\cdot}TuMP)^+$ complexes is of importance with several metal ions. Therefore, the stability constants $K^M_{M(H\cdot TuMP)}$ and $K^M_{M(TuMP)}$ of the binary M^{2+}/TuMP systems were determined under the conditions described above and in ref 29, but the stability constants were computed for each pair of titrations with a curve-fitting procedure;[31] this became satisfactory by taking into account the

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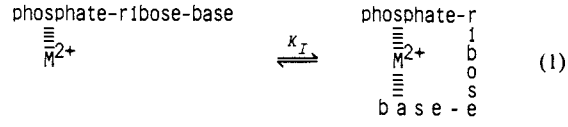
species H^+, $H_2(TuMP)^{\pm}$, $H(TuMP)^-$, $TuMP^{2-}$, M^{2+}, $M(H{\cdot}TuMP)^+$, and M(TuMP). The evaluation of the data in the upper pH range was stopped as usual[29] at that point where hydrolysis of M^{2+}_{aq} begins; this point was evident from the titrations of the solutions containing M^{2+} but no TuMP.

(4) To obtain reliable equilibrium constants from a representative set of data, which in several instances was collected independently by two different people, for all systems at least 10 independent pairs of titration curves were recorded for the acidity constants and the results were averaged. Similarly, the stability constants were determined from at least four (usually six) titration pairs.

Results and Discussion

1. Recognition of Macrochelate Formation and Definition of the Evaluation Procedure. Any kind of base backbinding or macrochelate formation enhances complex stability.[13,20,32] For the complexes of the alkaline earth metal ions, the divalent ions of the second half of the 3d series, and Zn^{2+}, as well as Cd^{2+}, it was shown[29] that D-ribose 5'-monophosphate ($RibMP^{2-}$) behaves as a simple phosphate monoester ($R{-}MP^{2-}$), i.e., just like methyl phosphate; hence, the stability of these complexes is solely determined by the basicity of the phosphate group in $RibMP^{2-}$. A possibly increased stability of the complexes of AMP^{2-} or its derivatives (Figure 1) must therefore be attributed to the participation of the base residue in complex formation; hence the following intramolecular equilibrium has to be considered:



(1)

The position of equilibrium 1 is quantified by the dimension-less equilibrium constant K_1 of eq 2, if the "open" isomer is defined as $M(NMP)_{op}$ and the "closed" species as $M(NMP)_{cl}$. The

$$K_1 = [M(NMP)_{cl}]/[M(NMP)_{op}] \quad (2)$$

reaction between a metal ion and a nucleoside 5'-monophosphate (NMP^{2-}) thus produces equilibrium 3. The equilibrium constant,

$$M^{2+} + NMP^{2-} \rightleftharpoons M(NMP)_{op} \rightleftharpoons M(NMP)_{cl} \quad (3)$$

$K^M_{M(NMP)_{op}}$, for the formation of the open complex is given by eq 4, and the (overall) equilibrium constant, $K^M_{M(NMP)}$, which is di-

$$K^M_{M(NMP)_{op}} = [M(NMP)_{op}]/([M^{2+}][NMP^{2-}]) \quad (4)$$

rectly accessible by experiments, covering both open and closed species is defined by eq 5.[32] Hence, the intramolecular and

$$K^M_{M(NMP)} = \frac{[M(NMP)]}{[M^{2+}][NMP^{2-}]} = \frac{([M(NMP)_{op}] + [M(NMP)_{cl}])}{[M^{2+}][NMP^{2-}]}$$

$$= K^M_{M(NMP)_{op}} + K_1 K^M_{M(NMP)_{op}} = K^M_{M(NMP)_{op}}(1 + K_1) \quad (5)$$

dimensionless equilibrium constant K_1 may be calculated according to eq 6, provided that values for $K^M_{M(NMP)_{op}}$ are obtainable, allowing

$$K_1 = \frac{K^M_{M(NMP)}}{K^M_{M(NMP)_{op}}} - 1 = 10^{\log\Delta} - 1 \quad (6)$$

the calculation of the crucial difference given in eq 7.[13]

$$\log \Delta = \log K^M_{M(NMP)} - \log K^M_{M(NMP)_{op}} = \log (1 + E) \quad (7)$$

The value for $10^{\log\Delta}$ is identical with the so-called[32,33] stability enhancement factor $(1 + E)$. Obviously the reliability of any calculations for K_1 (eq 6) depends on the accuracy of the difference given in eq 7, i.e., on the experimental error in the constants, which becomes more important the more similar the two constants are. Therefore, only clearly defined error limits allow a quantitative evaluation of the extent of a possibly formed macrochelate (eq 2, 6). Evidently, $100K_1/(1 + K_1)$ results in the percentage for the closed isomer of the concentration-independent equilibrium 1.

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Table I. Correlations for Metal Ion–Phosphate Coordination and Phosphate Group Basicity[a,b]

M^{2+}	m	b	SD
Mg^{2+}	0.224 ± 0.027	0.174 ± 0.167	0.014
Ca^{2+}	0.156 ± 0.039	0.487 ± 0.239	0.018
Sr^{2+}	0.089 ± 0.034	0.691 ± 0.206	0.016
Ba^{2+}	0.073 ± 0.036	0.706 ± 0.217	0.017
Mn^{2+}	0.250 ± 0.048	0.607 ± 0.293	0.022
Co^{2+}	0.230 ± 0.057	0.510 ± 0.345	0.024
Ni^{2+}	0.282 ± 0.045	0.201 ± 0.275	0.021
Cu^{2+}	0.453 ± 0.056	0.055 ± 0.340	0.026
Zn^{2+}	0.321 ± 0.057	0.125 ± 0.345	0.027
Cd^{2+}	0.317 ± 0.042	0.467 ± 0.253	0.019

[a] The slopes (m) and intercepts (b) for the straight base lines (log $K^M_{M(R\text{-}MP)}$ versus $pK^H_{H(R\text{-}MP)}$) are calculated from the equilibrium constants determined earlier[29] for the simple phosphate monoesters: 4-nitrophenyl phosphate, phenyl phosphate, *n*-butyl phosphate, D-ribose 5'-monophosphate, uridine 5'-monophosphate, and thymidine 5'-monophosphate (I = 0.1 M, $NaNO_3$; 25 °C). The column at the right lists the standard deviations (SD) resulting from the differences between the experimental and calculated values for the mentioned six ligand systems.[b] [b] The data are abstracted from Tables V and VI in ref 29. Straight-line equation: $y = mx + b$, where x represents the pK_a value of any phosphate monoester; the errors given with m and b correspond to 1 standard deviation (1σ). The listed SD values (column at the right) times 2 or 3 are considered as reasonable error limits for any stability constant calculation in the pK_a range 5–7.

The enhanced complex stability, log Δ (eq 7), may be obtained by comparing the observed stability constant, log $K^M_{M(NMP)}$ (eq 5), with that expected for the corresponding complex with a sole phosphate coordination, log $K^M_{M(NMP)_{op}}$ (eq 4). This latter constant is not directly accessible by experiments, but may be calculated from recent results.[29] The pK_a values for the deprotonation of monoprotonated phosphate groups of monoesters ($R{-}MP^{2-}$) differ;[21,29] therefore the metal ion affinity of different phosphate groups in dependence on their basicity had to be determined. The correlation[32] between metal ion–phosphate coordination and phosphate group basicity was obtained by plotting log $K^M_{M(R\text{-}MP)}$ versus $pK^H_{H(R\text{-}MP)}$ for a series of simple phosphate monoesters.[29] The parameters of the resulting straight base lines are summarized in Table I. This achievement[29] now allows the calculation of the stability constant for a pure phosphate coordination in M(R–MP) with the acidity constant of the corresponding H(R–MP) species and also for the M(NMP) complexes formed with the ligands of Figure 1. Therefore, we are next considering the acidity constants of these NMP species.

