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A Stability Concept for Metal Ion Coordination to Single-Stranded Nucleic Acids and Affinities of Individual Sites

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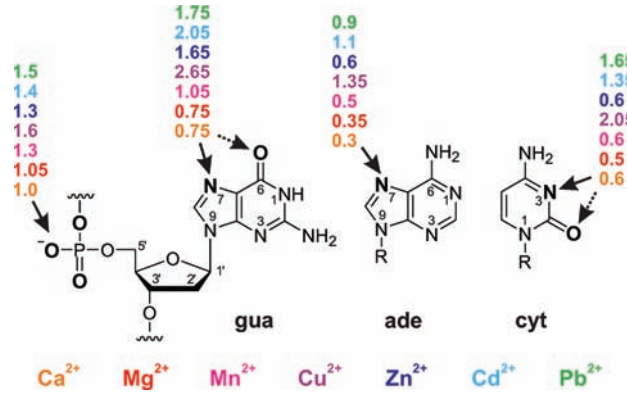
CONSPECTUS

The three-dimensional architecture and function of nucleic acids strongly depend on the presence of metal ions, among other factors. Given the negative charge of the phosphate−sugar backbone, positively charged species, mostly metal ions, are necessary for compensation. However, these ions also allow and induce folding of complicated RNA structures. Furthermore, metal ions bind to specific sites, stabilizing local motifs and positioning themselves correctly to aid (or even enable) a catalytic mechanism, as, for example, in ribozymes. Many nucleic acids thereby exhibit large differences in folding and activity depending not only on the concentration but also on the kind of metal ion involved. As a consequence, understanding the role of metal ions in nucleic acids requires knowing not only the exact positioning and coordination sphere of each specifically bound metal ion but also its intrinsic site affinity. However, the quantification of metal ion affinities toward certain sites in a single-stranded (though folded) nucleic acid is a demanding task, and few experimental data exist.



In this Account, we present a new tool for estimating the binding affinity of a given metal ion, based on its ligating sites within the nucleic acid. To this end, we have summarized the available affinity constants of Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} for binding to nucleobase residues, as well as to mono- and dinucleotides. We have also estimated for these ions the stability constants for coordinating the phosphodiester bridge. In this way, stability increments for each ligand site are obtained, and a clear selectivity of the ligating atoms, as well as their discrimination by different metal ions, can thus be recognized.

On the basis of these data, we propose a concept that allows one to estimate the intrinsic stabilities of nucleic acid-binding pockets for these metal ions. For example, the presence of a phosphate group has a much larger influence on the overall affinity of Mg^{2+}, Ca^{2+}, or Mn^{2+} compared with, for example, that of Cd^{2+} or Zn^{2+}. In the case of Cd^{2+} and Zn^{2+}, the guanine N7 position is the strongest intrinsic binding site. By adding up the individual increments like building blocks, one derives an estimate not only for the overall stability of a given coordination sphere but also for the most stable complex if an excess of ligating atoms is available in a binding pocket saturating the coordination sphere of the metal ion. Hence, this empirical concept of adding up known intrinsic stabilities, like building blocks, to an estimated overall stability will help in understanding the accelerating or inhibiting effects of different metal ions in ribozymes and DNAzymes.

1. Introduction

Nucleic acids are macromolecules carrying a negative charge due to their phosphate diester backbone. Consequently, the nucleotide units present require an equal amount of cations. These are mostly Na^+, K^+, and Mg^{2+}, though some other divalent metal ions or positively charged organic residues may also be present.

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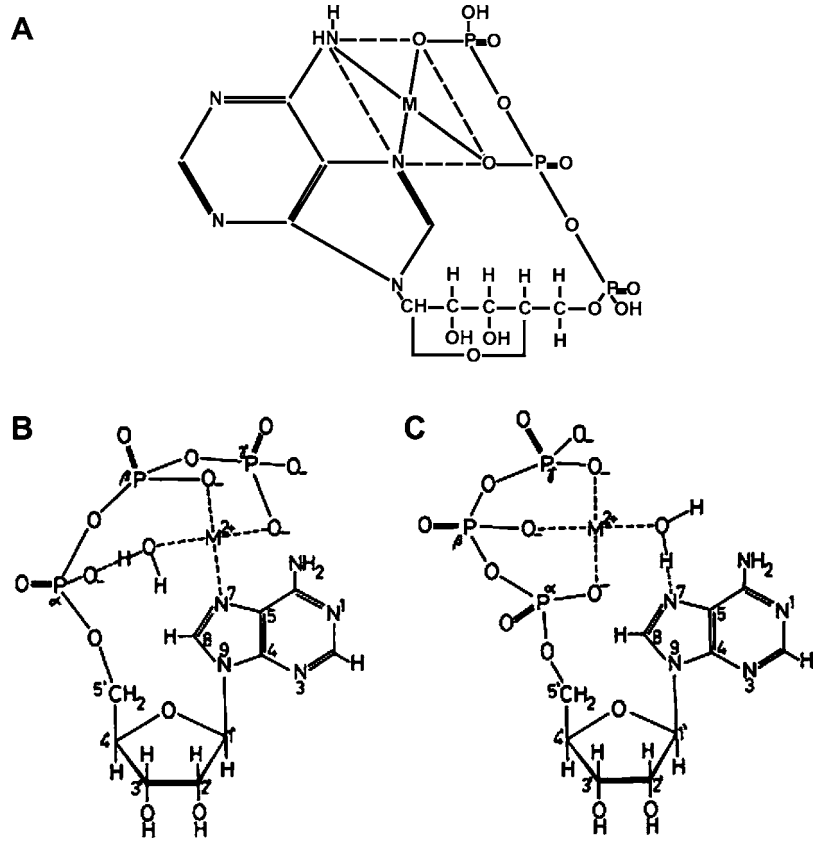


FIGURE 1. (A) Quadridentate Mg^{2+} coordination to H_2ATP^{2-}, as suggested by Szent-Györgyi[7] in 1956 (redrawn) and (B, C) Tentative and simplified structure for macrochelated inner-sphere (B) and outer-sphere (C) $M(ATP)^{2-}$ isomers (reproduced by permission of the Federation of European Biochemical Societies (FEBS) from *Eur. J. Biochem.*, ref 8). Note, inner-sphere and outer-sphere refer here only to the M^{2+}–N7 coordination. The type of phosphate coordination depends on the M^{2+} involved.[8,10,11] For example, evidence exists (see ref 10) that phosphate binding in $Mg(ATP)^{2-}$ occurs as a mixture of β,γ-bidentate and α,β,γ-tridentate complexation.

The "intensity" of the interaction between metal ions and nucleic acids can be described by, for example, calculations based on the nonlinear Poisson–Boltzmann equation quantifying the interaction of "diffuse" (fully hydrated) ions with nucleic acids[1−3] or adsorption isotherms, which provide kind of "averaged" binding constants.[4] Yet such "average" affinities are not very helpful because, for example, Mg^{2+} may be directly involved in the three-dimensional folding of nucleic acids and in ribozymes it may participate in reaction processes;[5,6] hence, such metal ions are bound to distinct sites, but their affinities have hardly been quantified.

