Complex Formation of Divalent Metal Ions with Uridine 5′-*O*-Thiomonophosphate or Methyl Thiophosphate: Comparison of Complex Stabilities with Those of the Parent Phosphate Ligands

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The stability constants of the 1:1 complexes formed in aqueous solution between Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} (M^{2+}) and methyl thiophosphate ($MeOPS^{2-}$) or uridine 5′-O-thiomonophosphate ($UMPS^{2-}$) ($PS^{2-} = MeOPS^{2-}$ or $UMPS^{2-}$) have been determined (potentiometric pH titrations; 25 °C; I = 0.1 M, $NaNO_3$). Comparison of these results for M(PS) complexes with those known for the parent M(PO) phosphate species, where $PO^{2-} = CH_3OPO_3^{2-}$ or UMP^{2-} (uridine 5′-monophosphate), shows that the alkaline earth metal ions, as well as Mn^{2+}, Co^{2+}, and Ni^{2+} have a higher affinity for phosphate groups than for their thio analogues. However, based on the linear $\log K^{M}_{M(R\text{-}PO_3)}$ versus $pK^{H}_{H(R\text{-}PO_3)}$ relationships ($R\text{-}PO_3^{2-}$ = simple phosphate monoester or phosphonate ligands with a non-interacting residue R) it becomes clear that the indicated observation is only the result of the lower basicity of the thiophosphate residue. In contrast, the thio complexes of Zn^{2+} and Cd^{2+} are more stable than their parent phosphate ones, and this despite the lower basicity of the PS^{2-} ligands. This stability increase is identical for M(MeOPS) and M(UMPS) species and amounts to about 0.6 and 2.4 log units for Zn(PS) and Cd(PS), respectively. Since no other binding site is available in $MeOPS^{2-}$, this enhanced stability has to be attributed to the S atom. Indeed, from the mentioned stability differences it follows that Cd^{2+} in Cd(PS) is coordinated by more than 99% to the thiophosphate S atom; the same value holds for Pb(PS), which was studied earlier. The formation degree of the S-bonded isomer amounts to 76 ± 6% for Zn(PS) and is close to zero for the corresponding Mg^{2+}, Ca^{2+}, and Mn^{2+} species. It is further shown that $Zn(MeOPS)(aq)^{2+}$ releases a proton from a coordinated water molecule with $pK_a \approx 6.9$; i.e., this deprotonation occurs at a lower pH value than that for the same reaction in $Zn(aq)^{2+}$. Since Mg^{2+}, Ca^{2+}, Mn^{2+}, and Cd^{2+} have a relatively low tendency for hydroxo complex formation, it was possible, for these M^{2+}, to also quantify the stability of the binuclear complexes, $M_2(UMPS - H)^+$, where one M^{2+} is thiophosphate-coordinated and the other is coordinated at $(N3)^-$ of the uracil residue. The impact of the results presented herein regarding M^{2+}/nucleic acid interactions, including those of ribozymes (rescue experiments), is briefly discussed.

KEYWORDS:

metal ion complexes · nucleotides · phosphorothioates · ribozymes · stability constants

1. Introduction

The use of structurally altered nucleotides as probes provides one way to study biological reactions which involve nucleotides. Indeed, all of the three units of a nucleotide, the base, the sugar, and the phosphate groups, have been modified (see citations in refs. [1 – 3]). At present we are interested in nucleotide analogues where a sulfur atom replaces a phosphate oxygen atom; such compounds were first synthesized in 1966[4] and they are known as thionucleotides[5] or nucleoside phosphorothioates.[4]

Initially these compounds were employed in studies regarding the mechanisms of enzymatic reactions,[6] and indeed, for this they are still in use.[7] However, more recently, with the development of the antisense strategy[8] and the observation that oligonucleotides with a sulfur modification at the phosphodiester linkage are usually more resistant toward nuclease degradation than natural oligonucleotides,[9, 10] phosphorothioate oligodeoxynucleotides became the most widely used compounds for sequence-specific inhibition of gene expression by the antisense strategy.[10] Several preclinical and clinical studies involving phosphorothioate compounds are currently under way[11, 12] and one of the products is already a commercially available drug.[12] Yet, there is a caveat here that apparently has been little appreciated so far: phosphorothioate analogues of oligonucleotides may undergo exonucleolytic degradation in inter- and intracellular media with the release of nucleoside 5′-*O*-thiomonophosphates, which give rise to biological effects in their own right.[9b, 13]

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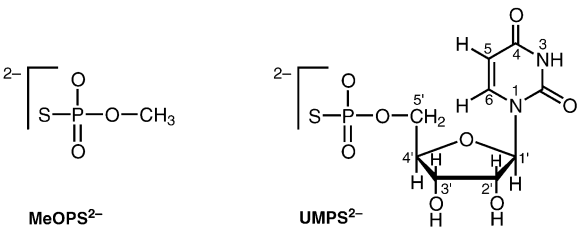
A further aspect which promotes the interest in thionucleotides is the increasing focus on RNA-catalyzed reactions.[14] Here, thio analogues are important tools for studying the role of metal ions in ribozyme folding and activity as well as in attempts to understand the mechanisms of ribozymic reactions[15] and to identify those phosphate oxygen atoms which are important for catalysis.[16] Considering that these studies are based on the different affinities of metal ions toward phosphate groups and their thio analogues, it is surprising to find that only very little information is available on the acid-base and the metal-ion-coordinating properties of these ligands.[17–19] So far only a few studies with thio derivatives of nucleotides have been reported, namely of adenosine 5′-mono-,[1, 20] di-, and triphosphate[5] and kinetically labile metal ions. In addition, very recently we completed a study[3] on the Pb(II)-binding properties of adenosine 5′-*O*-thiomonophosphate ($AMPS^{2-}$), uridine 5′-*O*-thiomonophosphate ($UMPS^{2-}$), and methyl thiophosphate ($MeOPS^{2-}$). Furthermore, the kinetically inert Pt(II) yields, in the form of chloro(diethylenetriamine)platinum(II) chloride, by reaction with AMPS exclusively a (phosphorothioate)platinum(II) complex.[21] In accord herewith, *cis*-$(NH_3)_2PtI_2$ forms with guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S) a macrochelate in which Pt(II) bridges the phosphorothioate S and guanine N7 atoms.[22] There is also a study[23] of *p*-nitrophenyl thiophosphate in which the effects of Mg^{2+} and Cd^{2+} on the rate of hydrolysis of this ester are compared.

In this work we report the stability constants for the 1:1 complexes formed in aqueous solution (25 °C; ionic strength $I =$ 0.1 M, $NaNO_3$) between the thiophosphate ligands (PS^{2-}) $UMPS^{2-}$ or $MeOPS^{2-}$ (Scheme 1)[24] and the divalent metal ions Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} (M^{2+}). The corresponding stability constants of the complexes formed with the parent phosphate ligands (PO^{2-}), i.e., uridine 5′-monophosphate (UMP^{2-})[25] and methyl phosphate ($CH_3OPO_3^{2-}$),[26] are known from previous studies. These results together allow a comparison between the stability of the M(PO) and M(PS) complexes and provide thus a better understanding of the changes which occur in the metal ion affinity in studies where natural nucleotides are replaced by nucleoside phosphorothioates.