2. Acidity Constants for $H_3(AMP)^+$ and Its Derivatives. The NMPs shown in Figure 1 can accept three protons, two at the phosphate group and one at the base. Accordingly, the following three deprotonation equilibria have to be considered:

$$H_3(NMP)^+ \rightleftharpoons H_2(NMP) + H^+ \quad (8a)$$

$$K^H_{H_3(NMP)} = [H^+][H_2(NMP)]/[H_3(NMP)^+] \quad (8b)$$

$$H_2(NMP)^{\pm} \rightleftharpoons H(NMP)^- + H^+ \quad (9a)$$

$$K^H_{H_2(NMP)} = [H^+][H(NMP)^-]/[H_2(NMP)] \quad (9b)$$

$$H(NMP)^- \rightleftharpoons NMP^{2-} + H^+ \quad (10a)$$

$$K^H_{H(NMP)} = [H^+][NMP^{2-}]/[H(NMP)^-] \quad (10b)$$

The release of the first proton (eq 8) from monoesterified derivatives of phosphoric acid occurs at a very low pH;[24,29] for $H_3(AMP)^+$ in aqueous solution $pK^H_{H_3(AMP)} = 0.4 \pm 0.2$ (I = 0.1, $NaNO_3$; 27 °C).[21] It is safe to assume similar values for $H_3(TuMP)^+$ and $H_3(\epsilon\text{-}AMP)^+$. This is important because it shows that the first proton from the phosphoric acid residue in $H_3(NMP)^+$ is completely released at pH ≥2.5; it does not therefore affect equilibria 9 and 10 (at pH >3) or the complex formation between M^{2+} and $H(NMP)^-$ or NMP^{2-}, which occurs only at pH >4.

Table II. Negative Logarithms of the Acidity Constants (Eq 9 and 10) of Monoprotonated D-Ribose 5′-Monophosphate and of the 2-Fold Protonated Nucleoside 5′-Monophosphates Considered in This Study (Figure 1) As Determined by Potentiometric pH Titrations in Water at 25 °C and I = 0.1 M ($NaNO_3$)[a]

protonated species	$pK^H_{H_2(NMP)}$[b]	$pK^H_{H(NMP)}$[c]
$H(RibMP)^-$		6.24 ± 0.01
$H_2(TuMP)^\pm$	5.28 ± 0.02	6.32 ± 0.01
$H_2(AMP)^\pm$	3.84 ± 0.02	6.21 ± 0.01
$H_2(\epsilon\text{-AMP})^\pm$	4.23 ± 0.02	6.23 ± 0.01

[a] The range of error given with the constants is 3 times the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] This pK_a value quantifies the release of the proton from the base residue; this proton is located at N-1 in $H_2(TuMP)^\pm$ and $H_2(AMP)^\pm$,[21] and at N-6 in $H_2(\epsilon\text{-AMP})^\pm$.[19,20] [c] The proton in $H(NMP)^-$ is at the phosphate group. The value for $H(RibMP)^-$ is from ref 29.

In the order of the above equilibria, the next proton is released from the base residue of the zwitterionic species $H_2(NMP)^\pm$ (eq 9), while $H(NMP)^-$ releases its proton from the phosphate group (eq 10).[21] The corresponding acidity constants are listed in Table II together with the value[29] for $H(RibMP)^-$. All constants are in the expected order and of high precision.

3. Evaluation of the Overlapping Equilibria for $H_2(TuMP)^\pm$ via Micro Acidity Constants. For $H_2(AMP)^\pm$ and $H_2(\epsilon\text{-AMP})^\pm$ the two acidity constants $pK^H_{H(NMP)}$ and $pK^H_{H_2(NMP)}$ are two or more log units apart (Table II), and therefore, there is practically no overlap between equilibria 9 and 10. In fact, the $pK^H_{H(NMP)}$ values for $H(RibMP)^-$, $H(AMP)^-$, and $H(\epsilon\text{-AMP})^-$ are nearly identical.

However, the difference $pK^H_{H(NMP)} - pK^H_{H_2(NMP)}$ for $H_2(TuMP)^\pm$ equals only 1.04 log units (Table II), and hence in this case the two equilibria 9 and 10 are overlapping. This means that in correlations between complex stability and ligand acidity (see Section 5) a microconstant,[34-36] e.g., for $H(TuMP)^-$, should be employed which is corrected for any influence of the in part still protonated base residue. Such a value is normally not easy to assess, but in the mentioned case it is certainly well described by $pK^H_{H(NMP)}$ from $H(RibMP)^-$ or $H(AMP)^-$.

In Figure 2 we have summarized the equilibrium scheme defining the microconstants (k) and giving their interrelation with the macro acidity constants (K). There are three independent equations (a), (b), and (c), but four unknown microconstants;[35] however, by use of the acidity constants of $H(RibMP)^-$ or $H(AMP)^-$, as indicated above for $pk^{TuMP}_{TuMP\cdot H}$, the other three microconstants can be calculated. The corresponding results are given on the arrows in Figure 2.

From the two values calculated for the microconstant, $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$, it is evident that here a rather large error is involved despite the similarity of the two values employed for $pk^{TuMP}_{TuMP\cdot H}$, which differ only by 0.03 log unit. However, considering the fact that the mentioned microconstant is calculated via equation (a) of Figure 2, i.e., by $k^{H\cdot TuMP}_{H\cdot TuMP\cdot H} = K^H_{H_2(TuMP)} - k^{TuMP\cdot H}_{H\cdot TuMP\cdot H} = 10^{-5.28} - 10^{-5.36(\text{or } -5.39)}$, the large error is not surprising anymore because the calculation involves a small difference resulting from two very similar numbers.

Despite the indicated handicap, one may attempt to estimate the ratio R of the monoprotonated and isocharged species $TuMP\cdot H^-$ and $H\cdot TuMP^-$, which carry the proton at the phosphate group or at N-1 of the base residue, respectively:

$$R = \frac{[TuMP\cdot H^-]}{[H\cdot TuMP^-]} = \frac{k^{TuMP\cdot H}_{H\cdot TuMP\cdot H}}{k^{H\cdot TuMP}_{H\cdot TuMP\cdot H}} = \frac{10^{-5.36(\text{or } -5.39)}}{10^{-6.05(\text{or } -5.93)}} = 10^{0.69(\text{or } 0.54)} = \frac{5}{1}\left(\text{or } \frac{3.5}{1}\right)$$

Clearly, $TuMP\cdot H^-$ is dominating: it occurs to about 80%, while $H\cdot TuMP^-$ forms only to about 20%. Certainly, this result is only an estimation, but it still proves that both isomeric forms of $H(TuMP)^-$ occur simultaneously in appreciable amounts.

