Cyclic phosphates of some antiviral acyclonucleosides: relationship between conformation and substrate/inhibitor properties in some enzyme systems*

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Solution conformations, and substrate/inhibitor properties towards several phosphodiesterases and other nucleolytic enzymes, have been investigated for the cyclic phosphates of various acyclonucleosides, some with known antiviral activity, including 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG) and its carbocyclic congener, 9-(3,4-dihydroxybutyl)guanine (DHBG), 9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (2HM-HBG), and 9-(2,3-dihydroxypropyl)guanine (HPG).

Conformations of the cyclic phosphate rings were derived from analysis of the $^1$H–$^1$H and $^1$H–$^3$P vicinal coupling constants in the $^1$H n.m.r. spectra, subsequently optimized by minimalization of the internal energy. The resulting structures were examined with respect to their ability to recognize various specific phosphodiesterases and nucleases, and some structural parameters were delineated for acyclonucleotide interactions. Qualitative data are presented for inhibitory properties of the acyclonucleoside cyclic phosphates, and, in those instances where they were substrates, kinetic constants were evaluated. An unusual finding was the apparent ability of nuclease P1 to hydrolyse a five-membered cyclic phosphate ring of an acyclonucleoside.

The discovery of Acyclovir (ACV, 9-(2-hydroxyethoxymethyl)-guanine) is an effective antiherpes agent [1], now widely applied clinically, has stimulated the synthesis of a

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1 Abbreviations employed: ACV, 9-(2-hydroxyethoxymethyl)guanine; DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; DHPG-cmp, DHPG-3′,5′-cyclic phosphate; DHPAde, adenine analogue of DHPG; DHPAde-cmp, DHPAde-3′,5′-cyclic phosphate; C-DHPG, carbo-DHPG, 9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine; C-DHPG-cmp, C-DHPG-4′,3′-cyclic phosphate; DHBG, 9-(3,4-dihydroxybutyl)guanine; DHBG-cmp, DHBG-3′-cyclic phosphate; HPG, 9-(2,3-dihydroxypropyl)guanine, HPG-cmp, HPG-2′,3′-cyclic phosphate; 2HM-HBG, 9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine; 2HM-HBG-cmp, 2HM-HBG-2′,3′-cyclic phosphate; 2HM-HBG-MP, 2HM-HBG-4′-monophosphate; RNase, ribonuclease; PDase, phosphodiesterase; cPDase, cyclic nucleotide phosphodiesterase; BH, beef heart; BB, bovine brain; SV, snake venom; PT, potato tuber; TK, thymidine kinase; HCMV, human cytomegalovirus; TSP, 3-trimethylsilyl-(2,2,3,3-H4-propionate) sodium salt; Me3Si, trimethylsilyl
multitude of purine and pyrimidine acyclonucleosides and nucleotides as potential antiviral agents [2, 3].

The antiviral activities of most acyclonucleosides are dependent on their intracellular phosphorylation largely [4], but not exclusively [5], by viral-coded thymidine kinase (TK). One notable exception is the chemically synthesized 3':5'-cyclic phosphate of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG-cMP, see Fig. 1), a potent broad-spectrum antiviral agent, the mechanism of action of which, although not yet clarified, is independent of viral or cellular TK [6, 7]. It was, therefore, proposed that DHPG-cMP, which is readily taken up by cells [7], may be active as such, i.e. as a structural analogue of a purine nucleoside 3':5'-cyclic phosphate, e.g. cGMP [5]. And, in fact, there are a number of reports to the effect that replication of viruses is modulated by intracellular levels of cAMP and cGMP [5], recently extended to include vesicular stomatitis and herpes simplex viruses [8] and human immunodeficiency virus (HIV) [9].

The desirability of elucidating the mechanism of antiviral activity of DHPG-cMP prompted us to undertake the synthesis of cyclic phosphates of acyclonucleosides with more than one hydroxyl on the acyclic chain, and to examine their substrate/inhibitor properties towards various nucleolytic enzymes [10, 11]. We now report an extension of these studies, including attempts to correlate the conformations of these compounds with their substrate/inhibitor properties.

Interest in the foregoing is further enhanced by the known activity of not only DHPG-cMP, but also its parent acyclonucleoside DHPG, against human cytomegalovirus (HCMV) [5]. HCMV does not code for thymidine kinase, but DHPG is phosphorylated in HCMV-infected cells. Two independent reports have now appeared [12, 13], demonstrating that DHPG is phosphorylated by an HCMV-coded protein.

