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Phosphorylation of the Enantiomers of the Carbocyclic Analog of 2′-Deoxyguanosine in Cells Infected with Herpes Simplex Virus Type 1 and in Uninfected Cells. Lack of Enantiomeric Selectivity with the Viral Thymidine Kinase

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SUMMARY

CdG, the carbocyclic analog of 2′-deoxyguanosine, is active against herpes, hepatitis B, and human cytomegaloviruses. We have studied the interaction of the tritiated enantiomers of CdG with the herpes simplex virus type 1-specific thymidine kinase (HSV-1 TK) and have examined their metabolism in uninfected and HSV-1-infected cells. D- and L-CdG were equally effective competitive inhibitors of the phosphorylation of thymidine (dThd) by the partially purified HSV-1 TK (K_i values were 2.1 and 3.4 μM, respectively) and were also equal as substrates (K_m values were 17 and 26 μM, respectively, and V_{max} values of the enantiomers were equal and about 50% greater than the V_{max} for dThd). The partially purified enzyme preparation, which contained cellular nucleotide kinase activities (pyruvate kinase also was present in the assay medium), converted D-CdG almost exclusively to the triphosphate and L-CdG almost exclusively to the monophosphate. Similarly, in virus-infected cells the D-enantiomer was converted predominantly to the triphosphate and the L-enantiomer predominantly to the monophosphate. In uninfected cells the results were qualitatively similar. In CEM cells deoxycytidine (dCyd) kinase (EC 2.7.1.74) seemed to be the enzyme principally responsible for the phosphorylation of both enantiomers, as shown by competition studies. Thus, both the HSV-1 TK and cellular dCyd kinase (of CEM cells) showed no selectivity for the enantiomers of CdG. This lack of enantiomeric specificity has obvious implications for the design of inhibitors of both viral proliferation and cellular metabolism.

CdG is of interest because of demonstrated activity against herpes (1), hepatitis B (2), and human cytomegaloviruses (3). We have resolved the enantiomers of CdG and have reported that the anti-HSV activity of CdG is associated primarily with the D-enantiomer (4). In cells infected with HSV-1, the D-enantiomer was converted predominantly to the triphosphate and incorporated into viral and cellular DNA, whereas no triphosphate was detected in virus-infected cells grown in the presence of the L-enantiomer (5, 6). In uninfected HEp-2 cells, D-CdG was also converted to a small extent to the triphosphate (5). We report here a comparison of the phosphorylation of the tritiated enantiomers of CdG by both HSV-1 TK and intact cells. A preliminary report of some of these results has been presented (7).

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Experimental Procedures

Materials. D- and L-CdG were prepared as described (4). The D-enantiomer corresponds in configuration to dGuo. Both enantiomers were tritiated by Moravek Biochemicals (Brea, CA) to yield [8-^{3}H]-D-CdG (250 mCi/mmol) and [8-^{3}H]-L-CdG (900 mCi/mmol). The samples had an initial radiopurity of >99%, but on storage they slowly accumulated some free guanine, presumably because of radiation-induced decomposition. [*methyl*-^{3}H]Thymidine (70–85 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). BzDAG, which was synthesized in our laboratories (8), was provided by Dr. S. Niwas (Kettering-Meyer Laboratory, Birmingham, AL). Pyruvate kinase, GMP kinase (from pig brain), dCyd, and dGuo were obtained from Sigma Chemical Co. (St. Louis, MO). HeLa (BU25) cells, a line deficient in TK, were kindly provided by Dr. Y.-C. Cheng, Yale University. HeLa (BU25) cells and HEp-2 cells were grown in Eagle's minimum essential medium supplemented with bovine calf serum. CEM cells were grown in RPMI 1640 medium supplemented with 10% fetal calf

ABBREVIATIONS: CdG, the carbocyclic analog of 2′-deoxyguanosine or [1*R*-(1α,3β,4α)]-2-amino-1,9-dihydro-9-[3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purin-6-one; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; TK, thymidine kinase; TK$^-$, thymidine kinase-deficient; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; dThd, thymidine; BzDAG, 9-benzyl-9-deazaguanine; HPLC, high performance liquid chromatography; SAX, strong anion exchange; DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; PNP, purine nucleoside phosphorylase; DHBG, 9-(3,4-dihydroxybutyl)guanine; carbovir, the carbocyclic analog of 2′,3′-dideoxy-2′,3′-didehydroguanosine; C-BVDU, the carbocyclic analog of 5-(2-bromovinyl)-2′-deoxyuridine; C-IDU, the carbocyclic analog of 5-iodo-2′-deoxyuridine; C-dThd, the carbocyclic analog of thymidine.

serum. The S148 strain of HSV-1, which encodes a dThd kinase, was used as the source of HSV-1 TK and in studies with virus-infected cells. This virus was grown in HEp-2 cells for use in HEp-2 or HeLa (BU25) cells. A TK^- strain of HSV-1 (BW10168), also used in our previous study (5), was provided by Dr. David R. Scholl, Ohio University, Athens, OH.

Isolation of HSV-1 TK. HSV-1 TK was isolated from HeLa (BU25) cells infected with the S148 strain of HSV-1 at a multiplicity of infection of 10 $CCID_{50}$ per cell (1 $CCID_{50}$ = the number of virions required to infect 50% of the cells). The procedure for isolation was described by Balzarini *et al.* (9) for the isolation of virus-specific TK from a TK^- HeLa cell line infected with the KOS strain of HSV-1.

Assays of nucleosides as substrates and inhibitors of HSV-1 TK. HSV-1 TK was incubated for 20 min at 37° in a total volume of 50 μl containing 50 mM Tris (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 9 mM KF, 5 mM phosphoenolpyruvate, 2.8 μg of pyruvate kinase, 10 mM β-mercaptoethanol, 5 μl of TK, and various concentrations of unlabeled and radiolabeled nucleosides (Figs. 1 and 2). Reactions were terminated by spotting 40 μl of the reaction mixture on DE-81 filter disks (Whatman Lab Sales, Inc., Hillsboro, OR), after which the disks were washed with ethanol. Determination of radioactivity in the phosphorylated product remaining on the disk was accomplished by transferring the disk to a vial, adding Complete Counting Cocktail 4a20 (Research Products International Corp., Mount Prospect, IL), and counting in a Packard Tri-Carb liquid scintillation spectrometer (Packard, Downers Grove, IL).