It was Szent-Györgyi who (to the best of our knowledge) first pointed out more than 50 years ago the relevance of nucleobase–metal ion interactions by proposing a Mg^{2+} chelate of ATP (Figure 1A).[7] This structure has severe shortcomings:[8] (i) At the physiological pH of about 7.5, especially if a metal ion is coordinated, the triphosphate group no longer carries any protons. (ii) More importantly, (C6)NH_2 of the adenine residue does not bind metal ions. This group has no basic but rather acidic properties and may release a proton with $pK_a \approx 17$.[9] (iii) Of a more subtle nature, binding of Mg^{2+} to N7 does not occur significantly inner-sphere but rather outer-sphere, that is, a coordinated H_2O forms a hydrogen bond to N7.[8,10] A more realistic coordination pattern is shown in Figure 1B,C.[8,10,11] Nevertheless, Szent-Györgyi's structure[7] had a tremendous influence because it triggered the interest of biochemists and coordination chemists in the metal ion binding properties of nucleotides[11,12] and thus indirectly in nucleic acids.

Evidently, nucleobase residues must be involved in the formation of metal ion-binding pockets, for example, in ribozymes. Therefore, we propose an empirical stability concept for metal ion binding to single-stranded nucleic acids and summarize first the M^{2+} affinities of nucleobases toward Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} as they follow from measurements with nucleosides. This allows development of affinity sequences for the nucleobases shown in Figure 2. Next, the M^{2+} affinity of the phosphodiester bridge was estimated and integrated into the sequences. The stability increments obtained in this way were thereafter complemented by

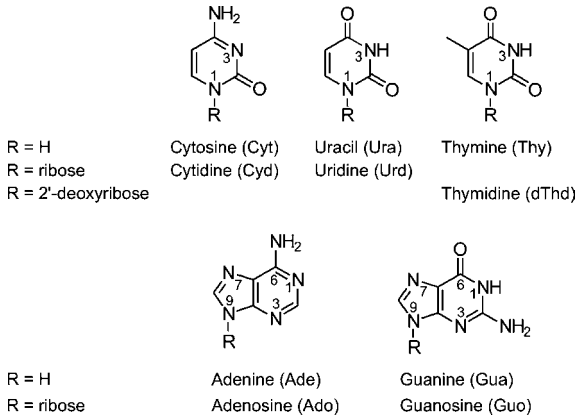


FIGURE 2. Structures of the nucleobases (R = H) occurring in nucleic acids and in their nucleosides (R = ribose; for dThd, R = 2′-deoxyribose).

data obtained from the M^{2+}-binding properties of dinucleotides. These results are then extrapolated to obtain estimates of the stability constants for binding pockets in nucleic acids.

2. Metal Ion Affinity Sequences for Nucleobase Residues

The nucleotide units of DNA contain mostly adenine, guanine, cytosine, and thymine. In RNA, thymine is replaced by the related uracil. The two purine and the cytosine residues contain imidazole- and pyridine-type nitrogens well-suited for M^{2+} binding (Figure 2). There is nothing similar in uracil or thymine as long as (N3)H is not deprotonated, and this requires exceptional conditions at physiological pH.[13] Hence, uracil and thymine offer commonly only their carbonyl groups, which bind M^{2+} very weakly with the assistance of a primary binding site.[13,14]

Consequently we need to consider the M^{2+}-binding properties of the adenine, guanine, and cytosine residues. These are best quantified as their nucleosides (Ns) according to equilibrium 1:

$$M^{2+} + Ns \rightleftharpoons M(Ns)^{2+} \quad (1a)$$

$$K^{M}_{M(Ns)} = [M(Ns)^{2+}] / ([M^{2+}][Ns]) \quad (1b)$$

Table 1 summarizes the results, mostly obtained via potentiometric pH titrations.[15−21]

The nucleoside-complex stability decreases in the order Guo > Cyt > Ado for all seven M^{2+} considered. Since these complexes are formed with neutral nucleosides, the effect of the negative charge of the phosphodiester bridge, RO−$P(O)_2^-$−OR′, on M^{2+} coordinated at a nucleobase residue is not yet considered. A correction is possible by using as mimics phosphate-monoprotonated nucleoside 5′-monophosphate complexes with M^{2+} at the nucleobase, $(M \cdot NMP \cdot H)^+$ (eq 2):

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

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

Consequently, the $M(Ns)^{2+}$ stability constants (Table 1) need to be corrected (i) for the small difference in basicity between the Ns and $(NMP \cdot H)^-$ species,[10] and (ii) for the charge effect of the phosphodiester bridge. The latter is well represented by the RO−$P(O)_2^-$−OH residue in the $(M \cdot NMP \cdot H)^+$ complexes and amounts to 0.40 ± 0.15 log unit.[22,23] The corrected values for the $M(Ns)^{2+}$ complexes representing now the stabilities of the "open" $(M \cdot NMP \cdot H)^+$ species are listed in Table 2; note, no macrochelate formation involving the monoprotonated phosphate group is considered.[18]

The so-called micro stability constants (k) of Table 2 quantify the M^{2+} affinities of nucleobases and the affinity sequences summarized in Figure 3 (lower part; black values) evolve from these constants. Though the absolute sizes of the values differ for the various M^{2+}, the order within the sequences remains the same. However, the affinity change from site to site may differ much depending on the M^{2+} involved, thus indicating selectivity.

3. Inclusion of the Phosphodiester Bridge into the Affinity Sequences for Nucleic Acids

Unfortunately, no stability constants of relevant phosphodiesters are known. Therefore we estimated stability constants for the M^{2+} considered here in three ways:

(i) Because the charge of a phosphodiester, $(RO)_2PO_2^-$, corresponds to that of formate, HCO_2^-, and acetate, $CH_3CO_2^-$, their complex stability constants[24,25] gave one data series.

TABLE 1. Metal Ion Affinity of Nucleobase Residues in Nucleosides[a]

M^{2+}	Guo[b]	Cyd[h]	Ado[j]
Mg^{2+}	0.35 ± 0.25[c]	0.12 ± 0.04	−0.06 ± 0.15
Ca^{2+}	0.35 ± 0.26[d]	0.18 ± 0.06	−0.12 ± 0.12
Mn^{2+}	0.57 ± 0.2[d]	0.19 ± 0.08	0.04 ± 0.09
Cu^{2+}	2.12 ± 0.14[e]	1.56 ± 0.06	0.84 ± 0.04
Zn^{2+}	1.16 ± 0.11[f]	0.20 ± 0.11	0.15 ± 0.04
Cd^{2+}	1.53 ± 0.07[c]	0.91 ± 0.07	0.64 ± 0.03
Pb^{2+}	1.25 ± 0.17[g]	1.20 ± 0.07[i]	0.4 ± 0.3[g]