![Diagram of acyclonucleoside analogues of guanine](image)

**Fig. 1. Cyclic phosphates of acyclonucleoside analogues of guanine.**
Note that the acyclic chain of DHPG-cMP (top left) mimics only the "upper" portion of the pentose ring of a nucleoside, whereas the acyclic chain of C-DHPG-cMP (top, centre and right) may mimic either the upper (centre) or lower (right) portion of the pentose ring. The three other compounds mimic the lower portion of the pentose ring.
Cyclic phosphates of acylonucleosides

Values of $K_m$ and $V_{\text{max}}$ were calculated with the aid of the Eisenthal – Cornish – Bowden algorithm, using an IBM PC program [19] as previously reported [11]. Percent inhibition of enzyme activity was determined by incubation of the appropriate enzyme with 2 mM each of substrate and inhibitor in 200 μl of 0.1 M buffer at optimal pH for each enzyme.

RESULTS

Conformation of cyclic phosphate rings

For the various compounds embraced in this study, the proton-proton and proton-phosphorus vicinal coupling constants are listed in Table 1; for purposes of simplicity the protons of the cyclic phosphate rings are numbered as in Fig. 2. The dihedral angles and the conformer populations were calculated from coupling constants using the Karplus relationship with appropriate parametrization for $^1\text{H}^-^1\text{H}$ [15] and $^1\text{H}^-^3\text{P}$ [16] couplings.

Five-membered rings. The conformation of a five-membered cyclic phosphate ring was deduced from the pseudorotational model of Altona & Sundaralingam [20]. The sets of 5 observed coupling constants for HPG-cMP and DHBG-cMP (Table 1) do not, in either case, correspond to a unique conformation, but rather to a dynamic equilibrium (rapid on the n.m.r. time scale) of two conformers, a dominant one with the substituted carbon atom displaced endo from the plane of the other atoms (pseudorotational angle $\Phi = 18^\circ$, see Fig. 2A, left), and a minor one with the neighbouring carbon displaced endo ($\Phi = 162^\circ$, see Fig. 2A, right). The population of the major conformer varies from about 90% for (R)-DHBG-cMP in (C$^2\text{H}_3$)$_2$SO to 80% for (R)- and (S)-DHBG-cMP in $^2\text{H}_2$O and to 60% for HPG-cMP in $^2\text{H}_2$O. The amplitude of pucker of the rings, $\Phi_m = 35^\circ - 40^\circ$, is typical of that for 5-membered rings of pentose nucleosides [21].

Six-membered rings. These exhibit a dynamic equilibrium of two chair forms, with the phosphorus atom and the substituted carbon atom displaced in opposite directions from the plane of the remaining atoms, with the substituent in one form in the axial orientation (Fig. 2B, left), and in the other equatorial (Fig. 2B, right). DHPG-cMP exhibits a single conformer with
Table 1

<table>
<thead>
<tr>
<th>Coupling constant</th>
<th>(S)-HPG-cMP</th>
<th>(S)-DHBG-cMP</th>
<th>(R)-DHBG-cMP</th>
<th>DHPG-cMP</th>
<th>C-DHPG-cMP</th>
<th>(R,S)-2HM-HBG-cMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^2$H$_2$O</td>
<td>$^2$H$_2$O</td>
<td>$^2$H$_2$O</td>
<td>$^2$H$_2$O</td>
<td>$^2$H$_2$O</td>
<td>$^2$H$_2$O</td>
</tr>
<tr>
<td>J(1,2)</td>
<td>6.4</td>
<td>7.2</td>
<td>7.2</td>
<td>7.7</td>
<td>2.0</td>
<td>5.9</td>
</tr>
<tr>
<td>J(1,3)</td>
<td>6.2</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>J(1,4)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
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<td>J(1,5)</td>
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<td></td>
<td></td>
<td></td>
<td>7.5</td>
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<tr>
<td>J(4,6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.2</td>
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<tr>
<td>J(4,7)</td>
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<td></td>
<td></td>
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<td>7.5</td>
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<tr>
<td>J(5,6)</td>
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<td>7.7</td>
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<td>J(5,7)</td>
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<td></td>
<td></td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>J(1,F)</td>
<td>a</td>
<td>5.8</td>
<td>5.8</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(2,F)</td>
<td>10.4</td>
<td>8.1</td>
<td>8.2</td>
<td>6.5</td>
<td>19.9</td>
<td>11.8</td>
</tr>
<tr>
<td>J(3,F)</td>
<td>11.4</td>
<td>13.4</td>
<td>13.5</td>
<td>14.6</td>
<td>3.6</td>
<td>11.8</td>
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<td>J(6,F)</td>
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<td>12.1</td>
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<td>J(7,F)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Not determined because of overlapping of signal by water; \(^b\) value approximate due to overlapping of signals.

