Biochemical Pharmacology, Vol. 40, No. 7, pp. 1515–1522, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

PHOSPHORYLATION OF THE CARBOCYCLIC ANALOG OF 2′-DEOXYGUANOSINE IN CELLS INFECTED WITH HERPES VIRUSES

L. LEE BENNETT, JR.,* Y. FULMER SHEALY, PAULA W. ALLAN, LUCY M. ROSE, WILLIAM M. SHANNON and GUSSIE ARNETT

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL 35255, U.S.A.

(*Received* 9 *August* 1989; *accepted* 13 *March* 1990)

Abstract—The carbocyclic analog of 2′-deoxyguanosine [(±)-2-amino-1,9-dihydro-9-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purine-6-one] (2′-CDG) is highly active in cell culture against strains S148 and E377 of herpes simplex virus type 1 (HSV-1), both of which code for thymidine kinase, and much less active against strain BW10168 which is deficient in this enzyme activity. Antiviral activity is associated primarily with the D-enantiomer; the L-enantiomer has much lower but significant activity. The metabolism of racemic 2′-CDG and its D- and L-enantiomers was studied in uninfected HEp-2 cells and in HEp-2 cells infected with the S148 or BW10168 strains of HSV-1. Nucleotides were separated by HPLC, and their elution was monitored by spectrophotometry. The chromatograms of extracts of cells infected with the S148 strain and treated with (±)-2′-CDG or D-2′-CDG included a new peak which appeared in the triphosphate region. This peak, the area of which exceeded that of the GTP peak, was shown to be due to the triphosphate of 2′-CDG. The new peak was not observed by HPLC of extracts of uninfected cells treated with (±)-2′-CDG or either of its enantiomers, cells infected with the S148 strain and treated with L-2′-CDG, or cells infected with the BW10168 strain and treated with (±)-2′-CDG or either of its enantiomers. The results were similar when these studies were performed with uninfected Vero cells and with Vero cells infected with strain S148 of HSV-1. In experiments with D-[8-^{3}H]-2′-CDG, small amounts of phosphates of 2′-CDG could also be detected in uninfected HEp-2 cells and in cells infected with the BW10168 strain of HSV-1. Thus, 2′-CDG apparently is a good substrate for the virus-coded kinase and a very poor substrate for cellular phosphorylating enzymes. The selective phosphorylation of 2′-CDG by the virus-specific kinase presumably is critical for its antiviral activity as it is for that of acyclovir and other acyclic derivatives of guanine.

The carbocyclic analog of 2′-deoxyguanosine[(±)-2-amino-1,9-dihydro-9-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purin-6-one] (2′-CDG†) has high activity against herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) replicating in cell cultures [1] and has also shown antiviral activity *in vivo*.‡ The enantiomers of 2′-CDG have been prepared; as expected, the D-enantiomer (Fig. 1) had high activity against herpes viruses, but the L-enantiomer also had a lesser but definite antiviral activity [2]. A number of derivatives of guanine, namely acyclovir and related acyclic compounds, are active antiherpetic agents; the activity of such compounds results from their conversion to phosphates, a reaction that is catalyzed specifically by virus-coded thymidine kinase [3–5]. It would, therefore, appear likely that the antiviral activity of 2′-CDG is also due to its selective phosphorylation by the virus-specific kinase. However, 2′-CDG differs sufficiently in structure from the 9-acyclic derivatives of guanine that it is by no means certain that it, too, would be phosphorylated specifically by the virus-coded kinase. To obtain information on the biochemical basis for the antiviral activity of 2′-CDG, we examined the metabolism of racemic 2′-CDG and its D- and L-enantiomers in uninfected and HSV-infected cells.

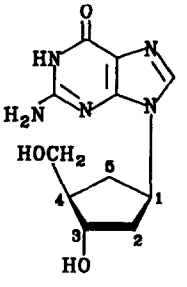

Fig. 1. Structure of [1*R*(1α,3β,4α)]-2-amino-1,9-dihydro-9-[3-hydroxy-4-(hydroxymethyl) cyclopentyl]-6*H*-purin-6-one(D-2′-CDG).

* Address Correpondence to: Dr. L. Lee Bennett, Jr., Southern Research Institute, P.O. Box 55305, Birmingham, AL 35255-5305.