Scheme 1. *Chemical structures of methyl thiophosphate ($MeOPS^{2-}$) and uridine 5′-O-thiomonophosphate ($UMPS^{2-}$) in its* anti *conformation.*[24]

2. Results and Discussion

2.1. Definition of the considered equilibria

$UMPS^{2-}$ and $MeOPS^{2-}$ (PS^{2-} ligands; see Scheme 1) can accept two protons at the thiophosphate group; hence, the following two deprotonation Equilibria (1 a) and (2 a) need to be considered:

$$H_2(PS) \rightleftharpoons H(PS)^- + H^+ \quad (1\,a)$$

$$K^H_{H_2(PS)} = [H(PS)^-][H^+]/[H_2(PS)] \quad (1b)$$

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

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

$UMPS^{2-}$ can be further deprotonated at its (N3)H site according to Equilibrium (3a):

$$UMPS^{2-} \rightleftharpoons (UMPS - H)^{3-} + H^+ \quad (3a)$$

$$K^H_{UMPS} = [(UMPS - H)^{3-}][H^+]/[UMPS^{2-}] \quad (3b)$$

The analogous equilibria also hold for the parent compounds of $UMPS^{2-}$ and $MeOPS^{2-}$, i.e., for UMP^{2-} and $CH_3OPO_3^{2-}$. The acidity constants for all four mentioned ligands are summarized in Table 1; these values were determined by potentiometric pH titrations and are taken from our earlier work.[3, 25, 26] However, the values for $pK^H_{H(MeOPS)}$, $pK^H_{H(UMPS)}$, and pK^H_{UMPS} have been confirmed in the study described herein, where constants identical within the error limits with those given in Table 1 were obtained. The acid-base properties of these species,[3] including those of AMPS,[3, 20] and the location of the proton(s), which is in the $-OP(S)(O)_2H^-$ residue at one of the terminal oxygen atoms ($-OP(S)(O)(OH)^-$) have previously been discussed. In the $-OP(S)(O)_2^{2-}$ moiety one of the two negative charges is located at the sulfur atom.[20, 27] The main conclusion from Table 1, of relevance in the context of this study, is the observation that $H(PS)^-$ is by a ΔpK_a value of 1.4 more acidic than $H(PO)^-$ (Table 1, Column 3), and also that the acidities of the (N3)H sites in $UMPS^{2-}$ and UMP^{2-} are identical within the error limits, which is in accord[3] with expectations.

Table 1. *Negative logarithms of the acidity constants of H_2(MeOPS), H_2(UMPS), and their parent acids [Eqs. (1) – (3)] as determined by potentiometric pH titrations in aqueous solution (25 °C; I = 0.1 M, $NaNO_3$).*[a]

Acid	$pK^H_{H_2(PS/PO)}$	$pK^H_{H(PS/PO)}$	$pK^H_{UMPS/UMP}$	Ref.
H_2(MeOPS)	0.62 ± 0.09	4.96 ± 0.02		[3]
$CH_3OP(O)(OH)_2$	1.1 ± 0.2	6.36 ± 0.01		[26]
H_2(UMPS)		4.78 ± 0.02	9.47 ± 0.02	[3]
H_2(UMP)	0.7 ± 0.3	6.15 ± 0.01	9.45 ± 0.02	[25]

[a] The error limits given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger.

The stability constants of the M(PS) complexes were also determined by potentiometric pH titrations by working with an excess of M^{2+} compared to the concentration of PS. The experimental data obtained under these conditions (25 °C; $I = 0.1$ M, $NaNO_3$) can be completely explained

in the pH range above 3 by taking into account Equilibria (2a) and (4a),

$$M^{2+} + PS^{2-} \rightleftharpoons M(PS) \quad (4a)$$

$$K^{M}_{M(PS)} = [M(PS)]/([M^{2+}][PS^{2-}]) \quad (4b)$$

provided the evaluation is not carried into the pH range where deprotonation of $M(aq)^{2+}$ or the (N3)H site of UMPS occurs (see Section 4.3).

With the Zn^{2+}/MeOPS system an interesting observation was made: in this system an additional proton is released before the onset of the deprotonation in the $Zn(aq)^{2+}$ system. This means Zn(MeOPS) forms a hydroxo complex according to Equilibrium (5a):

$$Zn(MeOPS)(aq) \rightleftharpoons Zn(MeOPS)(OH)^{-} + H^{+} \quad (5a)$$

$$K^{H}_{Zn(MeOPS)(aq)} = [Zn(MeOPS)(OH)^{-}][H^{+}]/[Zn(MeOPS)(aq)] \quad (5b)$$

In the potentiometric pH titrations of the UMPS systems containing metal ions like Mg^{2+} or Cd^{2+}, which have a relatively low tendency for the formation of hydroxo complexes, the pH range could be reached where deprotonation of the (N3)H unit occurs and this then gives rise to Equilibrium (6a):

$$M(UMPS) \rightleftharpoons M(UMPS-H)^{-} + H^{+} \quad (6a)$$

$$K^{H}_{M(UMPS)} = [M(UMPS-H)^{-}][H^{+}]/[M(UMPS)] \quad (6b)$$

The formation of the $M(UMPS-H)^{-}$ species, where the metal ion is located at the thiophosphate group and where the $(N3)^{-}$ site carries a negative charge, allows also the formation of 2:1 complexes by the involvement of this site because the experiments are done with an excess of M^{2+} over UMPS, and this leads to Equilibrium (7a):

$$M^{2+} + M(UMPS-H)^{-} \rightleftharpoons M_2(UMPS-H)^{+} \quad (7a)$$

$$K^{M}_{M_2(UMPS-H)} = [M_2(UMPS-H)^{+}]/([M^{2+}][M(UMPS-H)^{-}]) \quad (7b)$$

Since Equilibria (6a) and (7a) are coupled as far as the release of the proton is concerned, the relevant constants cannot be determined independently.[28] Fortunately, based on previous experience[29–31] an estimate for Equation (6b) can be made, i.e., $pK^{H}_{M(UMPS)} = 9.02 \pm 0.15$ (see Section 4.3). This value should be independent of the kind of metal ion coordinated to the thiophosphate group since here simply a charge neutralization occurs.[29] Keeping the mentioned value fixed in the evaluation process allows calculation of the constants for Equilibrium (7a). That this procedure is justified is proven in Section 2.6 by comparisons with the stability constants of $M(Urd-H)^{+}$ complexes, where Urd = uridine.

2.2. Stabilities of M(PS) complexes

The results obtained for the stability constants of the M(UMPS) and M(MeOPS) complexes according to Equilibrium (4) for the metal ions Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} are listed in Table 2, including our very recent results[3] regarding Pb(MeOPS) and Pb(UMPS). The Cu^{2+}/PS systems were not studied because from an earlier investigation of the Cu^{2+}/AMPS system it is known[1] that after mixing the reactants the solution turns slightly milky, which indicates the formation of a precipitate possibly involving a redox reaction. The stability constants of the various M(PO) parent complexes, known from previous studies,[25, 26] are given in Columns 3 and 5 of Table 2. Hence, these data permit a direct comparison of the absolute stabilities of the phosphate complexes with those of their thio analogues.

Table 2. *Logarithms of the stability constants of M(UMPS) and M(MeOPS) complexes [Eq. (4)]. For comparison the constants of the corresponding parent species, M(UMP) and $M(CH_3OPO_3)$, are also listed.*[a]

M^{2+}	$\log K^{M}_{M(UMPS)}$	$\log K^{M}_{M(UMP)}$	$\log K^{M}_{M(MeOPS)}$	$\log K^{M}_{M(CH_3OPO_3)}$
Mg^{2+}	1.24 ± 0.05	1.56 ± 0.02	1.33 ± 0.07	1.67 ± 0.05
Ca^{2+}	1.19 ± 0.10	1.44 ± 0.05	1.25 ± 0.06	1.49 ± 0.02
Sr^{2+}	1.04 ± 0.10	1.25 ± 0.04	1.10 ± 0.11	1.25 ± 0.04
Ba^{2+}	1.04 ± 0.07	1.13 ± 0.06	1.11 ± 0.06	1.23 ± 0.03
Mn^{2+}	1.82 ± 0.10	2.11 ± 0.02	1.82 ± 0.08	2.20 ± 0.02
Co^{2+}	1.59 ± 0.05	1.87 ± 0.05	1.69 ± 0.06	1.99 ± 0.03
Ni^{2+}	1.54 ± 0.08	1.97 ± 0.05	1.62 ± 0.05	1.94 ± 0.04
Cu^{2+}		2.77 ± 0.06		2.94 ± 0.03
Zn^{2+}	2.21 ± 0.06	2.02 ± 0.07	2.34 ± 0.05	2.22 ± 0.03
Cd^{2+}	4.37 ± 0.08	2.38 ± 0.04	4.50 ± 0.06	2.52 ± 0.03
Pb^{2+}	4.63 ± 0.03	2.80 ± 0.04	4.78 ± 0.06	2.98 ± 0.10

[a] All constants were determined by potentiometric pH titrations and refer to aqueous solution (25 °C; $I = 0.1$ M, $NaNO_3$). For the error limits, see footnote [a] of Table 1. The stability constants for the M(UMP) and $M(CH_3OPO_3)$ complexes are from Refs. [25] and [26], respectively. The constant for Pb(UMP) is from ref. [32] and those for $Pb(CH_3OPO_3)$, Pb(UMPS) and Pb(MeOPS) are from ref. [3].

