Properties of the Ternary (Dien)Pt(PMEA-N7) Complex Containing Diethylenetriamine (Dien) and the Antiviral 9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA).

Synthesis, Biological Screening, Acid-Base Behaviour, and Metal Ion-Binding in Aqueous Solution

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Intramolecular Equilibria, Isomeric Complexes, Mixed Metal Ion Complexes

The synthesis of (Dien)Pt(PMEA-N7), where Dien = diethylenetriamine and PMEA2- = dianion of 9-[2-(phosphonomethoxy)ethyl]adenine, is described. No useful biological activity could be discovered for this complex which is in contrast to the known antiviral properties of PMEA itself. The acidity constants of the twofold protonated $H_2[(Dien)Pt(PMEA-N7)]^{2+}$ complex were determined (UV spectrophotometry and potentiometric pH titration): The release of the proton from the -P(O)=O-H group is only slightly affected by the N7-coordinated (Dien)Pt2+ unit, whereas the acidity of the (N1)H+ site is strongly enhanced. The stability constants of the $M[(Dien)Pt(PMEA-N7)]^{2+}$ complexes with the metal ions M2+ = Mg2+, Ca2+, Mn2+, Co2+, Ni2+, Cu2+, Zn2+, and Cd2+ were measured by potentiometric pH titrations in aqueous solution at 25 °C and $f = 0.1 \text{M (NaNO}_3\text{)}$. Application of previously determined straight-line plots of $\log K_{M(\text{R-P}O_2)\text{H}^-}$ versus pH for simple phosph(on)ate ligands, $\text{R-P}O_2$ where $\text{R}$ represents a non-inhibiting residue without an affinity for metal ions, proves that the primary binding site of the complex-ligand, (Dien)Pt(PMEA-N7), with all the metal ions studied is the phosphonate group; in most instances the expected stability is actually reduced by about 0.4 log units due to the N7-bound (Dien)Pt2+ unit. Only for the Cu[(Dien)Pt(PMEA-N7)]2+ and the Zn[(Dien)Pt(PMEA-N7)]2+ systems the formation of some 5-membered chelates involving the ether oxygen atom of the -CH2-O-CH2-P=O residue could be detected; the formation degrees are 52 ± 9% and 32 ± 14%, respectively. The metal ion-binding properties of (Dien)Pt(PMEA-N7) differ considerably from those of PMEA2-, yet they are relatively similar to those of pyrimidine-nucleoside 5’-monophosphates. The structures of the various complex species in solution are discussed and compared.

1. Introduction

Cisplatin, cis-(NH3)2PtCl2 [1], is a powerful anti-tumor drug [2] and PMEA, adenine(N9)-CH2-CH2-O-CH2-P=O3–, a nucleotide analogue with pronounced antiviral [3] and anticancer [4] properties. Therefore, we considered it as interesting to combine platinum(II) with PMEA to a new compound. We selected Dien as a primary ligand for Pt2+ because N7-coordination of Pt(Dien)2+ to the adenine moiety of PMEA leads then to a ternary complex in which the kinetically inert Pr2+ has a saturated coordination sphere. The structure of (Dien)Pt(PMEA-N7) is shown in Fig. 1.

Aside from screening the biological properties of (Dien)Pt(PMEA-N7), a view on the structure of...
this complex (Fig. 1) gives immediately rise to two questions: (i) How does the N7-bound Pt$^{2+}$ affect the basicity of the phosphonate group of PMEA? (ii) What is the effect of the N7-bound Pt$^{2+}$ on the metal ion affinity of the phosphonate group?

An answer to both questions is of general interest regarding purine nucleotides since the solution structure [5] of PMEA$^{2-}$ is closely related to that of AMP$^{2-}$, which is not true for the complexes of the two ligands [6, 7]. In M(AMP) complexes, where M$^{2+}$ represents a divalent metal ion like Zn$^{2+}$, to some extent macrochelate formation occurs due to an interaction of the phosphate-coordinated metal ion with N7 of the purine moiety [7], whereas in the M(PMEA) complexes the interaction of the phosphonate-coordinated metal ion with the ether oxygen atom is important [6, 8]; an interaction with the adenine residue (via N3) [9] occurs only exceptionally [6b]. The involvement of the ether oxygen atom in M(PMEA) complexes is expressed schematically in eq. (1):

$$\text{R—O—C—P—O}^\text{M}^2+$$

It was recognized already some time ago [10] that this ether oxygen atom is compulsory for an antiviral activity, and an explanation for this observation has been offered recently [6, 11]. Hence, the question: Does equilibrium (1) also play a role in the quaternary M[(Dien)Pt(PMEA-N7)]$^{2+}$ complexes? With the above questions in mind we have determined now via potentiometric pH titrations the stability constants of several quaternary complexes involving divalent and kinetically labile metal ions like Mg$^{2+}$, Mn$^{2+}$ or Zn$^{2+}$. In addition, since the acid-base and metal ion-binding properties of PMEA have previously been studied [6], direct comparison of the equilibrium data becomes possible.

2. Results and Discussion

2.1. Synthesis and biological properties of (Dien)Pt(PMEA-N7)

This ternary complex was prepared by mixing aqueous solutions of [(Dien)Pt(H$_2$O)](NO$_3$)$_2$ and PMEA, both adjusted to pH 1.3. Under these conditions N1 of PMEA is protonated ($p_{OH}^{H}(\text{PMEA}) = 4.16$) [6] and consequently Pt(Dien)$^{2+}$ coordinates at N7 of the adenine residue giving the desired complex (Section 4.1). That (Dien)Pt$^{2+}$ is coordinated to N7 of the adenine residue of PMEA (and not to N1) was proven by $^1$H-$^1$H-ROESY and $^{195}$Pt-$^1$H-HMQC NMR spectroscopy (Section 4.2).

