Monophosphates and Cyclic Phosphates of Some Antiviral Acyclonucleosides: Synthesis, Conformation and Substrate/Inhibitor Properties in Some Enzyme Systems

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Chemical and enzymatic procedures are described for the synthesis of the monophosphates and cyclic phosphates of the antiviral acyclonucleoside 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG), its 3-hydroxymethyl-4-hydroxybutyl analogue, the (*R*)- and (*S*)-epimers of 9-(3,4-dihydroxybutyl)guanine, and 9-(2,3-dihydroxypropyl)guanine.

The structures, and some conformational features, of all the foregoing, were determined by ^{1}H and ^{31}P NMR spectroscopy. Their substrate/inhibitor properties have been examined in several enzyme systems, including ribonucleases, snake venom phosphodiesterase, beef heart and higher plant cyclic nucleotide phosphodiesterases, nuclease P1, and 3′- and 5′-nucleotidases. The enzymatic results are considered in relation to the mechanism of the antiviral activity of the cyclic phosphate of DHPG.

Introduction

Since the discovery over 10 years ago of the anti-herpes activity of 9-(2-hydroxyethoxymethyl)-guanine (Acyclovir, ACV), an acyclonucleoside analogue of guanosine [1], a multitude of purine and pyrimidine acyclonucleosides has been synthesized and tested for activity against various viruses (see *e.g.* [2, 3]). Interest in these unusual compounds has been further enhanced by the finding that some of them are potent inhibitors of enzymes involved in nucleic acid metabolism [4–7].

Whereas the antiviral activity of ACV, and some other acyclonucleosides is dependent on prior intracellular activation, *viz.* phosphorylation by viral-coded thymidine kinase (TK) in infected cells, there are others whose mode of action involves alternative pathways [8]. One of the most interesting is the cyclic phosphate of DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG-cMP, see Scheme 1), which is a broad-spectrum *in vitro* antiviral agent, the mechanism of action of which is not dependent on viral kinases and, indeed, is active against viruses which do not express such kinases [9].

The foregoing prompted us to prepare the monophosphates and cyclic phosphates of DHPG, C-DHPG, DHBG and HPG, the two latter of which may exist in two enantiometric forms, (*R*) and (*S*) (see Scheme 1), and to examine their conformational features, as well as their substrate/inhibitor properties in several relevant enzyme systems. This is an extension of previous investigations [10–12], based on the ability of the acyclic chains to mimic the conformation of some fragment of the pentose ring of the corresponding parent nucleoside, *e.g.* the acyclic chains of DHBG and HPG may be regarded as structural analogues of the "lower" portion of the 2′-deoxyribose ring. Such studies should contribute to a better understanding of the biological activities of these compounds, including their antiviral activities. It is also worth noting that the cyclic phosphates of DHPG, C-DHPG, DHBG and HBG may be considered as structural analogues of the signal molecule cGMP.

Abbreviations employed: ACV, 9-(2-hydroxyethoxymethyl)guanine; DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; DHPG-cMP, DHPG-3′:5′-cyclic phosphate; C-DHPG, carbo-DHPG, 9-(3-hydroxymethyl-4-hydroxybutyl)guanine; C-DHPG-cMP, C-DHPG-4′:4″-cyclic phosphate; DHPAde, adenine analogue of DHPG; DHPAde-cMP, DHPAde-3′:5′-cyclic phosphate; DHBG, 9-(3,4-dihydroxybutyl)guanine; DHBG-cMP, DHBG-3′:4′-cyclic phosphate; DHBG-MP, DHBG-4′-monophosphate; HPG, 9-(2,3-dihydroxypropyl)guanine; HPG-cMP, HPG-2′:3′-cyclic phosphate; PDase, phosphodiesterase; cPDase, cyclic nucleotide phosphodiesterase; RNase, ribonuclease.

