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QUARTERLY

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-^{31}P vicinal coupling constants in the ^{1}H n.m.r. spectra, subsequently optimalized 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 P_1 to hydrolyse a five-membered cyclic phosphate ring of an acyclonucleoside.

The discovery that 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':4''-cyclic phosphate; DHBG, 9-(3,4-dihydroxybutyl)guanine; DHBG-cMP, DHBG-3':4'-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':4'-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 venome; PT, potato tuber; TK, thymidine kinase; HCMV, human cytomegalovirus; TSP, 3-trimethylsilyl-(2,2,3,3-^{2}H$_4$-propionate) sodium salt; Me$_4$Si, tetramethylsilane

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

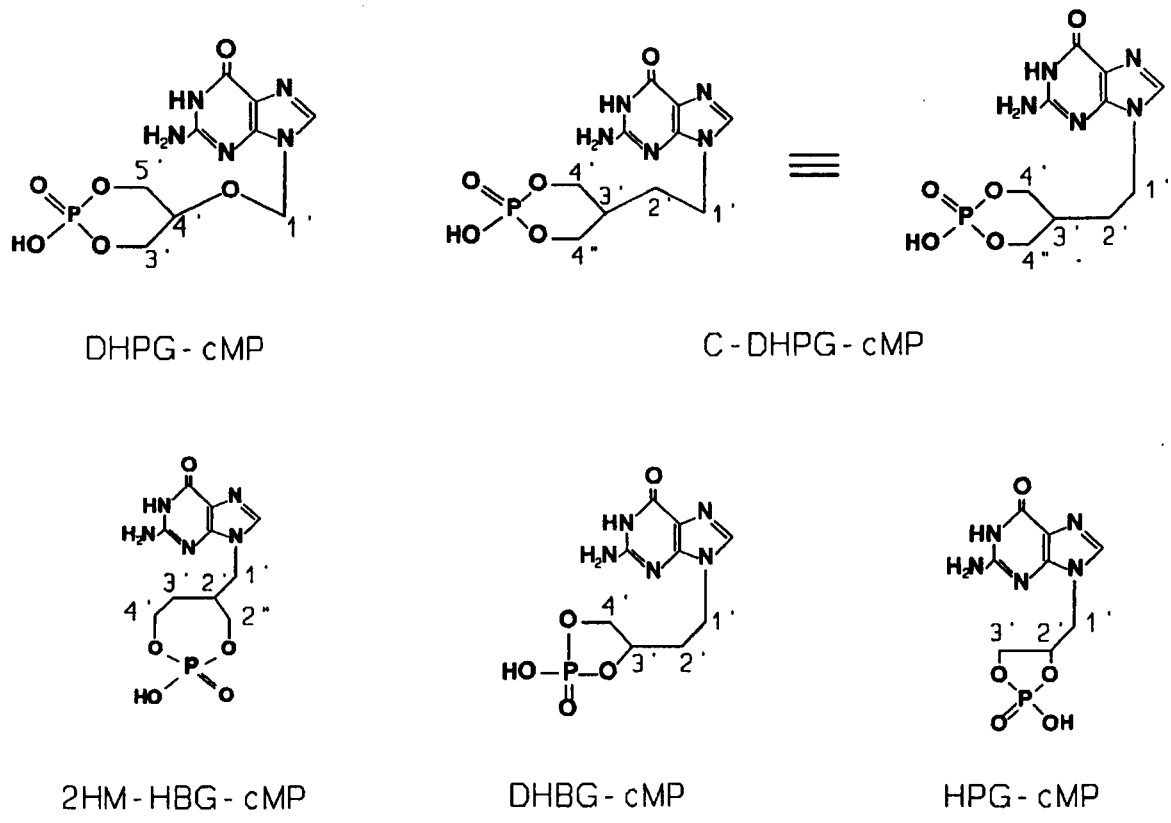

Fig. 1. Cyclic phosphates of acyclonucleoside analogues of guanine.
Note that the acyclic chain of DHPG-cMP (top left) mimics only the "upper" portion of the pentose ring of a nucleoside, whereas the acyclic chain of C-DHPG-cMP (top, centre and right) may mimic either the upper (centre) or lower (right)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

(which is not thymidine kinase) with the sequence properties of a protein kinase, suggesting that the conformation of DHPG itself may mimic some conformational region of a protein kinase substrate.