Metabolism of D- and L-CdG in uninfected and HSV-1-infected cells. Metabolic studies were performed in uninfected HeLa (BU25), HEp-2, or CEM cells and in HEp-2 or HeLa (BU25) cells infected with HSV-1. For studies in virus-infected cells, the cells were infected with the S148 strain of HSV-1 at a multiplicity of infection of 10. The medium was decanted 1 hr after addition of the virus and the cells were washed with phosphate-buffered saline. The cells (either uninfected or virus-infected) were placed in fresh medium containing tritium-labeled D- or L-CdG (8 μM; 2 μCi/ml). After 8 hr the cells were harvested and washed free of medium with Puck's saline G. The cells were then extracted with ice-cold 0.5 N perchloric acid for 0.5 hr. The extract was neutralized with potassium bicarbonate and the resulting precipitate was removed by centrifugation. The supernatant was lyophilized to dryness and the residue was dissolved in water. This solution was then subjected to HPLC analysis as described below.

HPLC. HPLC analyses were performed with a Series 4 liquid chromatograph (Perkin Elmer, Norwalk, CT). Nucleotides were separated on a Partisil-10 SAX column (Whatman, Inc., Clifton, NJ) using a 50-min linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) at a flow rate of 2 ml/min. Elution was monitored by determining absorbance at 254 nm or by the measurement of radioactivity. In experiments involving radioactivity, 2 ml samples were collected directly into counting vials. To each vial was added 13 ml of Safety Solve (high flash point mixture) (Research Products International Corp., Mount Prospect, IL), after which the vials were assayed for radioactivity by liquid scintillation spectrometry.

Results

D- and L-CdG inhibition of the phosphorylation of dThd catalyzed by HSV-1 TK. Both enantiomers of CdG competitively inhibited the phosphorylation of dThd by the partially purified HSV-1 TK (Fig. 1) with K_i values of 2.1 μM for D-CdG and 3.4 μM for L-CdG. The K_i value for inhibition of dThd phosphorylation by racemic CdG was 3.0 μM. dGuo also was a competitive inhibitor of the phosphorylation of dThd with a K_i value of 10.5 μM (results not shown).

D- and L-CdG as substrates for HSV-1 TK. Tritium-labeled D- and L-CdG were assayed as substrates for viral TK using the disk method of assay. Lineweaver-Burk plots for both

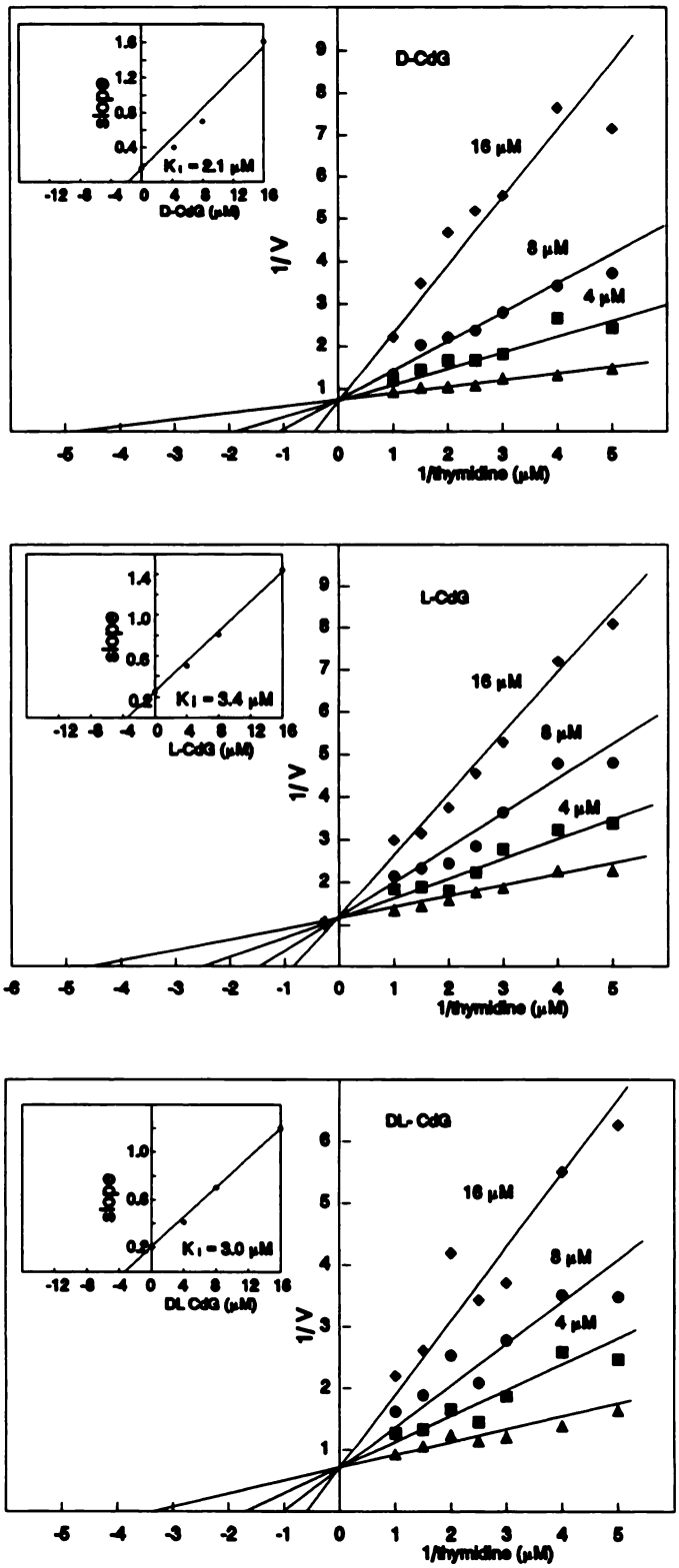
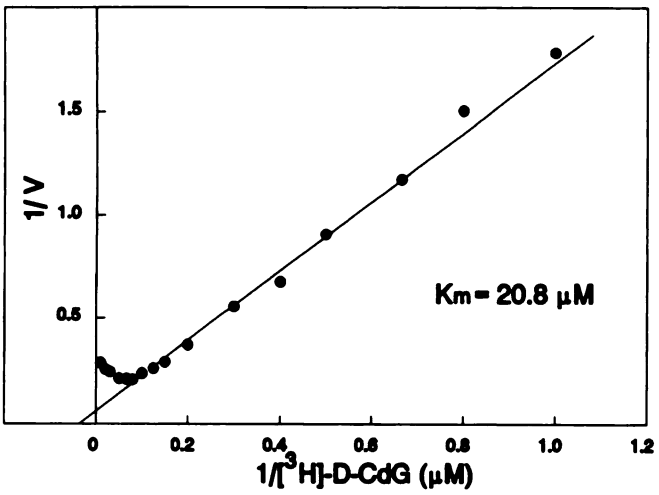