The antiviral activity of (Dien)Pt(PMEA-N7) was examined with herpes simplex viruses (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV), the vaccinia virus (VV) and the retroviruses HIV-1 and HIV-2. The antiviral assays, except for HIV-1, were based on virus-induced cytopathicity in cell cultures (see Section 4.3). Inhibition of HIV-1-induced cytopathicity was studied in human T-lymphoblastoid cells (CEM cells) (Section 4.3). In none of these experiments any significant antiviral activity or toxicity on the host cells by the complex could be proven. Similarly, in vitro cytostatic tests the suppression of the cell growth compared to the control cultures was only between 0 and 10% (see Section 4.3). Hence, no hint about a useful biological activity of the ternary (Dien)Pt(PMEA-N7) complex was obtained.

2.2. Acidity constants of $H_2[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+}$

The nucleotide analogue PMEA$^{2-}$ accepts in the pH range $\geq 0$ three protons; two at the phosphonate group and one at the N1 site of the adenine ring [5]. The same may be surmised for the N7-platinated species (Dien)Pt(PMEA-N7), but as known from related nucleotide complexes it is expected that the protons are released at a lower pH [12 - 14]. Considering that deprotonation of the -P(O)(OH)$_2$ residue of H$_3$(PMEA)$^+$ occurs with $p_{OH}^{H}(\text{PMEA}) = 1.22 \pm 0.13$ [5], one may estimate that the same proton from
Fig. 2. UV absorption spectra measured in 2-cm cells of (Dien)Pt(PMEA-N7) (2.8 \times 10^{-5} \text{ M}) in aqueous solution in dependence on pH; \textit{i.e.}, the pH values were varied from 0.07, 0.34, 0.75, 1.04, 1.08, 1.24, 1.48, 1.73, 1.80, 1.90, 2.13, 2.30, 2.52, 2.75, 3.16, 3.68 to 4.01 (25 °C; \(I = 0.1 \text{ M}, \text{NaClO}_4\), in those instances where [HClO\(_4\)] < 0.1 M; see also Section 4.5).

H\(_3\)[(Dien)Pt(PMEA-N7)]\(^{3+}\) (see Fig. 1) is released with \(pK_a \simeq 0.8\) \([15]\), hence, at very low pH. Of interest in the present context remains therefore the H\(_2\)[(Dien)Pt(PMEA-N7)]\(^{2+}\) species, in which one proton has to be located at the N1 site and the other at the phosphonate group. This means, the following two deprotonation reactions need to be considered:

\[
\begin{align*}
H_2[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} & \rightarrow H[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^+ + H^+ \quad (2a) \\
K_{(2a)}^H & = \frac{[H[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^+][H^+]}{[H_2[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+}]} \quad (2b) \\
H[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^+ & \rightarrow (\text{Dien})\text{Pt}(\text{PMEA-N7}) + H^+ \quad (3a) \\
K_{(3a)}^H & = \frac{[(\text{Dien})\text{Pt}(\text{PMEA-N7})][H^+]}{[H[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^+]} \quad (3b)
\end{align*}
\]

Eq. (2) defines the release of the proton from the (N1)H\(^+\) site and eq. (3) the one from the -P(O)\(_2\)(OH)\(^-\) group of H\(_2\)[(Dien)Pt(PMEA-N7)]\(^{2+}\) (see also below).

Preliminary potentiometric pH titrations indicated that the first deprotonation of H\(_2\)[(Dien)Pt(PMEA-N7)]\(^{2+}\) occurs with \(pK_a < 2\) (eq. (2)) and that this reaction involves the adenine residue, \textit{i.e.} N1; hence, UV spectrophotometry should be applicable \([16]\) for the determination of this acidity constant. Indeed, from Fig. 2 it is evident that the absorption of the complex in aqueous solution depends on the pH; furthermore, as seen in Fig. 3, plots of the absorption at various wavelengths \textit{versus} pH give typical “titration curves”, the evaluation of which provides the acidity constant for eq. (2a).
Table 1. Negative logarithms of the acidity constants of H$_2$[(Dien)Pt(PMEA-N7)]$^{2+}$ (eq. (2), (3)) together with the corresponding values of some related systems as determined by potentiometric pH titrations in aqueous solution (25 °C; $I = 0.1$ M, NaN$_3$)\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Protonated species</th>
<th>$p$K$_{a1}$</th>
<th>$p$K$_{a2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(9MeAde)$^+$</td>
<td>4.10 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>H(PME)$^+$</td>
<td>7.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>H$_2$(PMEA)$^+$</td>
<td>4.16 ± 0.02</td>
<td>6.90 ± 0.01</td>
</tr>
<tr>
<td>H$_2$[(Dien)Pt-(PMEA-N7)]$^{2+}$</td>
<td>1.80 ± 0.10d</td>
<td>6.46 ± 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The error limits given are three times the standard error of the mean value or the sum of the probable systematic and random errors, whichever is larger; \textsuperscript{b} so-called practical, mixed or Brønsted constants are listed (see also the last paragraph in Section 4.7); \textsuperscript{c} this work; \textsuperscript{d} the above value was determined by UV spectrophotometry (25°C; $I = 0.1$ M, NaClO$_4$); this result agrees well with the one obtained from potentiometric pH titrations, $p$K$_{H^+}$[(Dien)Pt(PMEA-N7)] = 1.8 ± 0.3 (Section 4.7).

This result, together with those obtained from potentiometric pH titrations for eq. (3a), are listed in Table 1, where also data for some related systems are given [6a, 17]. From the acidity constants of the monoprotonated species of 9-methyladenine (9MeAde) and (phosphonomethoxy)ethane, CH$_3$CH$_2$-O-CH$_2$-PO$_2$~ (PME$^2$~), given in Table 1 follows clearly that the first deprotonation in H$_2$(PMEA)$^+$ occurs at the (N1)H$^+$ site and the second one at the -P(O)$_2$(OH)$^-$ group [6]; consequently, the same order of deprotonations is followed by H$_2$[(Dien)Pt(PMEA-N7)]$^{2+}$.