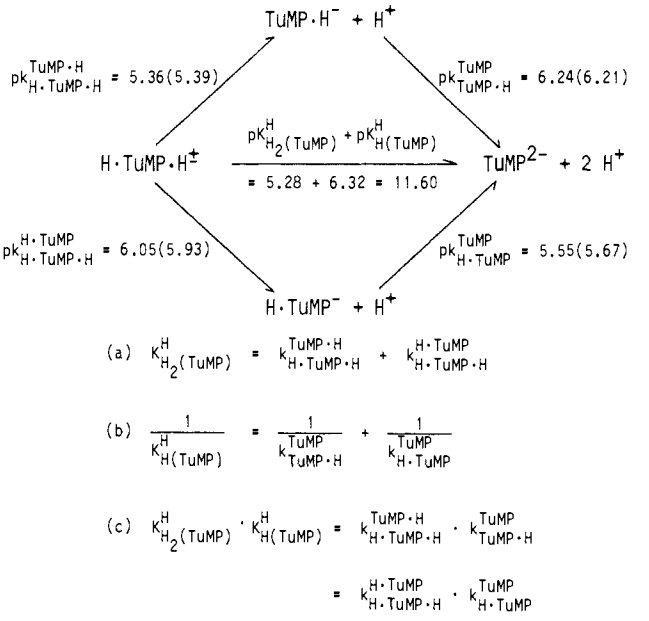

Figure 2. Equilibrium scheme defining the micro acidity constants (k) and showing their interrelation with the macro acidity constants (K) and also the interrelation between $TuMP\cdot H^-$ and $H\cdot TuMP^-$ and the other species present. In $TuMP\cdot H^-$ the proton is bound to the phosphate group, and in $H\cdot TuMP^-$ it is at N-1 of the base residue (Figure 1); $H\cdot TuMP\cdot H^\pm$ is also often written as $H_2(TuMP)^\pm$, it carries a proton each at N-1 and the phosphate group. The arrows indicate the direction for which the acidity constants are defined. By using for the microconstant $pk^{TuMP}_{TuMP\cdot H}$ the value measured for monoprotonated D-ribose 5′-monophosphate, $pK^H_{H(RibMP)} = 6.24$ (see Table II), the other microconstants can be calculated with equations a, b, and c; the microconstants given in parentheses are correspondingly based on $pK^H_{H(AMP)} = 6.21$ (Table II). For further details see text in Section 3.

4. Stabilities of Tubercidin 5′-Monophosphate Complexes. The experimental data of the potentiometric pH titrations of the M^{2+}/TuMP systems are completely described by equilibria 9–12, if the evaluation is not carried into the pH range where formation of hydroxo complexes occurs. The acidity constant of the connected equilibrium 13 may be calculated with eq 14.

$$M^{2+} + H(NMP)^- \rightleftharpoons M(H\cdot NMP)^+ \quad (11a)$$

$$K^M_{M(H\cdot NMP)} = [M(H\cdot NMP)^+]/[M^{2+}][H(NMP)^-] \quad (11b)$$

$$M^{2+} + NMP^{2-} \rightleftharpoons M(NMP) \quad (12a)$$

$$K^M_{M(NMP)} = [M(NMP)]/[M^{2+}][NMP^{2-}] \quad (12b)$$

$$M(H\cdot NMP)^+ \rightleftharpoons M(NMP) + H^+ \quad (13a)$$

$$K^H_{M(H\cdot NMP)} = [H^+][M(NMP)]/[M(H\cdot NMP)] \quad (13b)$$

$$pK^H_{M(H\cdot NMP)} = pK^H_{H(NMP)} + \log K^M_{M(H\cdot NMP)} - \log K^M_{M(NMP)} \quad (14)$$

The constants for equilibria 11–13 of the M^{2+}/TuMP systems are listed in Table III. In light of the discussion in Section 3, it should be emphasized here that the use of macroconstants is enough for a thermodynamic description[35,36] of the formation of metal ion complexes. Clearly, the analysis of potentiometric pH titrations yields only the amount and distribution of species of a net charge type, e.g. of $M(H\cdot TuMP)^+$, and additional information is required to locate the binding sites of the proton and the metal ion.

In this connection a comparison between the acidity constants for $M(H\cdot TuMP)^+$ ($pK^H_{M(H\cdot TuMP)}$, Table III) and $H_2(TuMP)^\pm$ ($pK^H_{H_2(TuMP)} = 5.28$, Table II; see also $pk^{TuMP\cdot H}_{H\cdot TuMP\cdot H} = 5.36$, Figure 2) is helpful. The proton in $M(H\cdot TuMP)^+$ is released with about the same or a slightly lower pK_a than the proton from N-1 in $H_2(TuMP)^\pm$; this indicates that in $M(H\cdot TuMP)^+$ the metal ion

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Table III. Logarithms of the Stability Constants of $M(H\cdot TuMP)^+$ (Eq 11) and M(TuMP) Complexes (Eq 12) As Determined by Potentiometric pH Titrations in Aqueous Solution, together with the Negative Logarithms of the Acidity Constants (Eq 13 and 14) of the Corresponding $M(H\cdot TuMP)^+$ Complexes at 25 °C and I = 0.1 M ($NaNO_3$)[a]

M^{2+}	log $K^M_{M(H\cdot TuMP)}$	log $K^M_{M(TuMP)}$	$pK^H_{M(H\cdot TuMP)}$[c]
Mg^{2+}	0.5 ± 0.2[b]	1.54 ± 0.06	5.3 ± 0.2
Ca^{2+}	0.4 ± 0.2[b]	1.43 ± 0.06	5.3 ± 0.2
Sr^{2+}	0.2 ± 0.2[b]	1.24 ± 0.04	5.3 ± 0.2
Ba^{2+}	0.1 ± 0.2[b]	1.13 ± 0.06	5.3 ± 0.2
Mn^{2+}	1.00 ± 0.14	2.11 ± 0.05	5.2 ± 0.2
Co^{2+}	0.96 ± 0.15	1.94 ± 0.04	5.3 ± 0.2
Ni^{2+}	0.85 ± 0.16	2.04 ± 0.08	5.1 ± 0.2
Cu^{2+}	1.75 ± 0.15	2.90 ± 0.08	5.2 ± 0.2
Zn^{2+}	0.93 ± 0.16	2.11 ± 0.05	5.1 ± 0.2
Cd^{2+}	1.39 ± 0.14	2.42 ± 0.07	5.3 ± 0.2

[a] The errors given are 3 times the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. The values of the error limits for $pK^H_{M(H\cdot TuMP)}$ were calculated according to the error propagation after Gauss. [b] These values are only estimates, as the formation degree of the corresponding $M(H\cdot TuMP)^+$ species is low (≤5%). [c] These values were calculated according to eq 14 by using the acidity constants of Table II and the stability constants listed above.

is mainly coordinated to the phosphate group and the proton to N-1. The large distance[37] of approximately 10 Å between the phosphate group and N-1 in the anti conformation[21] of TuMP can lead only to a small acidification of the proton at N-1 by a metal ion at the phosphate group. Indeed, this is observed and is also in accord with the mutual influence of protons in related NMPs, which leads to an acidification of about 0.15 log unit.[38] Furthermore, the metal ion affinity of N-1 is lower[39,40] than that of the phosphate group;[29] in fact, from general experience[41] this is expected, at least for the alkaline earth metal ions. More importantly, for the coordination at N-1 a pronounced pyridine-like[42] Irving–Williams sequence[43] is expected but not observed: the stability order for the $M(H\cdot TuMP)^+$ complexes (Table III) corresponds to that typical for phosphate complexes.[29]

Certainly, a final and quantitative analysis of the metal ion/proton distribution in M(H·TuMP) is only possible after a determination of M(tubercidin)$^{2+}$ stability constants. However, the above suggestion agrees with the analysis in the next section and especially with the observations[44] made at Cu(2,2′-bipyridyl)-$(H\cdot TuMP)^+$ and Cu(1,10-phenanthroline)$(H\cdot TuMP)^+$: the pK_a values for these two mixed-ligand complexes are about 0.35 log unit below $pK^H_{Cu(H\cdot TuMP)}$ due to the shorter distance between N-1 and Cu^{2+}, which is the result of an intramolecular ligand–ligand stack between the aromatic ring systems.[44]

5. Comparison of the Stabilities of the $M(H\cdot TuMP)^+$ and M(TuMP) Species with the Stabilities of Simple Phosphate Monoester Complexes. To evaluate the stability constants of Table III according to the concept described in Section 1, the basicity-adjusted binding strengths of $H(TuMP)^-$ and $TuMP^{2-}$ must be considered (see Section 3). We are asking the question: Is there any evidence of an increased complex stability, as defined by log Δ in eq 7, for $M(H\cdot TuMP)^+$ or M(TuMP), i.e., for a base backbinding or macrochelate formation?