Fig. 1. Lineweaver-Burk and Dixon plots of the effects of D-CdG, L-CdG, and DL-CdG on the phosphorylation of [*methyl*-^{3}H]thymidine by partially purified HSV-1 TK. Controls contained the enzyme, the standard incubation mixture (see text), and various concentrations of [*methyl*-^{3}H] thymidine; treated experiments contained these additions plus D-CdG, L-CdG, or DL-CdG at the concentrations shown in the charts.



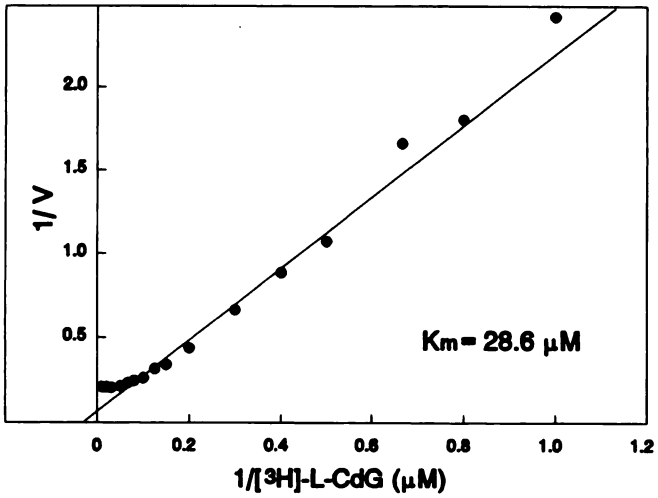


Fig. 2. Lineweaver-Burk plots for the phosphorylation of D-CdG and L-CdG by partially purified HSV-1 TK. The assay mixtures contained the standard constituents (see text) plus D-CdG or L-CdG at the concentrations shown in the charts. The units of the velocities are nanomoles/hr/mg of protein.

TABLE 1

Kinetic constants for dThd, D-CdG, and L-CdG as substrates for HSV-1 TK

Substrate	K_m	Relative V_{max}[a]
	μM	
dThd	0.32 ± 0.076[b]	100
D-CdG	16.7 ± 3.5[c]	145 ± 0.7[d]
L-CdG	26.0 ± 3.7[d]	148 ± 8.4[d]

[a] Relative to V_{max} for dThd taken as 100.
[b] Mean ± standard deviation of nine determinations.
[c] Mean ± standard deviation of five determinations.
[d] Mean ± standard deviation of two determinations.

enantiomers showed nonlinear kinetics at the higher concentrations (Fig. 2). Similar results at higher substrate concentrations have been reported for dThd and acyclovir as substrates for HSV-1 TK (10–12). The lines (Fig. 2) were extrapolated from the linear portions of the double reciprocal plots to obtain the K_m values. Table 1 presents a summary of the kinetic constants for the enantiomers of CdG and dThd. The K_m and V_{max} values for L-CdG did not differ significantly from those for D-CdG. The K_m values for the CdG enantiomers were much higher than those for the dThd and the V_{max} values were about 50% greater than those for dThd. The K_m value of 0.32 μM for dThd is in the range of values reported by others (9, 10, 13). The phosphorylation of CdG by the HSV-1 TK was inhibited by dThd (result not shown).

The assays discussed above were accomplished by the disk method, which measures total radioactivity retained on the disk. Because of the unexpected finding that the L-enantiomer was as good as the D-enantiomer as a substrate, it was desirable to confirm nucleotide formation by an independent method. Accordingly, the reaction mixtures were examined by SAX HPLC. Analyses were performed of products from assays with both radioactive and nonradioactive enantiomers. Because these experiments were performed to characterize the products, the reactions were allowed to go to completion (1 hr reaction time). Fig. 3 shows the results of experiments in which the nonradioactive substrates were used and products were detected by absorbance at 254 nm. The principal product from D-CdG was the triphosphate, whereas the principal product from L-CDG was the monophosphate. From the results with the enantiomers, it was predicted that the racemate yielded approximately equal amounts of a monophosphate and a triphosphate (Fig. 3). Studies with the tritiated enantiomers were performed in a similar fashion. The results (not shown) indicated that the triphosphate accounted for 95% of the products from D-CdG and that the monophosphate accounted for 98% of the products from L-CdG. These findings indicate the presence, in the enzyme assay mixture, of nucleoside monophosphate and nucleoside diphosphate kinase activities. For the nucleoside monophosphate kinase, D-CdG monophosphate must be a good substrate and L-CdG monophosphate must be a poor substrate.

These nucleotide kinase activities are probably a result of cellular enzymes present in the partially purified viral TK preparation. However, it is possible that pyruvate kinase, present in the assay mixture, accounted for some or all of the phosphorylation of the diphosphates, because this enzyme has been shown to catalyze the phosphorylation of certain nucleoside diphosphates (14). The fact that one enantiomer of CdG was converted predominantly to the triphosphate and the other predominantly to the monophosphate should not have affected the results of the kinetic studies, because these assays were performed by the disk method and the disks would have retained all nucleoside phosphates present.