EXPERIMENTAL

2':3'-cAMP and 3':5'-cAMP were products of Sigma (St. Louis, Mo., U.S.A.). The syntheses of DHPG-cMP, DHBG-cMP, C-DHPG-cMP and HPG-cMP were previously described and their structures established by u.v. and n.m.r. spectroscopy [11]. Similar procedures were employed to synthesize 2HM-HBG-cMP by chemical phosphorylation with $POCl_3$, and isolation of the product by column chromatography [11]. 2HM-HBG-MP was synthesized by enzymatic phosphorylation of the acyclonucleoside with the wheat shoot phosphotransferase system [14], as previously described [11].

^{1}Hn.m.r. spectra were recorded on Bruker AM 270 and AM 500 instruments, as well as on a Jeol XL 400, at concentrations of 0.02 M at room temperature. Chemical shifts are expressed relative to internal TSP in ^2H_2O and *versus* internal Me_4Si in $(C^2H_3)_2SO$. ^{31}Pn.m.r. spectra were recorded on the Bruker AM 270 at a frequency of 109.35 MHz under conditions as for the ^{1}H spectra.

Conformations of the cyclic phosphate rings were derived from ^1H-^1H and ^1H-^{31}P vicinal coupling constants with the aid of appropriate parametrization of the Karplus relationships [15, 16]. The conformational data thus obtained were employed as the input to determine overall conformation of the molecules by successive minimalization of the energy to derive the energetically most favorable conformations, with the aid of our own computer program on an IBM PC, using the force field of the database of the program Amber 4 [17].

The cPDases from beef heart (EC 3.1.4.17) and bovine brain (EC 3.1.4.37) and snake venom PDase (EC 3.1.15.1), the RNases A (EC 3.1.27.5), T_1 (EC 3.1.4.8) and T_2 (EC 3.1.27.1), snake venom 5'-nucleotidase (EC 3.1.3.5), rye grass 3'-nucleotidase (EC 3.1.3.6) and nuclease P_1 (EC 3.1.30.1) were products of Sigma (St. Louis, Mo., U.S.A.). Potato tuber cPDase (EC 3.1.4.–) was a partially purified preparation, prepared as elsewhere described [18].

Values of K_m and V_{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 ^1H-^1H [15] and ^1H-^{31}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 P = 18°, see Fig. 2A, left), and a minor one with the neighbouring carbon displaced *endo* (P = 162°, see Fig. 2A, right). The population of the major conformer varies from about 90% for (*R*)-DHBG-cMP in $(C^2H_3)_2SO$ to 80% for (*R*)- and (*S*)-DHBG-cMP in ^2H_2O and to 60% for HPG-cMP in ^2H_2O. The amplitude of pucker of the rings, $\Phi_m = 35° - 40°$, 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 oposite 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

^1H-^1H and ^1H-^{31}P vicinal coupling constants (in Hz, ±0.1 Hz) for cyclophosphate rings