the substituent in an axial orientation. The ring dihedral angles about C – C and C – O bonds are close to 50°. Since completion of this study, we have succeeded in obtaining suitable crystals of this compound, the X-ray diffraction data for which (to be published elsewhere) are consistent with these findings. A similar conformation is exhibited by other acyclonucleoside cyclic phosphates in which the acyclic chain may mimic the "upper" portion of the pentose ring [10]. C-DHPG-cMP exhibits an equilibrium of both conformers, with comparable populations in $^2$H$_2$O, but with 65% of the form with an equatorial orientation of the substituent in (C$^2$H$_3$)$_2$SO.

**Seven-membered ring.** The predominant conformation of the cyclic phosphate ring of 2HM-HBG-cMP is that for which the phosphorus atom is displaced from the plane of the ring in a direction opposite to that of the C – C bond, with the substituent in an equatorial orientation (Fig. 2C, left). The internal dihedral angles about the C – O bonds are close to 100°, while the angle about the displaced C – C bond is distorted from the cisoidal orientation about 5°-10°, as a result of steric repulsions of the substituent and protons. For the minor conformer, with a population of about 20%, one carbon and the O – P bond diametrically opposite to it are displaced from the plane with the substituent in an axial orientation (Fig. 2C, right).

**Global conformation of acyclonucleoside cyclic phosphates.** The values of the coupling constants for the acyclic chains point to a dynamic equilibrium about the C – C bonds, with comparable populations of all classical gauche and trans forms [11], and a predominance of the trans conformers (i.e. with enhanced "rigidity") about the C – O bonds [10]. The values of the $^1$H-$^{13}$C coupling constants, determined for several analogues [10, 22], demonstrate, as might be anticipated, free rotation about the C – N glycosidic bond.

Bearing in mind the conformational "flexibility" of the acyclic chains of the foregoing analogues in solution, a key problem is the determination of the energy minima in the conformational range corresponding to the steric requirements of the enzymes involved, particularly cPDases. Attempts to achieve this
were based on application of a program for minimalization of the internal energy, the starting point being the experimentally determined conformations of the cyclic phosphate rings (see above). The global conformations obtained by this procedure are presented in Fig. 3. C-DHPG-cMP is shown with two conformational equilibria of the cyclic phosphate rings, with the substituent in the orientation axial (ax) and equatorial (eq).

**Substrate/inhibitor properties**

A qualitative evaluation was conducted on the inhibitory properties of the various cyclic phosphates versus those enzymes for which they are not substrates, by measurement of their influence on the rate of hydrolysis of a typical known substrate for each of the enzymes. The overall results are listed in Table 2. For comparison purposes, 2′:3′-cAMP and 3′:5′-cAMP were also examined as potential inhibitors. One rather unusual finding was the apparent activation of RNase A by some of these compounds, particularly 3′:5′-cAMP. This phenomenon was not further examined.

For those cyclic phosphates which were substrates, the appropriate $K_m$ and $V_{max}$ values
Fig. 3. Global conformations of cyclic phosphates of nucleosides and acyclonucleosides, derived from n.m.r. data and minimalization of internal energy. Note that C-DHPG-cMP exists as an equilibrium mixture of two conformers with axial (ax) and equatorial (eq) orientations of the 9-ethylguanine moiety relative to the cyclic phosphate ring, as also shown in Fig. 2B. Atoms are denoted as follows: ○ C, ● N, ● O, ● P

were determined as previously described [11], with results shown in Table 3.

**DHPG-cMP.** This compound was hydrolyzed only by PT cPDase, but at a rate too slow to readily permit determination of kinetic constants. An estimate of these values was arrived at with the use of the adenine analogue, DHPAde-cMP, which is also a substrate, but hydrolyzed 2 - 3-fold more rapidly (Table 3). For this compound, \( K_m = 11.7 \text{ mM} \), and \( V_{\text{max}} = 0.2 \text{ nM} \cdot \text{min}^{-1} \) (enzyme unit)^{-1}. The \( K_m \) value is about an order of magnitude higher than for other standard substrates of this enzyme [18]. DHPG-cMP itself did not markedly inhibit the other enzymes (Table 2).

