METAL ION-BINDING PROPERTIES OF THE NUCLEOTIDE ANALOGUE 1-[2-(PHOSPHONOMETHOXY)ETHYL]CYTOSINE (PMEC) IN AQUEOUS SOLUTION

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The acidity constants of the twofold protonated nucleotide analogue 1-[2-(phosphonomethoxy)ethyl]cytosine, $H_2(PMEC)^{\pm}$, as well as the stability constants of the $M(H;PMEC)^+$ and M(PMEC) complexes with the metal ions M^{2+} = Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} have been determined by potentiometric pH titrations in aqueous solution at I = 0.1 M ($NaNO_3$) and 25 °C. Comparison with previous results for the nucleobase-free compound (phosphonomethoxy)ethane, PME, and the parent nucleotides cytidine 5′-monophosphate (CMP^{2-}) and 2′-deoxycytidine 5′-monophosphate ($dCMP^{2-}$) shows that the metal ion-binding properties of $PMEC^{2-}$ resemble closely those of PME^{2-}: This means, the primary binding site is the phosphonate group and with all of the metal ions studied, 5-membered chelates involving the ether oxygen of the $-CH_2-O-CH_2-PO_3^{2-}$ chain are formed. The position of the isomeric equilibria between these chelates and the "open" complexes, $-PO_3^{2-}/M^{2+}$ is calculated; the degree of formation of the chelates is identical within the error limits for the M(PME) and M(PMEC) systems. Hence, like in M(CMP) and M(dCMP) no interaction occurs with the cytosine residue in the M(PMEC) complexes. However, the monoprotonated $M(H;PMEC)^+$ as well as the $M(H;CMP)^+$ and $M(dCMP)^+$ species carry the metal ion predominantly at the nucleobase, while the proton is at the phosph(on)ate group. The coordinating properties of $PMEC^{2-}$ and CMP^{2-} or $dCMP^{2-}$ differ thus only with respect to the possible formation of the 5-membered chelates involving the ether oxygen in M(PMEC) species, a possibility which does not exist in the complexes of the parent nucleotides. Possible reasons why PMEC is devoid of a significant antiviral activity are shortly discussed.
Key words: Acidity constants; (*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]cytosine; Metal ion complexes; Phosphonate complexes; 9-[2-(Phosphonomethoxy)ethyl]adenine; Stability constants; Nucleotides; Phosphates; Phosphonates; Chelates; Acyclic Nucleotide Analogues.

In the course of our studies concerning antivirally active nucleotide analogues[1–7], we have now investigated the proton- and metal ion-binding properties of the dianion of 1-[2-(phosphonomethoxy)ethyl]cytosine,

$PMEC^{2-}$ (Fig. 1, ref.[8]). This substance can be considered as an analogue of cytidine 5′-monophosphate (CMP^{2-}; Fig. 1) and of its 2′-deoxy derivative ($dCMP^{2-}$). PMEC is related to the highly potent antivirals 9-[2-(phosphonomethoxy)ethyl]adenine, (PMEA; Fig. 1), also known as adefovir, which is especially active against retroviruses and several DNA-viruses[9,10], and (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC; Fig. 1), also known as cidofovir[11–13], which is used in the treatment of cytomegalovirus-induced retinitis[14], severe laryngeal papillomatosis[15], progressive multifocal leukoencephalopathy[16], and acyclovir-resistant herpes simplex virus lesions[17]. Despite its close resemblance to these two nucleotide analogues, PMEC is devoid of any significant activity against the viruses tested[18].

It is well known that nucleotides enter enzymatic reactions in the form of metal ion complexes[19–22]. The same may be surmised for their analogues (Fig. 1) which are diphosphorylated by various cellular kinases[23–25]. In this

FIG. 1
Dianions of 1-[2-(phosphonomethoxy)ethyl]cytosine ($PMEC^{2-}$), cytidine 5′-monophosphate (CMP^{2-}), 9-[2-(phosphonomethoxy)ethyl]adenine ($PMEA^{2-}$), 1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine ($HPMPC^{2-}$), and (phosphonomethoxy)ethane (PME^{2-} = ethoxymethanephosphonate). The CMP^{2-} ligand is shown in its dominating *anti* conformation where the carbonyl oxygen points away from the ribose ring[8]

form, *i.e.* as PMEApp and HPMPCpp[26–28], they exert their antiviral activity *via* the inhibition of the viral DNA polymerases, while the corresponding cellular enzymes are inhibited to a lesser extent[29–31].

It is not known why PMEC fails to be active against viruses. The diphosphorylated derivative PMECpp binds to several viral DNA polymerases[18,32] and exerts an inhibiting effect, though being less potent than, *e.g.*, PMEApp. Reasons for the indicated failure could be that PMEC is not taken up by the infected cells, or that the kinases involved in the activation do not recognize PMEC as a substrate. In this context it is interesting to note that $PMEA^{2-}$ mimics adenosine 5′-monophosphate (AMP^{2-}) quite well[7,33] and that this may be of importance for its mechanism of action[34]. To see how $PMEC^{2-}$ behaves in this respect we are aiming to gather information about the solution properties of $PMEC^{2-}$ including its divalent metal ion (M^{2+}) complexes in order to compare them with those of the parent nucleotides CMP^{2-} and $dCMP^{2-}$, for which abundant information exists[35–37]. For the interpretation of the observed complex stabilities we shall also make use of data already available for the dianion of (phosphonomethoxy)ethane (PME^{2-}; Fig. 1, refs[1–3]), which is the nucleobase-free parent ligand of $PMEC^{2-}$ as well as of $PMEA^{2-}$.