† Abbreviations: 2′-CDG, the carbocyclic analog of 2′-deoxyguanosine [(±)-2-amino-1,9-dihydro-9-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purin-6-one]; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HSV(TK$^+$), the S148 stratin of HSV-1 which codes for thymidine kinase; HSV(TK$^-$), the BW10168 strain of HSV-1 which is deficient in thymidine kinase; and m.o.i., multiplicity of infection.

‡ Shannon WM, Arnett G, O'Dell CA and Shealy YF, The potent antiviral activity of the carbocyclic analog of 2′-deoxyguanosine (CDG) and related compounds. *American Society for Virology, Annual Meeting, Albuquerque, NM*, July 21–25, 1985.

Table 1. Antiviral activity of racemic and enantiomeric forms of 2′-CDG*

Compound	HSV-1 strain†	Host cells	VR‡	MIC_{50}§ (μM)
(±)-2′-CDG	S148	Vero	4.4	3
	S148	HEp-2	5.8‖	3‖
	E377	Vero	5.3–7.0¶	0.8–1.1¶
	BW10168	Vero	2.1	30
	BW10168	HEp-2	2.3	43
D-(+)-2′)CDG	S148	HEp-2	5.5	1.6
	E377	Vero	4.6–6.3**	0.4–1.1**
	BW10168	HEp-2	2.3	6.4
L-(−)-2′-CDG	S148	HEp-2	0.8	990
	E377	Vero	0.8–2.4**	147–968**
	BW10168	HEp-2	0.6	234

* Results shown are for single experiments except where otherwise noted.

† Strains E377 and S148 are TK^+; strain BW-10168 is TK-deficient. Values for the E377 strain have been reported previously [2].

‡ VR is the virus rating, which is a weighted measurement of antiviral activity that takes into account both the degree of inhibition of virus-induced cytopathogenic effects and the degree of cytotoxicity produced by the test compound. A VR equal or greater than 1 indicates significant antiviral activity, a VR of 0.5–0.9 indicates marginal to moderate antiviral activity, and a VR less than 0.5 usually indicates no significant antiviral activity [7].

§ The MIC_{50} is the concentration required to inhibit virus-induced cytopathogenic effects by 50%.

‖ Two assays.

¶ Seven assays.

** Four assays.

MATERIALS AND METHODS

DL-2′-CDG and its D- and L-enantiomers were prepared in our laboratories [1, 2]. Alkaline phosphatase from *Escherichia coli* was obtained from the Sigma Chemical Co. (St. Louis, MO). D-[8-^{3}H]-2′-CDG (250 mCi/mmol) was prepared by Moravek Biochemicals (Brea, CA) by catalytic exchange between tritium gas and a sample of D-2′-CDG provided by us. The radiopurity of the sample was 98.2%, as determined by HPLC on a reversed-phase column (see below).

HEp-2 cells were maintained and grown for experimental purposes in 150 cm^2 flasks in Eagle's Minimum Essential Medium containing 9% bovine calf serum; Vero cells were grown in the same medium but were maintained in this medium + 5% bovine calf serum. The viruses used were the S148 and E377 strains of HSV-1, which code for thymidine kinase, and the BW10168 strain (provided by Dr. David R. Scholl, Ohio University, Athens, OH), which is deficient in this enzyme activity [6]. The BW10168 strain was originally designated BW-R [6]. The S148 and BW10168 strains are hereinafter designated HSV(TK^+) and HSV(TK^-) respectively. For evaluation of (±)-2′-CDG and its enantiomers for antiviral activity, viruses were grown in Vero cells or in HEp-2 cells (see Table 1), and the compounds were evaluated, by methods described in detail elsewhere [7], for inhibition of cytopathogenic effects.