**DHBG-cMP.** This analogue exists as two optical isomers, \( R \) and \( S \). Both of these are substrates for BH cPDase, PT cPDase and nuclelease P1, with \( K_m \) values of 1.0 mM, 1.0 mM and 0.5 mM, respectively (Table 3). For PT cPDase, the \( K_m \) value is similar to, or even lower, than the \( K_m \) for other standard substrates [18]. The product of hydrolysis of either enantiomer was resistant to snake venom 5'-nucleotidase, but was slowly converted to the nucleoside by 3'-nucleotidase. Neither enantiomer exhibited inhibition of the other enzymes.

**C-DHPG-cMP.** This analogue was not a detectable substrate for any of the enzymes, and was a weak inhibitor only of beef heart cPDase (Table 3). It is, however, worth noting that C-DHPG is a good substrate for viral thymidine kinase, and is then further phosphorylated to the triphosphate by cellular enzymes [23].
Table 2
Inhibition, by acyclonucleoside cyclic phosphates (each at 2 mM), of activities of various nucleolytic enzymes versus typical substrates (each 2 mM).
Activities are expressed as % of activity observed in absence of inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>cPDase BB</th>
<th>cPDase BH</th>
<th>PDase SV</th>
<th>cPDase PT</th>
<th>RNase A</th>
<th>RNase T1</th>
<th>RNase T2</th>
<th>Nuclease P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPG-cMP</td>
<td>105</td>
<td>71</td>
<td>69</td>
<td>s</td>
<td>119</td>
<td>81</td>
<td>98</td>
<td>88</td>
</tr>
<tr>
<td>C-DHPG-cMP</td>
<td>94</td>
<td>51</td>
<td>65</td>
<td>98</td>
<td>119</td>
<td>79</td>
<td>95</td>
<td>88</td>
</tr>
<tr>
<td>DHBG-cMP</td>
<td>78</td>
<td>s</td>
<td>69</td>
<td>s</td>
<td>122</td>
<td>70</td>
<td>91</td>
<td>s</td>
</tr>
<tr>
<td>HPG-cMP</td>
<td>73</td>
<td>a</td>
<td>125</td>
<td>s</td>
<td>97</td>
<td>44</td>
<td>85</td>
<td>s</td>
</tr>
<tr>
<td>2HM-HBG-cMP</td>
<td>107</td>
<td>4</td>
<td>77</td>
<td>102</td>
<td>127</td>
<td>38</td>
<td>95</td>
<td>110</td>
</tr>
<tr>
<td>2HM-HBG-MP</td>
<td>nd</td>
<td>2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2'3'cAMP</td>
<td>s</td>
<td>a</td>
<td>89</td>
<td>s</td>
<td>132</td>
<td>e</td>
<td>s</td>
<td>31</td>
</tr>
<tr>
<td>3'5'cAMP</td>
<td>f</td>
<td>s</td>
<td>100</td>
<td>s</td>
<td>180</td>
<td>e</td>
<td>93</td>
<td>60</td>
</tr>
</tbody>
</table>

a. BH cPDase is specific for nucleoside 3'5'-cyclic phosphates; b. K1 = 0.1 mM, inhibition competitive; c. K1 = 0.8 mM for (R)-2HM-HBG-MP, and K1 = 0.3 mM for (S)-2HM-HBG-MP, inhibition non-competitive; nd, not determined; e. RNase T1 is specific for guanine nucleotides; f. BB cPDase is specific for nucleoside 2'3'-cyclic phosphates; s, inhibitor is a substrate.

Table 3
Values of Km (mM) and Vmax (nmol·min⁻¹·enzyme unit⁻¹) for hydrolysis of cyclic phosphates of acyclonucleosides by nucleolytic enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DHPG-cMP</th>
<th>DHBG-cMP</th>
<th>HBG-cMP</th>
<th>Km (Vmax)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH cPDase</td>
<td>1.0 (20.0)</td>
<td>1.0 (20.0)</td>
<td>1.0 (20.0)</td>
<td>1.0 (20.0)</td>
</tr>
<tr>
<td>PT cPDase (native)</td>
<td>11.7 (0.2)</td>
<td>1.0 (0.3)</td>
<td>0.1 (0.5)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>PT cPDase (selectively inactivated)</td>
<td>nd</td>
<td>1.0 (0.2)</td>
<td>0.1 (0.26)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>Nuclease P1</td>
<td>0.5 (750)</td>
<td>0.6 (209)</td>
<td>0.6 (209)</td>
<td>0.6 (209)</td>
</tr>
</tbody>
</table>