[a] Log stability constants according to eq 1 (aqueous solution, 25 °C, I = 0.1 M, $NaNO_3$). The error limits correspond to three times the standard error of the mean value. The acidity constants are $pK^{H}_{H(Guo)}$ = 2.11 ± 0.04 for $(N7)H^+$,[12,15] $pK^{H}_{H(Cyd)}$ = 4.24 ± 0.02 for $(N3)H^+$,[12,20] and $pk^{Ado/N7-N1}_{H(Ado)/H \cdot N7-N1}$ = 2.2 ± 0.2 for $(N7)H^+$ (N1 being deprotonated).[12] [b] Except for $Pb(Guo)^{2+}$, values for 2′-deoxyguanosine (dGuo) are listed because these are more widely available. Based on $pK_a = pK^{H}_{H(dGuo)} - pK^{H}_{H(Guo)} \approx 0.2$[15] and on log K versus pK_a slopes,[16] we estimate that the stability constants for the $M(Guo)^{2+}$ complexes are at most smaller by 0.1 log unit. [c] From ref 15. [d] Estimate based on the log K versus pK_a straight-line plot[16] for benzimidazoles by also considering the other listed values. [e] From ref 17. [f] Estimate from ref 18. [g] From ref 19. [h] From ref 20. [i] From ref 21. [j] Kapinos, L. E.; Sigel, H., results to be published.

TABLE 2. Logarithms of the Stability Constants for the "Open" $(M \cdot NMP \cdot H)^+$ complexes (Equation 2b)[a]

M^{2+}	$(M \cdot GMP \cdot H)^+$	$(M \cdot CMP \cdot H)^+$	$(M \cdot AMP \cdot H)^+$
Mg^{2+}	0.76 ± 0.29	0.52 ± 0.16	0.35 ± 0.21
Ca^{2+}	0.75 ± 0.30	0.58 ± 0.16	0.28 ± 0.19
Mn^{2+}	1.04 ± 0.25	0.60 ± 0.17	0.48 ± 0.17
Cu^{2+}	2.66 ± 0.21	2.03 ± 0.16	1.33 ± 0.16
Zn^{2+}	1.66 ± 0.19	0.62 ± 0.19	0.62 ± 0.16
Cd^{2+}	2.04 ± 0.17	1.33 ± 0.17	1.11 ± 0.15
Pb^{2+}	1.77 ± 0.23	1.64 ± 0.17	0.9 ± 0.35

[a] See text in section 2. The error limits of these derived data were calculated according to the error propagation after Gauss taking into account the error limits of Table 1 plus ±0.15 log unit (see text).

(ii) Next, we extrapolated the known log $K^{M}_{M(R-PO_3)}$ versus $pK^{H}_{H(R-PO_3)}$ straight-line plots,[11,14,21,23] which hold for phosphate monoesters, $R-PO_3^{2-}$, in the pK_a range from about 4.5 to 8, to $pK_a = 1$, the approximate acidity constant[26] of a phosphate diester, giving another set of constants.

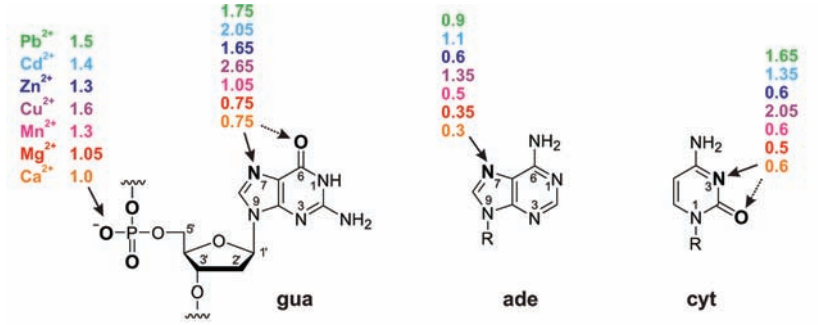
(iii) Finally, the logarithmic stability differences between complexes formed with diphosphate monoesters ($R-DP^{3-}$) and their monoprotonated form, log $K^{M}_{M(R-DP)}$ − log $K^{M}_{M(H;R-DP)}$,[27] which reflect the effect of the proton, were subtracted from the stability constants, log $K^{M}_{M(R-PO_3)}$ (valid for $pK_a = 6.2$),[11,14,21,23] of phosphate monoesters.

The three sets of stability constants turned out to be rather similar for a given M^{2+} allowing us to average the values (though in part weighted). The estimated log stability constant for $M[(RO)_2PO_2]^+$ of Mg^{2+} is log $K^{Mg}_{Mg[(RO)_2PO_2]} = 0.45$; for the other M^{2+}, they are 0.4 (Ca^{2+}), 0.7 (Mn^{2+}), 1.0 (Cu^{2+}), 0.7 (Zn^{2+}), 0.8 (Cd^{2+}), and 0.9 (Pb^{2+}) (25 °C, $I = 0.1$ M). That these values are reasonable is confirmed by a previous estimate for $Pb[ROP(O)_2OH]^+$ (log $K^{Pb}_{Pb[ROP(O)_2OH]} = 0.7 \pm 0.4$)[19] and by the measured[28] Ni^{2+}-affinity constant of monoprotonated D-ribose 5-monophosphate, log $K^{Ni}_{Ni(H;RibMP)} = 0.7$, because the stability of Ni^{2+}−phosphate complexes is commonly similar to those with Mn^{2+} and Zn^{2+}.[12]

However, there is another point: compared with the individual nucleobases present in a nucleic acid, the phosphodiester bridge occurs in excess because with each nucleobase a diester bridge is connected. If one assumes, to make matters easy to handle, that the four main nucleobases of RNA and DNA occur in about equal amounts, then the anionic phosphodiester bridge has a 4-fold excess. Application of this statistical factor leads to eq 3:

$$\log k = \log K^{M}_{M[(RO)_2PO_2]} + 0.6 \qquad (3)$$

The resulting micro stability constants quantifying the affinity of the phosphodiester bridge, in combination with the stability data in Table 2, provide the individual log affinity constants of the various sites (Figure 3, upper part). The lower part of Figure 3 contains the affinity sequences for each M^{2+} for the five nucleobases and the singly negatively charged phosphodiester bridge, which has a higher affinity for Mg^{2+}, Ca^{2+}, and Mn^{2+} than any of the nucleobases. For Zn^{2+}, the affinity of the guanine and the phosphate unit are relatively similar, whereas, for example, Cu^{2+} has a much higher affinity toward guanine and cytosine. This selectivity is of high relevance for the properties of nucleic acids in the presence of metal ions and it explains, for example, why Cu^{2+} penetrates into the double helix of DNA in contrast to Mg^{2+}.[29]



Ca2+: PO2− (log k = 1.0) > gua-N7(O6) (0.75) ~ cyt-N3(O2) (0.6) > ade (0.3) > ura ~ thy

Mg2+: PO2− (log k = 1.05) > gua-N7(O6) (0.75) > cyt-N3(O2) (0.5) ~ ade (0.35) > ura ~ thy

Mn2+: PO2− (log k = 1.3) > gua-N7(O6) (1.05) > cyt-N3(O2) (0.6) ~ ade (0.5) > ura ~ thy

Cu2+: gua-N7(O6) (log k = 2.65) >> cyt-N3(02) (2.05) > PO2− (1.6) > ade (1.35) > ura ~ thy

