Why is the antiviral nucleotide analogue 9-[2-(phosphonomethoxy)ethyl]adenine in its diphosphorylated form ($PMEApp^{4-}$) initially a better substrate for polymerases than (2′-deoxy)adenosine 5′-triphosphate ($dATP^{4-}$/ATP^{4-})? Considerations on the mechanism of nucleic acid polymerases

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The observation that the antivirally active PMEA in its diphosphorylated form ($PMEApp^{4-}$) is initially a better substrate for polymerases than $dATP^{4-}$ (ATP^{4-}) can be rationalized by (i) the increased basicity of the phosphonyl group (compared to a phosphoryl group) and (ii) the participation of the ether O atom of $PMEApp^{4-}$ in metal ion binding; both effects together favor M^{2+} binding at the α group and thus its nucleophilic attack.

Since adenosine 5′-triphosphate is at the crossroad of many metabolic processes, the search for analogues which can be employed as therapeutic agents is long-standing.[1] A promising attempt is presently focusing on 9-[2-(phosphonomethoxy)-ethyl]adenine (PMEA) and related derivatives,[1,2] which can be considered as acyclic analogues of adenosine 5′-monophosphate (AMP^{2-}; Fig. 1)[3] as well as of its 2′-deoxy or 2′,3′-dideoxy derivatives. PMEA shows antiviral properties and is active, after its diphosphorylation by kinases,[4] against a variety of DNA viruses and retroviruses (*e.g.* human immunodeficiency viruses; HIV).[1,4]

The triphosphate analogue ($PMEApp^{4-}$) is recognized by nucleic acid polymerases as substrate and incorporated in the growing nucleic acid chain, which is then terminated due to the lack of a 3′-hydroxy group, which is present in the parent adenosine 5′-triphosphate (ATP^{4-}) and 2′-deoxyadenosine 5′-triphosphate ($dATP^{4-}$) nucleotides.[4,5] Indeed, $PMEApp^{4-}$ is initially an excellent substrate, *e.g.* for reverse transcriptases, which are effectively inhibited even in the presence of a 20-fold excess of $dATP^{4-}$; similar observations have been made for other DNA polymerases.[6] Why are $PMEApp^{4-}$ and its relatives excellent substrates for polymerases? We are suggesting below that this is due to the special metal ion-binding properties of these nucleoside 5′-triphosphate (NTP) analogues.

Kinetic studies of the M^{2+}-promoted dephosphorylation of ATP^{4-} and other triphosphates have shown[7] that in the most reactive species one metal ion is coordinated to the α,β-phosphate groups and one to the terminal γ-phosphate group. This transphosphorylation mechanism was recently confirmed in biological systems by an X-ray structural study of *Escherichia coli* phosphoenolpyruvate carboxykinase.[8] The mentioned kinetic studies[7] have also led to the conclusion that the two activating metal ions 'may interact not only in a M(α,β)–M(γ)-like way but that a M(α)–M(β,γ) coordination can also be enforced (by an enzyme) and this would then lead to a reactive species ready for the transfer of . . . a nucleoside monophosphate' unit.[7a] Indeed, X-ray studies of nucleic acid polymerases have confirmed that two metal ions are involved in this process and corresponding mechanisms were proposed.[9]

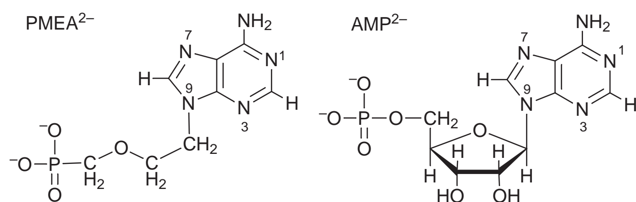
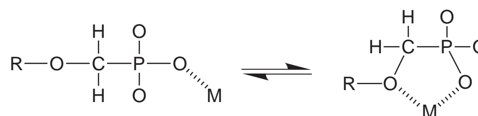