EXPERIMENTAL

Materials

The free acid of 1-[2-(phosphonomethoxy)ethyl]cytosine was synthesized according to published procedures[38]. The aqueous stock solutions of the ligand were freshly prepared daily just before the titration experiments by dissolving the substance in deionized, ultrapure (MILLI-Q185 PLUS; from Millipore S. A., 67120 Molsheim, France), CO_2–free water and adding 2 equivalents of NaOH.

The disodium salt of 1,2-diaminoethane-*N*,*N*,*N′*,*N′*-tetraacetic acid (Na_2EDTA), potassium hydrogen phthalate, HNO_3, NaOH (Titrisol), and the nitrate salts of Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} (all of analytical grade) were from Merck AG, Darmstadt, Germany. All solutions were prepared with ultrapure, CO_2–free water.

The buffer solutions (pH 4.00, 7.00, 9.00, based on the NBS scale; now NIST) for calibration were from Metrohm AG, Herisau, Switzerland.

Potentiometric pH Titrations

The pH titrations for the determination of the equilibrium constants in aqueous solutions were recorded with a Metrohm E 536 potentiograph connected to a Metrohm E 535 dosimat and a Metrohm 6.0202.100 (NB) combined macro glass electrode. The pH calibration of the instrument was done with the buffers mentioned above. The titre of the NaOH used for the potentiometric pH titrations was determined with potassium hydrogen phthalate.

Determination of the Acidity Constants

The exact concentration of the ligand solutions was in each experiment newly determined by the evaluation of the corresponding titration pairs described below. Direct pH readings were used in the calculation of the acidity constants; *i.e.*, these constants are so-called practical, mixed or Brønsted constants[39]. Their negative logarithms given for aqueous solutions at I = 0.1 M ($NaNO_3$) and 25 °C may be converted into the corresponding concentration constants by subtracting 0.02 from the listed pK_a values[39]; this conversion term contains both the junction potential of the glass electrode and the hydrogen ion activity[39,40].

The acidity constants $K^H_{H_2(PMEC)}$ and $K^H_{H(PMEC)}$ of $H_2(PMEC)^{\pm}$ were determined by titrating under N_2 50 ml of an aqueous 0.0006 M HNO_3 in the presence and absence of 0.0003 M $PMEC^{2-}$ with 1 ml of 0.033 M NaOH (25 °C). The ionic strength of 0.1 M was adjusted with $NaNO_3$. As the difference in NaOH consumption between pairs of solutions, *i.e.* with and without ligand[39], is evaluated, the ionic product of water (K_w) and the mentioned conversion term do not enter into the calculations.

The acidity constants were calculated with an IBM-compatible desk computer[6] using a Newton–Gauss nonlinear-least-squares fitting procedure. The calculations were carried out between about 30 and 100% neutralization with respect to the equilibrium $H_2(PMEC)^{\pm}/H(PMEC)^-$ and between 0 and about 97% with respect to the equilibrium $H(PMEC)^-/PMEC^{2-}$. The results are the averages of 24 pairs of independent titrations.

Determination of the Stability Constants

The exact concentrations of the M^{2+} stock solutions were determined by potentiometric pH titration *via* their EDTA complexes.

The conditions for the determination of the stability constants $K^M_{M(H;PMEC)}$ and $K^M_{M(PMEC)}$ were the same as given above for the determination of the acidity constants, but $NaNO_3$ was now partly or fully replaced by $M(NO_3)_2$ (I = 0.1 M; 25 °C). The M^{2+} : ligand ratios were 111 : 1 or 89 : 1 for the alkaline earth metal ions, 56 : 1 or 28 : 1 for Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}, and 11 : 1 or 5.6 : 1 for Cu^{2+}. The stability constants were calculated with the above mentioned computer facility by a curve-fitting procedure, taking into account the species H^+, $H_2(PMEC)^{\pm}$, $H(PMEC)^-$, $PMEC^{2-}$, M^{2+}, $M(H;PMEC)^+$, and M(PMEC). The experimental data were used every 0.1 pH unit starting from about 5% complex formation regarding M(PMEC) to a neutralization degree of about 90% with respect to the species $H(PMEC)^-$, or until the beginning of the hydrolysis of $M(aq)^{2+}$, which was evident from the titrations without ligand. The individual stability constants showed no dependence on pH or the metal ion concentration. The results are in each case the averages of at least seven independent pairs of titrations.

RESULTS AND DISCUSSION

1. Definition of Equilibrium Constants, Acid-Base Properties of $H_2(PMEC)^{\pm}$, and Stability of $M(H;PMEC)^+$ and M(PMEC) Complexes

It is well known that nucleobases and their derivatives can undergo self-association *via* π-stacking[41,42]. Therefore, the experimental conditions

for the determination of the equilibrium constants were selected carefully, like in previous studies (*e.g.* ref.[1]), to ascertain that the results obtained refer to monomeric species.

In the pH range of the present study, $PMEC^{2-}$ can take up two protons. The most basic site in $PMEC^{2-}$ is the phosphonate residue, $–PO_3^{2-}$. The resulting $H(PMEC)^-$ species may then be protonated at N3 of the nucleobase (see Fig. 1) to form the species $H_2(PMEC)^{\pm}$. The second deprotonation of the twofold protonated phosphonate group is expected to occur also with $pK_a \approx 1.2$ as determined for $H_3(PMEA)^+$ and some related ligands[4]. This means that this proton is already completely released at pH > 3.5, the pH range needed for the collection of data in the present study. The deprotonation steps of $H_2(PMEC)^{\pm}$ are defined below, where the abbreviation PC^{2-} is used for ligands composed of a phosph(on)ate group and a cytosine residue.