To find an answer for the $M(H\cdot TuMP)^+$ species we assume that the metal ion is bound to the phosphate group and the proton to N-1 of the base residue (see Section 4), and then we have to consider the metal ion affinity of the phosphate group in N-1

Table IV. Stability Constant Comparisons Based on the Microconstant Evaluations of Figure 2 and the Base-Line Equations of Table I for N-1-Protonated and Phosphate-Coordinated Complexes $(H\cdot TuMP\cdot M)^+$

M^{2+}	log $k^M_{H\cdot TuMP\cdot M}$		log Δ = log k_{exptl} − log k_{calcd}[b,d]
	exptl[a,b,d]	calcd[b-d]	
Mg^{2+}	1.27 (1.15)	1.53 (1.50) ± 0.04	−0.26 (−0.35)
Ca^{2+}	1.17 (1.05)	1.43 (1.41) ± 0.05	−0.26 (−0.36)
Sr^{2+}	0.97 (0.85)	1.23 (1.22) ± 0.05	−0.26 (−0.37)
Ba^{2+}	0.87 (0.75)	1.15 (1.14) ± 0.05	−0.28 (−0.39)
Mn^{2+}	1.77 (1.65)	2.12 (2.09) ± 0.07	−0.35 (−0.44)
Co^{2+}	1.73 (1.61)	1.90 (1.87) ± 0.07	−0.17 (−0.26)
Ni^{2+}	1.62 (1.50)	1.91 (1.87) ± 0.06	−0.29 (−0.37)
Cu^{2+}	2.52 (2.40)	2.80 (2.74) ± 0.08	−0.28 (−0.34)
Zn^{2+}	1.70 (1.58)	2.07 (2.03) ± 0.08	−0.37 (−0.45)
Cd^{2+}	2.16 (2.04)	2.38 (2.35) ± 0.06	−0.22 (−0.31)

[a] Calculated according to log $k^M_{H\cdot TuMP\cdot M}$ = log $K^M_{M(H\cdot TuMP)}$ + $(pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H} - pK^H_{H_2(TuMP)})$; the acidity constant is from Table II, the stability constants are from Table III, and for the microconstant see Figure 2. The error range of these log k values is on the order of ±0.4 log unit.[b] [b] See text in Section 5. [c] Calculated with the base-line equations in Table I and the microconstant $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$ of Figure 2; the given error ranges are 3 times the SD values listed in the column at the right in Table I. [d] The values in parentheses are calculated with $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$ = 5.93, and the others with 6.05 (see Figure 2).[b]

protonated $H\cdot TuMP^-$. With the corresponding micro acidity constant (see lower left hand corner in Figure 2) and the base-line equations of Table I, the expected stability of the $(H\cdot TuMP\cdot M)^+$ complexes with a sole phosphate coordination can be calculated (third column of Table IV). The calculation has been carried out with both values given in Figure 2 for $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$; the stability constants in parentheses are based on $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$ = 5.93, and the others on 6.05 (Table IV).

It has now to be recalled that the thermodynamic stability constants $K^M_{M(H\cdot NMP)}$ (Table III), as discussed in Section 4, are based on the macro acidity constant, $K^H_{H_2(NMP)}$ (Table II), which refers to the release of a proton mainly from N-1. Consequently, for the anticipated comparison via log Δ (eq 7), the experimental values for log $K^M_{M(H\cdot TuMP)}$ have to be "corrected" by adding the difference between the micro and macro acidity constants, i.e. $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H} - pK^H_{H_2(TuMP)}$; these basicity-adjusted experimental values are listed in the second column of Table IV. The error limit is rather large, i.e., approximately ±0.4 log unit: the constants, log $K^M_{M(H\cdot TuMP)}$ (Table III), already have an error range of about ±0.2 log unit (as the formation degree of $M(H\cdot TuMP)^+$ is usually below 10%), and the uncertainty in $pk^{H\cdot TuMP}_{H\cdot TuMP\cdot H}$ (see Section 3 and Figure 2) probably adds another ±0.2 log unit.

The stability differences, log Δ (eq 7), calculated for the $M(H\cdot TuMP)^+$ complexes are negative and zero within the indicated error limits (Table IV). There is no indication for an *increased* stability of $M(H\cdot TuMP)^+$ species, and therefore also not for any macrochelates (eq 1). It has to be mentioned again that this analysis is based on the reasonable assumption that in $M(H\cdot TuMP)^+$ the metal ion is mainly phosphate coordinated and the proton is N-1 bound (see Section 4).

The corresponding and more important analysis for the M(TuMP) complexes is simpler and without any ambiguity, as in this case the metal ion–phosphate coordination is certain. The question is again: is there any indication for an increased complex stability, i.e., for a macrochelate formation? From the scheme in Figure 2 it is evident that the sums of the pK_a values quantifying the different double steps of deprotonation from $H_2(TuMP)^\pm$ to $TuMP^{2-}$ are identical. Hence, the experimentally determined constants, log $K^M_{M(TuMP)}$, may directly be compared with the calculated stability constants representing pure phosphate coordination. These latter values are calculated with the microconstant, $pk^{TuMP}_{TuMP\cdot H}$ (again both values of Figure 2 are used), and the base-line equations of Table I; they reflect the basicity-adjusted metal ion binding strength of $TuMP^{2-}$. Table V contains the experimentally determined and the calculated stability constants and the differences, log Δ according to eq 7, which are zero within their error limits. Hence, there is *no* indication for any kind of

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(40) The above conclusion is still correct, if the basicity difference of N-1 in AMP and TuMP (Table II) is taken into account by using slopes of about 0.3 for the log K^M_{ML} versus pK^H_{HL} plots.
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Table V. Stability Constant Comparisons for the M(TuMP) Complexes between the Measured Stability Constants (Exptl) from Table III and the Calculated Stability Constants (Calcd) Based on the Basicity of the Phosphate Group (See Figure 2) and the Base-Line Equations of Table I

M^{2+}	log $K^{M}_{M(TuMP)}$ exptl	log $K^{M}_{M(TuMP)}$ calcd[a,b]	log Δ = log K_{exptl} − log K_{calcd}[a-c]
Mg^{2+}	1.54 ± 0.06	1.57 (1.57) ± 0.04	−0.03 (−0.03) ± 0.07
Ca^{2+}	1.43 ± 0.06	1.46 (1.46) ± 0.05	−0.03 (−0.03) ± 0.08
Sr^{2+}	1.24 ± 0.04	1.25 (1.24) ± 0.05	−0.01 (0.00) ± 0.06
Ba^{2+}	1.13 ± 0.06	1.16 (1.16) ± 0.05	−0.03 (−0.03) ± 0.08
Mn^{2+}	2.11 ± 0.05	2.17 (2.16) ± 0.07	−0.06 (−0.05) ± 0.09
Co^{2+}	1.94 ± 0.04	1.95 (1.94) ± 0.07	−0.01 (0.00) ± 0.08
Ni^{2+}	2.04 ± 0.08	1.96 (1.95) ± 0.06	0.08 (0.09) ± 0.10
Cu^{2+}	2.90 ± 0.08	2.88 (2.87) ± 0.08	0.02 (0.03) ± 0.11
Zn^{2+}	2.11 ± 0.05	2.13 (2.12) ± 0.08	−0.02 (−0.01) ± 0.09
Cd^{2+}	2.42 ± 0.07	2.45 (2.44) ± 0.06	−0.03 (−0.02) ± 0.09

[a] The values in parentheses are calculated with $pk^{TuMP}_{TuMP\cdot H}$ = 6.21, and the others with 6.24 (see Figure 2); the given error ranges are 3 times the SD values listed in the column at the right in Table I.[b] [b] See text in Section 5. [c] The error limits for these differences were calculated according to the error propagation after Gauss.

base-backbinding (eq 1); $TuMP^{2-}$ shows the properties of a simple phosphate monoester; it coordinates to the considered metal ions in M(TuMP) only via the phosphate group. This result is very important with regard to the structural evaluation of the M(AMP) complexes to be considered in the next section.