2.3. Comparison of the acidity constants of H$_2$[(Dien)Pt(PMEA-N7)]$^{2+}$ with related data

The constant summarized in Table 1 reveal that N7-coordination of the (Dien)Pt$^{2+}$ unit leads to a significant acidification of the (N1)H$^+$ site, i.e. $\Delta p$K$_a = p$K$_{H^+}$[(Dien)Pt(PMEA-N7)] - p$K_{H^+}$[(Dien)Pt(PMEA-N7)] = (4.16 ± 0.02) - (1.80 ± 0.10) = 2.36 ± 0.10. The extent of this acidification is close to the one calculated [14] from data provided by Martin et al. [18] based on $^1$H NMR shift measurements for the Pd$^{2+}$ complex, H[(Dien)Pd(adenosine-N7)]$^{3+}$: $\Delta p$K$_a = p$K$_{H^+}$[(Ado)] - p$K_{H^+}$[(Dien)Pd(adenosine-N7)] = (3.78 ± 0.03) - (1.92 ± 0.1) = 1.9 ± 0.1 (34 °C; $I = 0.5$ M, KNO$_3$); similar results were obtained for related systems [14]. That the acidification of the (Dien)Pt$^{2+}$ unit on the nearby (N1)H$^+$ site (see Fig. 1) is much more pronounced than that on the more distant -P(O)$_2$(OH)$^-$ group (see below) is expected.

The acidification of the -P(O)$_2$(OH)$^-$. proton by the N7-bound (Dien)Pt$^{2+}$ in H[(Dien)Pt(PMEA-N7)]$^{2+}$, i.e. $\Delta p$K$_a = p$K$_{H^+}$[(Dien)Pt(PMEA-N7)] - p$K_{H^+}$[(Dien)Pt(PMEA-N7)] = (6.90 ± 0.01) - (6.46 ± 0.01) = 0.44 ± 0.04 [19]. A very similar result is obtained [12] for the Pd$^{2+}$ complex, H[(Dien)Pd(GMP)]$^+$, using the constants determined earlier [18], $\Delta p$K$_a = 0.39 ± 0.05$. However, this close similarity of the mentioned results may also indicate that the acidification observed for the -P(O)$_2$(OH)$^-$ proton of H[(Dien)Pt(PMEA-N7)]$^+$ is not solely a charge effect of the (Dien)Pt$^{2+}$ unit, but also an effect of outer-sphere macrochelate formation between the -PO$_2$~ residue and one of the -NH$_2$ groups of Dien (Fig. 1); deprotonation of the monoanionic phosphate group favors this type of macrochelate formation as shown for (Dien)Pt(GMP-N7) [20]. These outer-sphere macrochelates were first detected in aqueous solution by Martin et al. [18] for the complexes formed between (Dien)Pd$^{2+}$ and AMP$^{2-}$, IMP$^{2-}$, or GMP$^{2-}$, and confirmed [21] in the solid state and in solution for (En)Pt(GMP)$_2$. For the mentioned cis-(NH$_3$)$_2$Pt(dGuo-N7)(dGMP-N7) complex [19] the formation degree of the outer-sphere macrochelate was calculated [13] as being about 40%. For the present case of (Dien)Pt(PMEA-N7) no firm conclusion is yet possible in this regard, since so far no complex has been studied in which such a macrochelate formation can be excluded with certainty; such data would be needed for comparison.

2.4. Stability constants of the quaternary M[(Dien)Pt(PMEA-N7)]$^{2+}$ complexes

The experimental data of the potentiometric pH titrations in the presence of divalent metal ions (M$^{2+}$) may all be excellently fitted by considering equilibria (3a) and (4a), as long as the evaluation is not carried into the pH range where hydroxo complexes [22] form (Section 4.8).

\textsuperscript{2} M$^{2+}$ + (Dien)Pt(PMEA-N7) $\rightarrow$ M[(Dien)Pt(PMEA-N7)]$^{2+}$ (4a)
Table 2. Stability constant comparison for the M[(Dien)-Pt(PMEA-N7)]
complexes between the potentiometrically measured stability constants (exptl; eq. (4)) and the calculated stability constants (calcd) based on the basicity of the phosphonate group in (Dien)Pt(PMEA-N7) (pK_H^M[(Dien)Pt(PMEA-N7)] = 6.46 ± 0.01, Table 1) and the baseline equations established previously (see eq. (5) and Fig. 4) [6a, 7b, 8a], together with the stability differences log Δ_M/[(Dien)Pt(PMEA-N7) as defined by eq. (6) (aqueous solution; 25 °C; I = 0.1 M, NaN_3)^a.

<table>
<thead>
<tr>
<th>M^{2+}</th>
<th>log K_M^M[(Dien)Pt(PMEA-N7)]</th>
<th>Δ_M/[(Dien)Pt(PMEA-N7)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg^{2+}</td>
<td>1.22 ± 0.04</td>
<td>-0.40 ± 0.06</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>1.01 ± 0.12</td>
<td>-0.47 ± 0.13</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>1.79 ± 0.07</td>
<td>-0.43 ± 0.09</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>1.61 ± 0.08</td>
<td>-0.38 ± 0.10</td>
</tr>
<tr>
<td>Ni^{2+}</td>
<td>1.56 ± 0.16</td>
<td>-0.44 ± 0.17</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>2.89 ± 0.04</td>
<td>-0.10 ± 0.07</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>1.96 ± 0.05</td>
<td>-0.25 ± 0.08</td>
</tr>
<tr>
<td>Cd^{2+}</td>
<td>2.13 ± 0.09</td>
<td>-0.39 ± 0.10</td>
</tr>
</tbody>
</table>

For the error limits see footnote[a] of Table 1. The error limits (3σ) of the derived data, in the present case for column 4, were calculated according to the error propagation after Gauss.

K_M^M[(Dien)Pt(PMEA-N7)] = K_M^M[(Dien)Pt(PMEA-N7)]^2

The stability constants determined in this study are listed in column 2 of Table 2; none of these constants has been determined before. Interestingly, the results confirm the long-standing experience [23] that the stabilities of phosph(on)ate-metal ion complexes often do not strictly follow [5 - 8, 24] the Irving-Williams series [25]; i.e., as observed before [15b, 24] the Mn^{2+} complex is more stable than those with Co^{2+} and Ni^{2+}.