$$H_2(PC)^{\pm} \rightleftharpoons H(PC)^- + H^+ \qquad (1a)$$

$$K^H_{H_2(PC)} = [H(PC)^-][H^+]/[H_2(PC)^{\pm}] \qquad (1b)$$

$$H(PC)^- \rightleftharpoons PC^{2-} + H^+ \qquad (2a)$$

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

The results are given in Table I together with previous data for $H(PME)^-$ and several naturally occurring cytosine derivatives[1,35,36,43,44]. Evidently, both proton binding sites are somewhat more basic in $PMEC^{2-}$ than in CMP^{2-} or $dCMP^{2-}$. From a comparison with the acidity constants of monoprotonated cytosine and cytidine follows that this is mainly an effect of the excellent solvation properties of the sugar moiety; an observation made before[45]. Consequently, at the physiological pH of 7.5, $H(CMP)^-$ and $H(dCMP)^-$ are already deprotonated to a larger extent than $H(PMEC)^-$, ***i.e.***, CMP^{2-} and $dCMP^{2-}$ occur to about 95%, while $PMEC^{2-}$ exists only to about 78%. However, this has only little impact on the metal ion-binding abilities of the three ligands.

The experimental data of the potentiometric pH titrations of all M^{2+}/PMEC systems can be completely described by equilibria (*1a*) through (*4a*),

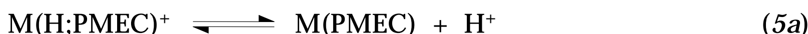
$$M^{2+} + H(PMEC)^- \rightleftharpoons M(H;PMEC)^+ \quad (3a)$$

$$K^{M}_{M(H;PMEC)} = [M(H;PMEC)^+]/([M^{2+}][H(PMEC)^-]) \quad (3b)$$

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

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

if the evaluation is not carried into the pH range where hydroxo complexes form. The acidity constant of the monoprotonated $M(H;PMEC)^+$ complex (Eqs (*5*)) can be calculated using Eq. (*6*):



$$M(H;PMEC)^+ \rightleftharpoons M(PMEC) + H^+ \quad (5a)$$

$$K^{H}_{M(H;PMEC)} = [M(PMEC)][H^+]/[M(H;PMEC)^+] \quad (5b)$$

$$pK^{H}_{M(H;PMEC)} = pK^{H}_{H(PMEC)} + \log K^{M}_{M(H;PMEC)} - \log K^{M}_{M(PMEC)} \quad (6)$$

TABLE I
Negative logarithms of the acidity constants of $H_2(PMEC)^{\pm}$ and related systems (aqueous solution; 25 °C; I = 0.1 M, $NaNO_3$)[a–c]

Protonated species	$pK^{H}_{H_2(PC)}$ or $pK^{H}_{(N1)H}$	$pK^{H}_{H(PC)}$ or $pK^{H}_{P(O)_3H}$
$H_2(PMEC)^{\pm}$	4.72 ± 0.01	6.95 ± 0.01
$H(PME)^-$		7.02 ± 0.01
$H_2(CMP)^{\pm}$	4.33 ± 0.04	6.19 ± 0.02
$H_2(dCMP)^{\pm}$	4.46 ± 0.01	6.24 ± 0.01
Cytidine	4.14 ± 0.02	
Cytosine	4.7	

[a] The errors given are three times the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] So-called practical, mixed or Brønsted constants are listed (see also Experimental)[39]. [c] The entries in rows 2, 3, 4, 5, and 6 are from refs[1,35,36,43,44], respectively.

The results listed in Table II show the usual trends: The stability of the M(PMEC) complexes for the alkaline earth ions decreases with increasing ionic radii. For the divalent 3d metal ions the long-standing experience[46,47] is confirmed that the stabilities of phosph(on)ate–metal ion complexes[1,3,35,36,48,49] often do not strictly follow the Irving–Williams sequence[50]. For the monoprotonated $M(H;PMEC)^+$ complexes, no unequivocal conclusions can be drawn in this respect due to the relatively large error limits of the corresponding stability constants which are a consequence of the low degree of formation of these species. Application of the determined equilibrium constants allows to calculate the formation degree of the various species in dependence on pH. Two representative examples are shown in Fig. 2; the concentrations used are among those employed in the experiments.

The analysis of potentiometric pH titrations yields only the amount and distribution of species of a net charge type, such as $M(H;PMEC)^+$, and further information is required to locate the binding sites of the proton and metal ion (see Section 2). Similarly, the stability constants of the M(PMEC) complexes also require a more detailed analysis (see Section 3).

TABLE II
Logarithms of the stability constants of the $M(H;PMEC)^+$ and M(PMEC) complexes (Eqs (*3*), (*4*)) together with the negative logarithms of the acidity constants of the $M(H;PMEC)^+$ species (Eqs (*5*), (*6*)) (aqueous solution; 25 °C; I = 0.1 M, $NaNO_3$)[a]

M^{2+}	$\log K^{M}_{M(H;PMEC)}$	$\log K^{M}_{M(PMEC)}$	$pK^{H}_{M(H;PMEC)}$
Mg^{2+}	0.5 ± 0.3	1.88 ± 0.03	5.6 ± 0.3
Ca^{2+}	0.3 ± 0.4	1.67 ± 0.04	5.6 ± 0.4
Sr^{2+}	0.0 ± 0.5	1.41 ± 0.04	5.5 ± 0.5
Ba^{2+}	0.0 ± 0.5	1.38 ± 0.05	5.6 ± 0.5
Mn^{2+}	0.6 ± 0.3	2.53 ± 0.03	5.0 ± 0.3
Co^{2+}	0.5 ± 0.3	2.30 ± 0.02	5.15 ± 0.3
Ni^{2+}	0.6 ± 0.4	2.26 ± 0.05	5.3 ± 0.4
Cu^{2+}	2.20 ± 0.11	3.73 ± 0.06	5.42 ± 0.13
Zn^{2+}	0.95 ± 0.19	2.67 ± 0.03	5.23 ± 0.19
Cd^{2+}	1.40 ± 0.05	3.00 ± 0.04	5.35 ± 0.06

[a] Regarding the error limits, see footnote [a] of Table I. The error limits of column 4 were calculated according to the error propagation after Gauss.