Phosphorylation of enantiomers of CdG in intact cells. Experiments on the phosphorylation of the enantiomers of CdG in intact cells were performed with HEp-2, CEM, and HeLa (BU 25) cells. In HEp-2 cells the phosphorylation of both enantiomers was examined in both uninfected and infected cells; in CEM cells, the enantiomers were compared in uninfected cells only; and in HeLa (BU 25) cells, only the L-enantiomer was examined. In infected HEp-2 cells and uninfected CEM cells, the D- and L-enantiomers were studied in the same experiments; in uninfected HEp-2 cells the enantiomers were studied in separate experiments. Overall the results were similar to the results with the isolated HSV-1 TK; in both infected and uninfected cells, the predominant product of D-CdG was the triphosphate and the predominant product of L-CdG was the monophosphate (Table 2, Fig. 4). In HEp-2 cells, infection with HSV-1 greatly increased the phosphorylation of both enantiomers, but the phosphorylation of D-CdG was in-

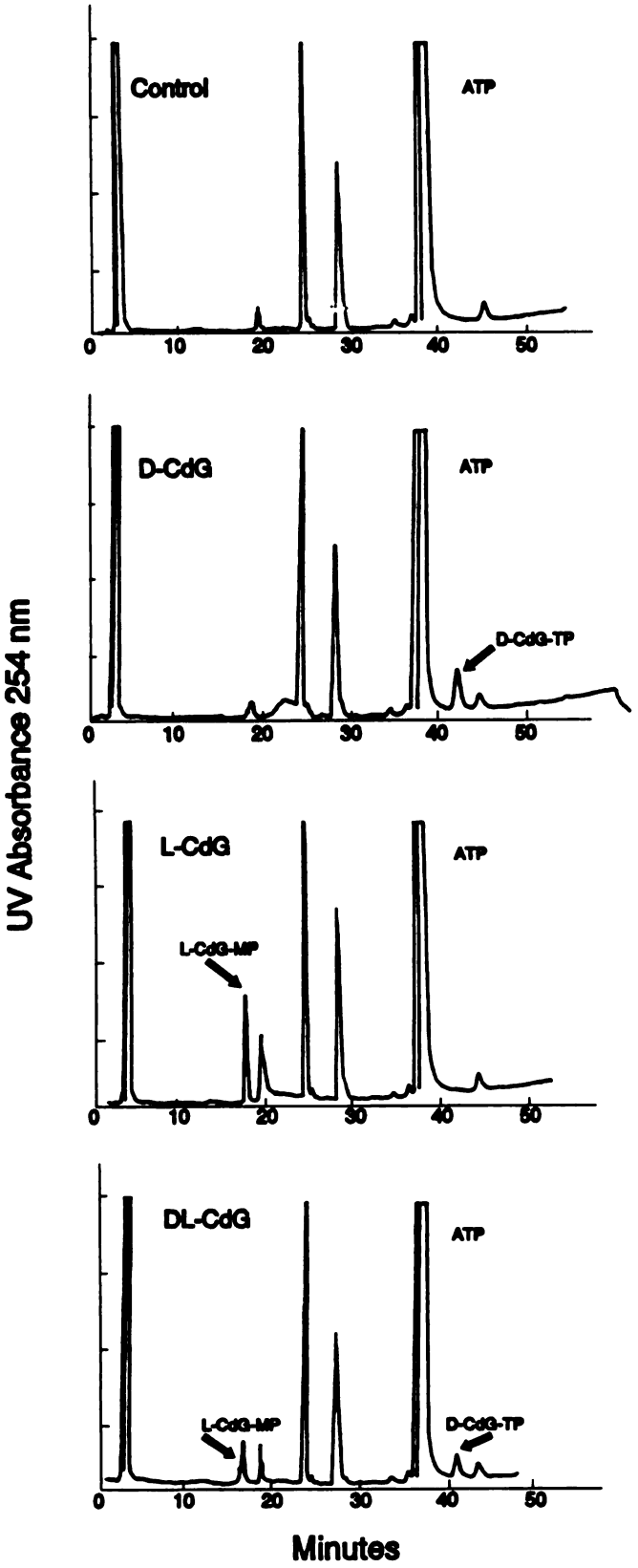


Fig. 3. HPLC analysis of the products of phosphorylation of racemic CdG and its enantiomers by partially purified HSV-1 TK. The assay mixture was the standard mixture (see text) except that ATP was at 0.5 mM to facilitate HPLC assay. After 60 min of incubation with the substrates (50 μM) and enzyme (0.89 mg/ml) the reaction was stopped by immersion of the assay tube in a boiling water bath. After centrifugation, the supernatant solution was subjected to SAX HPLC, which was performed as described in the text except that there was a 10 min delay before beginning the 50 min gradient (to provide better resolution of the monophosphates). An incubation mixture to which no CdG was added served as control. Detection was by determination of absorbance at 254 nm. The new peaks appearing on the chromatograms of assay mixtures containing CdG were scanned to determine that they had ultraviolet spectra characteristic of guanine.

TABLE 2

Phosphorylation of enantiomers of CdG in uninfected cells and in cells infected with HSV-1

[^{3}H]-L-CdG were added to uninfected cells or to cells infected with HSV-1 (strain S148) at a concentration of 8 μM (2 μCi/ml). Eight hr after addition of the labeled compounds, the cells were harvested and extracted, and the extract was subjected to SAX HPLC. Fractions of 2 ml each were collected and assayed for radioactivity by liquid scintillation spectrometry. Results shown are for single experiments.

Cell type and treatment	CdG enantiomer	Concentrations		
		MP[a]	DP[a]	TP[a]
		pmol/10^6 cells		
HEp-2				
Uninfected	D[b]	0.06	0.09	0.41
	L	0.47	0.07	0.15
Infected	D	6.03	20.7	99.4
	L	7.89	0.53	1.28
CEM				
Uninfected	D	0.15	0.34	1.09
	L	2.65	0.33	0.36
HeLa (BU25)				
Uninfected	L	1.05	0.12	0.15
Infected	L	4.72	0.22	0.44

[a] MP, monophosphate; DP, diphosphate; TP, triphosphate.
[b] The data for D-CdG in uninfected HEp-2 cells were derived from a previously published experiment (5).

creased much more than phosphorylation of L-CdG. Thus, in uninfected HEp-2 cells the concentrations (picomoles/10^6 cells) of the total phosphates of D- and L-CdG were 0.56 and 0.69, respectively, and in infected cells the corresponding concentrations were 126 and 9.7, respectively. Uninfected CEM cells produced more phosphates from L-CdG than did uninfected HEp-2 or HeLa (BU 25) cells, and more phosphates from L-CdG than from D-CdG (Fig. 5, Table 2). Thus, both D-CdG and L-CdG are substrates for one or more cellular-phosphorylating enzymes.

One experiment was performed on the metabolism of L-CdG in HEp-2 cells infected with the BW10168 strain of HSV-1. Cells infected with this TK$^-$ strain did not phosphorylate L-CdG to any greater extent than did uninfected cells (results not shown). We have reported similar results with D-CdG (5).