Experiments designed to examine the metabolism of 2′-CDG were performed with both HEp-2 and Vero cells. For each set of experiments the following cultures were prepared with both the HSV(TK^+) and HSV(TK^-) strains: (a) untreated control cells; (b) cells treated with (±)-2′-CDG or its D- or L-enantiomer; (c) cells infected with virus; and (d) virus-infected cells treated with (±)-2′-CDG or its D- or L-enantiomer. Cells were infected with either virus at a multiplicity of infection (m.o.i.) of 10 $CCID_{50}$/cell (1 $CCID_{50}$ = the number of virions required to infect 50% of the cells). One hour after infection with virus, the medium was decanted and the cell layers were washed with phosphate-buffered saline. To one virus-infected and one uninfected culture, (±)-2′-CDG, or one of its enantiomers, was added to a final concentration of 50 μM (total volume 60 mL); one uninfected and one virus-infected culture served as controls (60 mL of medium added). Eight hours after the addition of 2′-CDG, the cells were harvested and washed twice with Puck's saline G. The cells were then extracted with 0.5 N $HClO_4$ and the residue was separated by centrifugation. To the supernatant solution, $KHCO_3$ was added and the resulting precipitate of $KClO_4$ was removed by centrifugation. The supernatant fraction was lyophilized to dryness and the residue was dissolved in H_2O. This solution was then subjected to analysis by HPLC. These analyses were performed with a Series 4 liquid chromatograph (Perkin–Elmer, Norwalk, CT). Nucleotides were analyzed on a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ); elution was achieved with a 50-min gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 μM $NH_4H_2PO_4$ (pH 3.7) at a flow rate of 2 mL/min. Nucleosides were analyzed on a reversed-phase Spherisorb ODS 5 μm column (Keystone Scientific, State College, PA) with a solvent consisting of 5 μM $NH_4H_2PO_4$ (pH 5.5) and acetonitrile (95:5, v/v) at