6. Properties of M^{2+}/Adenosine 5′-Monophosphate Systems and Extent of Macrochelate Formation in M(AMP) Species. The experimental data of the potentiometric pH titrations can be analyzed by taking into account the species H^+, $H_2(AMP)^{\pm}$, $H(AMP)^-$, AMP^{2-}, M^{2+}, and M(AMP) (eq 9, 10, 12); i.e., under the present experimental conditions no evidence is obtained for $M(H\cdot AMP)^+$ complexes in the pH range ≥4.4. The affinity of the adenine residue (Figure 1) for protons or metal ions is too low to allow formation of significant amounts of $M(H\cdot AMP)^+$ complexes; although there is evidence for their existence,[18] they are usually not observed.[45,46] The stability constants determined now for the M(AMP) complexes (Table VI) are in average about 0.3 log unit lower than those given in ref 45, but they agree well with other earlier studies.[7,18,46,47]

A comparison of the stability constants, log $K^{M}_{M(NMP)}$, for the M(TuMP) and the M(AMP) complexes (Tables III and VI) indicates for several metal ions that the AMP complexes are more stable. This is reflected in Figure 3, where log $K^{M}_{M(R-MP)}$ versus $pK^{H}_{H(R-MP)}$ plots are shown for Mg^{2+}, Mn^{2+}, and Cu^{2+} and for several simple phosphate monoester ligands ($R-MP^{2-}$): it is evident that the data for M(TuMP) fit for all three examples on the reference line in accordance with the conclusions of Section 5. However, the data of the M(AMP) complexes fit the line only for Mg^{2+} and certainly not for Cu^{2+}, while the situation with Mn^{2+} is more difficult to assess (see below). The vertical distance between the point due to Cu(AMP) and the base line is identical with log Δ (eq 7) and quantifies the increased stability of Cu(AMP).

A quantitative evaluation for an increased stability of the M(AMP) complexes is possible by calculating with the base-line equations of Table I and the acidity constant of $H(AMP)^-$, $pK^{H}_{H(AMP)}$, the expected stability for the AMP^{2-} complexes with a sole phosphate coordination, i.e., the stability of the open isomer (eq 1 and 4); these results are listed in the third column of Table VI. The differences, log Δ (eq 7), which are a quantitative measure for an enhanced complex stability (fourth column), show that the alkaline earth metal ions form M(AMP) complexes with phosphate coordination only; i.e., the log Δ values are zero within the error limits, and thus at the most, traces of base-backbound

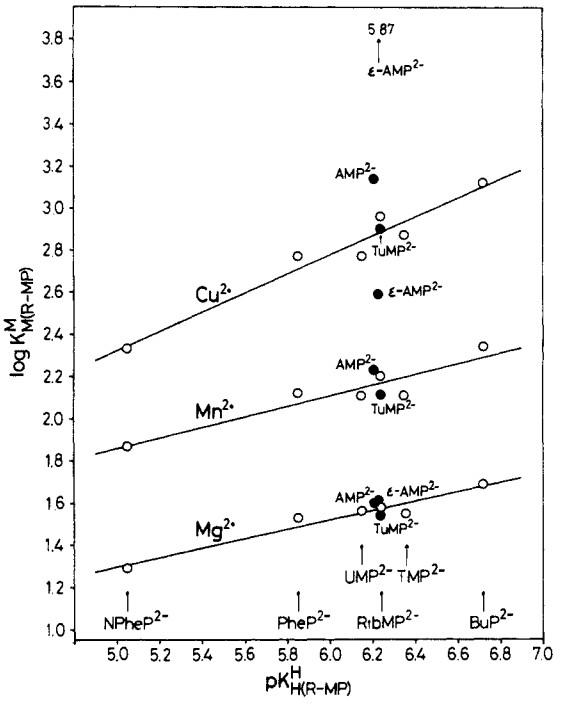

Figure 3. Relationship between log $K^{M}_{M(R-MP)}$ and $pK^{H}_{H(R-MP)}$ for the 1:1 complexes of Mg^{2+}, Mn^{2+}, and Cu^{2+} with some simple phosphate monoester ligands ($R-MP^{2-}$): 4-nitrophenyl phosphate ($NPheP^{2-}$), phenyl phosphate ($PheP^{2-}$), uridine 5′-monophosphate (UMP^{2-}), D-ribose 5′-monophosphate ($RibMP^{2-}$), thymidine 5′-monophosphate (TMP^{2-}) and *n*-butyl phosphate (BuP^{2-}) (from left to right) (○). The least-squares lines are drawn through the corresponding data sets, which are taken from ref 29; the equations for these base lines are given in Table I. The points due to the complexes formed with $TuMP^{2-}$, AMP^{2-} and ϵ-AMP^{2-} (●) are inserted for comparison; the stability constants for the M(TuMP) complexes are plotted versus the microconstant $pk^{TuMP}_{TuMP\cdot H}$ = 6.24 (Figure 2); the other equilibrium constants for these three NMPs are from Tables II, III, VI, and VII. All plotted equilibrium constant values refer to aqueous solutions at 25 °C and I = 0.1 M ($NaNO_3$).

isomers can occur. With the 3d metal ions and Zn^{2+} or Cd^{2+} an increased complex stability is observed, demonstrating that the macrochelated isomer (eq 1) is formed in appreciable amounts. Most importantly, the only structural difference between $TuMP^{2-}$ and AMP^{2-} is the presence of N-7 in the latter nucleotide (see Figure 1); this proves unequivocally that N-7 is responsible for the base-backbinding properties of AMP^{2-}.