It is interesting to note from the data in Table 2 that all of the phosphate (PO^{2-}) complexes of Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} are between 0.1 and 0.4 log units more stable than those of their thiophosphate (PS^{2-}) analogues. However, for Zn^{2+} and especially for Cd^{2+}, as well as Pb^{2+}, the thiophosphate complexes are significantly more stable than the phosphate ones. Pb(PS) is about 1.8 log units more stable than Pb(PO) and Cd(PS) about 2 log units more stable than Cd(PO).

At this point it is important to recall that the basicity of a thiophosphate group is considerably lower than that of a phosphate group (Table 1, Column 3). Furthermore, it is well known that the stability of phosphate complexes depends on the basicity of the corresponding phosphate groups.[25] Indeed, for a series of phosph(on)ate ligands and several metal ions straight-line correlations have been established[31] for plots of $\log K^{M}_{M(R\text{-}PO_3)}$ versus $pK^{H}_{H(R\text{-}PO_3)}$, where $R\text{-}PO_3^{2-}$ represents phosphate monoesters[25] and phosphonate[31] ligands in which R is unable to interact with the metal ion [Eq. (8)]:

$$\log K^{M}_{M(R\text{-}PO_3)} = m \cdot pK^{H}_{H(R\text{-}PO_3)} + b \quad (8)$$

The corresponding straight-line parameters, i.e., the values for the slopes *m* and the intercepts *b*, for the Pb(R-PO_3) complexes are given in ref. [32];[3] those for the other M(R-PO_3) complexes are listed in ref. [31].[33–35]

Plots of $\log K^{M}_{M(R\text{-}PO_3)}$ versus $pK^{H}_{H(R\text{-}PO_3)}$ according to Equation (8) are shown in Figure 1 for the 1:1 complexes of Ba^{2+}, Mg^{2+}, Zn^{2+}, and Cd^{2+} with eight simple R-PO_3^{2-} ligands (which include UMP^{2-}; crossed points) allowing only a phosph(on)ate–M^{2+} coordination. The solid points in the figure refer to the M^{2+} complexes of $MeOPS^{2-}$ and $UMPS^{2-}$. The data points for the Cd(PS) and Zn(PS) complexes are far above their reference line, which proves an increased stability for these thiophosphate systems, whereas the points for the Ba(PS) and Mg(PS) complexes fit on their reference lines. This observation can be evaluated quantitatively by calculating, with the straight-line Equation (8) and the $pK^{H}_{H(PS)}$ values (Table 1, Column 3), the expected (calcd) stabilities for the M(PS) complexes assuming that these stabilities correspond to a phosphate–M^{2+} coordination. The corresponding results are listed in the fourth column of Table 3 (see below).

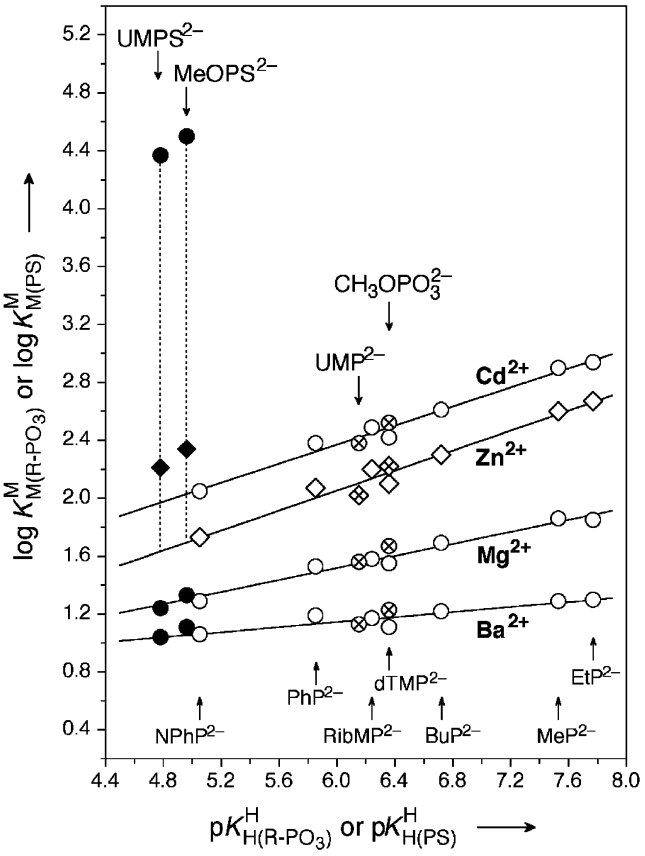


Figure 1. *Evidence for enhanced stability of the M(PS) complexes (PS^{2-} = $UMPS^{2-}$ or $MeOPS^{2-}$) of Zn^{2+} (◆) and Cd^{2+} (●) and for the lack of increased or decreased stability of the Mg(PS) and Ba(PS) (●) complexes as well as for all the corresponding M(PO) complexes (PO^{2-} = UMP^{2-} or $CH_3OPO_3^{2-}$; ⊗, ⊗). This evidence is based on $pK^{H}_{H(PS)}$ and $\log K^{M}_{M(PS)}$ values as well as on the linear relationship between $\log K^{M}_{M(R\text{-}PO_3)}$ and $pK^{H}_{H(R\text{-}PO_3)}$ established previously[31, 33–35] for the 1:1 complexes of the eight simple phosphate monoester or phosphonate ligands (R-PO_3^{2-}; ○): 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 ($dTMP^{2-}$), nbutyl phosphate (BuP^{2-}), methanephosphonate (MeP^{2-}), and ethanephosphonate (EtP^{2-}) (from left to right). The least-squares lines are drawn through the corresponding eight data sets (○) [including UMP^{2-} (⊗, ⊗)], which are taken from refs. [25] and [31]. The data points for the $UMPS^{2-}$, $MeOPS^{2-}$, UMP^{2-}, and $CH_3OPO_3^{2-}$ systems are based on the constants given in Tables 1 and 2. The vertical broken lines emphasize the stability differences to the corresponding reference lines; they are equal to $\log \Delta_{M/PS}$ [Eq. (9)], the values of which are listed in Column 5 of Table 3. All the plotted equilibrium constants refer to aqueous solutions at 25 °C and I = 0.1 M ($NaNO_3$).*

2.3. Further evaluations of the M(PS) complex stabilities

There are two more observations to be taken from Figure 1 that warrant emphasis: 1) The data points for the Zn^{2+}, Cd^{2+}/$CH_3OPO_3^{2-}$ and Zn^{2+}, Cd^{2+}/UMP^{2-} systems (crossed points) fit on the reference lines, which proves that the M(CH_3OPO_3) and M(UMP) species with Zn^{2+} and Cd^{2+} have the properties of simple phosphate monoester complexes. Consequently, this result means that the uridine moiety has no influence and that the increased stabilities of the corresponding M(MeOPS) and M(UMPS) complexes must be due to an interaction between Zn^{2+} or Cd^{2+} and the S atom of the thiophosphate residue. 2) The data given for the Ba^{2+} and Mg^{2+} systems demonstrate that the affinity of these alkaline earth ions toward sulfur is small; the data points with the thiophosphate ligands fit on the reference lines! This result also clearly shows that the lower stabilities observed for the M(PS) complexes (solid points) of Mg^{2+} and Ba^{2+} in comparison with those for the M(PO) complexes (crossed points) is only due to the lower basicity of the thiophosphate ligands. In other words, $UMPS^{2-}$ and $MeOPS^{2-}$ behave toward these metal ions in the same way as simple phosphate ligands.