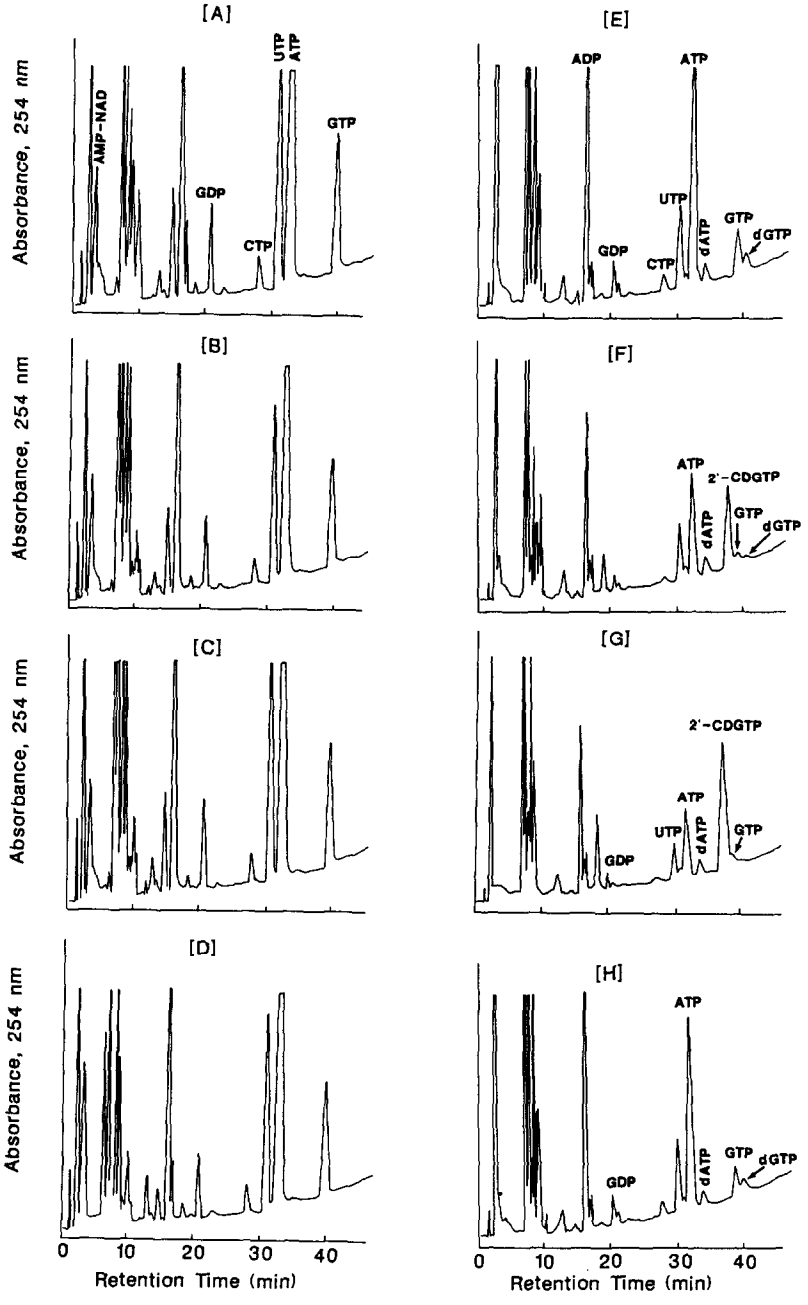

Fig. 2. Metabolism of 2′-CDG in uninfected and HSV(TK$^+$)-infected HEp-2 cells. Cells were infected with virus at an m.o.i. of 10 and, after a 1-hr period for virus adsorption, either (±)-2′-CDG, D-2′-CDG or L-2′-CDG was added to the culture medium to a final concentration of 50 μM. Eight hours after addition of the carbocyclic analogs, the cells were harvested, after which a cold $HClO_4$ extract was prepared and subjected to HPLC on an anion exchange column (see text). (A) Control cells; (B) cells treated with (±)-2′-CDG; (C) cells treated with D-2′-CDG; (D) cells treated with L-2′-CDG; (E) cells infected with HSV(TK$^+$); (F) cells infected with HSV(TK$^+$) and treated with (±)-2′-CDG; (G) cells infected with HSV(TK$^+$) and treated with D-2′-CDG; and (H) cells infected with HSV(TK$^+$) and treated with L-2′-CDG. 2′-CDGTP = the triphosphate of 2′-CDG.

a flow rate of 1 mL/min. The UV absorption spectra of selected peaks were determined with a rapid spectral detector (LKB, Gaithersburg, MD). Similar methods were used to study the metabolism of D-[8-^{3}H]2′-CDG, except that during HPLC analysis 1-mL samples were collected for radioassay.

RESULTS

Table 1 shows the activity of 2′-CDG against the viruses used in this study; the table also contains, for comparison, previously reported [2] results with HSV-1 strain E377. (±)-2′-CDG and its D-enantiomer were highly effective in preventing the