The question that arises for the M[(Dien)Pt(PMEA-N7)]^{2+} complexes is: To what extent does the N7-bound (Dien)Pt^{2+} at the adenine residue (Fig. 1) affect metal ion binding at the phosphonate group? Should charge repulsion of the two twofold positively charged metal ions within the complex occur, then its stability should be reduced. Therefore, it is necessary to define the stability of a pure, unaffected -PO_3^2- /M^{2+} interaction. This can be done by applying the previously defined [6a, 7b, 8a, 24b] straight-line correlations, which are based on log K_M^M[(R-PO_3)] versus pK_H^M[(R-PO_3)] plots for simple phosphate monoesters [26] and phosphonates [6]. These ligands are abbreviated as R-PO_3^{2-}, where R represents a non-coordinating and non-inhibiting residue. The parameters for the corresponding straight-line equations, which are defined by equation (5),

log K_M^M[(R-PO_3)] = m · pK_H^M[(R-PO_3)] + b

have been tabulated [6a, 7b, 8a, 24b], i.e., the slopes m and the intercepts b with the y-axis. Hence,
with a known pK_a value for the deprotonation of a -P(O)_{2} group an expected stability constant can be calculated for any phosph(on)ate-metal ion complex.

Plots of log K^M_{MR-PO_{2}} versus pK^H_{M-PO_{2}} according to eq. (5) are shown in Fig. 4 for the 1:1 complexes of Ca^{2+}, Ni^{2+}, and Cu^{2+}, as examples, with the data points (empty circles) of the eight simple ligand systems used [6a] for the determination of the straight baselines. The three solid points refer to the corresponding M[(Dien)Pt(PMEA-N7)]^{2+} complexes; those for the Ca^{2+} and Ni^{2+} species are far below their reference lines, whereas the one for the Cu^{2+} complex is only slightly below, but for all three complexes a reduced stability is evident.

2.5. Evaluation of the stabilities of the M[(Dien)Pt(PMEA-N7)]^{2+} complexes

Stability reductions like those expressed in Fig. 4 by the vertical broken lines can be quantified by the differences between the experimentally (exptl) measured stability constants and those calculated (calcd) according to eq. (5); this difference is defined in equation (6):

\[
\log \Delta_{M/\text{Dien}Pt(PMEA-N7)} = \log K^M_{M[(\text{Dien})Pt(PMEA-N7)]_{\text{exptl}}} - \log K^M_{M[(\text{Dien})Pt(PMEA-N7)]_{\text{calcd}}} \tag{6}
\]

In columns 2 - 4 of Table 2 the values for the three terms of eq. (6) are listed. It is evident that all values in column 4 carry a negative sign, thus proving that all M[(Dien)Pt(PMEA-N7)]^{2+} complexes are less stable than expected on the basis of the basicity of the phosphonate residue. A closer look at these data (Table 2, column 4) indicates that the values are actually identical within their error limits, except the ones for the Cu^{2+} and Zn^{2+} systems, which are somewhat less negative.

Does the above indicated relative stability increase of the Cu^{2+} and Zn^{2+} complexes mean that in these instances eq. (1) is of relevance and that the ether oxygen atom participates in metal ion binding? To evaluate this possibility we have listed in column 2 of Table 3 the stability enhancements log \Delta_{M/\text{PMEA-R}} (defined in analogy to eq. (6)), which are due to complexes formed with a 2-(phosphonomethoxy)ethyl chain which carries a non-coordinating residue R of the approximate size of a nucleobase [27]. These log \Delta_{M/\text{PMEA-R}} values differ somewhat from metal ion to metal ion; in fact, the tendency to form the 5-membered chelate seen in eq. (1) is expected to depend on the kind of metal ion involved.

Therefore, if the mentioned log \Delta_{M/\text{PMEA-R}} values (Table 3, column 2) are added to the calculated values (based on eq. (5)) listed in column 3 of Table 2, one obtains the 'corrected' stability constants listed in column 4 of Table 3 and these values reflect the expected stabilities of M(PMEA) complexes in which no nucleobase-metal ion coordination occurs. Formation of the difference between these values and those determined experimentally (Table 3, column 3) reflects then the charge repulsion of (Dien)Pt^{2+} at N7 on the overall stability of the M(PMEA) complexes involved in eq. (1). Of course, introduction of a positive charge at the adenine residue of PMEA^{2-} should affect complex formation with all divalent metal ions to the same extent. In fact, all the values given for log \Delta_{corr} in column 5 of Table 3 are identical within the error limits [the arithmetic mean of the 8 values gives log \Delta_{corr/av} = -0.59 ± 0.05 (3σ)]. This then proves that the stability of all M[(Dien)Pt(PMEA-N7)]^{2+} complexes is diminished to the same extent, indeed, provided the overall stability of the M(PMEA) com-

Table 3. Stability constant comparisons, log \Delta_{corr} (column 5), for the M[(Dien)Pt(PMEA-N7)]^{2+} complexes between the potentiometrically measured stability constants (exptl; eq. (4)) taken from column 2 in Table 2 and the calculated stability constants corrected (calcd/corr) for the metal ion-ether oxygen interaction\(^a\). The corrected, calculated stability constants were obtained by adding to the calculated values given in column 3 of Table 2 the stability enhancements, log \Delta_{M/\text{PMEA-R}} (column 2) \(^b\) which result from the M^{2+}-ether oxygen interaction (eq. (1)) in M(PMEA-R) complexes (aqueous solution; 25 °C; I = 0.1 M, NaNO_3)^c.