The last of the above-mentioned results is interesting because it is well known that, for example, Mg^{2+} has a lower affinity for sulfur than for oxygen atoms, and in a thiophosphate residue Mg^{2+} has only two possibilities to interact with an O atom as compared to the three possibilities in phosphates; hence, based on this statistical consideration, a stability *decrease* of 0.18 log unit is expected.[1] However, taking into account that also metal ions, just as the proton (see Section 2.1), may change the charge distribution, the lower electronegativity of sulfur compared to that of oxygen should cause in the complexes a higher charge density on the two oxygen atoms of PS^{2-} than on the three oxygen atoms of PO^{2-}.[1] Consequently, the oxygen atoms in PS^{2-} appear as being favored for Mg^{2+} binding, and therefore, an increase in stability is expected. The result seen in Figure 1 indicates that the two mentioned opposite effects evidently cancel each other. Indeed, in a recent ab initio study[36] of the interactions between phosphorothioate or phosphorodithioate and the, also "hard", Na^{+} ion it is concluded that a large destabilizing effect occurs only with the second sulfur substitution.

The described fact that Mg^{2+} and Ba^{2+} ions behave alike in their binding properties toward thiophosphates and phosphate groups is encouraging because it suggests that the stability data obtained for the other M(PS) complexes can be evaluated on the basis of their $\log K^{M}_{M(R\text{-}PO_3)}$ versus $pK^{H}_{H(P\text{-}PO_3)}$ reference lines; the resulting values are listed in Column 4 of Table 3 (see also the final paragraph of Section 2.2). Comparison of the results given in Columns 3 and 4 of Table 3 for the M(PS) complexes of Zn^{2+},

Table 3. *Stability constant comparison for the M(PS) complexes between the measured stability constants (exptl; Table 2, Columns 2 and 4) and the calculated ones (calcd), together with the stability differences $\log \Delta_{M(PS)}$ as defined by Equation (9).*[a]

PS^{2-}	M^{2+}	$\log K^{M}_{M(PS)exptl}$	$\log K^{M}_{M(PS)calcd}$	$\log \Delta_{M/PS}$
$UMPS^{2-}$	Mg^{2+}	1.24 ± 0.05	1.27 ± 0.03	-0.03 ± 0.06
	Ca^{2+}	1.19 ± 0.10	1.26 ± 0.05	-0.07 ± 0.11
	Sr^{2+}	1.04 ± 0.10	1.12 ± 0.04	-0.08 ± 0.11
	Ba^{2+}	1.04 ± 0.07	1.04 ± 0.04	0.00 ± 0.08
	Mn^{2+}	1.82 ± 0.10	1.82 ± 0.05	0.00 ± 0.11
	Co^{2+}	1.59 ± 0.05	1.62 ± 0.06	-0.03 ± 0.08
	Ni^{2+}	1.54 ± 0.08	1.59 ± 0.05	-0.05 ± 0.09
	Zn^{2+}	2.21 ± 0.06	1.63 ± 0.06	0.58 ± 0.08
	Cd^{2+}	4.37 ± 0.08	1.97 ± 0.05	2.40 ± 0.09
	Pb^{2+}	4.63 ± 0.03	2.23 ± 0.08	2.40 ± 0.09
$MeOPS^{2-}$	Mg^{2+}	1.33 ± 0.07	1.30 ± 0.03	0.03 ± 0.08
	Ca^{2+}	1.25 ± 0.06	1.29 ± 0.05	-0.04 ± 0.08
	Sr^{2+}	1.10 ± 0.11	1.14 ± 0.04	-0.04 ± 0.12
	Ba^{2+}	1.11 ± 0.06	1.05 ± 0.04	0.06 ± 0.07
	Mn^{2+}	1.82 ± 0.08	1.86 ± 0.05	-0.04 ± 0.09
	Co^{2+}	1.69 ± 0.06	1.66 ± 0.06	0.03 ± 0.08
	Ni^{2+}	1.62 ± 0.05	1.64 ± 0.05	-0.02 ± 0.07
	Zn^{2+}	2.34 ± 0.05	1.69 ± 0.06	0.65 ± 0.08
	Cd^{2+}	4.50 ± 0.06	2.03 ± 0.05	2.47 ± 0.08
	Pb^{2+}	4.78 ± 0.06	2.32 ± 0.08	2.46 ± 0.10

[a] The calculated stability constants are based on the basicity of the thiophosphate group in $UMPS^{2-}$ and $MeOPS^{2-}$ ($pK^{H}_{H(PS)}$; Table 1, Column 3) and the reference-line equations established previously[31] (see Eq. (8) and Figure 1) (aqueous solution; 25 °C; $I = 0.1$ M, $NaNO_3$). For the error limits, see footnote [a] of Table 1; the error limits of the derived data, in the present case for $\log \Delta_{M/PS}$, were calculated according to the Gaussian error propagation. The entries for the two Pb^{2+}/PS systems are from ref. [3].

Cd^{2+}, and Pb^{2+} confirms the already mentioned increased stabilities compared to those of the corresponding M(PO) complexes, and this despite the lower basicity of the former PS^{2-} ligands (see also Figure 1). This increase in stability can now be quantified by forming the difference between the experimentally determined (exptl) and the calculated (calcd) stability constants, as is expressed in Equation (9):

$$\log \Delta_{M/PS} = \log K^{M}_{M(PS)exptl} - \log K^{M}_{M(PS)calcd} \quad (9)$$

The values for $\log \Delta_{M/PS}$ are listed in the fifth column of Table 3 and it may be noted that these values correspond to the vertical differences (dotted lines) seen for the examples given in Figure 1 between the solid data points (Zn^{2+}, Cd^{2+}) and their reference lines.

From the data listed in Column 5 of Table 3, it follows that for the M(PS) complexes of all alkaline earth metal ions as well as for those of Mn^{2+}, Co^{2+}, and Ni^{2+}, the increase in stability is zero within the error limits, i.e., these M(PS) complexes behave like simple phosphate species despite their thiophosphate group. However, the Zn(PS), Cd(PS), and Pb(PS) complexes show stabilities which are beyond those expected on the basis of the basicity of the thiophosphate group; since the M(UMPS) and M(MeOPS) complexes behave alike, this proves that the sulfur of the thiophosphate residue participates in these instances in metal ion binding and is responsible for the enhanced stability.