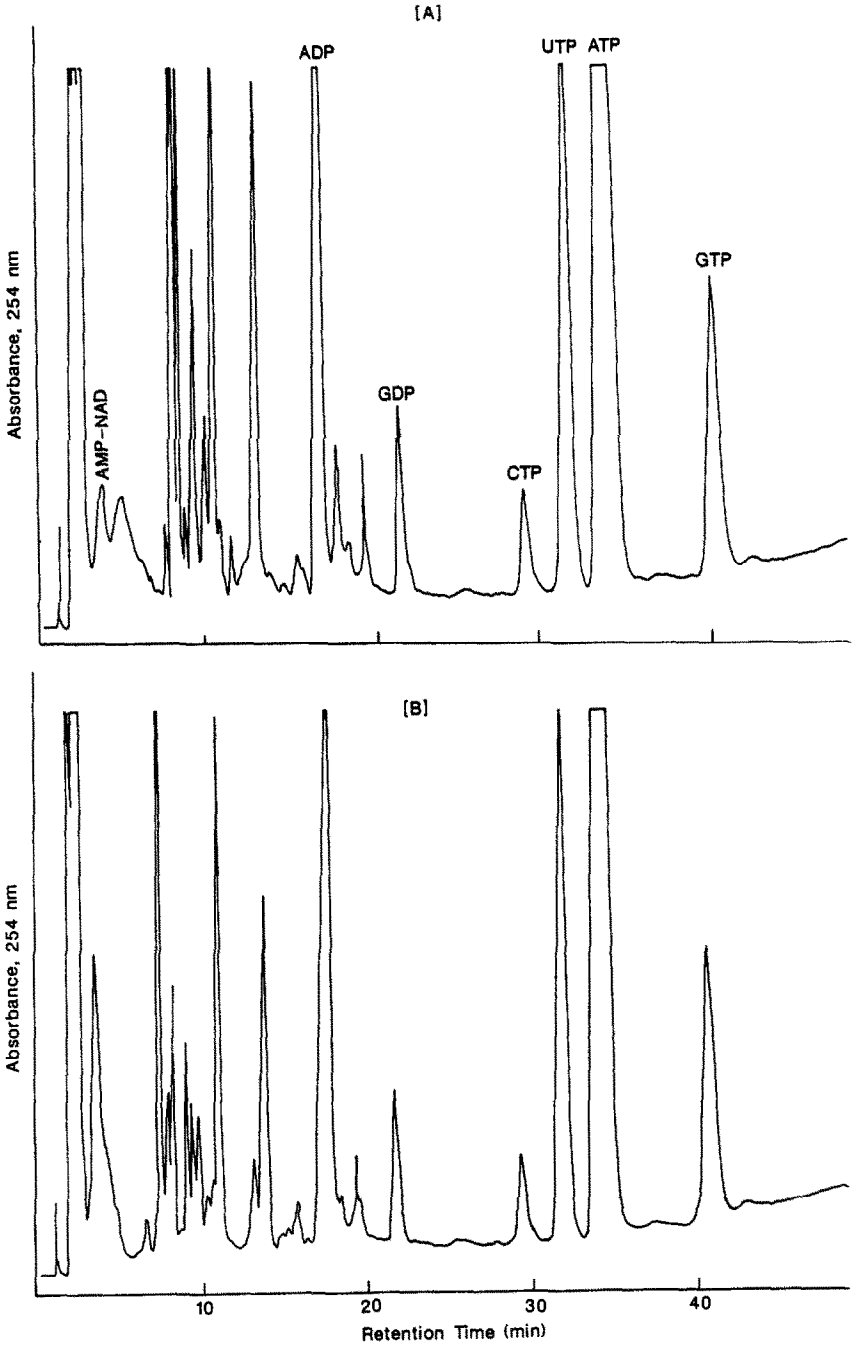

Fig. 3. Metabolism of 2′-CDG in uninfected and HSV(TK⁻)-infected HEp-2 cells. The experiments were performed as in those of Fig. 2, except that the TK⁻ strain of HSV was used. (A) Cells infected with HSV(TK⁻); and (B) cells infected with HSV(TK⁻) and treated with D-2′-CDG. Panel A of Fig. 2 serves as a control for these experiments.

cytopathogenic effects of HSV(TK⁺). The virus ratings for the racemate and the D-enantiomer were about the same, but the MIC_{50} (concentration required to inhibit virus-induced cytopathogenic effects by 50%) values were lower for the D-enantiomer. Against the kinase-deficient line (BW10168), the racemate and D-enantiomer showed a lower but still significant activity. The L-enantiomer also had some activity against both TK⁺ and TK⁻ strains, but was much less potent as shown by the high MIC_{50} values.

Figure 2 shows nucleotide profiles of control HEp-2 cells, HSV (TK⁺)-infected cells, and uninfected or HSV(TK⁺)-infected cells treated with (±)-2′-CDG or its D- or L-enantiomer. The nucleotide profile of cells treated with (±)-2′-CDG alone (Fig. 2B) or

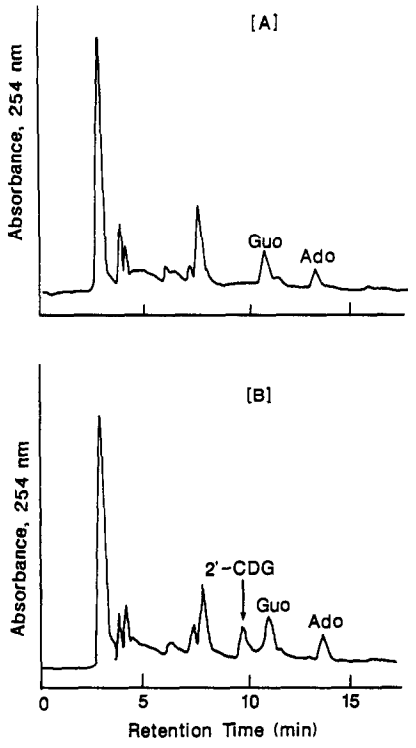

Fig. 4. Reversed-phase chromatography of phosphatase-treated nucleotides. HEp-2 cells were infected with HSV(TK$^+$) and exposed to (±)-2′-CDG as described in the text. After 8 hr, the cells were harvested and extracted, and the extract was subjected to ion-exchange chromatography as described in the legend of Fig. 2. The triphosphate area (peaks eluting from CTP through dGTP) was collected and freed of buffer salts and then treated with alkaline phosphatase; the resulting solution of nucleosides was subjected to reversed-phase HPLC (see text). (A) Extract from control cells; and (B) extract from HSV(TK$^+$)-infected cells exposed to 2′-CDG.