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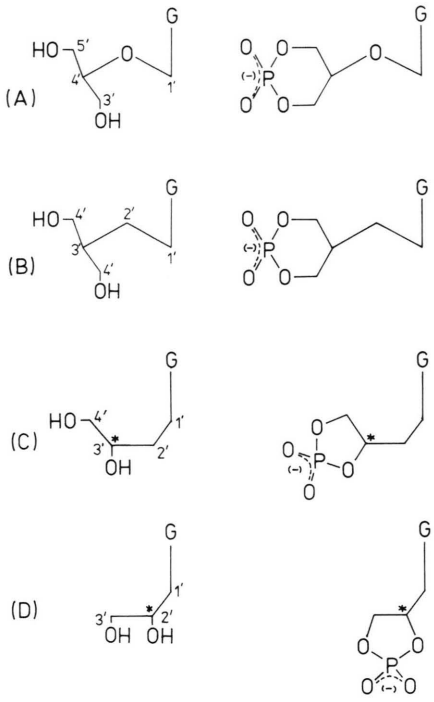


Scheme 1. Structures of investigated acyclonucleosides (left) and their cyclic phosphates (right). G = guanine; and a star indicates an asymmetric carbon: (A) DHPG and its 3′:5′-cyclic phosphate; (B) C-DHPG and its 4′:4″-cyclic phosphate; (C) (*R*)- and (*S*)-DHBG and their 3′:4′-cyclic phosphates; (D) (*S*)-HPG and its 2′:3′-cyclic phosphate.

Note: The numbering system for the acyclic chain carbons is in line with that for the carbons of the corresponding pentafuranose ring.

Materials and Methods

^{1}H NMR spectra of 0.02 M solutions were recorded on Bruker 270 and 400 MHz instruments at room temperature.

Thin-layer chromatography made use of Merck (Darmstadt, F.R.G.) F_{254} plates with the solvent systems: (A) 1 M ammonium acetate:96% EtOH (2:5, v/v); (B) isopropanol:conc. NH_4OH:H_2O (7:1:2, v/v).

Sephadex DEAE A-25 was a product of Pharmacia (Uppsala, Sweden), Sephadex XAD-4 and Dowex 50W 1 × 8 were from Serva (Heidelberg, F.R.G.). $POCl_3$ was purified by simple distillation, trimethylphosphate by distillation under reduced pressure, and acetonitrile by successive distillation over P_2O_2 and CaH_2.

DHPG was a gift from Dr. J. G. Moffat. C-DHPG, (*R*)- and (*S*)-DHBG were gifts of Dr. N. G. Johansson, and (*S*)-HBG was a gift from Dr. A. Holy.

The cyclic phosphate of DHPAde, DHPAde-cMP, was prepared as previously described [12].

Beef beart cPDase (EC 3.1.4.17), snake venom PDase (EC 3.1.15.1), nuclease P1 (EC 3.1.30.1), RNases A (EC 3.1.27.5), T1 (EC 3.1.4.8) and T2 (EC 3.1.27.1), 3′-nucleotidase and 5′-nucleotidase were products from Sigma (St. Louis, U.S.A.). Potato tuber cPDase was a partially purified preparation, elsewhere described [13]. Values of K_m and V_{max} were obtained from Lineweaver-Burk plots with the aid of the Bisenthal-Cornish-Bowden algorithm, using an IBM PC program elsewhere reported [14].

(R)- and (S)-DHBG-MP. (*R*)-DHBG and (*S*)-DHBG were each phosphorylated with the aid of the wheat shoot nucleoside phosphotransferase system as elsewhere described [15]. The resulting 4′-monophosphates were isolated from the reaction mixture by chromatography on Whatman paper 3 MM with solvent B (R_f of monophosphates 0.15, of DHBG 0.71). The products were eluted with water, and the eluates concentrated under vacuum and lyophilized (yields 40–50%).

(R)-DHBG-cMP. To a suspension of 480 mg (2 mmol) of (*R*)-DHBG in 10 ml triethylphosphate was added, portionwise and with constant stirring over a period of 5 h, 300 µl (3.3 mmol) $POCl_3$ [16]. The mixture was then poured onto ice and water, brought to neutrality with $NaHCO_3$, and loaded on a 3.3 × 30 cm column of DEAE-Sephadex A-25 (HCO_3^-). Elution was then conducted with a linear gradient of 0–0.8 M triethylammonium carbonate (2 × 21). Traces of non-reacted nucleoside were eluted with water, and the cyclic phosphate at 0.3–0.4 M, followed by ~5% of unidentified product. The cyclic phosphate was desalted, and freed from inorganic phosphate, on a Sephadex XAD-4 column by washing with water, and converted to the sodium salt on a column of Dowex 50W 1 × 8 (Na^+). Precipitation with ethanol yielded 465 mg (75%) as a white powder, R_f = 0.27 on TLC, with solvent A (R_f of (*R*)-DHBG = 0.52). The UV spectrum was identical with that of the starting product. ^{1}H NMR in $(C^2H_3)_2SO$ with internal Me_4Si: δ (ppm) 10.64 (NH), 7.67 (H-8), 6.64 (NH_2), 4.05 (H-1′), 4.15 (H-3′), 4.02 and 3.56 (H-4′), 1.89

(H-2′). ^{31}P NMR in $^{2}H_2O$ relative to external H_3PO_4: δ (ppm) 16.29. The product was resistant to alkaline phosphatase, but was hydrolyzed to the mononucleotide by potato tuber cPDase (see below).