Identity of the cellular enzymes responsible for phosphorylation of the enantiomers of CdG. The cellular enzymes most likely responsible for the phosphorylation of CdG are those known to catalyze the phosphorylation of dGuo, namely cytosolic dCyd kinase, which seems to be the enzyme primarily responsible for the phosphorylation of dGuo in mammalian cells (15, 16), and mitochondrial dGuo kinase, which is highly specific (17). Another possible candidate is 5′-nucleotidase, which has been shown to catalyze phosphorylation of several nucleoside analogs, such as carbovir, acyclovir, and 2′,3′-dideoxyguanosine (18–20). Competition studies in whole cells were performed to obtain information on the kinases involved in CdG phosphorylation. CEM cells were incubated with tritiated D- or L-CdG, plus dCyd or dThd, and the cellular content of CdG phosphates was determined by HPLC (Fig. 6). The formation of phosphates from both enantiomers was in-

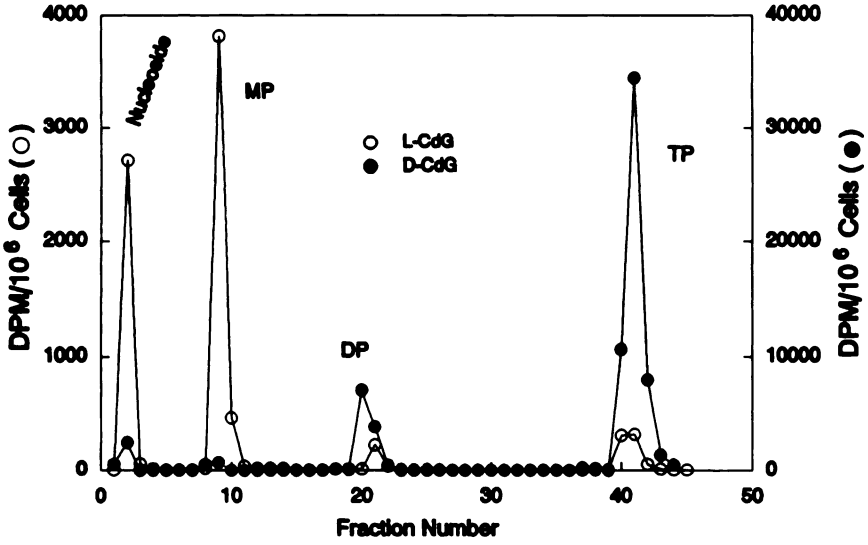


Fig. 4. Metabolism of [^{3}H]-L-CdG and [^{3}H]-D-CdG in HEp-2 cells infected with the S148 strain of HSV-1. Cells were infected with the virus 1 hr before the addition of the labeled nucleoside, which was present at a concentration of 8 μM (2 μCi/ml). Eight hr after the addition of the label, the cells were harvested, washed, and extracted with cold perchloric acid, after which the extract was subjected to SAX HPLC. Fractions (2 ml) were collected and assayed for radioactivity by liquid scintillation spectrometry. Note that different scales are used for the ordinates for the D- and L-CdG. MP, monophosphate; DP, diphosphate; TP, triphosphate; DPM, disintegrations per minute.

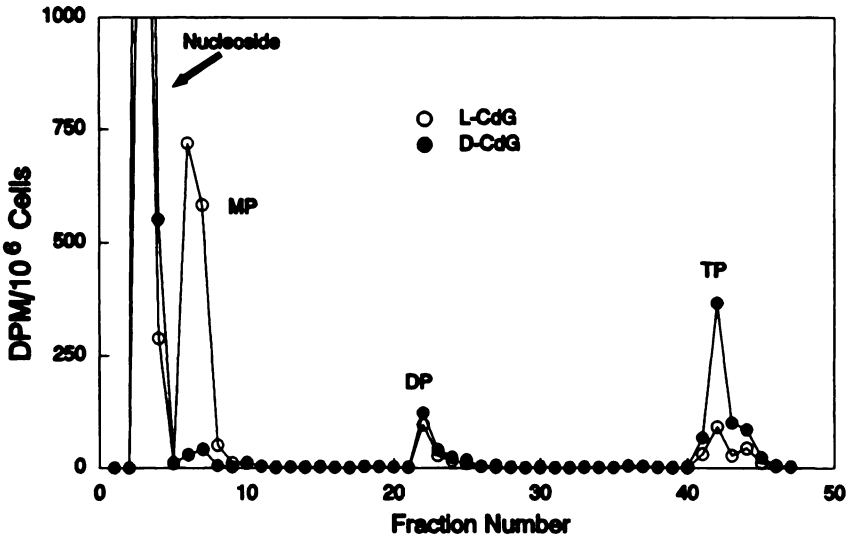


Fig. 5. Metabolism of [^{3}H]-D-CdG and [^{3}H]-L-CdG in CEM cells. Tritiated L-CdG or D-CdG were added to logarithmically growing cultures of CEM cells to a final concentration of 8 μM (2 μCi/ml). After 8 hr the cells were harvested and an extract was prepared and subjected to analysis by SAX HPLC as described in Fig. 4.

hibited strongly by dCyd and was increased by dThd. The stimulation by dThd probably results from the known capacity of dTTP to activate dCyd kinase (21). Similar experiments were performed with HEp-2 and HeLa (BU25) cells. In neither of these cell lines did dThd decrease the phosphorylation of CdG; dCyd produced only a small decrease in phosphorylation in HeLa(BU 25) cells and little or no decrease in HEp-2 cells (results not shown). In CEM cells the high effectiveness of dCyd rules out 5′-nucleotidase as a primary contributor to the phosphorylation, and also eliminates mitochondrial dGuo kinase, for which dCyd does not have detectable substrate activity (17). Other than dCyd kinase, the only kinase for which dCyd has good substrate activity is mitochondrial dThd kinase (22). This enzyme is an unlikely candidate because dGuo does not have detectable substrate activity (22). Moreover, it is eliminated from consideration by the failure of dThd to decrease the phosphorylation of CdG. Thus, it seems that dCyd kinase is the principal catalyzing enzyme in the phosphorylation of CdG in CEM cells. The identities of the enzymes phosphorylating CdG in uninfected HeLa (BU 25) and HEp-2 cells remain to be determined.