For assignments of protons see Fig. 2

Coupling constant	(*S*)-HPG--cMP	(*S*)-DHBG--cMP	(*R*)-DHBG-cMP		DHPG-cMP	C-DHPG-cMP		(*R,S*)-2HM -HBG-cMP
	^2H_2O	^2H_2O	^2H_2O	$(C^2H_3)_2SO$	^2H_2O	^2H_2O	$(C^2H_3)_2SO$	^2H_2O
J(1,2)	6.4	7.2	7.2	7.7	2.0	5.9	7.4	6.8
J(1,3)	6.2	5.9	5.9	5.9	2.0	2.9	3.5	1.8
J(1,4)								4.0
J(1,5)								7.5
J(4,6)								2.2
J(4,7)								7.5
J(5,6)								7.7
J(5,7)								2.0
J(1,P)	[a]	5.8	5.8	4.3				
J(2,P)	10.4	8.1	8.2	6.5	19.9	11.8	9.1	12.1
J(3,P)	11.4	13.4	13.5	14.6	3.6	11.8	14.2	12.1
J(6,P)								8.5
J(7,P)								15[b]

[a]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 succeded 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 ^2H_2O, but with 65% of the form with an equatorial orientation of the substituent in $(C^2H_3)_2SO$.

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 ^1H-^{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