(S)-DHBG-cMP. This was obtained by phosphorylation of (*S*)-DHBG (60 mg, 0.25 mmol) as for the (*R*)-epimer, above. In this instance, however, column chromatography led to a clear separation of the product from a minor unidentified product (~2% yield). The cyclic phosphate was precipitated with ethanol to give 35 mg (45% of a white amorphous powder, chromatographically homogeneous, with solvent A, R_f = 0.27; UV, ^{1}H and ^{31}P NMR spectra identical with those of the (*R*)-epimer.

C-DHPG-cMP. Phosphorylation of 127 mg (0.5 mmol) of C-DHPG, as described above for (*S*)-DHBG, yielded 60 mg (35%) of a white amorphous powder, chromatographically homogeneous (R_f 0.28; for C-DHPG, R_f 0.58).

Alternative procedure, using the technique of Prisbe *et al.* [17]: to a solution of 127 mg (0.5 mmol) of C-DHPG in 100 ml acetonitrile was added 85 µl $SnCl_4$ in 5 ml ethylene chloride. To the resulting clear solution was added portionwise, with stirring, pyrophosphoryl chloride in 50 ml acetonitrile, prepared by a procedure other than that of Prisbe *et al.* [17], *viz.* equimolar quantities (2.5 mmol) of $POCl_3$ and water dissolved in 5 ml acetonitrile, and the solution diluted to 50 ml with acetonitrile. The reaction mixture was left for 24 h at room temperature, following which starting substance disappeared, with formation of one product. Chromatography on a column of DEAE-Sephadex A-25 was followed by desalting on a column of Sephadex XAD-4, and conversion to the Na^+ salt, as above. Precipitation with ethanol gave 106 mg product (62% yield) in the form of white amorphous powder, chromatographically homogeneous, with a ^{1}H NMR spectrum in $(C^2H_3)_2SO$ identical with that of the product described in the preceding paragraph: δ (ppm *vs.* internal Me_4Si) 10.55 (NH), 7.70 (H-8), 6.48 (NH_2), 3.95 (H-1′), 3.88 and 3.72 (H-4′, H-4″ four protons), 1.63 (H-2′), 1.56 (H-3′). ^{31}P NMR in $^{2}H_2O$: δ (ppm *vs.* external H_3PO_4) −2.68.

(S)-HBG-cMP. Phosphorylation of 56 mg (0.25 mmol) of HBG, as described above, yielded 42 mg (60%) of a white amorphous powder of the cyclic phosphate and 9 mg of an additional, unidentified product. The main product was chromatographically homogeneous, R_f = 0.31 with solvent A (R_f of (*S*)-HBG = 0.47). The UV spectrum was identical with that of the substrate. ^{1}H NMR in $^{2}H_2O$: δ (ppm *vs.* internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$ propionate) 7.88 (H-8), 4.37 and 4.31 (H-1′), ~4.7 under H_2O (H-2′), 4.41 and 4.07 (H-3′).

DHPG-cMP. Phosphorylation of 252 mg (1 mmol) of DHPG, as described above, yielded 180 mg (56%) of the cyclic phosphate, 32 mg (9%) of the monophosphate mixture of 3′- and 5′-epimers, and 83 mg (17%) of 3′,5′-*bis*-monophosphate.

Phosphorylation of DHPG using the method of Prisbe *et al.* [12], as for C-DHPG gave 22 mg (18%) of the cyclic phosphate chromatographically and spectrophotometrically identical with that of Yoshikawa phosphorylation (see above). TLC with solvent A, R_f = 0.28 (R_f of DHPG 0.56). ^{1}H NMR in $^{2}H_2O$: δ (ppm *vs.* internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate): 7.96 (H-8), 5.63 (H-1′), 4.24 (H-3′, H-5′ equatorial), 4.38 (H-3″, H-5″ axial), 3.84 (H-4′).