Fig. 1 $PMEA^{2-}$ in comparison to AMP^{2-} which is depicted in its dominating *anti* conformation; the structure of $PMEA^{2-}$ is analogous.[3]

The crucial step in the polymerase mechanism indicated above is to force a metal ion into the α position of the triphosphate chain[7] of an NTP^{4-}. Hence, one might suspect that $PMEApp^{4-}$, being initially an excellent substrate, has in this respect an advantage over $dATP^{4-}$ or ATP^{4-}. Indeed, methylphosphonate is somewhat more basic than methyl phosphate; this follows from the release of the primary proton from the twofold protonated species which occurs with $pK^{H}_{CH_3P(O)(OH)_2}$ = 2.10 ± 0.03 (ref. 10) and $pK^{H}_{CH_3OP(O)(OH)_2}$ = 1.1 ± 0.2,[11] respectively. This increased basicity of a phosphonyl compared to a phosphoryl group should favor metal ion binding.

To verify the above assumption, we compared for Mg^{2+}, Mn^{2+} and Zn^{2+} (M^{2+}) the metal ion-binding properties of methyl phosphonylphosphate, $CH_3–P(O)_2^-–O–PO_3^{2-}$ ($MePP^{3-}$),[12,13] with those of methyl diphosphate and other diphosphate monoesters, $R–OP(O)_2^-–O–PO_3^{2-}$ ($R–DP^{3-}$), where R is a noncoordinating residue. The results summarized in Fig. 2, where the logarithms of the measured stability constants are plotted in dependence on the pK_a values of $H(R–DP)^{2-}$ or $H(MePP)^{2-}$, show that the $Mg(MePP)^-$ and $Mn(MePP)^-$ complexes are somewhat more stable than is expected on the basis of the basicity of the terminal phosphate group of $MePP^{3-}$. The stability increases, which correspond to the vertical broken lines seen in Fig. 2, are $\log \Delta_{Mg(MePP)} = 0.08 \pm 0.04$ and $\log \Delta_{Mn(MePP)} = 0.16 \pm 0.04$; not shown in Fig. 2 is $\log \Delta_{Zn(MePP)} = 0.16 \pm 0.04$.[14] Hence, the higher basicity of a phosphonyl unit, compared to that of a phosphoryl group, leads to an increased complex stability!

In the present context one must also mention that the ether oxygen of $PMEA^{2-}$ (see Fig. 1) participates in M^{2+} binding[15,16] which gives rise to the following intramolecular equilibrium:



Of course, the formation of the indicated five-membered chelate is also reflected in an increased complex stability (based on log *K versus* pK_a correlation lines)[16] which amounts for the M(PMEA) complexes[15a] of Mg^{2+}, Mn^{2+} and Zn^{2+} to $\log \Delta_{Mg(PMEA)} = 0.16 \pm 0.05$, $\log \Delta_{Mn(PMEA)} = 0.21 \pm 0.08$, and log