L-CdG monophosphate as a substrate for GMP kinase. L-CdG monophosphate seemed to be, at best, only poorly converted to higher phosphates. Therefore, it was desirable to examine it as a substrate for GMP kinase, the kinase most likely to act on it. Because the monophosphate was not available, we generated it by administering tritiated L-CdG to cells. An extract of the cells was incubated with GMP kinase under conditions described by Buccino and Roth (23). An excess of GMP kinase was used and the reaction was allowed to proceed for 1 hr. Under these conditions, GMP was phosphorylated completely, whereas only a part of the L-CdG was phosphorylated (results not shown). The relatively poor activity of L-CdG monophosphate as a substrate for the kinase is in accord with the poor conversion of L-CdG to polyphosphates in cells. The fact that some activity was observed indicates that the small amounts of polyphosphates formed in cells incubated with L-CdG may represent true phosphates of the L-enantiomer, rather than phosphates from a small amount of D-enantiomer contaminating the L-enantiomer.

Comparison of dGuo and CdG as substrates for HSV-1 TK. It was of interest to compare CdG and its natural counterpart, dGuo, as substrates for the viral kinase. Because the preparation of the viral enzyme was purified only partially, it was necessary to take into account the possible presence of interfering enzymes. The kinase preparation was in fact found

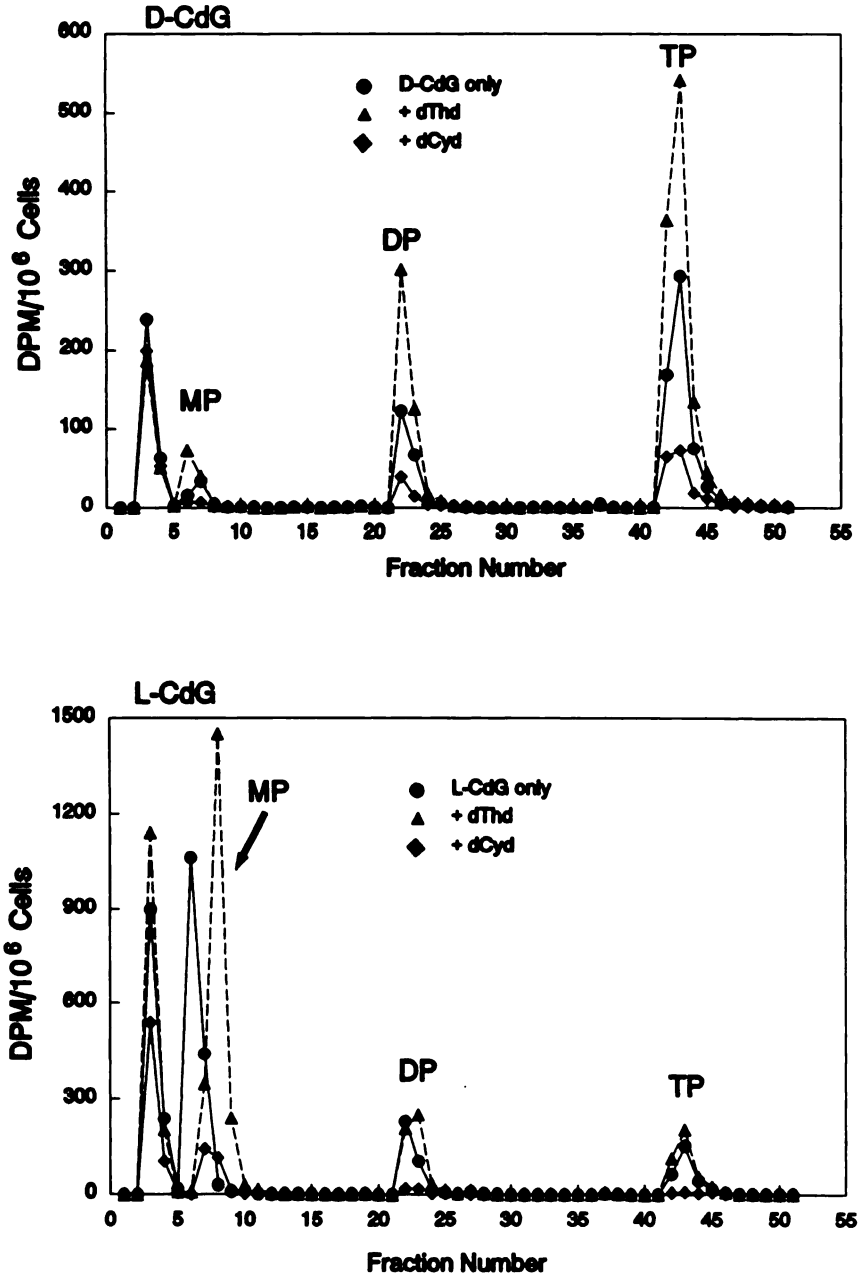


Fig. 6. Effects of dCyd and dThd on the phosphorylation of D-CdG and L-CdG in CEM cells. To cultures of logarithmically growing CEM cells (4×10^5 cells/ml) were added: [^{3}H]-D-CdG (8 μM), [^{3}H]-D-CdG (8 μM) plus dThd (50 μM), [^{3}H]-D-CdG (8 μM) plus dCyd (50 μM), [^{3}H]-L-CdG (8 μM), [^{3}H]-L-CdG (8 μM) plus dThd (50 μM), or [^{3}H]-L-CdG (8 μM) plus dCyd (50 μM). dThd and dCyd were added 2 hr before addition of the labeled compounds. Eight hr after the additions, the cells were harvested and extracted, and the extract was analyzed by SAX HPLC. Fractions (2 ml) were collected and assayed for radioactivity by liquid scintillation spectrometry. The variability in retention times of the L-CdG monophosphates was probably a result of variability introduced by the use of relatively large (2 ml) samples for radioactivity determinations.

to contain some PNP activity, which could interfere with phosphorylation of dGuo by cleaving it to guanine and deoxyribose 1-phosphate. Therefore, we added BzDAG, a potent inhibitor of PNP, to the incubation mixture (8). The phosphorylation of CdG proceeded at a much higher rate than that for dGuo (Fig. 7). Similar experiments performed in the absence of BzDAG yielded about the same results with D-CdG and showed, at best, a slight decrease in the rate of phosphorylation of dGuo (results not shown). Thus, although PNP activity was present in the enzyme preparation, it was not significantly limiting in the phosphorylation of dGuo.