2. Structural Considerations on Monoprotonated $M(H;PMEC)^+$ Complexes

Binding of a metal ion to a protonated ligand commonly leads to a more or less pronounced acidification of the ligand-bound proton. The acidity constants of the $M(H;PMEC)^+$ complexes are by 1.3 to 2 log units smaller than $pK^H_{H(PMEC)}$, but 0.3 to 0.9 log units larger than $pK^H_{H_2(PMEC)}$ (Tables I and II). This indicates that the proton in the $M(H;PMEC)^+$ complexes is mainly bound to the phosphonate group because only under this assumption, an acidification occurs. With this conclusion in mind one may then assume that the metal ion is bound preferentially to the nucleobase, since a mono-

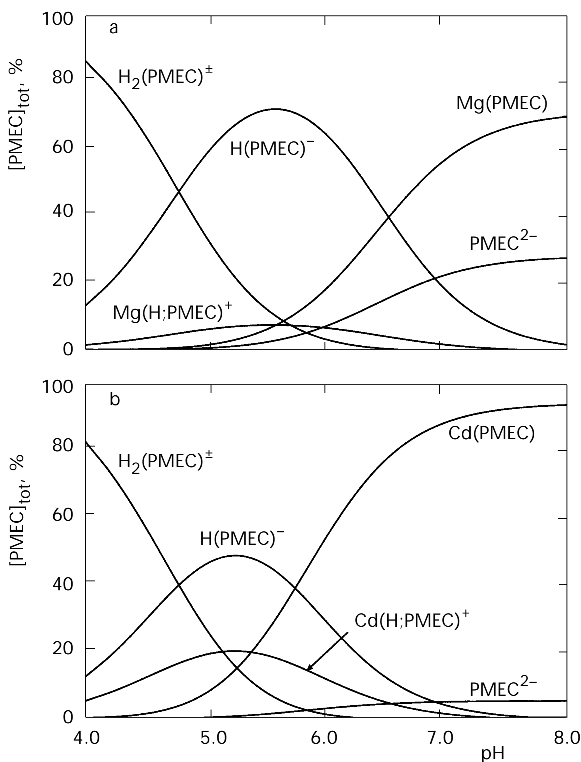


FIG. 2

Effect of pH on the concentration of the species present in the Mg^{2+} (a) and Cd^{2+} (b) systems with PMEC. The results are given as percentages of the total ligand concentration present. The calculations were carried out with the determined acidity (Table I) and stability constants (Table II) by using $[PMEC]_{tot}$ = 0.0003 M and $[Mg^{2+}]$ = 0.03333 M or $[Cd^{2+}]$ = 0.01667 M (concentrations which correspond to the experimental conditions)

protonated phosphonate group is only a weak binding site and at least the $M(H;PMEC)^{+}$ complexes of Cu^{2+}, Zn^{2+}, and Cd^{2+} are relatively stable (Table II).

One way to examine the mentioned assumption is to calculate "expected" stability constants for the coordination of the considered metal ions to the cytosine residue in $H(PMEC)^{-}$. This can be done by using the known[43] stability constants for the 1 : 1 complexes formed between cytidine (Cyd) and a divalent metal ion (M^{2+}); these values are given in the second column of Table III. These values need to be corrected for the different basicities of the N3 sites in $H(PMEC)^{-}$ and Cyd; *i.e.*, $\Delta\, pK_a = pK^{H}_{H_2(PMEC)} - pK^{H}_{H(Cyd)} = (4.72 \pm 0.01) - (4.14 \pm 0.02) = 0.58 \pm 0.02$ (Table I). By using the slopes of the correlation lines for the log *K versus* pK_a plots given earlier[43,44,51] for *o*-aminopyridine-like ligands (see column 3 of Table III) and $\Delta\, pK_a = 0.58$, one may calculate „expected" stability constants for metal ion binding to the cytosine residue in $H(PMEC)^{-}$. Comparison of these calcu-

TABLE III
Estimation of the stability of monoprotonated $M(H;PMEC)^{+}$ complexes based on the known[43] stability of the corresponding $M(Cyd)^{2+}$ complexes and comparison of the calculated (calc) stability constants with those experimentally (exp) determined[a]

M^{2+}	$\log K^{M}_{M(Cyd)}$ [b]	m [c]	$\log K^{M}_{M(H;PMEC)}$		$\log \Delta^{*}$ [f]
			calc[d]	exp[e]	
Mn^{2+}	0.19 ± 0.08	0.262	0.34 ± 0.22	0.6 ± 0.3	0.26 ± 0.37
Co^{2+}	0.03 ± 0.08	0.204	0.15 ± 0.24	0.5 ± 0.3	0.35 ± 0.38
Ni^{2+}	0.14 ± 0.12	0.335	0.33 ± 0.21	0.6 ± 0.4	0.27 ± 0.45
Cu^{2+}	1.56 ± 0.06	0.456	1.82 ± 0.09	2.20 ± 0.11	0.38 ± 0.14
Zn^{2+}	0.20 ± 0.11	0.367	0.41 ± 0.21	0.95 ± 0.19	0.54 ± 0.28
Cd^{2+}	0.91 ± 0.07	0.332	1.10 ± 0.17	1.40 ± 0.05	0.30 ± 0.18