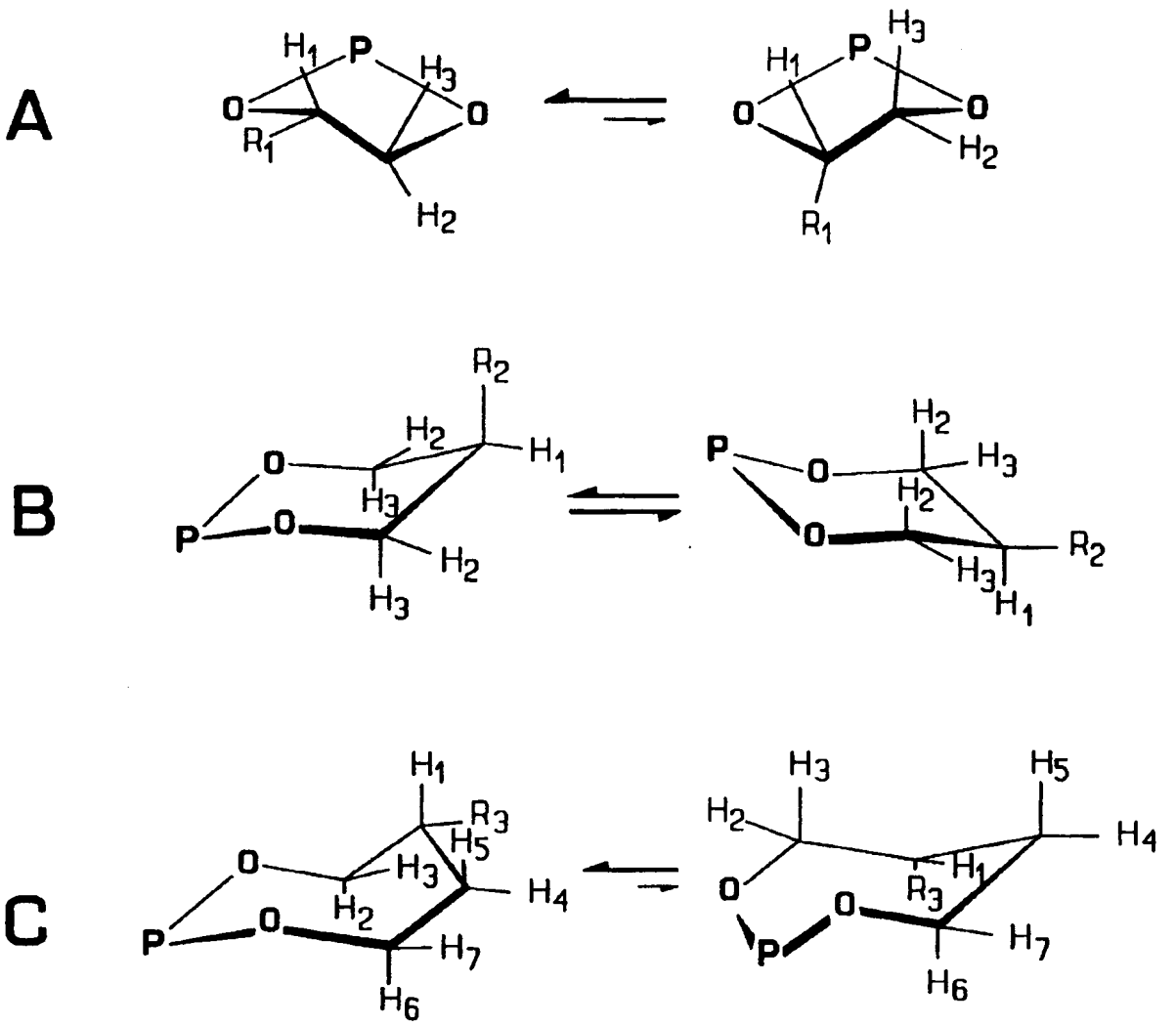

Fig. 2. Conformations of the cyclic phosphate rings of acyclonucleoside cyclic phosphates: (A) Five-membered rings; (B) Six-membered rings; (C) Seven-membered rings. R_1 = 9-ethylguanine (DHBG) or 9-methylguanine (HPG); R_2 = 9-methoxyguanine (DHPG) or 9-ethylguanine (C-DHPG); R_3 = 9-methylguanine (2HM-HBG)