The observation based on the data in Table 3 (Column 5) that the values of $\log \Delta_{M/PS}$ for the M(UMPS) and M(MeOPS) systems are identical within the error limits is important because it allows us to calculate the stability constants for other M(PS) complexes of PS^{2-} ligands for which the acidity constants of the monoprotonated thiophosphate group are known. Evidently, for M(PS) complexes of the alkaline earth metal ions as well as for those of Mn^{2+}, Co^{2+}, or Ni^{2+} the straight-line parameters [Eq. (8)][31–35] can directly be used to calculate the corresponding stability constants since these metal ions behave equally toward thiophosphate and phosphate groups. In the cases of Zn^{2+}, Cd^{2+}, and Pb^{2+} the same calculation procedure may be used but in these instances the $\log \Delta_{M/PS/av}$ values given below in Equations (10) – (12) need to be added to obtain the expected stabilities of the M(PS) complexes.

$$\log \Delta_{Zn/PS/av} = 0.62 \pm 0.11 \quad (10)$$

$$\log \Delta_{Cd/PS/av} = 2.44 \pm 0.11 \quad (11)$$

$$\log \Delta_{Pb/PS/av} = 2.43 \pm 0.09 \quad (12)$$

The mentioned $\log \Delta_{M/PS/av}$ values are the averages of the stability increases observed for the M(UMPS) and M(MeOPS) complexes.

As mentioned in Section 2.2, values for the stability constants of Cu(PS) complexes were not determined, however, the increase in stability for such complexes has been estimated in a previous study:[1] $\log \Delta_{Cu/PS} = 6 \pm 2$. In the same work[1] an estimation for $\log \Delta_{Pb/PS}$ was also derived in a complicated procedure; the result, $\log \Delta_{Pb/PS} = 2.35 \pm 0.29$, is in excellent agreement with the value that follows from the results listed in Table 3, $\log \Delta_{Pb/PS/av} = 2.43 \pm 0.09$ [see also Eq. (12)].

2.4. Extent of sulfur – metal-ion binding in the M(PS) complexes

From the evaluations in Section 2.3 it has become clear that the sulfur atom of the thiophosphate group in PS^{2-} may participate in metal ion binding. Therefore, the question arises: What is the formation degree of the sulfur-coordinated species?

As indicated before, the stability differences $\log \Delta_{M/PS}$ (Table 3, Column 5) solely quantify the stability increase, if any, due to the participation of the sulfur atom in metal ion binding. If we define the sulfur-coordinated species as (PS·M) and the oxygen-bound ones as (PO·M), we may further define the intramolecular equilibrium constant $K_{I/S}$ or the ratio R as given in Equation (13a). The remaining terms in Equation (13) may be derived in analogy to other intramolecular equilibria (see ref. [1]):

$$R = K_{I/S} = \frac{[(PS \cdot M)]}{[(PO \cdot M)]} \quad (13a)$$

$$= \frac{K^{M}_{M(PS)exptl}}{K^{M}_{M(PS)calcd}} - 1 = 10^{\log \Delta_{M/PS}} - 1 \quad (13b)$$

Of course, the percentage of the sulfur-bound isomer follows from Equation (14):

$$\%(PS \cdot M) = 100 \cdot R/(1 + R) \quad (14)$$

Since the values for $\log \Delta_{M/UMPS}$ and $\log \Delta_{M/MeOPS}$ listed in Column 5 of Table 3 are so similar, we calculated their averages, which we consider to be the best representatives for M(PS) complexes. These values are given in Column 2 of Table 4. It may be mentioned that most of these values are identical, within the error limits, with those derived earlier[1] from M^{2+}/AMPS systems. Application of the $\log \Delta_{M/PS/av}$ values to Equation (13) provides the *R* values listed in the third column of Table 4, and from these follow, according to Equation (14), the percentages for the (PS·M) isomers.

Table 4. *Average values of the stability increase, $\log \Delta_{M/PS/av}$ observed for the M(UMPS) and M(MeOPS) complexes and attributed to a sulfur–metal-ion coordination in the M(PS) species, together with the extent of this interaction as quantified by* R *[Eq. (13)] and the percentage [Eq. (14)] of the sulfur-coordinated species, (PS·M), for aqueous solutions at 25 °C and I = 0.1 M ($NaNO_3$).*[a]

M(PS)	$\log \Delta_{M/PS/av}$[b]	*R*	% (PS·M)
Mg(PS)	0.00 ± 0.09	0.00 ± 0.21	0 (≤ 21)
Ca(PS)	− 0.06 ± 0.05	≈ 0	≈ 0
Sr(PS)	− 0.06 ± 0.06	≈ 0	≈ 0
Ba(PS)	0.03 ± 0.09	≈ 0 (0.07 ± 0.22)	≈ 0 (7 ± 19)
Mn(PS)	− 0.02 ± 0.06	≈ 0	≈ 0 (≤ 9)
Co(PS)	0.00 ± 0.09	0.00 ± 0.21	0 (≤ 21)
Ni(PS)	− 0.04 ± 0.05	≈ 0	≈ 0 (≤ 2)
Zn(PS)	0.62 ± 0.11	3.17 ± 1.06	76 ± 6
Cd(PS)	2.44 ± 0.11	274 ± 70	99.6 ± 0.1
Pb(PS)	2.43 ± 0.09	268 ± 56	99.6 ± 0.1

[a] For the error limits, see footnote [a] in Table 3. [b] These values are the averages of the $\log \Delta_{M/PS}$ values given in Column 5 of Table 3 for the M(UMPS) and M(MeOPS) systems.

From Column 4 in Table 4 it is evident that in Cd(PS) and Pb(PS) the metal ions are nearly completely S-coordinated to the thiophosphate residue. In Zn(PS) about three quarters of all species are also sulfur-coordinated. This contrasts with the other M(PS) complexes of the remaining metal ions listed in Table 4, where overwhelmingly oxygen coordination to the thiophosphate group occurs.

2.5. Some comments on the formation of the $Zn(MeOPS)(OH)^-$ species

As indicated already in Section 2.1, it was surprising to observe that Zn(MeOPS) forms a hydroxo complex, i.e., $Zn(MeOPS)(OH)^-$, in a pH range where $Zn(aq)^{2+}$ itself does not yet form a hydroxo species. Consequently, according to Equilibrium (5a), the following acidity constant was measured (Section 4.3):

$$pK^{H}_{Zn(MeOPS)(aq)} = 6.9 \pm 0.2 \quad (15)$$

The result is in excellent agreement with an analogous observation made for the Zn^{2+}/AMPS system; in this case $pK^{H}_{Zn(AMPS)(aq)} = 6.8 \pm 0.4$ was determined.[1] This result confirms the earlier conclusion[1] regarding lowering of the pK_a value of a Zn^{2+}-bound water molecule in a complex containing a sulfur-binding site in its coordination sphere.

It has previously been concluded[37] that a reduction of the coordination number from 6 to 4 makes water bound to Zn^{2+} considerably more acidic; hence, such a reduction of the coordination number may also be assumed for the formation of the $Zn(MeOPS)(OH)^-$ species. The formation of fourfold coordinated Zn^{2+} is apparently driven[37] by the Lewis basicity of the donor atoms: If the coordinating ligand is a strong Lewis base, the coordination number of Zn^{2+} drops and the bond length shortens, i.e., the metal–ligand bond becomes more of the covalent type. This hypothesis would explain why an S donor promotes the formation of a fourfold-coordinated Zn^{2+} more effectively than an O donor. A possible structure for the $Zn(MeOPS)(OH)^-$ complex considered herein could therefore consist of an innersphere coordination of OH^- and the S atom of the thiophosphate group to Zn^{2+}, as well as of an outersphere interaction to an O atom of the thiophosphate group via a Zn^{2+}-bound water molecule, thus giving rise to the formation of a six-membered "semi-chelate",[25] the remaining fourth position at the Zn^{2+} being occupied by water.

In any case, the above example regarding the lowering of the pK_a value for a $Zn(aq)^{2+}$ complex containing also a sulfur-binding site is certainly meaningful for biological systems, since the deprotonation reaction occurs in the physiological pH range also relevant for catalytically active Zn(II).