Zn2+: gua-N7(O6) (log k = 1.65) > PO2− (1.3) >> cyt-N3(O2) (0.6) ~ ade (0.6) > ura ~ thy

Cd2+: gua-N7(O6) (log k = 2.05) >> PO2− (1.4) ~ cyt-N3(O2) (1.35) > ade (1.1) > ura ~ thy

Pb2+: gua-N7(O6) (log k = 1.75) ~ cyt-N3(O2) (1.65) ~ PO2− (1.5) >> ade (0.9) > ura ~ thy

FIGURE 3. Upper part, individual M^{2+} affinities for the various binding sites within nucleotide residues in single-stranded nucleic acids based on the data of Table 2 (rounded values) and eq 3 and **lower part**, M^{2+}-affinity sequences for single-stranded nucleic acids with the phosphodiester groups highlighted in red: "~" means that the complex stability difference is below 0.2 log unit, ">" indicates a difference larger than 0.2 log unit, and "≫" indicates a stability difference of more than 0.5 log unit.

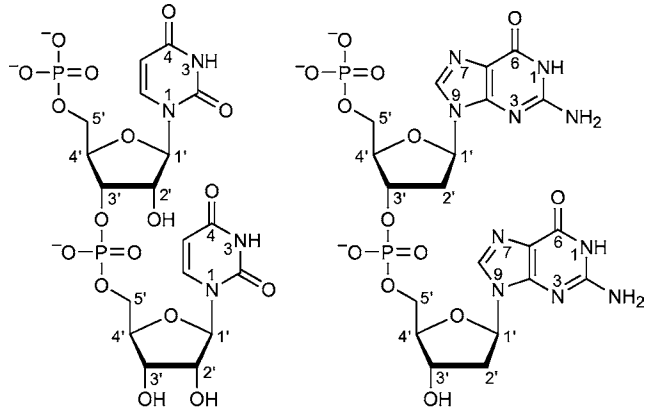


FIGURE 4. Structures of the trianions of uridylyl-(5′→3′)-5′-uridylate ($pUpU^{3-}$) and 2′-deoxyguanylyl-(5′→3′)-2′-deoxy-5′-guanylate ($d(pGpG)^{3-}$) with the two nucleoside units in each dinucleotide in the predominant *anti* conformation.

4. Neighboring Phosphate Units Stabilize Metal Ion Binding!

So far we considered only the M^{2+} affinity of individual binding sites. However, experience[10,11,30] teaches that nucleotide units may interact with M^{2+} via more than just one coordinating atom (Figure 1). For neighboring nucleotide units, this should even be more true, but so far, quantitative studies only exist for two dinucleotides (Figure 4).[12,18,26]

Because the uracil residue has no remarkable M^{2+} affinity,[27,31] as long as (N3)H is not deprotonated,[13] one expects M^{2+} binding to $pUpU^{3-}$ at the terminal phosphate group with the potential to form 10-membered chelates involving the neighboring phosphodiester bridge. Since any additional interaction of the terminal phosphate-coordinated M^{2+} must be reflected in an increased complex stability,[32] it is best to insert the results[26] for the $M^{2+}/pUpU^{3-}$ systems into straight-line plots of log $K^{M}_{M(R-PO_3)}$ versus $pK^{H}_{H(R-PO_3)}$, which hold for simple phosphate monoester and phosphonate ligands.[11,14,23,26]

In Figure 5, the negatively charged $M(pUpU)^-$ complexes for Mg^{2+}, Zn^{2+}, and Pb^{2+}show an increased stability compared with their straight reference lines representing uncharged $M(R-PO_3)$ species. With the known parameters for the straight-line plots, the expected stabilities based on $pK^{H}_{H(pUpU)} = 6.44 \pm 0.02$ can be calculated.[26] These values are listed in Table 3 (column 3) together with the observed stability enhancements (column 4) as defined in eq 4, where $(d)pNpN^{3-}$ represents any dinucleotide:

$$\log \Delta_{M(d)pNpN} = \log K^{M}_{M(d)pNpN} - \log K^{M}_{M(R-PO_3)} \qquad (4)$$

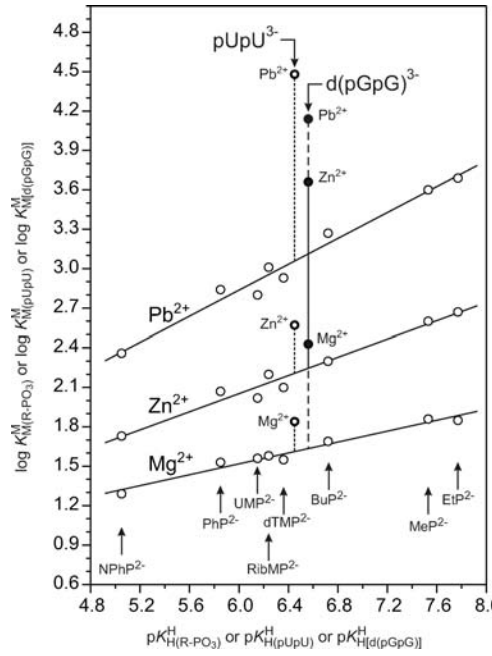


FIGURE 5. Evidence for an enhanced stability of the $M(pUpU)^-$ and $M[d(pGpG)]^-$ complexes of Mg^{2+}, Zn^{2+}, and Pb^{2+}, based on log $K^{M}_{M(R-PO_3)}$ versus $pK^{H}_{H(R-PO_3)}$ plots for $M(R-PO_3)$ complexes where $R-PO_3^{2-}$ = (from left to right) 4-nitrophenyl phosphate ($NPhP^{2-}$), phenyl phosphate (PhP^{2-}), uridine 5′-monophosphate (UMP^{2-}), D-ribose 5-monophosphate ($RibMP^{2-}$), thymidine [= 1-(2′-deoxy-β-D-ribofuranosyl)thymine] 5′-monophosphate ($dTMP^{2-}$), *n*-butyl phosphate (BuP^{2-}), methanephosphonate (MeP^{2-}), and ethanephosphonate (EtP^{2-}). The least-squares lines are drawn through the corresponding eight data sets;[23,31] the straight-line parameters are listed in refs 14, 23, and 26. The data for the $M^{2+}/H^+/pUpU^{3-}$ and $M^{2+}/H^+/d(pGpG)^{3-}$ systems are taken from refs 26 and 18. The vertical dotted lines emphasize the stability differences to the reference lines, log $\Delta_{M/pUpU}$ (defined in eq 4) for $M(pUpU)^-$ complexes. The vertical full (Zn^{2+}) and broken (Pb^{2+}, Mg^{2+}) lines describe the situation for the $M[d(pGpG)]^-$ complexes. All plotted constants refer to aqueous solutions at 25 °C and $I = 0.1$ M ($NaNO_3$). This is an altered version of Figure 2 in ref 18; reproduced with permission; copyright 2007 Wiley-VCH, Weinheim, Germany.