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

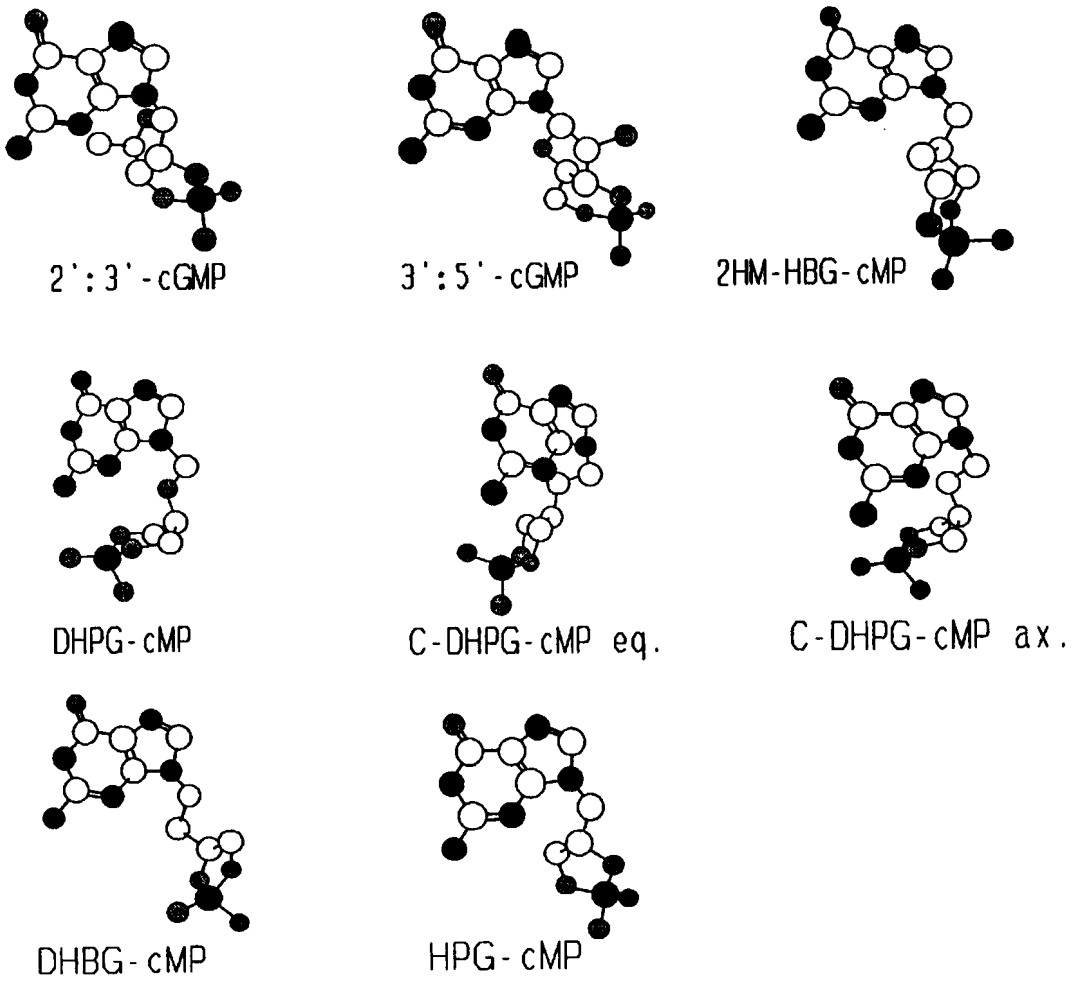

Fig. 3. Global conformations of cyclic phosphates of nucleosides and acyclonucleosides, derived from n.m.r. data and minimalization of internal energy. Note that C-DHPG-cMP exists as an equilibrium mixture of two conformers with axial (ax) and equatorial (eq) orientations of the 9-ethylguanine moiety 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 mM, and V_{max} = 0.2 nM · 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 nuclease P_1, 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

Inhibitor	Enzyme and typical substrate							
	cPDase BB	cPDase BH	PDase SV	cPDase PT	RNase A	RNase T_1	RNase T_2	Nuclease P_1
	2′:3′-cAMP	3′:5′-cAMP	ApC	2′:3′-cAMP	2′:3′-cCMP	2′:3′-cGMP	2′:3′-cAMP	ApC
DHPG-cMP	105	71	69	s	119	81	98	88
C-DHPG-cMP	94	51	65	98	119	79	95	88
DHBG-cMP	78	s	69	s	122	70	91	s
HPG-cMP	73	a	125	s	97	44	85	s
2HM-HBG-cMP	107	4[b]	77	102	127	38	95	110
2HM-HBG-MP	nd	2[c]	nd	nd	nd	nd	nd	nd
2′:3′-cAMP	s	a	89	s	132	e	s	31
3′:5′-cAMP	f	s	100	s	180	e	93	60

a, BH cPDase is specific for nucleoside 3′:5′-cyclic phosphates; [b]K_i = 0.1 mM, inhibition competitive; [c]K_i = 0.8 mM for (*R*)-2HM-HBG-MP, and K_i = 0.3 mM for (*S*)-2HM-HBG-MP, inhibition non-competitive; nd, not determined; e, RNase T_1 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 K_m (mM) and V_{max} ($nmol \cdot min^{-1} \cdot (enzyme\ unit)^{-1}$) for hydrolysis of cyclic phosphates of acyclonucleosides by nucleolytic enzymes

Enzyme	DHPG--cMP	DHBG--cMP	HBG-cMP
	K_m (V_{max})		
BH cPDase		1.0 (20.0)[a]	
PT cPDase (native)[b]	11.7 (0.2)[c]	1.0 (0.3)	0.1 (0.5)
PT cPDase (selectively inactivated)[b]	nd	1.0 (0.2)	0.1 (0.26)
Nuclease P_1		0.5 (750)	0.6 (209)

[a]In this case $nmol \cdot min^{-1} \cdot (mg\ enzyme)^{-1}$; [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; [c]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 P_1, with K_m values of 0.1 mM and 0.6 mM (Table 3), and was a feeble inhibitor only of RNase T_1 (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 T_1.

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 Schaeffer [24] with the adenosine deaminase system, which subsequently led to the development of the antiherpes 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, T_1 and T_2 (Table 3). Even RNase T_2, 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 T_1 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-cNMP'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-cMP 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 beef 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 P_1. 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, T_1 and T_2 [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 K_m values (Table 3). It is to be further noted that, wheres 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 P_1 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 acyclonucleoside 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, nuclease P_1 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 containes a seven-membered cyclic phosphate ring which is not found in any natural nucleoside cyclic phosphates.