with its D- or L-enantiomer (Fig. 2, C and D) did not differ from that of control cells (Fig. 2A). In cells infected with the HSV(TK$^+$), the ribonucleotide pools were reduced, and new peaks appeared that eluted just after ATP and GTP (Fig. 2E). The retention times of these new peaks correspond to those of dATP and dGTP, and their UV absorption spectra (determined in a separate experiment) were not distinguishable from those of standards of dATP and dGTP assayed in the same buffer. Treatment of virus-infected cells with (±)-2′-CDG (Fig. 2F) resulted in a marked decrease in the ATP and GTP peaks and the appearance of a new peak eluting just before GTP and incompletely resolved from GTP. This same peak appeared, and in even greater amount, on chromatograms of extracts of cells treated with D-2′-CDG (Fig. 2G) but was absent from chromatograms of extracts of cells treated with L-2′-CDG (Fig. 2H). Figure 3 shows results of some of the experiments with cells infected with HSV(TK$^-$). The nucleotide profile of HSV(TK$^-$)-infected cells treated with D-2′-CDG (Fig. 3B) did not differ from that of untreated HSV(TK$^-$)-infected cells (Fig. 3A); thus, there was no evidence for formation of phosphates of D-2′-CDG in these virus-infected cells. Experiments were also performed with (±)-2′-CDG and L-2′-CDG in HSV(TK$^-$)-infected cells to yield a complete set of experiments analogous to those of Fig. 2; these other charts are not shown since they did not differ from Fig. 3B. Similar experiments were performed with (±)-2′-CDG in Vero cells and in Vero cells infected with strain HSV(TK$^+$). These experiments yielded nucleotide profiles (not shown) similar to those obtained in the corresponding experiments with HEp-2 cells (Fig. 2), and thus indicated that in Vero cells, as in HEp-2 cells, 2′-CDG is phosphorylated only in HSV-infected cells, presumably by the virus-coded kinase.

The new peak appearing on chromatograms of extracts of cells infected with HSV(TK$^+$) and treated with (±)-2′-CDG and its D-enantiomer would be presumed, from its retention time, to be the triphosphate of 2′-CDG. To obtain material for characterization, a separate experiment was performed with HEp-2 cells infected with HSV(TK$^+$) and treated with (±)-2′-CDG. In this experiment, as in Fig. 2G, the new peak was not completely resolved from GTP. The combined peaks were scanned at multiple points; all the spectra obtained were indistinguishable from those of guanine nucleotides assayed in the same buffer. For further characterization, the entire triphosphate area (peaks eluting from CTP through dGTP) was collected as a single fraction. After the sample had been freed of buffer salts by passage through a charcoal (Norit-A) column, the adsorbed nucleotides were eluted with ammoniacal-ethanol [8], and the eluate was lyophilized to dryness. The residue was taken up in water and the resulting solution was treated with alkaline phosphatase (4 hr, 37°). The reaction was stopped by immersion of the tube in a boiling water bath; the protein was removed by centrifugation, and the supernatant solution was subjected to HPLC on a reversed-phase column. As shown in Fig. 4, chromatography showed the presence of a new peak eluting just before guanosine; this peak had the absorption spectrum characteristic of guanine nucleosides and had a retention time the same as that of 2′-CDG determined in a separate experiment (not shown). For further characterization, an experiment was performed in which 2′-CDG was added to the sample before reversed-phase chromatography; the new peak shown in Fig. 4B was specifically reinforced by this treatment (result not shown). Thus, all the evidence (depicted in Figs. 2 and 4) shows that this new peak is due to the triphosphate of 2′-CDG.

^{3}H-Labeled D-2′-CDG became available during the course of these experiments, and a single confirmatory experiment was performed with this compound in uninfected HEp-2 cells and in HEp-2 cells infected with HSV(TK$^+$) or with HSV(TK$^-$). The results (Fig. 5) confirm the results obtained with the unlabeled D-2′-CDG in showing a large peak of radioactivity in the triphosphate area in the experiment with HSV(TK$^+$)-infected cells. In addition, the greater sensitivity of the radioassay made possible the detection of radioactive peaks that were not detected by spectrophotometric monitoring. Control cells formed detectable amounts of the triphosphate