Results and Discussion

Chemical phosphorylation

Phosphorylation of DHPG with $POCl_3$ in triethylphosphate [16], as previously applied to phosphorylation of DHPAde [12], yielded as the major product the cyclic phosphate, DHPG-cMP (56%), and two minor products: 3′,5′-*bis*-monophosphate and a mixture of 3′- and 5′-monophosphates. The structures were confirmed by ^{1}H NMR spectroscopy [12]. The structure of the cyclic phosphate was additionally confirmed by the identity of its chromatographic properties and ^{1}H NMR spectrum with those of the product of phosphorylation by the alternative procedure of Prisbe *et al.* [17], which leads to the cyclic phosphates of acyclonucleosides.

The foregoing results are at variance with those reported for phosphorylation of DHPG by Tolman *et al.* [9] and DHPAde by MacCoss and Tolman [18], the major products being the 3′,5′-*bis*-monophosphates, accompanied by low yields of the 3′:5′-cyclic phosphates. This discrepancy, previously alluded to [12], now appears due to differ-

ences in phosphorylating conditions. In our approach (see Experimental), $POCl_3$ was added portionwise to a solution of the acyclonucleoside in $(CH_3O)_3PO$, whereas in the procedure of Tolman *et al.* [9] and MacCoss and Tolman [18], the acyclonucleoside was added to a solution of $POCl_3$ in $(CH_3CH_2O)_3PO$ (M. MacCoss, personal communication). This difference in phosphorylation conditions may therefore be profited from to prepare either the *bis*-monophosphates or the cyclic phosphates. Furthermore, under our experimental conditions, cyclic phosphate formation clearly must proceed *via* a monophosphate intermediate, followed by rapid nucleophilic attack at the phosphate moiety by the adjacent hydroxyl, *i.e.* under conditions where the $POCl_3$ concentration is low relative to the intermediate monophosphate.

Phosphorylation of C-DHPG with Yoshikawa method [16] yielded a single product, 4′:4″-cyclic phosphate (C-DHPG-cMP), the structure of which was confirmed by: (a) ^1H and ^{31}P NMR spectroscopy in $(C^2H_3)_2SO$ demonstrating the absence of OH groups and the characteristic coupling constants ~12 Hz between ^{31}P and *all* the four protons at C(4′) and C(4″). (b) Resistance of the product to alkaline phosphatase; (c) identity of the chromatographic properties and ^1H NMR spectrum with those of the product of phosphorylation by the procedure of Prisbe *et al.* [17].

Further application of our phosphorylation method to the (*R*)- and (*S*)-enantiomers of DHBG, and the (*S*)-enantiomer of HPG again yielded, as the major products, the cyclic phosphates: (*R*)- and (*S*)-DHBG-3′:4′-cMP and (*S*)-HPG-2′:3′-cMP respectively, the latter previously reported by Holy [19].

It should be noted that, in contrast to the cyclic phosphates of DHPG and C-DHPG, the cyclic phosphates of the two DHBG enantiomers are 5-membered rings (linking a primary to a secondary hydroxyl) analogous to the 2′:3′-cyclic phosphates of nucleosides. The structures of the DHBG cyclic phosphates were established by: (a) ^1H and ^{31}P NMR spectroscopy in $(C^2H_3)_2SO$, demonstrating the absence of OH groups, the presence of the phosphate group and the characteristic couplings between ^{31}P and neighbouring protons; (b) resistance of both products to alkaline phosphatase; (c) hydrolysis of the cyclic phosphate rings by specific PDases to give the monophosphates (see below). In the case of DHBG and HPG, formation of the cyclic phosphates was accompanied by appearance of minor products (1%–10%) the identity of which is under study.

Enzymatic phosphorylation

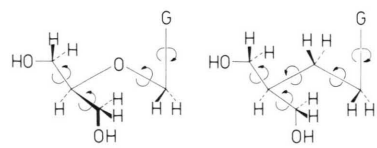
With the aid of the wheat shoot nucleoside phosphotransferase system [15], the (*R*)- and (*S*)-enantiomers of DHBG were converted to the 4′-phosphates in 40–50% yield.

Conformational aspects in solution

In contrast to analogues with a 1,3-dihydroxy-2-propoxy(methyl) chain, for which there is a preference *trans* for substituents about C–O bonds [12], acyclic chains which may also mimic the "lower" portion of the pentose ring exhibit greater conformational flexibility (Scheme 2). For all the acyclonucleosides, and their cyclic phosphates, the values of the vicinal coupling constants $^1H–^1H$ (4.5 Hz–9.5 Hz) point to existence of a dynamic equilibrium of classical conformers with substituents *gauche* and *trans* about C–C bonds, and with comparable populations. This permits adoption by a given acyclic chain of conformations resembling those of the pentose ring.