*In this case nmol · min⁻¹ · (mg enzyme)⁻¹, b native enzyme is active versus nucleoside 2'3'- and 3'5'-cyclic phosphates. Selectively inactivated enzyme is active only versus 2'3'-cyclic phosphates; these values are for the adenine analogue, DHPAde-cMP, which is a better substrate than DHPG-cMP; nd, no detectable activity.

(S)-HPG-cMP. This was a substrate only for PT cPDase and nuclease P1, with Km values of 0.1 mM and 0.6 mM (Table 3), and was a feeble inhibitor only of RNase T1 (Table 2).

(R,S)-2HM-HBG-cMP. This was resistant to all enzymes, but exhibited marked inhibition of BH cPDase, as did also its monophosphate (Table 2). It was also a weak inhibitor of RNase T1.

**DISCUSSION**

There are now numerous well-documented examples of the "recognition" by a variety of enzymes of acyclonucleosides, and their mono-, di- and triphosphates, in some instances with marked stereospecificity [5].

This is clearly due to the flexibility of the acyclic chains, which enables them to adopt a conformation that may mimic the "upper" and/or "lower" portion of the pentose ring of natural nucleosides (see Fig. 1). This was first demonstrated by Schaefer [24] with the adenosine deaminase system, which subsequently led to the development of the antithymosine agent Acyclovir, an acyclonucleoside recognised and
phosphorylated by viral-coded, but not cellular, thymidine kinase. The most recent, and probably most striking, example is the recognition of DHPG by a protein coded by human cytomegalovirus (HCMV), with the sequence properties of the catalytic domain of a protein kinase, but which recognizes and phosphorylates DHPG [12, 13] as though it were a nucleoside kinase.

DHPG-cMP was the first example of a nucleotide analogue, and the first cyclic phosphate of an acyclonucleoside, with potent biological (antiviral) activity. The fact that it acts as such, and not as a result of its intracellular metabolism, implies that it is "recognized" by some intracellular component(s) in virus-infected cells, perhaps a cyclic nucleotide-dependent protein kinase.

The five model acyclonucleoside cyclic phosphates embraced in this study were not substrates for beef brain cPDase, snake venom PDase and RNases A, T₁ and T₂ (Table 3). Even RNase T₂, with its known broad specificity, did not hydrolyse 1-(2,3-dihydroxypropyl)thymine-2'-3' cyclic phosphate, previously reported to be a substrate for several bacterial RNases [25]. The cyclic phosphates were also only moderate inhibitors of these enzymes, in that a concentration equimolar with that of substrate led to only approx. 40% inhibition. With RNase T₁ inhibition by HPG-cMP and 2HM-HBG-cMP was approx. 60%; for HPG-cMP this is likely due to its structural resemblance to the natural substrate: 2':3'-cGMP (Fig. 3). We now discuss the results for the remaining three enzymes in turn.

**Beef heart cPDase.** The known specificity of this enzyme for nucleoside 3':5'-cyclic phosphates [26] suggested it might hydrolyze DHPG-cMP and, perhaps, C-DHPG-cMP; both close structural analogues of cGMP (Fig. 1). Actually these are closer analogues of 2'-deoxy-cGMP, but both beef heart and beef brain cPDases recognize 2'-deoxy-cMP's, which are cleaved at 20 - 90% of the rate for ribose derivatives [26, 27], and are therefore also good "inhibitors" of the latter. Hence the absence of the 2'-OH (e.g. in DHPG-cMP) is of minor significance for recognition by these enzymes. Nonetheless the beef heart enzyme is more tolerant than that from beef brain with regard to absence of the 2'-OH, in that it hydrolyzes 2'-deoxy-cAMP two-fold faster, while the latter inhibits hydrolysis of cAMP almost 10-fold more effectively.

Surprisingly, neither DHPG-cCMP nor C-DHPG-cMP was a substrate or inhibitor. Quite unexpected was the observation that both enantiomers of DHBG-cMP, which include five-membered cyclic phosphate rings, were good substrates.