<table>
<thead>
<tr>
<th>M^{2+}</th>
<th>log \Delta_{M/\text{PMEA-R}} (^b)</th>
<th>log K^M_{M[(\text{Dien})Pt(PMEA-N7)]_{\text{exptl}}}</th>
<th>log K^M_{M[(\text{Dien})Pt(PMEA-N7)]_{\text{calcd/corr}}}</th>
<th>log \Delta_{corr} (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg^{2+}</td>
<td>0.16 ± 0.04</td>
<td>1.22 ± 0.04</td>
<td>1.78 ± 0.06</td>
<td>-0.56 ± 0.07</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>0.12 ± 0.05</td>
<td>1.01 ± 0.12</td>
<td>1.60 ± 0.07</td>
<td>-0.59 ± 0.14</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>0.19 ± 0.06</td>
<td>1.79 ± 0.07</td>
<td>2.41 ± 0.08</td>
<td>-0.62 ± 0.11</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>0.20 ± 0.06</td>
<td>1.61 ± 0.08</td>
<td>2.19 ± 0.08</td>
<td>-0.58 ± 0.11</td>
</tr>
<tr>
<td>Ni^{2+}</td>
<td>0.14 ± 0.07</td>
<td>1.56 ± 0.16</td>
<td>2.14 ± 0.09</td>
<td>-0.58 ± 0.18</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>0.48 ± 0.07</td>
<td>2.89 ± 0.04</td>
<td>3.47 ± 0.09</td>
<td>-0.58 ± 0.10</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>0.29 ± 0.07</td>
<td>1.96 ± 0.05</td>
<td>2.50 ± 0.09</td>
<td>-0.54 ± 0.10</td>
</tr>
<tr>
<td>Cd^{2+}</td>
<td>0.30 ± 0.05</td>
<td>2.13 ± 0.05</td>
<td>2.82 ± 0.07</td>
<td>-0.69 ± 0.11</td>
</tr>
</tbody>
</table>

\(^a\) For the error limits (3σ) and the error propagation see footnote [a] of Table 2; \(^b\) these differences are defined in analogy to eq. (6); they are taken from Table IV in [27]; \(^c\) these values correspond to the differences between columns 3 and 4.
plexes occurring in eq. (1) is used as a basis for the comparisons.

2.6. Chelate formation in the \( \text{M}[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} \) complexes of \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \)

The insight described in Section 2.5 allows now to evaluate further the results listed in column 4 of Table 2. If one ignores the \( \Delta \log K_{M/(Dien)Pt(PMEA-N7)}^{2+} \) values for the \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \) complexes formed with (Dien)Pt(PMEA-N7), one obtains as the arithmetic mean of the remaining six values \( \Delta \log K_{M/(Dien)Pt(PMEA-N7)}^{2+} \) = \(-0.42 \pm 0.04 \) (3σ); in other words, the values for the \( \text{Mg}^{2+} \), \( \text{Ca}^{2+} \), \( \text{Mn}^{2+} \), \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \), and \( \text{Cd}^{2+} \) complexes are identical within the error limits with the given arithmetic mean. Consequently, the value for \( \Delta \log K_{M/(Dien)Pt(PMEA-N7)}^{2+} \) reflects the stability decrease expected for the situation when only the ‘open’ (op) isomer in eq. (1) is formed.

The above reasoning means that the difference of the differences as defined in eq. (7)

\[
\Delta \log \Delta = \log \Delta K_{M/(Dien)Pt(PMEA-N7)}^{2+} - \log \Delta K_{M/(Dien)Pt(PMEA-N7)}^{2+}
\]

is a reflection of the formation degree of the chelate in eq. (1). The value for \( \Delta \log \Delta \) for the \( \text{Cu}^{2+} \) species amounts to \((-0.10 \pm 0.07) - (-0.42 \pm 0.04) = 0.32 \pm 0.08 \) and for the \( \text{Zn}^{2+} \) complex to \((-0.25 \pm 0.08) - (-0.42 \pm 0.04) = 0.17 \pm 0.09 \). If we term the chelated isomer in eq. (1) as ‘closed’ (cl), the intramolecular equilibrium constant for eq. (1) is defined by eq. (8):

\[
K_{1} = \frac{[M[(Dien)Pt(PMEA-N7)]^{2+}_{\text{cl}}]}{[M[(Dien)Pt(PMEA-N7)]^{2+}_{\text{op}}]}
\]

As described previously [6, 7, 24, 28], based on the values \( \Delta \log \Delta \), the intramolecular equilibrium constant \( K_{1} \) and the formation degree of the chelated species can be calculated. The results are for the \( \text{Cu}[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} \) system \( K_{1} = 1.09 \pm 0.38 \), which gives a formation degree of 52 ± 9% for the chelated isomer in eq. (1), and for the \( \text{Zn}^{2+} \) system, \( K_{1} = 0.48 \pm 0.31 \), from which a formation degree of 32 ± 14% follows for the chelate.

To conclude, there is no evidence for chelate formation according to eq. (1) in the \( M[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} \) complexes of \( \text{Mg}^{2+} \), \( \text{Ca}^{2+} \), \( \text{Mn}^{2+} \), \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \), and \( \text{Cd}^{2+} \) whereas the corresponding complexes of \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \) do occur, albeit to a limited extent. This result is in line with the general experience in coordination chemistry that among the divalent metal ions of the second half of the 3d-transition series the complexes of \( \text{Cu}^{2+} \) are the most stable (usually) followed by those with \( \text{Zn}^{2+} \).

3. Conclusions

From the presented results it is evident that the metal ion-binding properties of (Dien)Pt(PMEA-N7) and PMEA\(^{2-} \) differ considerably; this is despite the fact that the basicity of the phosphonate residue is only slightly lowered by the N7-bound (Dien)Pt2+ unit (Table 1). For both ligands the phosphonate group remains the initial and stability determining binding site in complexes, but there is no indication for an interaction with the adenine residue in the \( M[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} \) complexes and the formation of the 5-membered chelates according to eq. (1) also occurs only exceptionally. It is especially this latter point which is responsible for the different structures of the complexes in solution: For the M(PMEA) species [6] eq. (1) is important for all divalent metal ions. In fact, it is eq. (1) which is mainly responsible for the different properties of the complexes formed [6] with PMEA\(^{2-} \) in comparison with those formed by its parent nucleotide AMP\(^{2-} \) [6a, 26].