Discussion

The inhibitory forms of antiherpetic nucleoside analogs are the triphosphates; their antiviral activity is a result of either inhibition of the virus-coded DNA polymerase or incorporation of the analog into viral DNA. Activity of nucleoside analogs for viral inhibition may therefore be determined by substrate activity for any of the enzymes leading to the triphosphate and by capacity of the triphosphates to act as inhibitors or substrates of the viral polymerase (24). The antiherpetic selectivity of most known nucleoside analogs results from their activities as substrates for the virus-specific dThd kinase and their lack of activity as substrates for cellular phosphorylating enzymes. We already have reported that the antiherpetic activity of CdG is associated primarily with the D-enantiomer (4). The results of the present paper indicate that the superior antiviral activity of D-CdG, compared with L-CdG, is not the result of selective phosphorylation by HSV-1 TK. However, there are dramatic differences in the further phosphorylation of their monophosphates and in the polymerase-inhibitory activity of their triphosphates. Very little triphosphate is formed from L-CdG (Fig.

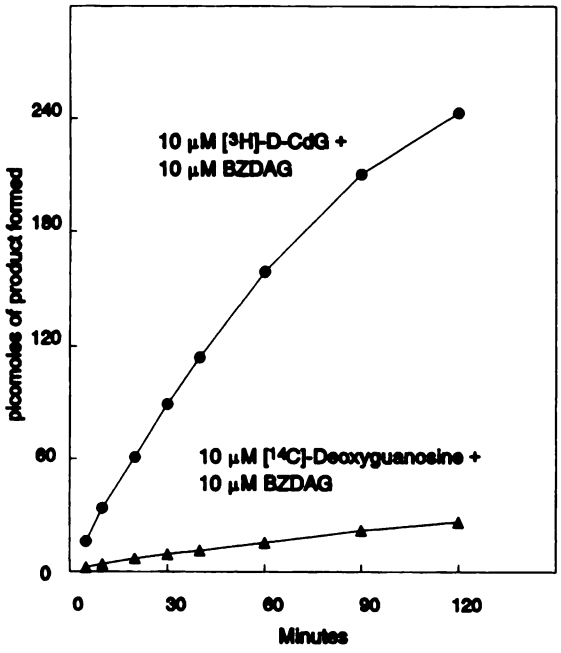


Fig. 7. Comparison of D-CdG and dGuo as substrates for partially purified HSV-1 TK. HSV-1 TK was incubated with the standard assay mixture (see text) containing 10 μM [^{3}H]-D-CdG plus 10 μM BzDAG or 10 μM [8-^{14}C]dGuo plus 10 μM BzDAG. Nucleotide formation was assayed by the disk method (see text). Within 2 hr, 68% of the D-CdG was phosphorylated.

4), and L-CdG-triphosphate is a poor inhibitor of HSV polymerase (6); either of these factors would render L-CdG a relatively ineffective antiherpetic agent.

Because only partially purified HSV-1 TK was used, and because uninfected cells had some capacity to phosphorylate CdG, the possibility exists that the phosphorylation by infected cells and by the isolated viral kinase was catalyzed by cellular enzymes induced by the viral infection. That enzyme induction is not involved is indicated by two lines of evidence: 1) infection with a TK$^-$ strain of HSV-1 (BW10168) did not increase the phosphorylation of CdG, and 2) dThd inhibited the phosphorylation of CdG by the isolated HSV-1 TK but not by uninfected cells. In addition to these observations, we have preliminary results with cell lines transfected with viral TK that also bear on this point. The FM3A and FM3A TK$^-$/HSV-1 TK$^+$ lines of a mouse mammary carcinoma (25) (provided by Drs. J. Balzarini and E. De Clercq, Katholieke Universiteit, Leuven, Belgium) were examined for sensitivity to CdG; the line containing the viral TK was inhibited by a concentration of CdG only 5% of that required to inhibit the parent line.[1] In other experiments, HEp-2 cells transfected with HSV-1 TK (degree of transfection undetermined) converted CdG to phosphates more extensively than did untransfected cells.[2]

The complete lack of selectivity of HSV-1 TK with respect to the enantiomers of CdG was surprising but not totally unexpected. Balzarini *et al.*(9) examined the interaction of several carbocyclic analogs of pyrimidine nucleosides with a TK isolated from the KOS strain of HSV-1. These investigators found that the phosphorylation of dThd by this HSV-1 TK was inhibited about equally by both enantiomers of C-BVDU, equally by both enantiomers of C-IDU, and also by both enantiomers of C-dThd. The ability of HSV-1 TK to phosphorylate these enantiomers was not reported, but, because both enantiomers of C-BVDU and both enantiomers of C-IDU had good antiviral activity, in each case both enantiomers probably were phosphorylated to a significant extent. However, this does not hold for the C-dThd enantiomers because the (−)-enantiomer had no anti-HSV activity (26). Thus, our results are generally similar to those of Balzarini *et al.*(9), but they differ in the type of kinase inhibition obtained with the "unnatural" enantiomers. The D-enantiomers (*i.e.*, the natural configurations, (+) in methanol) of C-BVDU and C-IDU competitively inhibited the phosphorylation of dThd by HSV-1 TK, whereas inhibition by the L-enantiomers was linear-mixed. In contrast, in our study inhibition by both enantiomers of CdG was competitive. The reason for this difference is not apparent, but it may reflect differences in enzyme interaction with pyrimidine moieties and the guanine moiety.

After our work was essentially complete, Spadari *et al.*(27) reported that HSV-1 (KOS strain) TK phosphorylated the D- and L-enantiomers of dThd at equal rates, and in addition that the L-enantiomers of other natural deoxynucleosides inhibited the phosphorylation of dThd by this enzyme. Thus, there is a considerable and accumulating body of evidence for the lack of enantiomeric specificity of the HSV-1 TK. However, other studies with other substrates have shown that kinases encoded by herpes viruses do not always fail to discriminate between enantiomers. For example, the enantiomers of 9-[2,3-(bishydroxymethyl)cyclobutyl]guanine were phosphorylated at different rates by HSV-1 (KOS) TK (28, 29) as were the enantiomers of DHBG by HSV-2 TK (30). Furthermore, in cells infected with HSV-1 (Patton strain), DHPG, which is not chiral until phosphorylated, yielded only one enantiomeric phosphate (31).