Equally unexpected was the finding that, in contrast to other analogues, 2HM-HBG-cMP, as well as (R)- and (S)-2HM-HBG-cMP, were reasonably good inhibitors of the bee heart cPDase (Table 2). The cyclic phosphate of 2HM-HBG consists of a 7-membered ring, whereas the cPDases embraced in this study are considered to be specific for 5- and/or 6-membered rings, consistent with the fact that none of them recognize 2HM-HBG-cMP as a substrate. It had previously been noted that synthetic araC-2':5'-cMP, which also contains a 7-membered cyclic phosphate ring, is not a substrate for PT cPDase [18].

**Nuclease P₁.** This enzyme, of fungal origin, is widely employed as a tool for the total hydrolysis of nucleic acids and oligonucleotides, and, in the case of ribonucleic acids, without the intermediate formation of 2':3'-cyclic phosphates [28], as is the case for RNases A, T₁ and T₂ [29]. It is consequently of considerable interest that it hydrolyses the five-membered cyclic phosphate rings of DHBG-cMP and HPG-cMP at appreciable rates and with relatively low Kₘ values (Table 3). It is to be further noted that, whereas 3':5'-cAMP is a moderate inhibitor of this enzyme, 2':3'-cAMP (with a five-membered cyclic phosphate ring) is considerably more effective (Table 2). This unusual behaviour of nuclease P₁ is clearly deserving of further investigation.

**Potato tuber cPDase.** Consistent with the known broad specificity of this enzyme towards 2':3'- and 3':5'-cyclic phosphates and some phosphate esters [18], it proved active versus DHPG-cMP, DHBG-cMP and HPG-cMP. Furthermore, the selectively inactivated enzyme, which fully retains activity versus nucleoside 2':3'-cyclic phosphates [18], retains activity versus DHBG-cMP and HPG-cMP, both with five-membered cyclic phosphate rings. The fact that C-DHPG and 2HM-HBG-cMP are neither substrates nor inhibitors is probably due to their "recognition" as analogues of the "lower" region of the pentose ring, with addi-
tional carbons, as in the case of beef heart cPDase (see above). It should be recalled that this enzyme also does not hydrolyze the seven-membered cyclic phosphate ring of 2':5'-araC-cMP [18], although the conformation of this compound is additionally quite different from those of substrates (Fig. 3).

Although the potato tuber cPDase is ubiquitous in higher plants, it is relevant to note that a cPDase with remarkably similar properties has been isolated from mammalian cells. It is probably this enzyme which is responsible for the observed low rate of hydrolysis of DHPG-cMP and other acylcyclopentosyl cyclic phosphates in crude extracts of various cells [7, 11].

Some general conclusions. It appears from the foregoing that the recognition, by a nucleolytic enzyme, of the acyclic chain which links the heterocyclic base with the cyclic phosphate ring as the "upper" or "lower" portion of a pentose ring is determined by the presence or absence of an ether oxygen linked to C(1'), corresponding to the O(4') of the pentose ring. It follows that only the acyclic chain of DHPG-cMP mimics the "upper" portion of a pentose ring, and the acyclic chains of the other compounds the lower portion of a pentose ring. This accounts for the weak interactions of C-DHPG-cMP and 2HM-HBG-cMP with the enzymes, in that their acyclic chains are analogues of a sugar with an additional carbon at the 2' and 3' positions, and attendant steric hindrance to their recognition as substrates or inhibitors. If this is so, HPG-cMP should be a good analogue of 2',3'-cGMP and, in fact, it is a good substrate for two of the enzymes, nucl ease P1 and PT cPDase. Its lack of recognition by beef brain cPDase would then be due to the stricter specificity of this enzyme with respect to the sugar ring, such as the presence of O(4') and/or 5'-OH, as well as the distance between the cyclic phosphate ring and the aglycon.

The size of the cyclic phosphate ring (whether five-, six- or seven-membered) appears to be of lesser significance, if we exclude enzymes strictly specific for 2':3'-cyclic phosphates. DHBG-cMP is hydrolyzed by beef heart cPDase, notwithstanding that it contains a five-membered ring in place of the six-membered ring of the natural substrate 3':5'-cGMP. By contrast, this enzyme does not hydrolyze DHPG-cMP and C-DHPH-cMP, both with six-membered cyclic phosphate rings. And 2HM-HBG-cMP is a competitive inhibitor of beef heart cPDase despite the fact that it contains a seven-membered cyclic phosphate ring which is not found in any natural nucleotide cyclic phosphates.

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REFERENCES