[a] For the error limits, see footnotes [a] of Tables I and II. [b] From ref.[43]. [c] Slopes *m* for $\log K^{M}_{ML}$ *versus* pK^{H}_{HL} correlations for *o*-aminopyridine-like ligands (L) as listed in Table 3 of ref.[43] or Table I of ref.[51]. Calculations with the slopes provided in ref.[44] lead to results very similar to those listed in column 4. [d] These values refer to complexes formed with a cytosine moiety that has $pK_a = 4.72$; *i.e.*, the difference 0.58 ($= \Delta\, pK_a = pK^{H}_{H_2(PMEC)} - pK^{H}_{H(Cyd)} = 4.72 - 4.14$) times the slope *m* was added to the $\log K^{M}_{M(Cyd)}$ values of column two (see also the text in Section 2). The error limits given with the above values were calculated by taking into account those of column 2 and 3 times the *SD* values given in refs[43,51]. [e] From column 2 of Table II. [f] $\log \Delta^{*} = \log K_{exp} - \log K_{calc}$ (see the two columns on the left).

lated constants given in column 4 of Table III with the measured ones in column 5 leads to the differences listed in column 6.

At this point it should be noted that the effect of a single negative charge as present in the $-P(O)_2(OH)^-$ residue on a twofold positively charged metal ion at N3 is larger than on the single-charged proton at the same site[52]. The latter effect has been taken into account in the calculations for column 4 of Table III by considering the difference in basicity but not yet the (–/2+) effect on metal ions. In previous considerations on a nucleotide ligand system with about the same distance between the charged sites, it was concluded[52] that metal ion binding should be promoted by about 0.4 ± 0.15 log units due to such an effect. Despite the large error limits of the log Δ^* values listed in column 6 of Table III, the trend is clear; all of them (on average 0.35 log unit) are in the expected order. Consequently, the increased stability of the $M(H;PMEC)^+$ complexes can be explained solely by a charge effect and there is no need to postulate, for example, chelate formation between an N3-bound metal ion and the $-P(O)_2(OH)^-$ residue.

From the given analysis follows that the metal ions in the $M(H;PMEC)^+$ complexes are bound to the cytosine residue. But where exactly do they bind to the nucleobase? Here one may assume that the same binding patterns hold as revealed earlier[43] for the $M(Cyd)^{2+}$ complexes in a detailed study. This means that for the binding of the alkaline earth ions, the carbonyl oxygen at C2 is important whereas Ni^{2+} and Cu^{2+} rather prefer N3; there are also indications that the adjacent amino group at C4 may exert steric effects and that for some metal ions, outer-sphere coordination is of relevance. In other words, the exact binding mode to the nucleobase depends much on the individual properties of the metal ion considered; for further details, ref.[43] should be consulted.

3. Evidence for Enhanced Stability of the M(PMEC) Complexes and Comparison with Properties of the M(PME) Species

$PMEC^{2-}$ offers three potential sites for the coordination of metal ions: The twofold negatively charged phosphonate group, the above mentioned donor atoms of the cytosine moiety (Section 2), and the ether oxygen of the $-CH_2-O-CH_2-PO_3^{2-}$ chain (see Fig. 1). The phosphonate group is clearly the primary binding site for the metal ions considered in this study and therefore any participation of one of the other potential sites has to be reflected in a relative stability increase[3,53]. Hence, what is needed is the stability of the M(PMEC) complexes in which the metal ion is solely coordinated to the

$-PO_3^{2-}$ group; these species are called "open" isomers, $M(PMEC)_{op}$, in contrast to possibly "closed" or chelated isomers, which are designated as $M(PMEC)_{cl}$.

The stability constant of the open isomer

$$K^{M}_{M(PMEC)_{op}} = [M(PMEC)_{op}]/([M^{2+}][PMEC^{2-}]) \qquad (7)$$

is not directly accessible by experiments, but may be calculated using the acidity constant, $K^{H}_{H(PMEC)}$ (Eqs (*2*)), which is due to the deprotonation of the $-P(O)_2(OH)^-$ residue in $H(PMEC)^-$, and the straight-line equations obtained previously for the correlation plots of log $K^{M}_{M(R-PO_3)}$ *versus* $pK^{H}_{H(R-PO_3)}$ which were based on simple phosphonate or phosphate monoester ligands, $R-PO_3^{2-}$, where R is a noncoordinating residue. These data are listed in Table 5 of ref.[1] or in Table 3 of ref.[54] and are also used now. Three examples of such plots are depicted in Fig. 3.

The solid points due to the M(PMEC) and M(PME) complexes in Fig. 3 are in all instances above their reference lines, thus proving an increased stability of these complexes. Since corresponding observations are made for the complexes of $PMEC^{2-}$ and the nucleobase-free PME^{2-}, it is clear that the ether oxygen of the (phosphonomethoxy)ethyl moiety (see Fig. 1) must participate in metal ion binding and hence, the following intramolecular equilibrium needs to be considered:

$$R-O-CH_2-P(O)_2-O\cdots M \rightleftharpoons \text{R–O}\cdots\text{M}\cdots\text{O–P(O)}_2\text{–CH}_2\text{–(chelate)} \qquad (8)$$

It is obvious that the vertical distance from the solid points in Fig. 2 to their reference lines reflects the "intensity" of the participation of the ether oxygen in metal ion binding; in other words, this distance corresponds to the degree of formation of the $M(PMEC)_{cl}$ or $M(PME)_{cl}$ species which appear on the right hand side in equilibrium (*8*). Using for $PMEC^{2-}$ and PME^{2-} (as well as for CMP^{2-} and $dCMP^{2-}$; see below) the abbreviation PM^{2-}, we may define a stability enhancement according to Eqs (*9*):

$$\log \Delta_{M/PM} = \log K^{M}_{M(PM)_{exp}} - \log K^{M}_{M(PM)_{calc}} \qquad (9a)$$

$$= \log K^{M}_{M(PM)} - \log K^{M}_{M(PM)_{op}} \qquad (9b)$$

$$= \log \Delta \qquad \text{(see Eq. (11); vide infra)}$$

The corresponding terms in Eqs (*9*) define their meaning and equivalence. Values for $K^{M}_{M(PM)_{op}} = K^{M}_{M(PM)_{calc}}$ can be calculated as indicated above by using the mentioned straight-line equations[1,3,54] and applying the experimentally determined acidity constants $K^{H}_{H(PM)}$ (Table I). The results are listed in the third column of Table IV, whereas the second column provides the experimentally determined stability constants; the resulting stability differences