Interestingly, because in the \( M[(\text{Dien})\text{Pt}(\text{PMEA-N7})]^{2+} \) complexes \( \text{M}^{2+} \) binding occurs largely only via the phosphonate group, the structures of the complexes with the biologically relevant metal ions Mg\(^{2+} \) and Ca\(^{2+} \) are very similar to those of AMP\(^{2-} \) [7, 24]; these complexes exist for both ligands only in the open phosph(on)ate-bound form of eq. (1). In fact, to a first approximation this is also true for the complexes of Mn\(^{2+} \) and Zn\(^{2+} \). Furthermore, if one ignores the structural differences of the nucleobases present in (Dien)Pt(PMEA-N7) and in the pyrimidine-nucleoside 5'-monophosphates (PNMP\(^{2-} \)) [6a, 26], the acid-base \( pK_{H}^{\beta}[(\text{Dien})\text{Pt}(\text{PMEA-N7})] = 6.46 \) (Table 1) versus \( pK_{H}^{\beta}(\text{PNMP}) \approx 6.2 \) [6a, 26] and also the metal ion-binding properties of all these ligands are very similar; they all form only the simple phosph(on)ate-bound species. Maybe, here is a reason why the ternary (Dien)Pt(PMEA-N7) complex is so little toxic and shows no pronounced biological activity (Section 2.1) in contrast to its platinum(II)-free parent ligand PMEA [3, 4, 10].
4. Experimental Section

4.1. Synthesis of [(Dien)Pt(PMEA-N7)]·HNO₃ · NaNO₃ · H₂O

K₂PtCl₄ was obtained from Heraeus GmbH, Hanau, Germany. PMEA was prepared as described ([29] and references therein). All the other chemicals used in the synthesis were from Merck GmbH, Darmstadt, Germany, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, or from Fluka AG, Buchs, Switzerland.

A solution of [(Dien)Pt(H₂O)](NO₃)₂ (0.55 mmol), obtained from [(Dien)PtII] [30] and AgNO₃ (2 equiv.) in water (20 ml) in the dark (24 h at 35 °C) and subsequent cooling to 4 °C followed by filtration of AgI, was adjusted to pH 1.3 with 1 M HNO₃. The same pH was adjusted in an aqueous solution (10 ml) of PMEA (0.55 mmol). The PMEA solution was slowly added to the other one during a period of 3 h and the combined solutions were then stirred for 24 h at 35 °C, before being brought to dryness (room temperature, N₂ stream). The pale yellow residue was treated twice with methanol (12 h, ambient temperature) and filtered. The isolated yield was 49%. Potentiometric pH titrations and NMR measurements were consistent with the composition [(Dien)Pt(PMEA-N7)]·HNO₃·NaNO₃·H₂O. Analysis for C₁₂H₂₆N₁₀O₁₁PtNa: calcd. C 19.6, H 3.6, N 19.0; found C 19.8, H 3.5, N 19.0. ¹H NMR (D₂O; pD 5.9): δ = 8.76 (s, H₈, PMEA), 8.32 (s, H₂, PMEA), 4.50 (t, N-CH₂, PMEA), 3.91 (t, O-CH₂, PMEA), 3.59 (d, O-CH₂-PO₃, PMEA).

4.2. Determination of the structure of (Dien)Pt-(PMEA-N7) by NMR

¹H NMR and ¹H,¹H-ROESY spectra were recorded on a Bruker DRX 400 instrument in D₂O with sodium-3-(trimethylsilyl)-1-propanesulfonate (TSP) as internal reference. ¹⁹⁵Pt NMR and ¹⁹⁵Pt,¹H HMOC spectra were obtained on a Bruker AC 200 FT spectrometer in D₂O with shifts referenced to external PtCl₆²⁻. Fig. 5 shows the ¹H,¹H-ROESY spectrum of the above compound at pD 5.9.

The cross peak between the resonances of the aromatic proton at 8.76 ppm and the N9-bound methylene moiety at 4.50 ppm unambiguously identifies the former signal as H₈. Consequently, the other signal in the aromatic region at 8.32 ppm has to correspond to the H₂ proton. It may be added that this assignment of the H₂ and H₈ protons is by making use of the fact that the H₈ proton of adenine and its derivatives is relatively acidic; i.e., in alkaline D₂O solution and at increased temperature a H-D exchange takes place. This effect should be enhanced by N7-coordinated Pt(II) and indeed, this is observed: After one day at 45 °C and pD 10.5 the signal at 8.76 ppm (H₈) of the N7-coordinated product was no longer detectable in the ¹H-NMR spectrum whereas no effect could be observed in the ¹H NMR spectrum [31] of the N1-coordinated compound.

In the ¹⁹⁵Pt,¹H HMOC spectra (not shown), the H₈ signal at 8.76 ppm couples with the ¹⁹⁵Pt NMR signal at ~2874 ppm, thus proving that the platination site is N7. A further indication that the assignment of the platination site is correct stems from the close similarity of the ¹H and ¹⁹⁵Pt NMR data obtained now and the data published [33] for the (Dien)Pt(adenosine-N7)²⁺ complex.

4.3. Biological screening of (Dien)Pt(PMEA-N7)

The antiviral activity of the ternary platinum(II) complex was examined with herpes simplex viruses, HSV-1 (KOS) and its TK⁻ mutants (VMW 1837, ACVr) and HSV-2, with cytomegalovirus (CMV) (Davis, AD-169), varicella zoster viruses (VZV) (TK⁻ OKA, YS, TK⁻ O7/1, YS/R), the vaccinia virus (VV), and the retroviruses HIV-1 and HIV-2. The antiviral assays, other than HIV-1 were based on the inhibition of virus-induced cytopathicity in either E₆SM (HSV-1, HSV-2, VV) or HEL
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(VZV, CMV) cell cultures, following previously established procedures (see [29] and references therein). Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compound. Inhibition of HIV-1-induced cytopathicity in CEM cells followed the methodology of the anti-HIV assays described previously [29]. In none of these experiments the complex exerted any significant activity or any toxicity on the host cells.

The cytostatic activity in vitro was established in HeLa, L-1210, P388 and CEM cells under standard conditions (see [34] and references therein). At 10 μM concentration of the suppressor, the suppression of the cell growth, compared to the control cultures, was not significant, i.e. 0 to 10% only.