2.6. Formation of binuclear $M_2(UMPS - H)^+$ complexes

As indicated already in Section 2.1, with those metal ions which have a low tendency to form hydroxo complexes it was possible in the potentiometric pH titrations to reach the pH range where deprotonation of the (N3)H site in the M(UMPS) species begins. Clearly, the availability of a negatively charged $(N3)^-$ unit allows then formation of binuclear complexes, $M_2(UMPS - H)^+$, according to Equilibrium (7a), where one M^{2+} is at the thiophosphate residue and the other at the $(N3)^-$ site. The stability constants of these complexes were calculated as indicated in Section 2.1 (see also Section 4.3) and the results are listed in the second column of Table 5; the relatively large errors of these constants result mainly from the error connected with $pK^{H}_{M(UMPS)} = 9.02 \pm 0.15$ (Sections 2.1 and 4.3).

The determination of the stability constants $K^{M}_{M_2(UMPS-H)}$ was handicapped by the need of a value for $pK^{H}_{M(UMPS)}$ [Eq. (7)], which

Table 5. *Comparison of the logarithms of the stability constants of the binuclear $M_2(UMPS - H)^+$ complexes [Eq. (7)] with those of the $M(Urd - H)^+$ species.*[a]

M^{2+}	$\log K^{M}_{M_2(UMPS-H)}$	$\log K^{M}_{M(Urd-H)}$
Mg^{2+}	0.80 ± 0.16	0.68 ± 0.08
Ca^{2+}	1.0 ± 0.3	0.78 ± 0.11
Mn^{2+}	1.40 ± 0.22	1.36 ± 0.05
Zn^{2+}	-	2.41 ± 0.14
Cd^{2+}	3.22 ± 0.20	3.15 ± 0.04

[a] The stability constants of the $M(Urd - H)^+$ species were taken from ref. [38]. All constants were determined by potentiometric pH titrations and refer to aqueous solution at 25 °C and $I = 0.1$ M ($NaNO_3$). For the error limits, see footnote [a] in Table 3.

could only indirectly be obtained (Sections 2.1 and 4.3). It was therefore of interest to validate the applied evaluation procedure in an independent manner. Since M^{2+} coordination at the thiophosphate group leads to charge neutralization, one obtains a situation that may be mimicked by the nucleoside uridine (Urd).

Indeed, the acidity constant for (N3)H deprotonation of uridine, $pK^{H}_{Urd} = 9.18 \pm 0.02$[38] is close to the mentioned $pK^{H}_{M(UMPS)} = 9.02 \pm 0.15$, which is due to M(UMPS) deprotonation. Moreover, since the complexes $M_2(UMPS-H)^+$ and $M(Urd)^+$ also carry the same charge, their stability should also be similar. The stability constants of the $M(Urd)^+$ complexes were recently measured[38] and these results are listed in the third column of Table 5. It is comforting to observe that the stability constants for the two types of complexes are, for a given metal ion, identical within the error limits. This result then confirms the appropriateness of our evaluation procedure and, more important, it also proves that metal-ion binding at the $(N3)^-$ site of the uracil residue is possible.

3. General Conclusions

As far as the metal ions important in biological systems are concerned, the stability constants determined in this study show that Mg^{2+} and Ca^{2+} ions coordinate to the thiophosphate group of a PS^{2-} ligand in a phosphate-type manner (Figure 1). This fact means that the presence of the sulfur atom does not significantly affect the kind of binding of these alkaline earth ions, except that the stabilities of their M(PS) complexes are somewhat smaller, if compared with those of the parent M(PO) complexes (Table 2), but this is simply the result of the reduced basicity of the thiophosphate group compared to that of the phosphate one (Table 1).

On the contrary, metal ions like Zn^{2+} (or the detrimental Cd^{2+} and Pb^{2+} ions even more so) show a significant binding affinity for the S atom of a thiophosphate group, i.e., the stability of their complexes is much larger than that of the corresponding M(PO) complexes despite the lower basicity of a PS^{2-} ligand compared to that of the corresponding PO^{2-} one. Metal ions like Mn^{2+} may possibly bind partially to the sulfur atom, i.e., more than in the case of Mg^{2+}, but this interaction is not very pronounced and is not manifested in the stability constants of Mn(PS) complexes.

It may be helpful in this context to consider the absolute metal ion affinity, based on measured stability constants, toward a phosphate monoester or its thiophosphate analogue by plotting the data according to the Irving – Williams sequence. Such a plot is shown in Figure 2 for the complexes of methyl phosphate ($CH_3OPO_3^{2-}$, ○) and methyl thiophosphate ($MeOPS^{2-}$, ●).[39] For the metal ions Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}, the M(PO) complexes are more stable than the M(PS) analogues, however, it should be recalled that, based on $\log K^{M}_{M(R\text{-}PO_3)}$ versus $pK^{H}_{H(R\text{-}PO_3)}$ plots (Figure 1), it was shown (Section 2.3) that this difference in stability is only the result of the lower basicity of the PS^{2-} ligands. In fact, these metal ions behave toward thiophosphates as they do toward phosphates of the same basicity.

The vertical distances between the points due to the M(MeOPS) and $M(CH_3OPO_3)$ complexes seen in Figure 2 can be

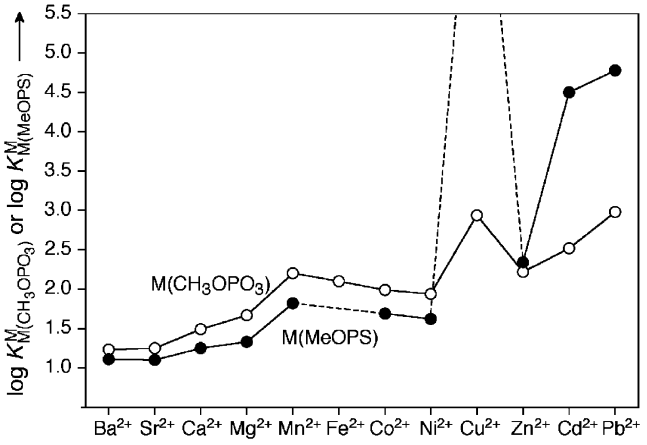


Figure 2. *Irving – Williams sequence-type plot for the 1:1 complexes of Ba^{2+} to Zn^{2+}, as well as Cd^{2+} and Pb^{2+}, formed with methyl phosphate ($CH_3OPO_3^{2-}$, ○) and its thiophosphate analogue ($MeOPS^{2-}$, ●). The data used in this plot are given in Table 2 (see Section 2.2). For the Fe(CH_3OPO_3) complex, one estimates[39] $\log K^{Fe}_{Fe(CH_3OPO_3)} = 2.1 \pm 0.1$; for Fe(MeOPS) no constant exists. For the Cu^{2+} complex of $MeOPS^{2-}$ an estimation of the stability constant is possible, based on the estimate[1] $\log \Delta_{Cu/PS} = 6 \pm 2$; this leads to $\log K^{Cu}_{Cu(MeOPS)} = \log K^{Cu}_{Cu(CH_3OPO_3)} + \log \Delta_{Cu/PS} = (2.94 \pm 0.03) + (6 \pm 2) = 9 \pm 2$; however, this estimated stability constant is far outside the log K range shown in this figure, as is indicated by the broken lines.*

expressed in terms of the differences in free energy, ΔG°. This free energy difference is connected with the stability differences by Equation (16a) and at 25 °C by Equation (16b).[35, 40]

$$\Delta G^\circ = -2.303 \cdot RT(\log K^{M}_{M(MeOPS)} - \log K^{M}_{M(CH_3OPO_3)}) \quad (16a)$$

$$= -5.71 \cdot \log \Delta_{M/MeOPS/CH_3OPO_3} \quad (16b)$$

Application of the stability differences $\log K^{M}_{M(MeOPS)} - \log K^{M}_{M(CH_3OPO_3)}$ (see Table 2) gives the following approximate ΔG° values at 25 °C: $+1\ kJ\,mol^{-1}$ for the Ca^{2+}, Sr^{2+}, and Ba^{2+} complexes, $+2\ kJ\,mol^{-1}$ for those of Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}, $-1\ kJ\,mol^{-1}$ for Zn^{2+}, and about $-11\ kJ\,mol^{-1}$ for the Cd^{2+} and Pb^{2+} complexes. Although the differences in energy for the complexes of the alkaline earth ions, as well as for those of Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} are small, they are significant in terms of enzymatic reactions where a slight change in energy may decide for the one or other substrate.[35, 41] This is important because most of the enzymes that use nucleotides as substrates are metal-ion-dependent and their mechanism of action is frequently studied by using thionucleotides.[6, 7]

The results summarized above are also of relevance regarding ribozymes because very often so-called rescue experiments[14, 42] are carried out which are based on the assumption that the affinity of, for example, Mn^{2+} toward S donors is higher than that of Mg^{2+} ions.[43] Indeed, Mn^{2+} has a higher overall affinity for thiophosphate residues than Mg^{2+} but also a higher affinity for phosphate groups (see Figure 2). In fact, Mn(PS) complexes have a stability which is very similar to that of Mg(PO) species and this stability is also expected for other structurally somewhat different S/O ligand analogues.