The smaller stability enhancement observed for Cu(AMP) in comparison with that for Ni(AMP) agrees with an earlier[19] less comprehensive evaluation and indicates that the geometry of the coordination sphere of the metal ion is probably playing a role. Assuming for Cu^{2+} a Jahn–Teller distorted octahedral coordination sphere with a strong tendency to coordinate donor atoms equatorially,[48] there are three equatorial positions at the Cu^{2+} left in a phosphate coordinated complex, but only the *two* cis positions are for steric reasons able to form a macrochelate with N-7. In the octahedral coordination sphere of Ni^{2+} there are five positions left after phosphate coordination and *four* of these are sterically suitable for N-7 coordination. Hence, Cu^{2+} backbinding to N-7 is statistically disfavored by a factor of $^1/_2$ corresponding to −0.3 log unit, and indeed this is close to the reduced stability enhancement of −0.26 log unit (= log $\Delta_{Cu/AMP}$ − log $\Delta_{Ni/AMP}$ = 0.27 − 0.53; Table VI) in comparison with the Ni^{2+} complex. The near identity of the stability enhancement (log Δ) of the complexes with Co^{2+}, Zn^{2+}, and Cd^{2+} (Table VI) is a reflection of the similar affinity of these ions toward imidazole nitrogen donors.[49]

The affinity of Mn^{2+} toward imidazole nitrogen is considerably lower[49] than that of the above-mentioned other 3d ions, but it is also considerably larger than that of the alkaline earth ions.[41]

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Table VI. Comparison of the Measured Stability, $K^{M}_{M(AMP)}$, of the M(AMP) Complexes[a] with the Calculated Stability, $K^{M}_{M(AMP)_{op}}$, for an Isomer with only a M^{2+}/Phosphate Coordination,[b] and Extent of the Intramolecular Macrochelate Formation (Eq 1) in the M(AMP) Complexes at 25 °C and I = 0.1 M ($NaNO_3$)

M^{2+}	log $K^{M}_{M(AMP)}$ (eq 3, 5, 12)[a]	log $K^{M}_{M(AMP)_{op}}$ (eq 4)[b]	log Δ (eq 7)[c]	K_I (eq 2, 6)	% $M(AMP)_{cl}$ (eq 1)
Mg^{2+}	1.60 ± 0.02	1.57 ± 0.04	0.03 ± 0.04	0.07 ± 0.10	0 (7 ± 9/≤15)
Ca^{2+}	1.46 ± 0.01	1.46 ± 0.05	0.00 ± 0.05	0.00 ± 0.12	0 (≤11)
Sr^{2+}	1.24 ± 0.01	1.24 ± 0.05	0.00 ± 0.05	0.00 ± 0.12	0 (≤11)
Ba^{2+}	1.17 ± 0.02	1.16 ± 0.05	0.01 ± 0.05	0.02 ± 0.13	0 (≤13)
Mn^{2+}	2.23 ± 0.01	2.16 ± 0.07	0.07 ± 0.07[e]	0.17 ± 0.19[e]	15 ± 14[e]
Co^{2+}	2.23 ± 0.02	1.94 ± 0.07	0.29 ± 0.07	0.95 ± 0.33	49 ± 9
Ni^{2+}	2.49 ± 0.02	1.95 ± 0.06	0.54 ± 0.06	2.47 ± 0.50	71 ± 4
Cu^{2+}	3.14 ± 0.01	2.87 ± 0.08	0.27 ± 0.08	0.86 ± 0.35	46 ± 10
Zn^{2+}	2.38 ± 0.07[d]	2.12 ± 0.08	0.26 ± 0.11	0.82 ± 0.45	45 ± 13
Cd^{2+}	2.68 ± 0.02	2.44 ± 0.06	0.24 ± 0.06	0.74 ± 0.25	43 ± 8

[a] Determined in aqueous solution by potentiometric pH titrations. The errors given are 3 times the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] Calculated with $pK^{H}_{H(AMP)}$ = 6.21 and the base-line equations of Table I; the error limits correspond to 3 times the SD values given in the column at the right in Table I. [c] The errors given here and in the other two columns at the right were calculated according to the error propagation after Gauss by using the errors listed in the second and third columns. [d] Regarding the experimental difficulties see the Experimental Section. The log $K^{Zn}_{Zn(AMP)}$ values determined in aqueous solution with $NaNO_3$ and $NaClO_4$ as background electrolyte (I = 0.1 M) were 2.41 ± 0.10 and 2.34 ± 0.06, respectively; the value given above is the overall average. [e] This result is probably significant; with 2σ as error limits, the data are log Δ = 0.07 ± 0.04, K_1 = 0.17 ± 0.11, and % $Mn(AMP)_{cl}$ = 15 ± 8 (see also text in Section 6).

Table VII. Comparison of the Measured Stability, $K^{M}_{M(\epsilon\text{-AMP})}$, of the M(ε-AMP) Complexes[a] with the Calculated Stability, $K^{M}_{M(AMP)_{op}}$, for an Isomer with only a M^{2+}/Phosphate Coordination,[b] and Extent of the Intramolecular Macrochelate Formation (Eq 1) in the M(ε-AMP) Complexes at 25 °C and I = 0.1 M ($NaNO_3$)

M^{2+}	log $K^{M}_{M(\epsilon\text{-AMP})}$ (eq 3, 5, 12)[a]	log $K^{M}_{M(\epsilon\text{-AMP})_{op}}$ (eq 4)[b]	log Δ (eq 7)	K_I (eq 2, 6)	% $M(\epsilon\text{-AMP})_{cl}$ (eq 1)
Mg^{2+}	1.61 ± 0.02	1.57 ± 0.04	0.04 ± 0.04	0.10 ± 0.10	9 ± 9 (≤17)
Mn^{2+}	2.59 ± 0.04	2.16 ± 0.07	0.43 ± 0.08	1.69 ± 0.50	63 ± 7
Co^{2+}	~3.5	1.94 ± 0.07	~1.6	~40	~98
Ni^{2+}	~4	1.96 ± 0.06	~2	~100	~99
Cu^{2+}	5.87 ± 0.02	2.88 ± 0.08	2.99 ± 0.08	976 ± 180	99.9 ± 0.1
Zn^{2+}	3.18 ± 0.04	2.12 ± 0.08	1.06 ± 0.09	10.5 ± 2.4	91 ± 2

[a] The values for the Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} systems were determined by potentiometric pH titrations in aqueous solution; they are taken from Table 2 in ref 19. The values for the Co^{2+} and Ni^{2+} complexes are estimates taken from Table 4 of ref 19. Regarding the error limits, the statements made in footnotes *a–c* of Table VI are also valid here. [b] Calculated with $pK^{H}_{H(\epsilon\text{-AMP})}$ = 6.23 and the base-line equations of Table I.

Indeed, the enhanced complex stability of Mn(AMP) is just at the limit of significance: two standard deviations give log $\Delta_{Mn/AMP}$ = 0.07 ± 0.04 (see also footnote *e* in Table VI). In addition, the following points support the conclusion that some N-7 backbound isomers (eq 1) are also present in Mn(AMP). (i) By analogy, macrochelates are formed to some extent in $Mn(ATP)^{2-}$ and $Mn(ADP)^{-}$ complexes.[12-14] (ii) Introduction of a further binding site into the adenine residue of AMP^{2-} via the 1,N^6-etheno bridge (Figure 1) enhances base backbinding considerably with Mn^{2+}, while there is no such effect for Mg(ε-AMP) (see Section 7). (iii) Replacement of AMP^{2-} in Mn(NMP) by GMP^{2-}, which has a more basic N-7,[39] promotes the extent of macrochelate formation considerably.[23] (iv) There are spectroscopic indications for a Mn^{2+}/N-7 interaction in solid Mn(AMP), though in this case probably polymeric structures are present,[15] and there is also X-ray evidence for such an interaction in $Mn(GMP)(H_2O)_5$.[50]

Finally, the log Δ values of the M(AMP) complexes may be used to calculate the intramolecular equilibrium constant, K_1 (eq 2 and 6), and also the percentage of the closed species formed in the concentration-independent equilibrium 1 (Table VI). It is satisfying to note that for those five metal ion systems which had already been studied earlier,[7,19] the percentages for $M(AMP)_{cl}$ agree well. Finally, the results of Table VI demonstrate that even a small increase in complex stability is connected with the presence of substantial amounts of macrochelated isomer.