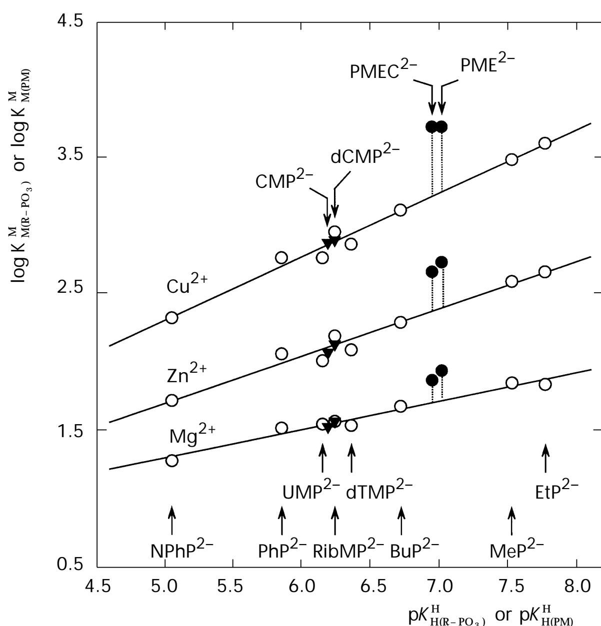


FIG. 3

Evidence for an enhanced stability of several M(PMEC) and M(PME) (●) complexes based on the relationship between log $K^{M}_{M(R-PO_3)}$ or log $K^{M}_{M(PM)}$ and p$K^{H}_{H(R-PO_3)}$ or p$K^{H}_{H(PM)}$ for the 1 : 1 complexes of Mg^{2+}, Zn^{2+}, and Cu^{2+} with some phosph(on)ate 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 ($dTMP^{2-}$), n-butyl phosphate (BuP^{2-}), methanephosphonate (MeP^{2-}), and ethanephosphonate (EtP^{2-}) (from left to right). The least-squares lines[1,3,54] are drawn through the corresponding eight data sets taken from ref.[35] for the phosphate monoesters and from ref.[1] for the phosphonates. The points due to the equilibrium constants for the M^{2+}/PMEC systems (●) are based on the constants given in Tables I and II, those for the M^{2+}/PME systems (●) are from ref.[1]. The vertical dotted lines correspond to the stability enhancements log $\Delta_{M/PM}$ as defined by Eq. (*9*). The equilibrium data for the M^{2+}/CMP and M^{2+}/dCMP systems (▼) are from ref.[36]. All the plotted equilibrium constants refer to aqueous solutions at 25 °C and I = 0.1 M ($NaNO_3$)

(Eqs (*9*)) are given in column 4 of Table IV. The latter values are identical within their error limits with those determined earlier[1] for the M(PME) complexes which appear in column 5; this means that the cytosine residue does not participate in metal ion binding and that the stability enhancements observed for the M(PMEC) complexes can be solely explained by equilibrium (*8*).

A careful comparison of the data listed in columns 4 and 5 of Table IV reveals an even finer detail: There is not a single case where a value for $\log \Delta_{M/PMEC}$ appears to have the tendency to be larger than that for $\log \Delta_{M/PME}$. It is the other way round! The $\log \Delta_{M/PMEC}$ values for the complexes formed with metal ions that are either small and/or prefer a regular octahedral coordination sphere, such as Mg^{2+}, (Ca^{2+}), Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+},

TABLE IV
Stability constant comparisons for the M(PMEC) complexes between the measured stability constants (exp; Table II, column 3) and the calculated stability constants (calc) based on the basicity of the phosphonate group in $PMEC^{2-}$ and the baseline equations established previously (see text in Section 3 and Fig. 2)[1,3]. The also previously determined[1] stability enhancements $\log \Delta_{M/PME}$ are given for comparison. The values for $\log \Delta_{M/PME-R}$ listed in the final column to the right are intended for future comparisons (see the final paragraph in Section 3) (aqueous solution; 25 °C; I = 0.1 M, $NaNO_3$)[a]

M^{2+}	$\log K^{M}_{M(PMEC)}$		$\log \Delta_{M/PMEC}$ [b]	$\log \Delta_{M/PME}$ [b]	$\log \Delta_{M/PME-R}$
	exp	calc			
Mg^{2+}	1.88 ± 0.03	1.72 ± 0.03	0.16 ± 0.04	0.22 ± 0.03	0.16 ± 0.04 [c]
Ca^{2+}	1.67 ± 0.04	1.55 ± 0.05	0.12 ± 0.06	0.14 ± 0.05	0.12 ± 0.05 [d]
Sr^{2+}	1.41 ± 0.04	1.30 ± 0.04	0.11 ± 0.06	0.07 ± 0.05	0.09 ± 0.05 [d]
Ba^{2+}	1.38 ± 0.05	1.23 ± 0.04	0.15 ± 0.06	0.10 ± 0.05	0.11 ± 0.05 [d]
Mn^{2+}	2.53 ± 0.03	2.34 ± 0.05	0.19 ± 0.06	0.27 ± 0.05	0.19 ± 0.06 [c]
Co^{2+}	2.30 ± 0.02	2.10 ± 0.06	0.20 ± 0.06	0.29 ± 0.06	0.20 ± 0.06 [c]
Ni^{2+}	2.26 ± 0.05	2.12 ± 0.05	0.14 ± 0.07	0.19 ± 0.05	0.14 ± 0.07 [c]
Cu^{2+}	3.73 ± 0.06	3.22 ± 0.06	0.51 ± 0.08	0.48 ± 0.07	0.48 ± 0.07
Zn^{2+}	2.67 ± 0.03	2.38 ± 0.06	0.29 ± 0.07	0.34 ± 0.06	0.29 ± 0.07 [c]
Cd^{2+}	3.00 ± 0.04	2.69 ± 0.05	0.31 ± 0.06	0.30 ± 0.05	0.30 ± 0.05