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REFERENCES

1. Elion, G.B., Furman, P.A., Fyfe, J.A., de Miranda, P., Beauchamp, L. & Schaeffer, H.J. (1977) Selectivity of action of an antiherpetic agent 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5716 - 5720.
2. De Clercq, E. (1984) Biochemical aspects of the selective antiherpes activity of nucleoside analogues. *Biochem. Pharmacol.* **33**, 2159 - 2169.
3. Shugar, D. (1985) Antiviral agents - Some current developments. *Pure Appl. Chem.* **57**, 423 - 440.
4. Furman, P.A., St. Clair, M.H. & Spector, T. (1984) Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. *J. Biol. Chem.* **259**, 9575 - 9579.
5. Shugar, D. (1992) Phosphorylating enzymes involved in activation of chemotherapeutic nucleosides and nucleotides; in *Molecular Aspects of Chemotherapy. Proceedings of the Third International Symposium, Gdańsk, Poland,* (Shugar, D., Rode, W. & Borowski, E., eds.) pp. 239 - 271. Springer Verlag, Berlin, Polish Scientific Publishers, PWN, Warszawa.
6. Oliver, S., Bubley, G. & Crumpacker, C. (1985) Inhibition of HSV-transformed murine cells by nucleoside analogues, 2′-NDG and 2′-nor-cGMP: mechanism of inhibition and reversal by exogenous nucleosides. *Virology* **145**, 84 - 95.
7. Tolman, R.L., Field, A.K., Karkas, J.D., Wagner, A.F., Germershausen, J., Crumpacker, C. & Scolnic, E.M. (1985) 2′-nor-cGMP: a seco-cyclic nucleotide with powerful anti-DNA viral activity. *Biochem. Biophys. Res. Commun.* **128**, 1329 - 1335.
8. Borghi, P., Di Marzio, P., Varano, B., Conti, L., Beraldelli, F. & Gessani, S. (1992) Cyclic AMP-mediated inhibition of vesicular stomatitis virus and herpes simplex virus replication in mouse macrophage-like cells. *J. Gen. Virol.* **73**, 2949 - 2954.