7. Enhanced Macrochelate Formation with 1,N^6-Etheno-adenosine 5′-Monophosphate. Comparison of Base Backbinding in M(ε-AMP) and M(AMP). M^{2+}/ε-AMP systems have been studied earlier[19] by potentiometric pH titration, and for several metal ions the stability constants have been determined for the $M(H\cdot\epsilon\text{-AMP})^{+}$ and M(ε-AMP) complexes. The binding site governing the stability of the $M(H\cdot\epsilon\text{-AMP})^{+}$ complexes is the phenanthroline-like N-6,N-7 unit (Figure 1), and the extent of backbinding to the monoprotonated phosphate group has been quantified.[19] In addition, the metal ion coordinating properties of 1,N^6-ethenoadenine derivatives have been reviewed,[20] and therefore we are restricting the present evaluation to M(ε-AMP) complexes and the comparison of their properties with those of the corresponding M(AMP) species.

The data points for the M(ε-AMP) complexes with Mg^{2+}, Mn^{2+}, and Cu^{2+} have been inserted into Figure 3. The vertical distance between these points and the corresponding base lines reflect the enhanced complex stability according to log Δ (eq 7). Mg(ε-AMP) shows no enhanced stability, and if at all, there are only traces of a backbound species present; this agrees with the low affinity of Mg^{2+} toward nitrogen donors.[41] However, the stability of Mn(ε-AMP) is not only clearly enhanced with regard to the base line but also with respect to Mn(AMP); hence the formation degree of the macrochelate (eq 1) must be considerable, and this supports our view that also with Mn(AMP) some macrochelation occurs (Section 6). Even more impressive is the stability increase for Cu(ε-AMP): the corresponding point is beyond the scale of Figure 3; i.e., the extent of macrochelation has to be very large.

This latter observation raises the question: should Cu(ε-AMP) be considered as a complex derived from a phosphate monoester or rather as a complex derived from 1,N^6-ethenoadenosine (ε-Ado)? The stability constants for the corresponding isolated binding sites may be represented by the stabilities of the Cu^{2+} complexes of D-ribose 5′-monophosphate and ε-adenosine: log $K^{Cu}_{Cu(Rib\text{-}MP)}$ = 2.96 ± 0.02 (ref 29) and log $K^{Cu}_{Cu(\epsilon\text{-}Ado)}$ = 2.81 ± 0.09 (ref 51). The affinities toward Cu^{2+} are very similar for both binding sites and arguments could be found to consider Cu(ε-AMP) as either derivative. For the other metal ions studied the situation is clear:[29,51] the metal ion affinity of the phosphate group

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is always larger, and therefore we are treating here Cu(ε-AMP) also as a complex derived from a phosphate monoester.

Comparison of the percentages for $M(\epsilon\text{-AMP})_{cl}$ (Table VII) with those for $M(AMP)_{cl}$ (Table VI) demonstrates that the incorporation of the 1,N^6-etheno bridge into the adenine residue drastically enhances the extent of metal ion backbinding, confirming the above conclusions based on Figure 3. Therefore, great care should be exercised in employing $\epsilon\text{-AMP}^{2-}$ as a probe for AMP^{2-} in the presence of metal ions.[20] In addition, the large stability increase (log Δ) observed for several of the M(ε-AMP) complexes is meaningful with regard to the structures of the macrochelates discussed in the next section.

General Considerations

The comparison of the properties of the M(AMP) and M(TuMP) complexes proves that macrochelation occurring with M(AMP) is due to the backbinding of the phosphate-coordinated metal ion to N-7 of the adenine residue (eq 1). The seemingly small structural alteration of the adenine residue by incorporating the 1,N^6-etheno bridge converts N-6 into an excellent donor atom and in combination with N-7 the phenanthroline-like N-6,N-7 binding unit is created (Figure 1), which is very attractive for transition elements, including Zn^{2+} and Cd^{2+}; therefore, $\%\ M(\epsilon\text{-AMP})_{cl} > \%\ M(AMP)_{cl}$.

Also of interest is a comparison of the extent of macrochelate formation in M(AMP) with that in $M(ADP)^-$ and $M(ATP)^{2-}$ complexes.[12-14] For all metal ions studied, the fraction of the total closed form depends on the number of phosphate groups present in the adenine nucleotide: $\%\ M(AMP)_{cl} < \%\ M(ADP)^-_{cl} > \%\ M(ATP)^{2-}_{cl}$.

Though it is now clear that N-7 has the decisive role in binding the metal ion back to the base in M(AMP) complexes, there remain three obvious possibilities for the actual structure of the resulting macrochelates. (i) The metal ion coordinates inner sphere to N-7 and outer sphere, i.e., via water, to the phosphate group. (ii) The metal ion interacts outer sphere via a coordinated and hydrogen-bonded water molecule with N-7 and directly with the phosphate group. (iii) Both binding sites, N-7 and the phosphate group, are inner sphere coordinated to the metal ion.

The possible binding modes for the open complex involving only phosphate binding have recently been summarized[29] and are not repeated here.[52]

On the basis of X-ray crystal-structure analysis of purine nucleoside 5′-monophosphates,[16,17] which show a metal ion coordinated inner sphere to N-7 while the phosphate group is outer sphere bound via coordinated water molecules, it was repeatedly concluded[7,8,53] for purine nucleotide metal ion complexes in aqueous solution that if macrochelates are formed, "α-phosphate binding is outer sphere when a metal is coordinated intramolecularly at N-7".[8] The involvement of water is attributed to steric hindrance, which "reduces the opportunity for both an α-phosphate and N-7 to be coordinated inner sphere in a macrochelate at the same time. One of the coordinate bonds is outer sphere: either N-7 inner and α-phosphate outer sphere or N-7 outer and α-phosphate inner sphere".[53] Thus, structures (i) and (ii) agree with these suggestions but structure (iii) does not.

However, there are indications from kinetic studies (mainly with Ni^{2+} and Co^{2+}) that both phosphate and base may bind inner sphere to a metal ion,[18,54] and indeed space-filling molecular models allow the construction of such a macrochelate without strain.[19,55] More importantly, kinetic and product studies[56] for the reaction between *cis*-$Pt(NH_3)_2^{2+}$ and 5′-(2′-deoxy)GMP^{2-} indicate a direct coordination of the metal ion to N-7 and the phosphate group; this suggestion was also independently confirmed in a careful NMR study[57] for *cis*-$Pt(NH_3)_2^{2+}$ and IMP^{2-} and GMP^{2-}. In addition, the M(ε-AMP) complexes with Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} are between 1 and 3 log units (= log Δ, eq 7) more stable than expected for a sole phosphate coordination (Table VII). It is difficult to see how such substantial stability increases could be achieved via outer-sphere interactions only; the sterically possible macrochelate formation with inner-sphere coordination of both the base and phosphate groups appears as much more likely.

Hence, one has to conclude that all three structures must be considered and that the involvement of water in structures (i) and (ii) may have reasons other than steric constraints. In an infrared study[58] on aqueous solutions with methyl phosphate it is concluded that Cu^{2+} and Zn^{2+} bind inner sphere to the phosphate, while for Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} outer-sphere structures dominate (the order being 60–80%). Indeed, a temperature-jump study[59] confirms for the Ni^{2+}-methyl phosphate system about 50% outer-sphere species. It is further concluded[59] that the higher the charge of a phosphate, the more predominant are inner-sphere complexes; for $Ni(ATP)^{2-}$ the ratio phosphate inner sphere to outer sphere is given as 260, i.e., Ni^{2+} coordinates to the phosphate chain of ATP^{4-} overwhelmingly in an inner-sphere fashion (regarding N-7 backbinding in $Ni(ATP)^{2-}$ see ref 12 and 13).