These differences are all positive but those for the Mg^{2+}, Mn^{2+}, and Cd^{2+} complexes are identical within their error limits. Considering that for these three M^{2+}, the binding affinities toward liganding sites differ considerably[10,24,25] (column 2), this result can only mean that this stability enhancement, on average log $\Delta_{M/pUpU/charge} = 0.24 \pm 0.04$,[26] reflects the charge effect of the neighboring phosphodiester unit on M^{2+} coordinated to the terminal phosphate group. Hence, only $Zn(pUpU)^-$ and $Pb(pUpU)^-$ are additionally stabilized (Table 3, column 4).

A stability enhancement beyond 0.24 log unit must result from an additional interaction,[32] that is, chelate formation according to equilibrium 5 (op = open; cl = closed/chelated):

$$M[(d)pNpN]^-_{op} \rightleftharpoons M[(d)pNpN]^-_{cl} \qquad (5)$$

TABLE 3. Stability Constant Comparisons for $M(pUpU)^-$ with $M(R-PO_3)$ Complexes and the Stability Enhancements Defined by Eq 4. The Resulting Formation Degrees of the Closed Species Are Given in the Lower Part[a]

M^{2+}	log $K^M_{M(pUpU)}$	log $K^M_{M(R-PO_3)}$	log $\Delta_{M/pUpU}$
Mg^{2+}	1.84 ± 0.04	1.61 ± 0.03	0.23 ± 0.05[†]
Mn^{2+}	2.49 ± 0.05	2.22 ± 0.05	0.27 ± 0.07[†]
Zn^{2+}	2.57 ± 0.03	2.20 ± 0.06	0.37 ± 0.07
Cd^{2+}	2.75 ± 0.03	2.52 ± 0.05	0.23 ± 0.05[†]
Pb^{2+}	4.45 ± 0.25	3.05 ± 0.08	1.40 ± 0.26

[†] average: log $\Delta_{M/pUpU/charge}$ = 0.24 ± 0.04
Zn^{2+}: log Δ^* = 0.13 ± 0.08 (eq 6) -- % $Zn(pUpU)^-_{cl}$ = 26 ± 14% (eq 8)
Pb^{2+}: log Δ^* = 1.16 ± 0.26 (eq 6) -- % $Pb(pUpU)^-_{cl}$ = 93 ± 4% (eq 8)

[a] All data are from ref 26 (aqueous solution, 25 °C; I = 0.1 M, $NaNO_3$). The error limits are three times the standard error of the mean value (3σ); those of the derived data (column 4) were calculated according to the error propagation after Gauss.

This extra stability enhancement is generally defined by eq 6a, and for $pUpU^{3-}$ by eq 6b:

$$\log \Delta^*_{M/(d)pNpN} = \log \Delta_{M/(d)pNpN} - \log \Delta_{M/(d)pUpU/charge} \quad (6a)$$

$$\log \Delta^*_{M/pUpU} = \log \Delta_{M/pUpU} - (0.24 \pm 0.04) \quad (6b)$$

The position of the intramolecular equilibrium 5 is given[26] by the dimensionless constant K_I of eq 7,

$$K_I = [M[(d)pNpN]^-_{cl}] / [M[(d)pNpN]^-_{op}] = 10^{\log\Delta^*} - 1 \quad (7)$$

and the formation degree of the closed species by eq 8,

$$\%M[(d)pNpN]^-_{cl} = 100K_I/(1 + K_I) \quad (8)$$

Formation degrees of about 26% and 93% for the 10-membered chelates follow for $Zn(pUpU)^-$ and $Pb(pUpU)^-$ (Table 3, lower part). Among the five systems for which data exist,[26] only these two can with certainty form a chelate between two neighboring phosphate groups. For the Mg^{2+}, Mn^{2+}, and Cd^{2+} complexes, the formation degrees are zero within the error limits, yet due to these errors, small amounts (<15%)[26] of the chelated species could still exist.

5. Studies of a Dinucleotide Allowing Macrochelate Formation

Macrochelate formation of guanosine 5′-phosphates involving N7 is well-known[10−12] and thus expected for the dinucleotide $d(pGpG)^{3-}$ (Figure 4) and also within a general GG sequence. Indeed, increased complex stabilities for the $M[d(pGpG)]^-$ complexes of Mg^{2+}, Zn^{2+}, and Pb^{2+} are observed (Figure 5).[18] Comparison of the experimentally obtained stability constants for the four systems studied (Table 4, column 2; defined in analogy to eq 1) with the values calculated for the $M(R-PO_3)$ complexes (column 3; based on $pK^H_{H[d(pGpG)]} = 6.56 \pm 0.03$)[18] gives according to eq 4 the stability differences listed in column 4. Correction of these values for the charge effect (eq 6) results in the stability enhancements (column 5). These enhancements are significantly higher for the complexes of Mg^{2+}, Zn^{2+}, and Cd^{2+} compared with those of the corresponding $M(pUpU)^-$ species (column 6), except for $Pb[d(pGpG)]^-$, which shows a lower stability enhancement.[18]

TABLE 4. Stability Constant Comparisons for Some $M[d(pGpG)]^-$ Complexes[a]

M^{2+}	log $K^M_{M[d(pGpG)]}$	log $K^M_{M(R-PO_3)}$	log $\Delta_{M/d(pGpG)}$	log $\Delta^*_{M/d(pGpG)}$	log $\Delta^*_{M/pUpU}$
Mg^{2+}	2.43 ± 0.06	1.64 ± 0.03	0.79 ± 0.07	0.55 ± 0.08	−0.01 ± 0.06
Zn^{2+}	3.66 ± 0.05	2.25 ± 0.06	1.41 ± 0.08	1.17 ± 0.08	0.13 ± 0.08
Cd^{2+}	4.01 ± 0.06	2.56 ± 0.05	1.45 ± 0.08	1.21 ± 0.08	−0.01 ± 0.06
Pb^{2+}	4.14 ± 0.10	3.11 ± 0.08	1.03 ± 0.13	0.79 ± 0.14	1.16 ± 0.26

[a] All data are from ref 18 (aqueous solution, 25 °C, I = 0.1 M, $NaNO_3$); those of column 6 follow according to eq 6b from the values listed in Table 3 (column 4). For the error limits see footnote *a* of Table 3. This table is adapted from Table 4 in ref 18 by permission; copyright 2007 Wiley-VCH, Weinheim, Germany.