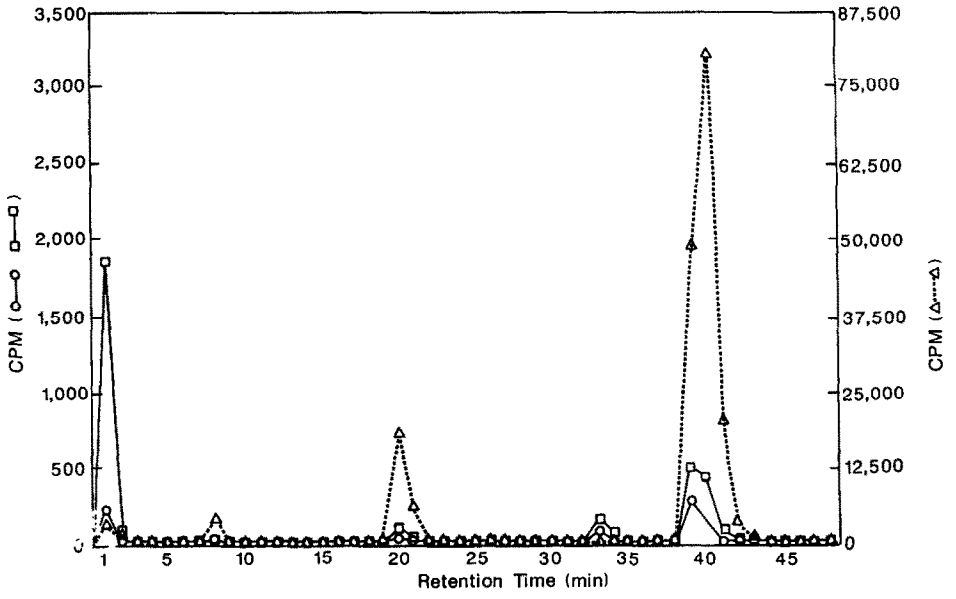

Fig. 5. Metabolism of D-[8-^{3}H]-2′-CDG. The experiments were performed as described in the legend to Fig. 2, except that D-[8-^{3}H]-2′-CDG (1.67 μCi/mL) was used as substrate. Key: (○——○) uninfected HEp-2 cells treated with D-[8-^{3}H]-2′-CDG; (△——△) HEp-2 cells infected with HSV(TK$^+$) and treated with D-[8-^{3}H]-2′-CDG; (□——□) HEp-2 cells infected with HSV(TK$^-$) and treated with D-[8-^{3}H]-2′-CDG. Note the difference in scales for the cells infected with HSV(TK$^+$) and for uninfected cells or cells infected with HSV(TK$^-$).

of 2′-CDG and, in addition, small peaks were detectable that had retention times close to those of GMP and GDP and hence are probably mono- and diphosphates of D-2′-CDG. The amount of triphosphate of [8-^{3}H]-2′-CDG found in uninfected cells was 0.3% of that found in cells infected with HSV(TK$^+$). Cells infected with HSV(TK$^-$) formed a little more triphosphate of [8-^{3}H]-2′-CDG than did uninfected cells, but the radioactivity in both cells was so low that the significance of this difference is questionable. A comparison of the levels of triphosphate found in cells infected with HSV(TK$^+$) and in cells infected with HSV(TK$^-$) indicates absence of virus-induced kinase activity in HSV(TK$^-$)-infected cells. Conversely, these findings support the status of BW10168 as a thymidine kinase-minus or kinase-deficient strain of HSV-1.

The cellular concentration of the triphosphate of D-[8-^{3}H]-2′-CDG found in the experiment of Fig. 5 was 80 μM (calculated on the basis of 10^9 cells = 1 mL). The amount formed in the experiment of Fig. 2 (in which non-labeled 2′-CDG was used) could not be calculated accurately because of incomplete resolution of 2′-CDG triphosphate and GTP; however, it was estimated at about 200 μM. These differences with the labeled and unlabeled precursor appear reasonable in view of the fact that the concentration of the tritiated compound (used undiluted) in the medium was 7 μM, whereas that of the unlabeled compound was 50 μM.