9. Nokta, M.A. & Pollard, R.B. (1992) Immunodeficiency virus replication: Modulation by cellular levels of cAMP. *Aids Res. & Human Retroviruses* **8**, 1255 - 1261.
10. Stolarski, R., Lassota, P., Kazimierczuk, Z. & Shugar, D. (1988) Solution conformation of some acyclo nucleoside and nucleotide analogues of antiviral acyclonucleosides, and their substrate/inhibitor properties in several enzyme systems. *Z. Naturforsch.* **43c**, 231 - 242.
11. Stolarski, R., Cieśla, J.M. & Shugar, D. (1990) Monophosphates and cyclic phosphates of some antiviral acyclonucleosides: synthesis, conformation and substrate/inhibitor properties in some enzyme systems. *Z. Naturforsch.* **45c**, 293 - 299.
12. Litter, E., Stuart, A.D. & Chee, M.S. (1992) Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature (London)* **358**, 160 - 162.
13. Sullivan, V., Talarico, C.L., Stanat, S.C., Davis, M., Coen, D.M. & Biron, K.K. (1992) A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature (London)* **358**, 162 - 164.
14. Giziewicz, J. & Shugar, D. (1975) Preparative enzymic synthesis of nucleoside 5'-phosphates. *Acta Biochim. Polon.* **22**, 87 - 89.
15. Haasnoot, C.A.G., de Leeuw, F.A.A.M. & Altona, C. (1980) The relationship between proton-proton NMR coupling constants and substituent electronegativities I. An empirical generalization of the Karplus equation. *Tetrahedron* **36**, 2783 - 2792.
16. Lankhorst, P.L., Haasnoot, C.A.G., Erkelens, C. & Altona, C. (1984) Carbon-13 NMR in conformational analysis of nucleic acid fragments 2. A reparametrization of the Karplus equation for vicinal NMR coupling constants in COOP and HOOP fragments. *J. Biomolec. Struct. Dyn.* **1**, 1387 - 1405.
17. Pearlman, D.A., Case, D.A., Caldwell, J., Seibel, G.L., Singh, U.C., Weiner, P.K. & Kollman, P.A. (1991) *Amber 4.0 (UCSF)*, University of California, San Francisco.
18. Zan-Kowalczewska, M., Cieśla, J.M., Sierakowska, H. & Shugar, D. (1987) Potato tuber cyclic-nucleotide phosphodiesterase: selective inactivation of activity *vs* nucleoside cyclic 3',5'-phosphate and properties of the native and selective inactivated enzyme. *Biochemistry* **26**, 1194 - 1200.
19. Kamiński, Z.W. & Domino, E.F. (1987) Computer program for calculating of kinetic and pharmacologic parameters using a "direct linear plot" derived algorithm. *Computer Meth. Progr. Biomed.* **24**, 41 - 45.
20. Altona, C. & Sundaralingam, M. (1972) Conformational analysis of the sugar ring in nucleosides and nucleotides. A new description using the concept of pseudorotation. *J. Am. Chem. Soc.* **94**, 8205 - 8212.
21. De Leeuw, H.P.M., Haasnoot, C.A.G. & Altona, C. (1980) Empirical correlations between conformational parameters in β-D-furanoside fragments derived from a statistical survey of crystal structures of nucleic acid constituents. *Israel J. Chem.* **20**, 108 - 126.
22. Stolarski, R., Kazimierczuk, Z., Lassota, P. & Shugar, D. (1986) Acyclo nucleosides and nucleotides: synthesis, conformation and other properties, and behaviour in some enzyme systems, of 2',3'-seco purine nucleosides, nucleotides and 3':5'-cyclic phosphates, analogues of cAMP and cGMP. *Z. Naturforsch.* **41c**, 758 - 770.
23. Tolman, R.L. (1989) Structural requirements for enzymatic activation of acyclonucleotide analogues; in *Nucleotide Analogues as Antiviral Agents* (Martin, J.C., ed.) ACS Symp. Series No. 401, pp. 35 - 50.
24. Schaeffer, H.J., Gurwara, S., Vince, R. & Bittner, S. (1971) Novel substrate of adenosine deaminase. *J. Med. Chem.* **14**, 367 - 369.
25. Holy, A. & Ivanova, G.S. (1974) Aliphatic analogues of nucleotides: synthesis and affinity towards nucleases. *Nucl. Acids Res.* **1**, 19 - 34.
26. Miller, J.P., Shuman, D.A., Scholten, M.B., Dimmitt, M.K., Stewart, C.M., Khwaja, T.A., Robins, R.K. & Simon, L.N. (1973) Synthesis and biological activity of adenosine 3',5'-cyclic phosphate. *Biochemistry* **12**, 1010 - 1016.
27. Miller, J.P., Boswell, K.H., Mian, A.M., Meyer, R.B., Jr, Robins, R.K. & Khwaja, T.A. (1976) 2'-Derivatives of guanosine and inosine cyclic 3',5'-phosphates. Synthesis, enzymic activity, and the effect of 8-substituents. *Biochemistry* **15**, 217 - 223.
28. Potter, B.V.L., Connolly, B.A. & Eckstein, F. (1983) Synthesis and configurational analysis of dinucleoside phosphate isotopically chiral at phosphorus. Stereochemical course of *Penicillium citrum* nuclease P_1 reaction. *Biochemistry* **22**, 1369 - 1377.
29. Uchida, T. & Egami, F. (1971) Microbial ribonucleases with special references to RNases T_1, T_2, N_1, and U_2; in *The Enzymes* (Boyer, P.D., ed.) vol. 4, pp. 205 - 220, Academic Press, New York & London.