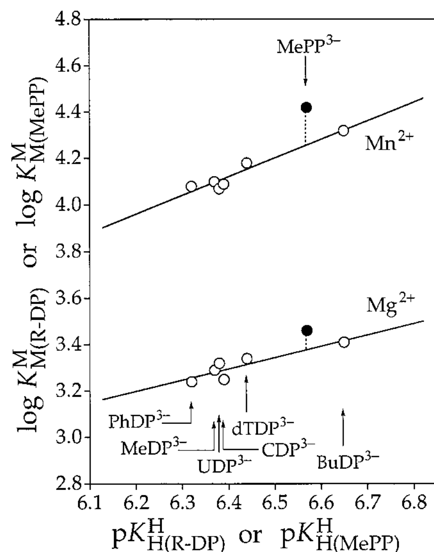


Fig. 2 Comparison of the stabilities of the $Mg(MePP)^-$ and $Mn(MePP)^-$ complexes (●) with those of the corresponding M^{2+} complexes formed with diphosphate monoesters ($R\text{–}DP^{3-}$) (○) based on the relationship between $\log K^{M}_{M(R\text{–}DP)}$ and $pK^{H}_{H(R\text{–}DP)}$, for the Mg^{2+} and Mn^{2+} 1 : 1 complexes of phenyl diphosphate ($PhDP^{3-}$), methyl diphosphate ($MeDP^{3-}$), uridine 5′-diphosphate (UDP^{3-}), cytidine 5′-diphosphate (CDP^{3-}), thymidine [= 1-(2′-deoxy-β-D-ribofuranosyl)thymine] 5′-diphosphate ($dTDP^{3-}$), and *n*-butyl diphosphate ($BuDP^{3-}$) (from left to right). The least-squares lines are drawn through the indicated six data sets; the corresponding straight-line equations are listed in Table 4 of ref. 13(*b*). The equilibrium constants for the M^{2+}–MePP systems are given in footnote 13(*a*). All plotted values refer to aqueous solution at 25 °C and I = 0.1 M ($NaNO_3$).

$\Delta_{Zn(PMEA)} = 0.30 \pm 0.10$,[17] respectively; the corresponding formation degrees of the five-membered chelates are 31(±8), 38(±11), and 50(±12)%, respectively.[15a]

The above mentioned two effects, *i.e.* the increased basicity of the phosphonyl group and the participation[15c] of the ether oxygen in metal ion binding, favor the coordination of a second metal ion at the α group which occurs under the 'guidance' of the enzyme.[7,9] The binding of both metal ions to $PMEApp^{4-}$ is depicted in Fig. 3 in comparison to the situation in ATP^{4-}. Of course, a higher formation degree of the structurally correct $M_2(PMEApp)$ species will also facilitate the nucleophilic attack

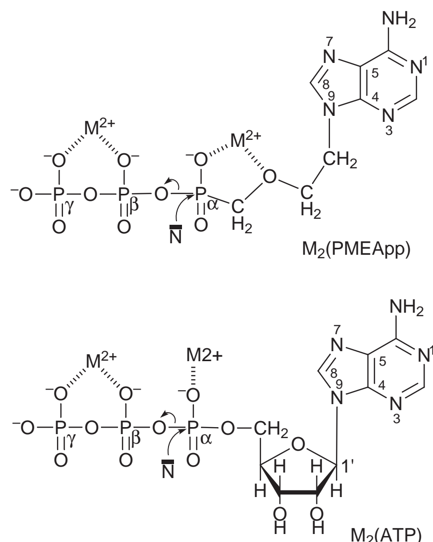


Fig. 3 Structures of the $M_2(PMEApp)$ and $M_2(ATP)$ intermediates ready for the attack of a nucleophile (N) and on their way to the transition state in nucleic acid polymerases. Metal ion binding to the α group is favored with $PMEApp^{4-}$ (top) due to the formation of the five-membered chelate involving the ether oxygen atom as well as by the enhanced basicity of the α-phosphonyl group. Both divalent metal ions (usually Mg^{2+}) are anchored to amino acid-side chains (see, *e.g.* ref. 9) of the protein. Of course, the adenine residue can also be replaced by other nucleobase moieties.

at the α group and thus favor the transfer of the phosphonyl unit with its nucleobase residue in the polymerase-catalyzed reaction and its incorporation into the growing nucleic acid chain and thus, the termination of the latter. The above given mechanistic considerations are further confirmed by the repeated observation that the ether oxygen of $PMEA^{2-}$ and of its (phosphonomethoxy)ethyl relatives is important for obtaining a biological effect:[1,6] its omission or replacement leads to a reduction or even loss of the antiviral activity.[18]

To conclude, in the search for new antivirally active nucleotide analogues the above gained insight should be kept in mind that favored metal ion-binding properties of the α-group are important for obtaining a high biological activity of the nucleotide analogues.

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18 It may be added that PMEApp is a *poorer* substrate than ATP for ATPases[4] and this agrees with the above conclusions because for ATPases, like for kinases,[7,8] a M(α,β)–M(γ)-like coordination is desirable. Clearly, M(α,β) binding of M^{2+} will be somewhat inhibited by the five-membered chelate formed with the α group and the ether O atom.

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