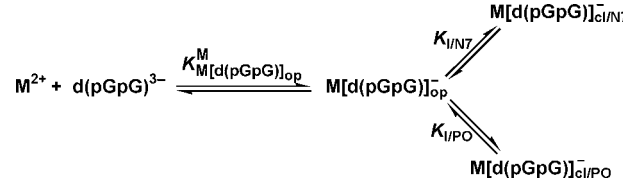


FIGURE 6. In the equilibrium scheme, $M[d(pGpG)]^-_{op}$ designates the "open" complex in which M^{2+} is only bound to the terminal phosphate group. The "closed" or macrochelated isomers involving either the phosphodiester bridge or the N7 sites are termed $M[d(pGpG)]^-_{cl/PO}$ or $M[d(pGpG)]^-_{cl/N7}$. Note, in contrast to our previous conclusion (ref 18), we are now convinced (see text in connection with Table 6) that due to self-stacking within $M[d(pGpG)]^-$, the dinucleotide is preorientated[30,33] allowing thus an interaction of M^{2+} coordinated at the terminal phosphate group with both N7 sites (see also Figure 7). Hence, additional equilibria exist and $M[d(pGpG)]^-_{cl/N7}$ represents all isomers containing N7 in two-, three-, or four-point interactions (cf. the crystal structure studies in section 7). Clearly, the analytical concentration of $M[d(pGpG)]^-$, determined in the experiments,[18] encompasses the sum of all isomeric species in the equilibrium scheme. Evidently, $K^M_{M[d(pGpG)]op} = [M[d(pGpG)]^-_{op}] / ([M^{2+}][d(pGpG)^{3-}])$.

From columns 5 and 6 of Table 4, it follows that Mg^{2+} and Cd^{2+} in $M[d(pGpG)]^-$ form only macrochelates with N7 (see also below) and no indication for the formation of the 10-membered chelate with the neighboring phosphodiester bridge exists. Pb^{2+} only forms the latter chelate, whereas for $Zn[d(GpG)]^-$, the equilibria depicted in Figure 6 exist.[18] The formation degrees of the various species were calculated by applying known procedures;[23] the results[18] are summarized in Table 5, together with estimations for $Ca[d(pGpG)]^-$. However, regarding the $M[d(pGpG)]^-_{cl/N7}$ formation, the above view is a simplification because two N7 positions of the two preoriented guanine residues (via stacking) in $M[d(pGpG)]^-$ are

TABLE 5. Percentages of Isomeric Species Formed in Intramolecular Equilibria with M[d(pGpG)]$^-$ Complexes[a]

M^{2+}	% $M[d(pGpG)]^-_{op}$	% $M[d(pGpG)]^-_{cl/N7}$	%$M[d(pGpG)]^-_{cl/PO}$
Mg^{2+}	28 ± 5	72 ± 5.5	~0
Ca^{2+}	~39[b]	~47[b]	~14[b]
Zn^{2+}	6.8 ± 1.4	91 ± 2.5	2.4 ± 1.8
Cd^{2+}	6 ± 2	94 ± 1.5	~0
Pb^{2+}	16 ± 5	~0	84 ± 6

[a] The data for Mg^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} are from ref 18 (aqueous solution, 25 °C, $I = 0.1$ M, $NaNO_3$); see also Figure 6 and text, and for the error limits footnote *a* of Table 3. [b] Estimates based on the following reasoning: The ionic radius of Ca^{2+} is only slightly smaller than the one of Pb^{2+}, and both metal ions have a very adaptable coordination sphere. Therefore one may expect that Ca^{2+} also bridges to some extent the two neighboring phosphate groups forming the 10-membered chelate. In the calculations, it is assumed that the connected stability enhancement (as kind of a lower limit) corresponds to that observed for Zn^{2+}, that is, $\log \Delta^*_{Ca/pUpU} = 0.13 \pm 0.08$ (cf. Table 3, lower part). The stability enhancement due to the formation of the N7-macrochelate with Ca^{2+} is assumed to be about one-half of that observed for Mg^{2+}: This assumption is based on the complexes formed with AMP^{2-}, IMP^{2-}, and GMP^{2-} (see Tables 7 and 9 in ref 11); hence, $\log \Delta_{Ca/N7} = (0.55 \pm 0.08)(1/2) = 0.28 \pm 0.08$. Therefore, one obtains overall $\log \Delta^*_{Ca/d(pGpG)} = (0.13 \pm 0.08) + (0.28 \pm 0.08) = 0.41 \pm 0.11$, and thus[18] $K_{l/tot} = 1.57$, with $K_{l/PO} = 0.35$ [from Zn(pUpU)],[27] and finally $K_{l/N7} = 1.22$ follows. Note, $K_{l/tot} = K_{l/N7} + K_{l/PO}$ (cf. ref 18) in accord with Figure 6.

TABLE 6. Comparison of Stability Enhancements and Formation Degrees of the Macrochelated Species Involving N7 for M[d(pGpG)]$^-$ and M(dGMP) Complexes[a]

M^{2+}	$\log \Delta^{**}_{M/d(pGpG)}$	% $M[d(pGpG)]^-_{cl/N7}$	$\log \Delta_{M/dGMP}$	% $M(dGMP)_{cl/N7}$
Mg^{2+}	0.55 ± 0.08	72 ± 5.5	0.23 ± 0.05	41 ± 7
Zn^{2+}	1.16 ± 0.09[b]	91 ± 2.5	0.84 ± 0.08	86 ± 3
Cd^{2+}	1.21 ± 0.09	94 ± 1.5	0.92 ± 0.11[c]	88 ± 3

[a] The values in columns 2 and 3 are from Tables 4 (column 5) and 5 (column 3). The M(dGMP) data are from ref 34 (aqueous solution, 25 °C, $I = 0.1$ M, $NaNO_3$). Regarding the error limits, see footnote *a* of Table 3. [b] This value is corrected for the interaction of Zn^{2+} with the neighboring phosphodiester bridge.[30] [c] Estimate from ref 30.

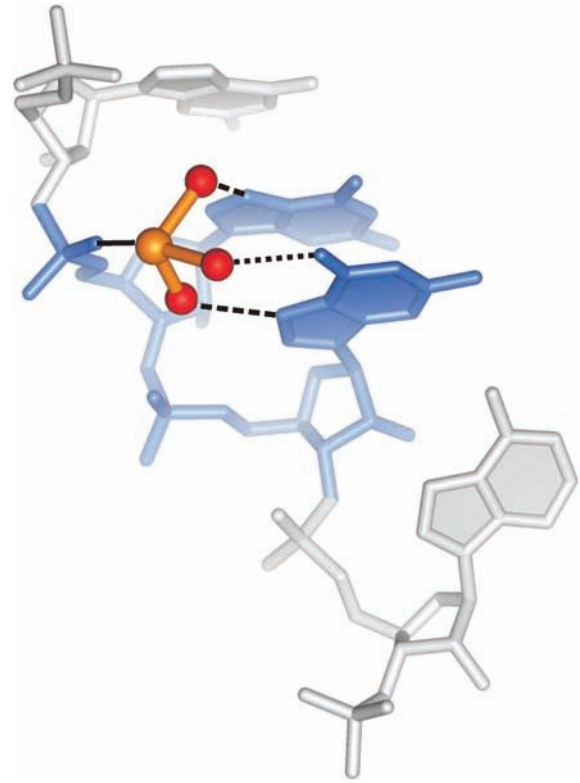


FIGURE 7. Three- and four-point interaction of a M^{2+} with two consecutive guanines as is known for Mg^{2+} in the ribosome.[35] Mg^{2+} is inner-sphere coordinated to a phosphodiester oxygen and five water molecules. Hydrogen bonding, that is, outer-sphere coordination, is observed to the two N7 atoms (broken lines), thus forming a three-point interaction. A third water molecule forms a hydrogen bond to (C6)O (dotted line) resulting in a four-point interaction (prepared with the MOLMOL[36] program and the PDB file 1S72).[35]

present (see also Figure 6, legend).[30,33] This is in accord with the data summarized in Table 6.[34]