Interestingly, a kinetic study[43] on the activity of the 3′ – 5′-exonuclease domain of *Escherichia coli* DNA polymerase I (the 3′

terminal phosphodiester linkage of a DNA oligonucleotide is cleaved) in the presence of Mg^{2+} or Mn^{2+} and a phosphorothiolate (bridging S) modified substrate, R′-S-P(O)$_2$-OR, showed that the cleavage rates of the thio-modified substrate are lower for both metal ions than those observed for the unmodified substrate, R′-O-P(O)$_2$-OR. However, the rate constant for cleavage of R′-S-P(O)$_2$-OR with Mn^{2+} ($7.7 \times 10^3\,M^{-1}\,s^{-1}$) is of the same order as that for the cleavage of R′-O-P(O)$_2$-OR in the presence of Mg^{2+} ($6.4 \times 10^3\,M^{-1}\,s^{-1}$), which agrees with the above-mentioned comparable size of the stability constants for Mn(PS) and Mg(PO) complexes.

Several crystal structures[44] of the above-mentioned exonuclease complexed with normal, as well as phosphorothioate- (terminal S) and phosphorothiolate-modified (bridging S) substrates in conjunction with various metal ions, led to the suggestion that the resistance of these thio analogues toward nuclease degradation (see also Section 1) may partly be due to the lower affinity of the metal ion in the active center for the S-substituted substrates in comparison with the unmodified oligonucleotides.[44] Taking into account that in most instances the native metal ion in these enzymes is Mg^{2+}, these suggestions constitute no surprise if compared with the results described in this work.

As far as $Cd^{2+}$and Pb^{2+} are concerned, one has to expect, based on the results described herein, that in a nucleic acid sequence into which an artificial thiophosphate group has been inserted by replacing an O by an S atom, it is this site to which Cd^{2+} or Pb^{2+} coordinate. In fact, with these two metal ions, "rescue" experiments are expected to be successful even if only a low portion of an active site phosphate has been transformed into its thio analogue[3] because the stability of S interactions with these two metal ions is much higher than that of the corresponding O interactions (Tables 2 and 3).

Finally, the observation of the formation of a Zn^{2+}-bound hydroxo group at a pH value of about 6.9, provided there is also an S^- site in the coordination sphere of the metal ion (see Section 2.5), is clearly of biological relevance because this $Zn(H_2O)^{2+}$ deprotonation occurs in the physiological pH region. Thus, it is not surprising that Zn^{2+} enzymes are so important for many hydrolytic processes occurring in nature[45] because this metal ion allows coupling of a deprotonation reaction with a change of its coordination sphere.[37] To what extent the observed (N3)H deprotonation of uracil residues, which is facilitated by metal ions (Section 2.6), is of biological relevance is more difficult to say because at a pH value of 7.6 the formation degree of such a deprotonated species only amounts to approximately 4%.

4. Experimental Section

4.1 Materials: The disodium salts of uridine 5′-*O*-thiomonophosphate and methyl thiophosphate were synthesized as described recently in detail;[3] in fact, the same batches of the compounds were used in this study. HNO_3, NaOH (Titrisol), the nitrate salts of the metal ions, the disodium salt of ethylenediaminetetraacetic acid (EDTA), and potassium hydrogen phthalate (all pro analysi) were obtained from Merck KGaA, Darmstadt, Germany. All solutions were prepared with deionized, ultrapure (MILLI-Q 185 PLUS, obtained from Millipore S. A., 67120 Molsheim, France), and CO_2-free water.

The aqueous stock solutions of the ligands were freshly prepared daily and their exact concentration was newly determined each time by titrations with NaOH. The titer of the NaOH used for the titrations was established with potassium hydrogen phthalate and the concentrations of the M^{2+} stock solutions were determined through their EDTA complexes.

4.2 Potentiometric pH titrations: The potentiometric pH titrations were carried out with a Metrohm E536 potentiograph equipped with a Metrohm E655 or E665 dosimat and 6.0222.100 combined double-junction macro glass electrodes from Metrohm AG, Herisau, Switzerland. Previously,[1, 3, 20] a "poisoning" effect was observed in titrations of adenosine 5′-*O*-thiomonophosphate and this problem was overcome by the use of separated electrodes: a pH measuring electrode (Metrohm 6.0133.100) in combination with an Ag/AgCl double-junction reference electrode (Metrohm 6.0726.100). In the titrations of UMPS and MeOPS both double-junction and separated electrodes were used and no differences between the experimental results were observed in these instances, and no "poisoning" effects were detected either.

In order to exclude dioxygen from the titration solutions and to avoid adding the thiophosphate ligand to strongly acidic solutions, the ligand was added to the titration mixture only after all the other components, except HNO_3, were kept under N_2 for about 2 minutes, and only thereafter was the necessary HNO_3 added.

Some of the titrations were carried out with a Metrohm DMS-Titrino 716 instrument directly connected to a Pentium computer with a Windows 95 system, and equipped with the TiNet 2.1 or TiNet 2.4 software obtained from Metrohm AG, Herisau, Switzerland. The titrations with the titrino gave the same results as those with the potentiograph.

The buffer solutions (pH 4.00, 7.00, and 9.00, based on the U.S. National Institute of Standards and Technology (NIST) scale) used for the pH calibrations were also obtained from Metrohm AG. In those cases where the titrations were pursued up to a high pH value (about 10), a buffer solution with pH 9.98 (25 °C, also based on the NIST scale) purchased from Merck KGaA, Darmstadt, Germany, was employed in addition (for further details see refs. [29, 46]).

4.3 Determination of stability constants: The conditions for the determination of the stability constants $K^{M}_{M(UMPS)}$ and $K^{M}_{M(MeOPS)}$ of the binary M(UMPS) and M(MeOPS) complexes [Eq. (4)], respectively, were the same as those recently described in ref. [3] for the acidity constants $K^{H}_{H(UMPS)}$ and $K^{H}_{H(MeOPS)}$ [Eq. (2)] (25 °C; $I = 0.1$ M, $NaNO_3$). Aqueous solutions (50 mL) of HNO_3 (0.56 mM) and $M(NO_3)_2$ were always titrated in the presence and absence of UMPS (0.27 mM) or MeOPS (0.3 mM) with NaOH (1 mL, 0.03 M). Whenever needed, $NaNO_3$ was added to maintain the ionic strength, *I*, at 0.1 M. Three extra experiments with the Cd^{2+}/UMPS system were done with HNO_3 (1.12 mM) and using NaOH (2 mL, 0.03 M) in the titrations. The metal ion concentrations employed in the titrations of UMPS were 33.33 mM (which gives an M^{2+}:PS ratio of 123:1) with Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}; 30.01 mM (111:1) with Mg^{2+}, Ca^{2+}, and Ni^{2+}; 26.67 mM (99:1) with Co^{2+} and Ni^{2+}; 25.01 mM (93:1) with Ca^{2+}; 20.00 mM (74:1) with Co^{2+}, 16.67 mM (62:1) with Mn^{2+}, Co^{2+}, and Ni^{2+}; 11.67, 8.34, and 6.67 mM (43:1, 31:1, and 25:1) with Zn^{2+}; 1.67, 1.00, and 0.67 mM (6.2:1, 3.7:1, and 2.5:1) with Cd^{2+}. For the experiments with MeOPS, the metal ion concentrations were 33.33 mM (111:1) with Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Ni^{2+}; 30.01 mM (100:1) with Sr^{2+} and Ba^{2+}; 26.67 mM (89:1) with Mg^{2+}, Ca^{2+}, and Ni^{2+}; 23.34 mM (78:1) with Co^{2+}; 16.67 mM (56:1) with Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}; 8.33 mM (28:1) with Mn^{2+} and Zn^{2+};