4.4. Materials for the UV spectrophotometric measurements and the potentiometric pH titrations

All solutions were prepared with deionized, ultrapure (MILLI-Q185 PLUS; from Millipore S. A., 67120 Molsheim, France) CO₂-free water. The disodium salt of 1,2-diaminoethane-N,N,N',N'-tetraacetic acid (Na₂H₂EDTA), potassium hydrogen phthalate, HNO₃, NaOH (Titrisol), HClO₄ (70 - 72%), NaClO₄, and the nitrate salts of Na²⁺, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺ and Cd²⁺ (all pro analysi) were from Merck AG, Darmstadt, Germany.

The buffer solutions (pH 4.00, 7.00, 9.00 based on the NBS scale; now NIST) used for calibration (Section 4.5 and 4.6) were from Metrohm AG, Herisau, Switzerland.

The exact concentrations of the stock solutions of the divalent metal ions were determined by potentiometric pH titrations via their EDTA complexes. The stock solutions of the ligand, i.e. of (Dien)Pt(PMEA-N7), were freshly prepared daily by dissolving the compound (Section 4.1) in ultrapure water with one equivalent of NaOH and adjusting the pH to 8.5; the exact concentration was each time newly determined by the evaluation of the corresponding titration pairs described below in Section 4.7.

4.5. Spectrophotometric determination of the first acidity constant of H₂[(Dien)Pt(PMEA-N7)²⁺]

The acidity constant (eq. (2)) $K_{H}^{\text{+}}$[(Dien)Pt(PMEA-N7)], which refers to the deprotonation of the (N1)H⁺ site (Fig. 1), was determined by recording UV spectra (sample beam: HClO₄, NaClO₄ and [(Dien)Pt(PMEA-N7)] = 2.8 × 10⁻⁵ M; reference beam: HClO₄ and NaClO₄) in aqueous solutions at 25 °C in dependence on pH with 2-cm quartz cells with a Cary 3C spectrophotometer connected to a Compaq 2000 5/166 PC. The ionic strength was adjusted to $I = 0.1$ M (NaClO₄) in those instances where [HClO₄] < 0.1 M; no adjustment was made in solutions with pH ≤ 1, i.e. where [HClO₄] ≥ 0.1 M. The pH of the solutions was adjusted with HClO₄ and measured with a Metrohm 713 digital pH meter using a Metrohm 60216100 (PC) glass electrode in the pH range ≥ 1 (see also Section 4.4); lower pH values (≤ 1) were calculated by determining the acidity of HClO₄ in these solutions as described below. Each solution was individually prepared. An example of an experimental series is shown in Fig. 2.

The spectrophotometric data were analyzed with an IBM compatible Pentium desk computer connected to an Epson Stylus 1500 printer (data) as well as a Hewlett-Packard Deskjet 1600CM printer (curves) by a curve-fitting program applying a Newton-Gauss non-linear least-squares fitting procedure. An example of such an evaluation is shown in Fig. 3. The final result given in Table 1 is the average of two independent series of experiments.

To obtain a well defined absorption of the twofold protonated species, H₂[(Dien)Pt(PMEA-N7)]²⁺, it was necessary to record several spectra at pH < 1 (see Fig. 3). Since the activity coefficients of perchloric acid in higher concentrations differ from 1, the following procedure was applied to calculate the pH of a solution: Values of the mean molar activity coefficients in dependence on the molality of HClO₄ are known [35]. To obtain activity coefficients in dependence on the molarity, the density of HClO₄ must be taken into account; these values were interpolated from data listed [36] for the dependence of the density on the weight percentage of HClO₄. In this way for each molarity the corresponding weight percentage of HClO₄ was obtained. The also needed density of water at 25 °C was interpolated from the data given in [37], where the density of water at different temperatures (but not 25 °C) is listed. Thereafter the mean molar activity coefficients could be calculated and now the activity of HClO₄ at the different concentrations given in [35] became known. From these data we interpolated the activity of HClO₄ at the concentrations used in our experiments; in the following the first value refers to the calculated pH and the second one given in parentheses to the concentration of HClO₄: pH = 0.07 [(HClO₄) = 1.08 M], 0.34 (0.60 M), 0.75 (0.24 M) and 1.04 (0.12 M). It may be added that from pH 1.08 [(HClO₄) = 0.07 M] on the values measured with the glass electrode were used (see also the last paragraph in Section 4.7).

4.6. 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 glass electrode. The pH calibration of the instrument was
done with the buffers mentioned above (Section 4.4). The titre of the NaOH used for the potentiometric pH titrations was determined with potassium hydrogen phthalate.

4.7. Determination of the acidity constants of 
\[ H_2[\text{(Dien)Pt(PMEA-N7)}]^2+ \]

The acidity constants \( K_{H^+}^{\text{M}} \) and \( K_{H^+}^{\text{M}} \) of \( H_2[\text{(Dien)Pt(PMEA-N7)}]^2+ \) (eq. (2), (3)), where one proton is at the nucleobase moiety (N1) and the other at the phosphonate group, were determined by titrating 20 ml, 24 ml and 28 ml of aqueous 0.006 M, 0.005 M and 0.0043 M HNO\(_3\), respectively, in the presence and absence of 0.00096 M, 0.00080 M and 0.00086 M (Dien)Pt(H;PMEA-/V7) under Na\(_2\) with 2 ml 0.07 M NaOH (25 °C; \( I = 0.1\) M, NaNO\(_3\)).

In another set of experiments only \( K_{H^+}^{\text{M}} \) (eq. (3)) was determined by titrating 30 ml of an aqueous 0.0003 M HNO\(_3\) in the presence and absence of 0.00032 to 0.00041 M (Dien)Pt(H;PMEA-N7) under Na\(_2\) with 1 ml 0.03 M NaOH (25 °C; \( I = 0.1\) M, NaNO\(_3\)).

The acidity constants were calculated with the mentioned computer facilities (Section 4.5) using a Newton-Gauss non-linear least-squares fitting procedure. The calculations were carried out between about 2 and 98% neutralization for the equilibrium of \( H_2[\text{(Dien)Pt(PMEA-N7)}]^+/\text{(Dien)Pt(PMEA-N7)} \) and about 81, 83 or 84 and 100% neutralization for the equilibrium of \( H_2[\text{(Dien)Pt(PMEA-N7)}]^2+ / H_2[\text{(Dien)Pt(PMEA-N7)}]^+ \); it is evident that the latter result is only an estimate. The final results for \( pK_{H^+}^{\text{M}} \) and \( pK_{H^+}^{\text{M}} \) are the averages of 18 and 3 independent pairs of titrations, respectively.