[a] For the error limits see footnotes [a] of Tables I and II. [b] See Eqs (*9*). [c] See also text in the final paragraph of Section 3. [d] The values for $\log \Delta_{M/PMEA}$ of ref.[1] were also taken into account.

signify the tendency to be slightly smaller than those for log $\Delta_{M/PME}$; this indicates that the cytosine residue exhibits in these instances a slight steric hindrance toward the metal ion interaction of the ether oxygen. Therefore, considering future studies of metal ion complexes to be made with the dianions of 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine ($PMEDAP^{2-}$) and 9-[2-(phosphonomethoxy)ethyl]guanine ($PMEG^{2-}$), it appears appropriate to define stability enhancements for complexes formed by a ligand consisting of the (phosphonomethoxy)ethyl chain and a noncoordinating residue R of the approximate size of a nucleobase, *i.e.* for $PME–R^{2-}$. Those values which we consider most appropriate and which can be used in future comparisons are listed in column 6 of Table IV. These stability differences are confirmed by the earlier results obtained for the Mg^{2+} complexes[7] of $PMEA^{2-}$, 3-deaza-$PMEA^{2-}$, and 7-deaza-$PMEA^{2-}$, as well as by those for the Mn^{2+} and Zn^{2+} (and Cd^{2+}) complexes[1] of $PMEA^{2-}$.

4. Extent of Chelate Formation in Aqueous Solution for M(PMEC) Complexes

With the results depicted in Fig. 2 in mind and knowing from the evaluations in Section 3 that the stability enhancements observed for the M(PMEC) complexes are solely due to chelate formation of the phosphonate-bound metal ions with the ether oxygen, the question as to the position of the intramolecular equilibrium (*8*) arises. The corresponding dimensionless equilibrium constant K_I is defined by Eq. (*10*); values for K_I may be calculated[1,3,5,53] using Eqs (*11*) which also show how log $\Delta_{M/PM}$ and K_I are interrelated.

$$K_I = [M(PM)_{cl}]/[M(PM)_{op}] \tag{10}$$

$$K_I = \frac{K^M_{M(PM)}}{K^M_{M(PM)_{op}}} - 1 \tag{11a}$$

$$K_I = 10^{\log \Delta} - 1 \tag{11b}$$

In this context it should be emphasized that the reliability of any calculation for K_I depends on the accuracy of the difference log $\Delta_{M/PM}$ which becomes the more important the more similar the two constants in Eqs (*9*)

are. Therefore, only well defined error limits allow a quantitative evaluation of the extent of a possibly formed chelate. Clearly, knowledge of K_I allows then to calculate, using Eq. (*12*), the percentages of the closed form, $M(PM)_{cl}$, occurring in equilibrium (*8*):

$$\% \ M(PM)_{cl} = 100 \ K_I/(1 + K_I) \qquad (12)$$

Application of the indicated procedure[1,3,5,53] yields the results of Table V. Substantial percentages of chelates are formed for all the M(PMEC) species including the complexes of the alkaline earth ions. In the column farthest to the right, the percentages[1] for $M(PME)_{cl}$ are listed. Comparison of these values with those for % $M(PMEC)_{cl}$ shows that the degree of formation of the chelates in both systems are identical within the error limits, confirming thus the conclusions given in Section 3 about the non-participation of the cytosine residue in the M(PMEC) complexes.

TABLE V
Extent of chelate formation according to equilibrium (*8*) for the M(PMEC) complexes as quantified by the dimensionless equilibrium constant K_I (Eqs (*10*), (*11*)) and the percentages of $M(PMEC)_{cl}$ (Eq. (*12*)); those for $M(PME)_{cl}$ (from ref.[1]) are given for comparison (aqueous solution; 25 °C; I = 0.1 M, $NaNO_3$)[a]

M^{2+}	log $\Delta_{M/PMEC}$	K_I	% $M(PMEC)_{cl}$	% $M(PME)_{cl}$
Mg^{2+}	0.16 ± 0.04	0.45 ± 0.14	31 ± 7	40 ± 4
Ca^{2+}	0.11 ± 0.06	0.32 ± 0.19	24 ± 11	28 ± 9
Sr^{2+}	0.11 ± 0.06	0.29 ± 0.17	22 ± 10	15 ± 10
Ba^{2+}	0.15 ± 0.06	0.41 ± 0.21	29 ± 10	21 ± 9
Mn^{2+}	0.19 ± 0.06	0.55 ± 0.21	35 ± 9	46 ± 7
Co^{2+}	0.20 ± 0.06	0.58 ± 0.23	37 ± 9	49 ± 7
Ni^{2+}	0.14 ± 0.07	0.38 ± 0.22	28 ± 12	35 ± 8
Cu^{2+}	0.51 ± 0.08	2.24 ± 0.63	69 ± 6	67 ± 5
Zn^{2+}	0.29 ± 0.07	0.95 ± 0.30	49 ± 8	54 ± 7
Cd^{2+}	0.31 ± 0.06	1.04 ± 0.30	51 ± 7	50 ± 6

[a] For the error limits see footnotes [a] of Tables I and II.