An apparent contradiction is implicit in the observation that whereas D- and L-CdG were equal in substrate activity for HSV-1 TK, in HSV-1-infected HEp-2 cells, the D-enantiomer was much more extensively phosphorylated than the L-enantiomer (Fig. 4, Table 2). The reason for this difference probably lies in the differences in the dynamics of the enzyme system and the intact cell system. In the latter, D-CdG is converted to the triphosphate, which is incorporated into DNA, whereas L-CdG accumulates as the monophosphate.

Our findings indicate that in contrast to the lack of specificity of the viral kinase, the cellular nucleotide kinases discriminate strongly between the enantiomers of CdG-monophosphate. This enzyme is presumed to be GMP kinase, which has been shown to catalyze the phosphorylation of monophosphates of other derivatives of guanine with antiviral activity (32). We have not yet prepared the monophosphates of the CdG enantiomers required for full study with GMP kinase, but we have used L-CdG monophosphate formed in cells to show that it is a substrate for GMP kinase, although a relatively poor one. Enantiomeric specificity for GMP kinase has been observed by others. For example, in studies with enantiomers of DHPG (31) and carbovir (24) it was found that one of each pair of enantiomeric monophosphates had very low substrate activity for GMP kinase.

In addition to its antiherpetic activity, CdG also has activity against hepatitis B virus because of the inhibition of the viral polymerase by CdG triphosphate (33). Because hepatitis B virus is not known to encode a nucleoside kinase, the initial phos-

[1] D. J. Adamson and W. B. Parker, unpublished results.
[2] E. Sorscher (Univ. of Alabama at Birmingham) and W. B. Parker, unpublished results.

phorylation of CdG in cells infected with hepatitis B virus must be catalyzed by cellular enzymes. The results of competition studies in CEM cells indicate that dCyd kinase catalyzes the phosphorylation of both enantiomers and is the principal enzyme responsible for phosphorylation. This apparent lack of enantiomeric specificity of a mammalian enzyme is perhaps even more surprising than that of the HSV TK, which was already known to catalyze phosphorylation of nucleoside analogs of diverse structure (32). The fact that these two enzymes have this lack of enantiomeric specificity in common may not be coincidental in light of evidence that HSV TK may have evolved from cellular dCyd kinase (34). There are other reports indicating that dCyd kinase lacks enantiomeric specificity toward certain substrates. Krenitsky *et al.* (35) found that for dCyd kinase from calf thymus, the L-enantiomer of arabinosylcytosine was an even better substrate than the D-enantiomer. More recently Chang *et al.* (36) have reported that cytoplasmic dCyd kinase from K562 or CEM cells showed little discrimination between enantiomers of 3′-thia-2′,3′-dideoxycytidine. Our results in whole CEM cells, together with those of Krenitsky *et al.* (35) and Chang *et al.* (36), seem to represent unique examples of a kinase either completely failing to discriminate between nucleoside enantiomers or discriminating in favor of the nucleoside in the unnatural configuration. However, there are multiple reports on the capacity of nucleosides or nucleoside analogs of unnatural configuration (i.e., other than β-D) to serve to some degree as substrates for phosphorylating enzymes. For example, L-nucleosides of the natural bases (37, 38), α-2′-deoxy-6-thioguanosine (39), and α-arabinosyl and α-L-lyxosyl derivatives of adenine (40) have been reported. A full elucidation of our observations with CdG must await a study of the phosphorylation of the enantiomers of CdG by purified mammalian dCyd kinase.

We have reported anti-HSV activity for L-CdG (4), but the possibility of contamination of the L-enantiomer with the D-enantiomer was not excluded as an explanation, nor is it excluded by the results of the present paper. Thus, although it is clear that the two enantiomers are metabolized differently (the D-enantiomer yielded predominantly the triphosphate and the L-enantiomer predominantly the monophosphate), it is not clear whether the small amounts of di- and triphosphates produced from the L-CdG sample are derived entirely from L-CdG or in part from a small amount of contaminating D-CdG. However, the results of the enzyme assay possibly may be used to estimate the maximum amount of the D-enantiomer that could be present in the sample of L-enantiomer. If one assumes that any D-CdG contaminating L-CdG would be metabolized almost exclusively to di- and triphosphates, then the amount of di- plus triphosphates formed from L-CdG would be a measure of the maximum contamination by D-CdG. The maximum thus calculated is 2%. Although these results do not elucidate the basis for the low degree of antiviral activity of L-CdG, it is apparent that its monophosphate has, at best, a low degree of antiviral activity. It also has little cytotoxicity, as evidenced by the fact that HSV-1-infected cells treated with L-CdG showed no evidence of the cytotoxicity seen in HSV-1-infected cells treated with D-CdG (5).

Among the antiviral nucleoside analogs, the one most closely related structurally to CdG is carbovir, and it is instructive to compare what is known about these two carbocyclic derivatives of guanine. CdG is obviously the more closely related structurally to dGuo. Carbovir has anti-HIV activity (3) but no anti-HSV activity,[3] whereas CdG is a potent anti-HSV agent but is devoid of anti-HIV activity (3); both agents are active against hepatitis B virus[4] (2). Carbovir is not a substrate for dCyd kinase and is phosphorylated by 5′-nucleotidase (19); only the (−)-enantiomer of carbovir was phosphorylated at a detectable rate (24). In contrast, the enantiomers of CdG are equal as substrates for phosphorylating enzymes in several cell culture lines (Table 2). However, the monophosphates of carbovir (24) and CdG are similar in that only one enantiomer is converted extensively to the higher phosphates.

A point of peripheral interest is the large difference in activities of dGuo and CdG as substrates for HSV-1 TK. It is unexpected that the replacement of the 4′-oxygen atom by a methylene group would improve substrate activity so markedly. It has been noted by Fyfe *et al.* (41) that dGuo is a poor substrate for this enzyme.

The lack of stereospecificity of HSV-1 TK and cellular dCyd kinase toward some substrates indicates the feasibility of introducing into cells phosphates of nucleoside analogs that have not been considered previously as potential chemotherapeutic agents. This lack of specificity has obvious implications for drug design, which have been noted by others (9, 27, 42). However, the fact that these enzymes lack stereospecificity toward some substrates but not toward others indicates that one cannot predict whether a nucleoside in the unnatural configuration will be phosphorylated. In addition, it seems from the results with CdG and carbovir (24) that the nucleotide kinases may be highly enantioselective with the result that some nucleosides in unnatural configurations will not be metabolized beyond the monophosphate level.

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[3] W. M. Shannon, unpublished results.

[4] Y.-C. Cheng (Yale Univ.), personal communication.

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