The stability enhancements $\log \Delta^{**}_{M/d(pGpG)}$ of the M[d(pGpG)]$^-$ complexes are on average by about 0.3 log unit larger than the ones determined for M(dGMP), $\log \Delta_{M/dGMP}$ (Table 6, column 4). Since the possibility of intranucleotide macrochelate formation of the phosphate-coordinated M^{2+} with guanine-N7 (possibly involving (C6)O in an outersphere manner)[12] is identical in both complexes, the additional stability increase by 0.3 log unit of M[d(pGpG)]$^-$ means that a further interaction must occur with the neighboring nucleotide. Self-stacking of guanine residues is well-known (e.g., ref 10) and, thus, may also be expected in M[d(pGpG)]$^-$ as it occurs in GpG^- (see in ref 33). Clearly, this leads to an orientation of the $d(pGpG)^{3-}$ ligand, giving rise to a second N7, possibly even (C6)O, interaction (Figure 7).[35,36] Indeed, similar interactions are found with Mg^{2+} in an X-ray structure of a large ribosomal subunit.[35] In other words, an additional interaction of the M^{2+}, coordinated at the terminal phosphate group in M[d(pGpG)]$^-$, occurs involving in total three or even four sites.

To summarize, the stability enhancements in Table 6 (column 4) hold for a single intranucleotide macrochelate formation within M[d(pGpG)]$^-$. Instead, the larger values of column 2 apply for a three- or four-point interaction involving the neighboring GMP unit (see Figure 7). If chelate formation in nucleic acids is considered, in both instances the charge effect of 0.24 ± 0.04 needs to be added (Table 3, lower part). A so-called three- or four-point interaction thereby denotes a coordination pattern involving one phosphate and two N7 sites, as well as possibly one (C6)O as the fourth liganding atom, whether mediated by a water molecule or not.

The mentioned orientation of the guanines due to stacking within M[d(pGpG)]$^-$ is also responsible for the reduced stability of Pb[d(pGpG)]$^-$ compared with $Pb(pUpU)^-$ (Table 4, columns 5,6). In the latter case, the dinucleotide can freely rotate around the bridging phosphodiester and thus adjusts easily to the steric requirements for the formation of the

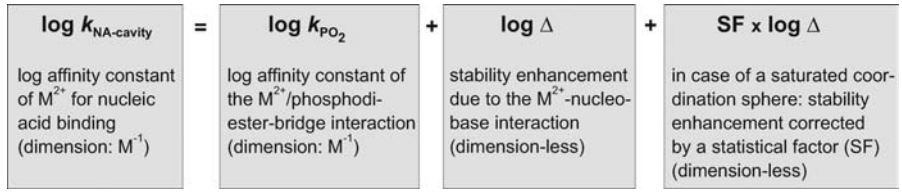


FIGURE 8. Empirical calculation of log stability constants for M^{2+}–nucleic acid interactions. If log Δ values (Table 4) are missing, one may tentatively apply the stability constants for M^{2+}–nucleobase interactions (Figure 3) but should note the change in dimension (see also ref 41).

10-membered chelate involving both neighboring phosphate units. With $d(pGpG)^{3-}$, the same kind of Pb^{2+} binding is inhibited because the intramolecular stack needs first to be "broken" or at least reduced to allow formation of the 10-membered chelate. This is a nice example how metal ion coordination may enforce a structural change in a nucleic acid.

6. Application of the Summarized Stability Increments to Nucleic Acids

In many nucleic acids, a metal ion is coordinated to a phosphodiester bridge (Figure 7), and we propose now that if one wants to consider, for example, Mg^{2+} binding to a phosphodiester unit and a nucleobase, one needs to add up the two increments of 1.05 for initial phosphate binding (Figure 3) and 0.79 for the nucleobase coordination (Table 4, column 4) resulting in a three- or four-point interaction, giving the intrinsic stability constant for a pGpG site of 1.8 log units. However, a hexacoordinating Mg^{2+} still has two to three further binding sites. For statistical reasons (and steric constraints or preorientations), we estimate that this additional stability increment only amounts to about 0.5 log units (i.e., about 3/5 from 0.79).[37] This gives an overall micro stability constant of 2.3 log units, which agrees perfectly with recent experimental data:[38] the different Mg^{2+}-binding sites present in branch-domain 6 of a group II intron ribozyme vary in their affinities between 2.14 ± 0.03 and 2.38 ± 0.06, possibly indicating that the coordination sphere of Mg^{2+} is not always completely filled by RNA-binding sites.

Similarly, the stability increments for Cd^{2+} would be 1.4 (phosphate unit; Figure 3) plus 1.45 (nucleobase; Table 4, column 4), giving a micro stability constant of about 2.85 log units for a three- or four-point interaction. If further coordination sites are used, another approximately 0.85 log units (i.e., about 3/5 from 1.45) may be added giving in total an intrinsic stability constant of about 3.7 log units for a fully coordinated Cd^{2+} in a nucleic acid. These values agree closely with recent experimental results obtained for a group II intron ribozyme:[39] the log stability constants for the binding of four Cd^{2+} ions to specific regions within domain 5 range from 2.80 ± 0.12 to 3.6 ± 0.2.[39]

An experimental value smaller than the sum of the increments considered here would indicate that not all coordination sites of M^{2+} are filled by sites of the nucleic acid; a larger value would indicate a preformed binding pocket.[40] In addition, (i) we estimate that the approximate error limit for the sum of the stability increments for a given binding pocket is about ±0.3 log unit, and (ii) less importantly, the values used are partly a combination of ribose and 2′-deoxyribose data because no other stability constants are available. However, we believe that this shortcoming is of relatively little influence and only on the order of about ±0.1 log unit (see Table 4 in ref 30).

The applied calculation procedure is summarized in Figure 8. Unfortunately, the known stability increments that need to be added to the stability constant due to the M^{2+}–phosphodiester interaction (Figure 3) are limited to Mg^{2+}, Zn^{2+}, and Cd^{2+} (Table 4, column 4). However, it may be noted that the stability enhancement, $\log \Delta_{Mg/d(pGpG)} = 0.79 \pm 0.07$, is very close to $\log k = 0.76 \pm 0.29$ estimated for the guanine-N7/Mg^{2+} interaction (Table 2, column 2, and Figure 3) (note, these two values have different dimensions). The values for Zn^{2+} also agree roughly within their error limits, that is, $\log \Delta_{Zn/d(pGpG)} = 1.41 \pm 0.08$ (Table 4, column 4) versus $\log k = 1.66 \pm 0.19$ (Table 2, column 2, and Figure 3). The agreement of the Cd^{2+} values is poorer but still not totally off, that is, $\log \Delta_{Cd/d(pGpG)} = 1.45 \pm 0.08$ (Table 4, column 4) versus $\log k = 2.04 \pm 0.17$ (Table 2 and Figure 3); hence, in the absence of $\log \Delta_{M/d(pGpG)}$ values, we recommend applying the affinity values of Figure 3.[41,42]

For example, for Mn^{2+} binding to a phosphodiester unit together with a three- or four-point interaction involving a GG unit, one obtains approximately 2.35 log units [1.3 (phosphate) + 1.05 (guanine); Figure 3] for the intrinsic micro stability constant and for a more saturated coordination sphere (plus 0.65; that is, 3/5 from 1.05) 3.0 log units. Should only a single macrochelate form, the value would be about 2.1 log

units (2.35 − 0.3; 0.3 log unit being the difference following from columns 2 versus 4 in Table 6).