CONCLUSIONS

In Section 2 we have seen that in the mentioned monoprotonated $M(H;PMEC)^+$ complex, the proton is located at the phosphonate group and the metal ions at the nucleobase. The same conclusion has previously been reached[36] for the corresponding $M(H;CMP)^+$ and $M(H;dCMP)^+$ complexes, proving that the cytosine residue is able to bind indeed metal ions; this result is in accord with a previous study[43] on cytidine.

Therefore, it is surprising that no evidence could be found for any metal ion binding of the cytosine residue in the M(PMEC) complexes (Sections 3, 4) which predominate in the physiological pH range and which do contain, however, in their stability a contribution from the interaction with the ether oxygen. The effects of this latter interaction can be formally eliminated by considering the differences between the $\log \Delta_{M/PM}$ values for the M(PMEC) and the M(PME) complexes. These differences are denoted as $\Delta \log \Delta_{PMEC/PME}$ and are defined in Eq. (*13*):

$$\Delta \log \Delta_{PMEC/PME} = \log \Delta_{M/PMEC} - \log \Delta_{M/PME} \qquad (13)$$

In other words, these differences (Eq. (*13*)) correspond to the $\log \Delta_{M/PM}$ values (Eqs (*9*)) of the M(dCMP) and M(CMP) complexes which solely reflect the influence, if any, of the cytosine residue because $dCMP^{2-}$ and CMP^{2-} contain next to the primary phosphate binding site no other sites with a potential for metal ion binding. The corresponding data are summarized in Table VI.

The results of Table VI show clearly that in all three series of complexes, no positive interaction with the cytosine moiety occurs; hence, in this respect, $PMEC^{2-}$ behaves quite alike as its parent nucleotides[35–37], CMP^{2-} and $dCMP^{2-}$. In the M(CMP) and M(dCMP) complexes, the absence of a nucleobase interaction can be attributed to the fact that these two nucleotides occur preferentially in the *anti* conformation (see Fig. 1, ref.[8]), where the phosphate group points away from the N3 site. In the case of the M(PMEC) complexes, the reasons for the lack of a nucleobase interaction are less clear, especially as the (phosphonomethoxy)ethyl chain is less rigid than the sugar moiety in (2′-deoxy)cytidine 5′-monophosphate. Clearly, one of the reasons is that another potent binding site, *i.e.* the ether oxygen, is within easy reach of a metal ion already coordinated to the phosphonate group. A simultaneous coordination of a metal ion to all three potential binding sites, the phosphonate group, the ether oxygen, and N3, is

sterically rather unfavorable as space-filling molecular models indicate. The alternative species involving a macrochelate of the phosphonate-coordinated metal ion with N3 does evidently not form. Since the percentages of the M(PMEC) complexes in which the metal ions are only phosphonate-coordinated are quite significant (Table V), $PMEC^{2-}$ evidently adopts in solution a conformation in which the N3 site points away from the phosphonate group; hence, $PMEC^{2-}$ appears to resemble CMP^{2-} and $dCMP^{2-}$ also in this respect (see Fig. 1).

Considering that the properties of $PMEC^{2-}$ and of its parent nucleotides $dCMP^{2-}$ and CMP^{2-} are so similar, it is surprising that PMEC is devoid of any significant antiviral activity[18]. Apparently PMEC is not recognized as a substrate by the enzymes in question. Since so-called weak interactions[42], like stacking, hydrogen bonding, *etc.*, play significant roles in the recognition of substrates as well as in their orientation in an active-site cavity, one has to assume that the absence of the sugar moiety, which allows hydrogen-bond formation, cannot be compensated by the remaining interactions possible with the cytosine residue, whereas in HPMPC, the presence of the

TABLE VI
Stability differences quantifying the influence of the cytosine residue in M(PMEC), M(dCMP), and M(CMP) complexes (aqueous solution; 25 °C, I = 0.1 M, $NaNO_3$)[a]

M^{2+}	$\Delta \log \Delta_{PMEC/PME}$ [b]	$\log \Delta_{M/dCMP}$ [c,d]	$\log \Delta_{M/CMP}$ [d,e]
Mg^{2+}	–0.06 ± 0.05	0.01 ± 0.04	–0.01 ± 0.06
Ca^{2+}	–0.02 ± 0.08		–0.04 ± 0.07
Sr^{2+}	0.04 ± 0.08		–0.07 ± 0.06
Ba^{2+}	0.05 ± 0.08		–0.05 ± 0.06
Mn^{2+}	–0.08 ± 0.08		–0.05 ± 0.06
Co^{2+}	–0.09 ± 0.08		–0.07 ± 0.08
Ni^{2+}	–0.05 ± 0.09		0.01 ± 0.08
Cu^{2+}	0.03 ± 0.11	0.01 ± 0.08	0.04 ± 0.11
Zn^{2+}	–0.05 ± 0.09	0.00 ± 0.08	–0.02 ± 0.08
Cd^{2+}	0.01 ± 0.08		–0.04 ± 0.09

[a] For error limits see footnotes [a] in Tables I and II. [b] Defined according to Eq. (*13*). [c] Ref.[36]. [d] Defined according to Eqs (*9*). [e] Ref.[37]; these values were calculated using the micro acidity constant pk = 6.15 (= $pK^{H}_{H(UMP)}$).

additional OH group (Fig. 1) can make up for the loss of the sugar moiety, allowing thus antiviral activity[11–17]. The fact that PMEA (Fig. 1) and HPMPA are both antivirally active[9–11,55] would then indicate that the larger purine residue, which allows intense stacking interactions[42] (next to hydrogen bonding), is able to compensate for the loss of the sugar moiety and allows binding of these nucleotide analogues in active-site cavities of enzymes.

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