4.17 mM (14:1) with Zn^{2+}; 1.67, 0.60, 0.42, and 0.33 mM (5.6:1, 2:1, 1.4:1, and 1.1:1) with Cd^{2+}. As can be seen, the metal:ligand ratios varied between 1.1:1 in systems with Cd^{2+} and 123:1 in systems with the alkaline earth metal ions.

The titration data for calculating $K^{M}_{M(UMPS)}$ and $K^{M}_{M(MeOPS)}$ were collected every 0.1 pH unit in the pH range of about 3.6 to 5.5 or 5.7 for Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} and from a pH value of about 3.4 to 5.5 for Cd^{2+}/UMPS, and 3.1 to 6.1 for Cd^{2+}/MeOPS. The evaluation of the experimental data was stopped before the onset of the hydrolysis of the metal ions, which was evident from the titrations without ligand, and in the case of $UMPS^{2-}$ also before deprotonation of the (N3)H site took place. In those instances where the M^{2+}:PS ratio was larger than 10:1, the data were evaluated with a curve-fitting procedure using a Newton–Gauss nonlinear least-squares program for each pair of titrations (i.e., with and without ligand) by calculating the apparent acidity constant K'_a. The stability contants were then calculated according to Equation (17).[1, 47]

$$K^{M}_{M(PS)} = (K'_a - K^{H}_{H(PS)})/(K^{H}_{H(PS)}[M^{2+}]) \quad (17)$$

For the systems where smaller M^{2+}:PS ratios were employed, the data were evaluated (again every 0.1 pH unit) by considering the concentration of all the species present in equilibrium, that is, H^+, $H(PS)^-$, PS^{2-}, M^{2+}, and M(PS).[48]

The pH ranges mentioned above correspond to formation degrees of the M(PS) complexes of about 2–30% with Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}; 5–50% with Mn^{2+}, Co^{2+}, and Ni^{2+}; 10–55% with Zn^{2+}; 59–97% of Cd(UMPS) (pH 3.4–5.5); and 40–98% of Cd(MeOPS) (pH 3.1–6.1). The values calculated individually for $\log K^{M}_{M(PS)}$ showed no dependence on pH or on the excess amount of M^{2+} ions. The final results are the averages of at least 4, usually 5 or 6, independent titration pairs.

In the Zn^{2+}/MeOPS system the titration curves showed that from Zn(MeOPS)(aq) a further proton is released before the onset of hydrolysis of $Zn(aq)^{2+}$ alone. Therefore, this system was also evaluated by considering the formation of a $Zn(MeOPS)(OH)^-$ species resulting from the deprotonation of a Zn^{2+}-bound water molecule in Zn(MeOPS)(aq); i.e., $K^{H}_{Zn(MeOPS)(aq)}$ [Eq. (5)] was calculated by a re-evaluation of the titration data in the pH range up to about 6.5 by taking into account the concentrations of H^+, $H(MeOPS)^-$, $MeOPS^{2-}$, Zn^{2+}, Zn(MeOPS), and $Zn(MeOPS)(OH)^-$. The final value for $K^{H}_{Zn(MeOPS)(aq)}$ is the average of 6 independent titration pairs.

Since in titrations of M^{2+}/UMPS systems deprotonation of the (N3)H site was observed, we performed some titrations with Mg^{2+}, Ca^{2+}, Mn^{2+}, and Cd^{2+} up to a pH value where this deprotonation could actually be measured. After the stability constants of the M(UMPS) complexes were calculated in the way described above, the experimental data were re-evaluated but this time up to a pH value of about 9.2 for Mg^{2+}, 9.5 for Ca^{2+}, 8.6 for Mn^{2+}, and 8.1 for the Cd^{2+} system. It became immediately clear that not only $M(UMPS-H)^-$ but also $M_2(UMPS-H)^+$ complexes are formed. Since the equilibria for the formation of these two complexes are coupled, it was not possible to determine equilibrium constants for both of them independently. We therefore estimated the acidity constant $pK^{H}_{M(UMPS)}$ for the release of the proton from the (N3)H site [Eq. (6)]; this release should be facilitated by M^{2+} coordination to the thiophosphate group. Indeed, such acidifications are independent of the kind of M^{2+} ion considered and they are always on the same order,[29, 30] i.e, with ΔpK_a values of about 0.40 ± 0.15. For the case described herein we used $\Delta pK_a = 0.45$; hence, by keeping constant $pK^{H}_{M(UMPS)} = pK^{H}_{UMPS} - 0.45 = 9.47 - 0.45 = 9.02 \pm 0.15$ (but taking the error into account) in the evaluations (as well as the already determined values for $\log K^{M}_{M(UMPS)}$), stability constants for the formation of $M_2(UMPS-H)^+$ complexes [Eq. (7)] could be calculated (again every 0.1 pH unit) by taking into account the species H^+, $H(UMPS)^-$, $UMPS^{2-}$, $(UMPS-H)^{3-}$, M^{2+}, M(UMPS), $M(UMPS-H)^-$, and $M_2(UMPS-H)^+$ (see also Section 2.6). The formation degree of the $M_2(UMPS-H)^+$ species amounted to a maximum of only about 6% for $M^{2+} = Mg^{2+}$ and Mn^{2+}, 14% for Ca^{2+}, and about 10% for Cd^{2+}. The $M(UMPS-H)^-$ complexes reach formation degrees of about 30% with Mg^{2+} and Ca^{2+}, 14% for Mn^{2+}, and 9% for Cd^{2+}.

The final results given for the $K^{M}_{M_2(UMPS-H)}$ constants are the averages of 4 independent titration pairs in the case of the Mg^{2+} system, 2 each for Ca^{2+} and Mn^{2+}, and 6 for Cd^{2+}.

Abbreviations and Definitions

$AMPS^{2-}$	adenosine 5′-*O*-thiomonophosphate
I	ionic strength
K_a	general acidity constant
M^{2+}	general divalent metal ion
$MeOPS^{2-}$	methyl thiophosphate
PO^{2-}	general phosphate ligand; mostly $CH_3OPO_3^{2-}$ and UMP^{2-}
PS^{2-}	general thiophosphate ligand; mostly $MeOPS^{2-}$ and $UMPS^{2-}$
$R\text{-}PO_3^{2-}$	simple phosphate monoester or phosphonate ligand, where R represents a noncoordinating residue (see also legend of Figure 1)
UMP^{2-}	uridine 5′-monophosphate
$UMPS^{2-}$	uridine 5′-*O*-thiomonophosphate
Urd	uridine

Species written without a charge either do not carry one or represent the species in general (i.e., independent of their protonation degree); which of the two possibilities applies is always clear from the context. A formula like $(UMP-H)^{3-}$ means that the ligand has lost a proton and it is to be read as UMP^{2-} *minus* H^+. The term (aq) is used to indicate that water is acting as a ligand.

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