The value of 11.8 Hz for the vicinal coupling constants $^1H–^{31}P$ in C-DHPG-cMP shows that the cyclic phosphate ring exists as an equilibrium of two classical chair forms with identical populations. For one of these, C(2′) is *gauche* with respect to the cyclic phosphate ring oxygens, as prevails also for the O(4′) in DHPG-cMP and DHPAde-cMP. In the second chair form, C(2′) is *trans* with respect to the ring oxygens as for the O(4′) in nucleoside 3′:5′-cyclic phosphates [12].

The conformation of the 5-membered phosphate ring of DHBG-cMP is in the envelope form with the phosphorus atom displaced from the plane of the remaining atoms, as deduced from the



Scheme 2. Conformational equilibria about C–C and C–O bonds, indicated by arrows.

coupling constants $^{1}H-^{31}P$ in $^{2}H_2O$, 5.8 Hz, 13.4 Hz and 8.1 Hz, respectively, for H(3′) and the two H(4′) protons. With HPG-cMP the $^{1}H-^{31}P$ coupling constants point to an equilibrium of several forms similar to those of nucleoside 2′:3′-cyclic phosphates [20]. More detailed conformational analyses are being undertaken.

Enzymatic aspects

None of the foregoing acyclonucleoside cyclic phosphates was a substrate for RNase A, T1 (which is specific for guanine residues) and T2, or for snake venom PDase. It should, however, be noted that the (*S*), but not the (*R*), enantiomer of the thymine analogue of HPG-cAMP, which can mimic the conformation of the corresponding D-ribonucleoside 2′:3′-cyclic phosphate, was earlier reported to be a substrate for several bacterial RNases [19]. The racemate of the foregoing was also claimed to be susceptible to RNase A [21], but the enormous concentrations of enzyme and substrate employed suggest that it is, at best, a very feeble substrate.

Higher plant cPDase. In line with previous observations that DHPAde-cMP, as well as 2′,3′-*seco*-3′:5′-cAMP, are substrates of potato tuber cPDase [11, 12], both enantiomers of DHBG-cMP, as well as (*S*)-HPG-cMP, were excellent substrates of this enzyme, each being converted to the monophosphate, readily dephosphorylated by alkaline phosphatase to the parent acyclonucleoside. From Lineweaver-Burk plots (see Fig. 1) the K_m values for both (*R*)- and (*S*)-DHBG-cMP were 0.5 mM, and for HPG-cMP 0.1 mM; while the V_{max} values were comparable to those for the natural substrates, 2′:3′- and 3′:5′-cAMP. By contrast, DHPAde-cMP was a poorer substrate (K_m ~10 mM and V_{max} about 10% that for DHBG-cMP); and DHPG-cMP was even less susceptible to hydrolysis, at a rate 2- to 3-fold lower than that for DHPAde-cMP.

Relevant to these findings are the known multisubstrate properties of potato (and other higher plant) cPDase, which hydrolyzes both 2′:3′- and 3′:5′-cyclic phosphates of nucleosides. We have elsewhere shown that mild alkaline treatment of the enzyme leads to selective loss of activity *vs.* 3′:5′-cyclic phosphates, with full maintenance of activity *vs.* 2′:3′-cyclic phosphates [13]. We now

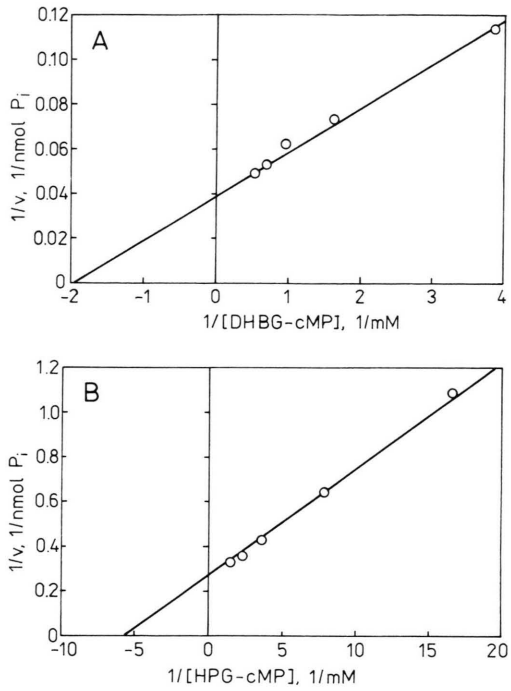


Fig. 1. Lineweaver-Burk double reciprocal plots for hydrolysis by potato tuber cPDase of the (*R*)- and (*S*)-enantiomers of DHBG-cMP (top) and (*S*)-HPG-cMP (bottom).