7. Mg^{2+} Binding in the Ribosome. Some Statistical Considerations

The large ribosomal subunit of *Haloarcula morismortui* was analyzed at a resolution of 2.4 Å with respect to its metal ion binding sites.[35] This subunit comprises 3045 nucleotides and thus the same number of negative charges. In total, 88 Na^+/K^+ could be identified, and 116 Mg^{2+} were localized in the structure determination. This means that 320 negative charges are neutralized corresponding to about 10.5% of the phosphodiester bridge charges. The 116 Mg^{2+} identified represent only a small fraction (7.6%) of the divalent metal ions needed for charge compensation; that is, the overwhelming part of these are loosely bound and not fixed in a certain site. However, the relatively tightly bound cations are crucial for the structural stability and reactivity of the RNA.

Only two of the 116 localized Mg^{2+} in this RNA subunit are exclusively protein-bound, but 108 interact with the phosphates in accordance with the prediction of Figure 3. From the 116 Mg^{2+}, 24 form an inner-sphere 10-membered chelate with a neighboring phosphate bridge. This corresponds to about 22%, which is somewhat above the upper limit of 15% estimated for aqueous solution (section 4). However, in such a complicated RNA fold as the ribosome, hydrogen bonding and stacking between the building blocks add steric restraints and rigidity to the backbone, increasing the chance of Mg^{2+} binding to two (or more) phosphates, though the reason could also be a simple reduction of the intrinsic permittivity giving rise to a (slightly) enhanced phosphate affinity.[5,10] The basic affinity of these 24 Mg^{2+} to two neighboring phosphate units amounts to about log $k = 1.4$, as deduced from the above established increments [1.05 (phosphate, Figure 3) + 0.24 (charge, Table 3) + 0.1 (chelate)].

Interestingly, 48 Mg^{2+} are bound to at least one phosphate moiety and any one N7-guanine position. Out of these, 26 are contacts of an intranucleotide type. Based on the 108 phosphate-bound Mg^{2+}, this corresponds to a formation degree of 24% for macrochelates. This is close to the 31% ± 7% found[30] in solution for $Mg(GMP)_{Cl/N7}$. Again, the stability of such intramolecular macrochelates (two-point interaction) can now be estimated to be about log $k = 1.55$ [1.05 (phosphate, Figure 3) + 0.79 (Table 4) − 0.3 (terminating sentence of section 6)]. Any further interaction with the RNA will lead to an increased stability, as is expected for 17 Mg^{2+} exhibiting a three-point macrochelation giving the estimated affinity constant log $k = 1.85$ [1.05 (phosphate, Figure 3) + 0.79 (Table 4, column 4)].

There are also 25 intranucleotide macrochelates out of 28 phosphate/N7 contacts involving an adenine residue but none containing N3 of a cytosine residue. This is understandable because in the more stable[20] *anti* conformation, N3 of cytosine points away from a metal ion at the phosphodiester bridge.

The carbonyl oxygens of the pyrimidine nucleobases can only be considered as minor binding sites; no inner-sphere coordination of cytosine-O2 to Mg^{2+} could be detected, and only one to uracil-O2 and three to uracil-O4. Taking outer-sphere coordination into account, uracil-O4 remains the major binding site among the pyrimidine carbonyls. Twenty-five Mg^{2+} coordinate through a water molecule to uracil-O4, six to uracil-O2, and nine to cytosine-O2.

8. Conclusions and Outlook

In this Account, we have first summarized the available quantitative information regarding M^{2+} binding to nucleobase residues. Next, we estimated stability constants for M^{2+} coordination to the phosphodiester bridge, and by taking into account the simultaneous binding of a metal ion to various sites as can occur in dinucleotides, we developed an empirical concept that allows to estimate intrinsic (or micro) stability constants for "cavities" formed by nucleic acids.

Comparison with the few available experimental data allowed us to verify the concept, which will be helpful to predict stability constants for given microenvironments within large nucleic acids. However, the outlined concept will need adjustments and fine-tuning, for example, by extending the data in Tables 4 and 6 to additional metal ions by actually measuring the various M^{2+} affinities of phosphate diesters and by studying complex stabilities of dinucleotides like $pGpU^{3-}$, $pUpG^{3-}$, $pGpA^{3-}$, and $pApG^{3-}$. Yet from the examples, the following is evident: The concept of adding up stability increments works and provides the intrinsic (or micro) stability constant of a certain cavity in a nucleic acid with a reasonable accuracy. Molecular dynamics simulations may in the future also be of further help here.[43]

Overall, the concept allows conclusions about which metal ions preferably bind at which sites of a nucleic acid[44] and in which cases the coordination sphere of a metal ion bound to a nucleic acid is only partially saturated. Finally, the concept should be helpful in improving our understanding of the inter-

actions and equilibria between metal ions and single-stranded nucleic acids and, consequently, especially toward RNAs and ribozymes.

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Helmut Sigel, Emeritus Professor at the University of Basel (Department of Chemistry, Inorganic Chemistry), endowed with numerous honors, has longstanding interests in metal ion−nucleotide interactions. Both authors edited previously with Astrid Sigel the series *Metal Ions in Biological Systems*, and since 2006 they have been editing the *Metal Ions in Life Sciences* series, now published by the Royal Society of Chemistry (Cambridge, U.K.).

FOOTNOTES

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41 There is one more point: In the context of the M^{2+} affinity for gua-N7(O6) and PO_2^- (Figure 3), one might want to consider statistical effects because fewer sites become available than in a "free" M^{2+}; for example, 5/6 or 4/6 depending on the participation of O6. This would give with 5/6 (in accord with crystal structures; see ref 32) for Zn^{2+} $\log k = 1.38 \pm 0.19$ and for Cd^{2+} with 4/6 (see ref 32) $\log k = 1.36 \pm 0.17$, improving the agreement with the stability enhancement $\log \Delta$ considerably. Furthermore, the proposed adding of log stability increments is simply based on intuition (in contrast to the procedure based on Tables 4 and 6) because it actually means that the microconstants of the individual binding sites are multiplied with each other. This approach differs from the recently described quantification of the chelate effect.[42] However, the present "multiplication" appears to be justified because it compensates for the chelate effect; that is, once M^{2+} is coordinated to one site in a nucleic acid, other potential binding sites are spatially close, thus facilitating their entrance into the M^{2+} coordination sphere.

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