DISCUSSION

These results show that 2′-CDG was phosphorylated with high selectivity by the HSV-coded nucleoside kinase and this fact is probably a prime factor of its antiviral activity, as it is for that of acyclic derivatives of guanine. That only the D-enantiomer was phosphorylated to an extent detectable by spectrophotometric monitoring is to be expected from the fact that natural nucleosides are in the D-configuration. Although these results rationalize the antiherpetic activity of 2′-CDG, they leave unexplained two unexpected observations: (a) the small but significant activity of 2′-CDG against HSV(TK$^-$) and (b) the small but significant activity of L-2′-CDG against both HSV(TK$^+$) and HSV(TK$^-$). A possible explanation of both of these observations is that the unphosphorylated nucleoside analogs themselves may have some activity. A second possible explanation is the production of small amounts of phosphates by the action of cellular enzymes. This, in fact, was observed with the D-enantiomer, but the amounts were so small that they were detectable only with the radioisotope-labeled sample. Phosphorylation of the L-enantiomer was not observed, but since radioisotope-labeled L-enantiomer was not available for study, a low degree of phosphorylation by cellular enzymes, similar to that observed with the D-enantiomer, would not have been detected. Although the L-enantiomer represents an "unnatural" configuration, the possibility of its phosphorylation cannot be excluded in light of the observations of Holý and his co-workers that L-nucleosides are phosphorylated in mice [9, 10]. That a very low level of phosphorylation by cellular enzymes may account for antiviral activity has been shown in studies with 2′,3′-dideoxy nucleosides against human immunodeficiency virus, which is not known to code for a kinase [11–13]. We have not

attempted to identify the enzyme(s) responsible for the phosphorylation of 2′-CDG in uninfected cells. As an analog of 2′-deoxyguanosine, 2′-CDG may be a substrate for deoxycytidine kinase. Alternatively, the phosphorylation may be catalyzed by cytoplasmic 5′-nucleotidase which has been shown to catalyse phosphorylation of inosine and guanosine [14], acyclovir [15], and tiazofurin [16].

Since the results show that antiviral activity is associated primarily with the D-enantiomer, it would be expected that the antiviral activity of the D-enantiomer would be about twice that of the racemate. Although the virus ratings for racemate and D-enantiomer were about the same (Table 1), the D-enantiomer was clearly the more potent agent (i.e. lower MIC_{50} values). We have no explanation for the similar antiviral selectivity of the D-enantiomer and the racemic mixture. However, that this is not a unique observation is shown by the fact that Herdewijn *et al.* [17] observed similar antiviral activities for racemic carbocyclic adenosine and its active enantiomer. It is probable that the cytotoxicity for the host cells, as well as the antiviral potency, is associated with the D-enantiomer. This would result in similar selectivity indexes or virus ratings for the racemate and D-enantiomer. It may also indicate that similar molecular targets are involved in the antiviral and cytotoxic effects of 2′-CDG. It is speculated that phosphorylated derivatives of this antiviral agent may inhibit the virus-induced DNA polymerase. Cellular DNA polymerases may also be inhibited to some extent.

The effects of HSV-1-infection and of 2′-CDG on nucleotide pools merit some comment. As shown in Fig. 2E, infection of HEp-2 cells with HSV(TK^+) caused the appearance of peaks with the retention times of dGTP and dATP; the size of these peaks changed little when the infected cells were treated with 2′-CDG. Increase in pools of deoxyribonucleoside triphosphates in HSV-1-infected cells has been noted by others, and it has also been observed that treatment of HSV-1-infected cells with acyclovir or bucyclovir further increases the size of the pools [18, 19]. In our study marked effects were also observed on the ribonucleotide pools. Virus infection produced a decrease in pools of CTP, UTP, ATP and GTP (Fig. 2E). These pools were reduced even further in virus-infected cells treated with (±)-2′-CDG (Fig. 2F) or D-2′-CDG (Fig. 2G). These results are in contrast to those of Karlsson *et al.* [19] who observed only minor effects of acyclovir or bucyclovir on the ribonucleotide pools of HL or GMK cells infected with HSV-1. The very low ribonucleotide pools found in the HSV(TK^+)-infected cells that were treated with 2′-CDG indicate that these cells were in a poor metabolic state. Since these pools are much lower than those of control cells infected with HSV(TK^+), it would apper that phosphates of 2′-CDG, produced by the virus-coded kinase, in addition to inhibiting virus replication also damage the host cells. This consideration raises the possibility that selective destruction of the cells in which the viruses are replicating may also be a factor in the antiviral activity of 2′-CDG.

Acknowledgements—This work was supported by Grant P01 CA 34200, National Cancer Institute, National Institutes of Health, DHHS, and by ViraChem, Inc., New York, NY.

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