It may be emphasized that the direct pH-meter readings were used in the calculations of the acidity constants; i.e., these constants are so-called practical, mixed or Brønsted constants [38]. Their negative logarithms may be converted into the corresponding concentration constants by subtracting 0.02 from the listed \( pK_a \) values [38]; this conversion term contains both the junction potential of the glass electrode and the hydrogen ion activity [38, 39]. As the difference in NaOH consumption between pairs of solutions, i.e., with and without ligand (see above) [38], is evaluated, the ionic product of water \( (K_w) \) and the mentioned conversion term do not enter into the calculations.

4.8. Determination of the stability constants of 
\[ M[\text{(Dien)Pt(PMEA-N7)}]^2+ \] complexes

The stability constant \( K_{M^+}^{\text{M}} \) of \( M[\text{(Dien)Pt(PMEA-N7)}]^2+ \) complexes (eq. (4)) was determined either under the conditions given above (Section 4.7) for the second acidity constant \( \{[\text{(Dien)Pt(H;PMEA-N7)}] = 0.00032 \text{ to } 0.00041 \text{ M}, \text{ with NaNO}_3 \text{ being partly or fully replaced by } M[\text{NO}_3]^2_2 (I = 0.1 \text{ M; } 25 \text{ °C}), \text{ or the solutions used for the determination of the acidity constant, } K_{H^+}^{\text{M}} \). The acidity constants \( K_{H^+}^{\text{M}} \) were used again because only small amounts of (Dien)Pt(PMEA-N7) were available; i.e., the solutions were acidified with the equivalent amount of HNO\(_3\) as NaOH had been used in the titration for the acidity constant, and then M(NO\(_3\))\(_2\) was added (volume 50 ml; \( I = 0.1\) M, NaNO\(_3\)) and the titration repeated with 0.03 M NaOH (the various dilutions being taken into account in the calculations). Application of this latter method to the Mg\(^{2+}\) and Ca\(^{2+}\) systems led to a somewhat higher ion strength, i.e., \( I = 0.12\) M; these results did not differ within the error limits from those obtained with \( I = 0.1\) M.

The Mg\(^{2+}\)-ligand ratios employed in the experiments were approximately 84:1 for Mg\(^{2+}\), 102:1 or 85:1 for Ca\(^{2+}\), 63:1 or 44:1 for Mn\(^{2+}\), 74:1 or 48:1 for Co\(^{2+}\), 68:1 or 46:1 for Ni\(^{2+}\), 64:1 or 53:1 or 41:1 for Zn\(^{2+}\), 46:1 or 29:1 for Cd\(^{2+}\), and 10:1 or 8:1 or 5:1 for Cu\(^{2+}\). The experimental data for all these Mg\(^{2+}\) systems were evaluated by determining with the curve-fitting procedure (Section 4.7) the so-called [15b, 40] “apparent” acidity constant, \( K_a \), for the deprotonation of \( H[\text{(Dien)Pt(PMEA-N7)}] \) which is valid only for the given conditions and in the presence of a significant excess of Mg\(^{2+}\), that is, \( [\text{Mg}^{2+}]_{\text{free}} \approx [\text{Mg}^{2+}]_{\text{total}} \) [41]. With the value determined for \( K_a \) and the acidity constant \( K_{H^+}^{\text{M}} \) determined previously (Section 4.7), the stability constant \( K_{M^+}^{\text{M}} \) (eq. (4)) can be calculated [15b, 40].

The stability constants calculated individually for the various experiments showed no dependence on the metal ion concentration. The final results are in each case the averages of at least two (usually three) different pairs of titrations.

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Abbreviations and definitions: Ado, adenosine; AMP$^{2-}$, adenosine 5'-monophosphate; av, average value (arithmetic mean); CEM cells, human T-lymphoblastoid cells; Cisplatin, cis-diaminedichloroplatinum(II); CMV, cytomegalovirus; Dien, diethylenetriamine (=3-azapentane-1,5-diamine); En, ethylenediamine (=1,2-diaminoethane); GMP$^{2-}$, guanosine 5'-monophosphate; Guo, guanosine; HIV, human immunodeficiency virus; HMOC, heteronuclear multiple quantum coherence spectroscopy; HSV, herpes simplex virus; IMP$^{2-}$, inosine 5'-monophosphate; $K_a$, acidity constant; M$^{2+}$, divalent metal ion; 9MeAden, 9-methyladenine; PDE$^{2-}$, diacid of (phosphonomethoxy)ethane (=ethoxymethanephosphonate); PMEA$^{2-}$, dianion of 9-[2-(phosphonomethoxy)ethyl]adenine; ROESY, rotating frame Overhauser effect spectroscopy; $R^-$PO$_2^-$, simple phosphate monoester or phosphonate ligand with $R$ representing a non-coordinating residue (see also legend of Fig. 4); TK, thymidine kinase; TSP, trimethylsilylpropane sulfonate; VV, vaccinia virus; VZV, varicella zoster virus. Species written without a charge either do not carry one (i.e., independent of their protonation degree); which of the two possibilities applies is always clear from the context.


[22] The beginning of the hydrolysis of M(aq)$^{2+}$ (e.g., with Cu$^{2+}$ or Zn$^{2+}$) was evident from the titrations with Cu$^{2+}$ or Zn$^{2+}$.) was evident from the titrations.
[41] For the 5:1 Cu²⁺ system one could argue that [Cu²⁺]_{free} ≃ [Cu²⁺]_{tot} is no longer valid, therefore we repeated the calculations by taking into account free Cu²⁺ as well as the formation of Cu[(Dien)Pt(PMEA-N7)²⁺]. The result of these calculations differed from the simplified ones only by 0.02 log unit; hence, the results are identical within their error limits.