These results allow rationalization of the formation of solid complexes, like barium adenosine 5′-monophosphate heptahydrate,[60] in which a completely hydrated Ba^{2+} interacts only in an outer-sphere manner with the nucleotide, i.e., especially via phosphate–hydrogen bonds of coordinated water molecules. Similarly, the solid complex $[Ni(AMP)(H_2O)_5]\cdot H_2O$, for which the crystal-structure analysis[61] shows an inner-sphere N-7 coordination to Ni^{2+} and an outer-sphere binding to the phosphate group, is no longer a surprise. Assuming that in an aqueous solution of Ni(AMP) a considerable fraction of Ni^{2+} is outer sphere bound to the phosphate group, as is the case for Ni^{2+}–methyl phosphate,[58,59] then crystallization of the above complex is not exceptional and no steric arguments are needed for an explanation. Furthermore, an ultraviolet spectral perturbation study[7] gives 80% for the closed isomer, $Ni(AMP)_{cl}$ (eq 1), in good agreement with the 71 ± 4% of Table VI; this suggests[7] that all Ni^{2+} in the closed isomer is inner sphere bound to N-7 and consequently structure (i) was originally suggested[7] for $Ni(AMP)_{cl}$. However, in accord with the kinetic studies[18a,18c,54a] (and in the absence of steric requests) one may now conclude that a substantial fraction of $Ni(AMP)_{cl}$ is actually present with structure (iii), i.e., N-7 and the phosphate group are inner sphere bound to Ni^{2+}; clearly, the sum of structures (i) and (iii) corresponds to the mentioned 80% (or more precisely, 71 ± 4%) of the closed form.

In addition, an early ultraviolet spectral perturbation study[62] of the Cu^{2+}/AMP^{2-} system provides evidence that in a substantial amount of $Cu(AMP)_{cl}$ N-7 is inner sphere coordinated. Considering further that Cu^{2+} is expected[58] to coordinate also in an

(52) For some of the simple phosphate–metal ion complexes furnishing the baselines (Table I) indications exist[29] for an equilibrium between inner-sphere and outer-sphere phosphate coordination. Clearly, in an NMP complex in the open form the metal ion will coordinate to the phosphate group in the same way as with simple phosphate monoesters. Should macrochelate formation in backbound M(NMP) alter the phosphate inner sphere/outer sphere ratio to some extent, then the percentage of the total closed form calculated for the corresponding M(NMP) complex (Tables VI and VII) would still be correct, as any alteration of the phosphate inner sphere/outer sphere ratio would be on account of the stability increase, i.e., log Δ (Tables VI and VII), on which the calculation for the extent of macrochelation is based.

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(55) The result of model building differs with nucleoside 5′-di- and triphosphates: inner-sphere coordination of N-7 and the β- and γ-phosphate groups makes direct coordination of the α-group more difficult; here the steric situation is facilitated with a water molecule between the metal ion and the α-phosphate group in accordance with the previous suggestions.[7,8,53]

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inner-sphere mode to the phosphate group, one may conclude that probably the larger part (or even all) of the 46 ± 10% of Cu(AMP)$_{cl}$ (Table VI) is present as a pure inner-sphere macrochelate, i.e., in the form of structure (iii).

The possible occurrence of structure (ii) with N-7 outer sphere coordinated via a water molecule and direct metal ion–phosphate binding should not lightly be dismissed. There is evidence for the formation of such species in M(ATP) systems,[7,12,13] and it is well known[60,63] that N-7 has a pronounced tendency to form hydrogen bonds to water molecules. Should there be traces of closed species present in the alkaline earth ion systems, like Mg(AMP)$_{cl}$ (Table VI) or Mg(ε-AMP)$_{cl}$ (Table VII), then N-7 binding may occur outer sphere as suggested[12,13] for the small fraction of Mg(ATP)$^{2-}_{cl}$. The question if structure (ii) is also playing a role in closed M(NMP) complexes of transition-metal ions has to be left open for the present; it can only be solved with additional experiments like those carried out[7] for Ni(AMP), and (as concluded above) in this latter case structure (ii) is not significant.

To conclude, the structures to be considered for M(NMP)$_{cl}$ species comprise the three described structures, (i), (ii), and (iii). Equilibria between these different forms have to be assumed, though depending on the metal ion, the one or other closed isomer may form only in traces or not at all. It is now evident that equilibrium 1 is a simplification as M(NMP)$_{cl}$ is not a well defined single isomer; hence, the K_1 values and the percentages for M(NMP)$_{cl}$ given in Tables VI and VII with regard to equations 2 and 6 are actually overall values which quantify the *total* formation of all closed species, and the macrochelate formation may be inner sphere or outer sphere at both the base and phosphate moieties.

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Trichodiene Biosynthesis and the Role of Nerolidyl Pyrophosphate in the Enzymatic Cyclization of Farnesyl Pyrophosphate†

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Abstract: Incubation of (1*Z*)-[1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate (**4a**) with a preparation of trichodiene synthetase isolated from the fungus *Trichothecium roseum* gave labeled trichodiene (**3**), which was shown by chemical degradation to carry the tritium label exclusively at H-11β. These results were consistent with a previously proposed isomerization–cyclization mechanism for the formation of trichodiene from farnesyl pyrophosphate (**1**). The absolute configuration of the enzymatically active enantiomer of nerolidyl pyrophosphate was determined by incubating a mixture of (3*S*)-(1*Z*)-[1-^{3}H]nerolidyl pyrophosphate and (3*RS*)-[12,13-^{14}C]nerolidyl pyrophosphate with trichodiene synthetase. The finding that the resulting trichodiene (**3**) was labeled only with ^{14}C established that the cyclase utilized only (3*R*)-nerolidyl pyrophosphate. Further support for the proposed cyclization mechanism was obtained by carrying out a competitive incubation of [1-^{3}H]farnesyl pyrophosphate (**1**) and [12,13-^{14}C]nerolidyl pyrophosphate (**4**) with trichodiene synthetase and examining the ^{3}H/^{14}C ratio of the resulting trichodiene as well as that of remaining farnesyl pyrophosphate and nerolidyl pyrophosphate as a function of time. The results of the latter experiment indicated that both allylic pyrophosphate substrates compete for the same active site in trichodiene synthetase, with one enantiomer of nerolidyl pyrophosphate having a V_{max}/K_m approximately 1.5–2 times that of farnesyl pyrophosphate.

Sesquiterpene synthetases provide a striking example of Nature's synthetic virtuosity and economy of catalytic design. On the basis of the cyclization of a single substrate, farnesyl pyrophosphate (**1**), these remarkable enzymes are able to mediate the formation of more than 200 distinct sesquiterpene hydrocarbons and alcohols.[1,2] According to the currently accepted hypothesis, all these cyclizations take place by variations on a simple mechanism involving ionization of the allylic pyrophosphate and intramolecular electrophilic attack of the resulting carbocation on either the central or distal double bond of the farnesyl skeleton (Scheme I). Subsequent cyclization or rearrangement processes followed by quenching of the cationic intermediates by deprotonation or capture of an external nucleophile such as water can, in principle, account for the formation of all the known sesquiterpene carbon skeletons. Completely analogous schemes have been invoked to account for the formation of cyclic monoterpenes from the universal acyclic precursor geranyl pyrophosphate (**2**).[3]

Over the last 10 years, substantial progress in the understanding of terpenoid cyclizations has come from the isolation and study of a variety of monoterpene and sesquiterpene cyclases.[1,3,4] One

† This paper is dedicated to Professor Duilio Arigoni on the occasion of his 60th birthday.

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