find that, following such selective inactivation of activity *vs.* nucleoside 3′:5′-cyclic phosphates, there is no change in activity of the enzyme *vs.* both enantiomers of DHBG-cMP, and only a small decrease in the rate of hydrolysis of (*S*)-HPG-cMP. It follows that hydrolysis of these compounds is due solely to the activity of the enzyme *vs.* 2′:3′-cyclic phosphates, consistent with the fact that they contain 5-membered cyclic phosphate rings. This was further conformed by the fact that the selectively inactivated enzyme did not hydrolyze DHPG-cMP or DHPAde-cMP, both with 6-membered cyclic phosphate rings.

Beef heart cPDase. Somewhat unexpected was the finding that both enantiomers of DHBG-cMP were converted to the mononucleotides by this mammalian enzyme, with K_m ~1 mM and V_{max} comparable to that for 3′:5′-cAMP. DHBG-cMP was also slowly hydrolyzed by a crude extract of Ehrlich ascites cells (see also below). HPG-cMP was not a substrate of the beef heart enzyme.

Nuclease P1: This enzyme, at pH 5.3, hydro-

lyzed both enantiomers of DHBG-cMP to the mononucleotide. At pH 7.2 there was also formation of the nucleosides, consistent with the known 3′-phosphatase activity of this enzyme at the more alkaline pH [22]. (*S*)-HPG-cMP was hydrolyzed at about 50% the rate for DHBG-cMP.

Other enzymes. Both enantiomers of DHBG-4′-monophosphate were resistant to rye grass 3′-nucleotidase, but were very slowly converted to the nucleosides by venom 5′-nucleotidase (at about 1% the rate for 5′-AMP). The 4′-monophosphates also inhibited hydrolysis of DHBG-cMP by potato cPDase ($K_i \sim 0.25$ mM).

The product of hydrolysis of DHBG-cMP by potato cPDase was resistant to venom 5′-nucleotidase, but was slowly converted to the nucleoside by 3′-nucleotidase. This suggests that the product of hydrolysis of DHBG-cMP by potato cPDase is the 3′-phosphate, for both the (*R*)- and (*S*)-epimers.

Quite surprising was the observation that the carba analogue of DHPG-cMP, *i.e.* C-DHPG-cMP, was resistant to all the foregoing enzymes, as well as to Ehrlich ascites cell extracts. This analogue also exhibited no inhibition of hydrolysis of 2′:3′-cAMP or 3′:5′-cAMP by potato cPDase.

Antiviral activity of DHPG-cMP. In contrast to the antiherpes activity of DHPG, DHPG-cMP exhibits potent activity not only against herpes, but also a number of other DNA, viruses [23]. But, whereas the activity of DHPG is dependent on intracellular phosphorylation by viral thymidine kinase, this is not the case for DHPG-cMP [24]. As previously observed by us [11], as well as in the present study, DHPG-cMP is slowly converted to the monophosphate by cell extracts. But, as reported by Germershausen *et al.* [24], further phosphorylation to the triphosphate cannot account for the antiviral activity of this compound, and the mechanism of action of DHPG-cMP as an antiviral reagent remains to be clarified.

Furthermore, the nature of the enzyme(s) which hydrolyze DHPG-cMP in mammalian cell extracts has not been established. We have previously drawn attention to reports on the existence in mammalian cells of cyclic nucleotide phosphodiesterases with properties similar to those of our purified potato tuber cPDase [13], and consider that such non-conventional cPDase(s) may account for the slow hydrolysis of DHPG-cMP in mammalian cells. However, since such hydrolysis is still inadequate to account for the antiviral activity of this compound, other potential pathways must be considered. One possibility which has hitherto not been envisaged is that DHPG-cMP may be the active species as such, perhaps by acting as a signal molecule, like cGMP. It will be of interest to determine whether the cyclic phosphates of other antiviral acyclonucleosides, such as DHBG-cMP, also exhibit antiviral activity.

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