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Differential Phosphorylation of (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine Monophosphate by Thymidylate Kinases from Herpes Simplex Viruses Types 1 and 2 and Varicella Zoster Virus

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SUMMARY

5-(2-Bromovinyl)-2′-deoxyuridine (BrVdUrd) is a potent and selective inhibitor of herpes simplex virus Type I (HS-I) and varicella zoster (VZ) virus replication but is much less potent against herpes simplex virus Type II (HS-II) replication. A possible enzymatic basis for this difference is reported here to involve the virus-coded dThd-dTMP kinases (EC 2.7.1.75) from the three virus strains. The thymidine kinases from the three virus strains were purified by affinity chromatography. In addition to catalyzing the phosphorylation of nucleosides, each of the three purified enzymes catalyzed the phosphorylation of thymidylate to its diphosphate but at strikingly different rates. The relative amounts of extractable virus-coded thymidylate kinases were estimated to be 100/2/40 for cells infected with HS-I, HS-II, and VZ viruses, respectively. Extracts of cells infected with HS-I virus catalyzed the phosphorylation of the monophosphate of BrVdUrd to its diphosphate. In contrast, the product was not detected with extracts from cells infected with HS-II virus. The ratios of rates with 0.5 mM BrVdUrd monophosphate versus 0.1 mM dTMP as substrates for each of the purified dThd-dTMP kinases from HS-I, HS-II, and VZ viruses and the dTMP kinase from host cells were, respectively, 0.09, <0.002, 0.03, and <0.0002. These observations correlate with the relative sensitivities of these viruses to BrVdUrd in cell culture and suggest that, if BrVdUrd exerts its effect as a triphosphate, the inefficient phosphorylation of the monophosphate contributes to the insensitivity of HS-II virus to this agent.

INTRODUCTION

An increasing number of nucleoside analogues are being found which selectively inhibit HS-I virus[1] replication (1). With acyclovir and 5-iodo-5′-amino-2′,5′-dideoxyuridine, evidence indicates that this selectivity exists in part because at therapeutic concentrations the analogues are phosphorylated to their respective triphosphates predominantly in infected cells (2–4). The enzyme that catalyzes the first step of the phosphorylation has been identified as the virus-coded dThd kinase (EC 2.7.1.75). Indirect evidence also supports this mechanism of activation for BrVdUrd and several other nucleoside analogues (1, 5, 6). For the purine nucleoside analogue acyclovir, the monophosphate appears to be phosphorylated to the di- and triphosphate by host-cell enzymes (4, 7). This mechanism of activation probably extends to the HS-II and VZ viruses. These viruses also induce virus-coded dThd kinases (8), and acyclovir has about the same activity against HS-I as against HS-II virus in cell culture (9–11); it is about 2- to 8-fold less active against VZ virus (10). In contrast, the dThd analogue BrVdUrd has been reported to be about 100-fold less active against HS-II virus than against HS-I virus (5). A possible explanation for this became apparent when, in the course of the present studies, a substantial dTMP phosphorylating activity was found to be associated with the virus-coded dThd kinases from HS-I and VZ viruses but only a small amount with the enzyme from HS-II virus. The bifunctional nature of the dThd-dTMP kinase had been observed with the enzyme from HS-I virus (12) but not from HS-II or VZ virus. The studies reported here examine the hypothesis that the virus-coded dTMP kinases are important in determining the antiviral effectiveness of BrVdUrd.

[1] The abbreviations used are: HS-I and HS-II virus, herpes simplex virus (Types I and II); VZ virus, varicella zoster virus; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; BrVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BrVdUMP and BrVdUDP, the 5′-monophosphate and 5′-diphosphate of BrVdUrd; HPLC, high-pressure liquid chromatography; RT, retention time.

MATERIALS AND METHODS

Most commercially available materials were from the same sources as those used previously (4). "Fatty acid-free" albumin crystallized from bovine serum (Sigma Chemical Company, St. Louis, Mo.) was used to avoid ATP-cleaving activity observed in "Fraction V" albumin. [α-^{32}P]ATP was purchased from Amersham (Arlington

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Heights, Ill.) and [2-^{14}C]dTMP from ICN (Irvine, Calif.). BrVdUrd and the 5'-mono- and 5'-diphosphates (BrVdUMP and BrVdUDP) of BrVdUrd were kindly provided by Dr. Erik De Clercq, Rega Institute (Leuven, Belgium). The phosphate derivatives were chemically prepared by Dr. T. Fukui in the laboratory of Dr. De Clercq. Vero, LMTK$^-$, and WI-38 cells were grown and infected with HS-I (Patton strain) virus, HS-II (MS strain) virus, or VZ (Ellen strain) virus, respectively, as previously described (3, 10, 13), and were kindly provided by Drs. Phillip Furman and Karen Biron of the Wellcome Research Laboratories.

Phosphorylation of dThd was measured with a DEAE-paper method as described previously (4) except that ATP·Mg was 2 mM, [2-^{14}C]dThd (50–70 cpm/pmole) was 0.1 mM, and a creatine phosphate-creatine kinase (EC 2.7.3.2) (2 mM creatine phosphate and 10 IU of creatine kinase/ml reaction) ATP-regenerating system was used. Phosphorylation of dTMP was measured by "procedure A" of Chen *et al.* (14). Reaction mixtures contained 0.1 mM [2-^{14}C]dTMP (90–100 cpm/pmole), 5 mM ATP·Mg, and 100 mM Tris·HCl (pH 7.5[2]), and 0.25 M formic acid-sodium formate at pH 3.4 was used to develop the thin-layer plates. In each case initial velocities were measured. Alternatively, a DEAE-paper method (14) with [2-^{14}C] dTMP was used to monitor activity from column fractions. These reaction mixtures contained 110 μM (100 cpm/pmole), 250 μM (10 cpm/pmole), or 50 μM (50 cpm/pmole) dTMP with column fractions from HS-I, HS-II, or VZ virus dThd kinase purifications.

Reaction velocities with nonradioactive BrVdUrd as substrate for the purified dThd kinases were determined by measuring the conversion of [α-^{32}P]ATP to [α-^{32}P]-ADP. Reaction mixtures (40 μl) contained 1 mM nucleoside, 1 mM [α-^{32}P]ATP (50 cpm/pmole), 1 mM $MgCl_2$, 100 mM Tris·HCl (pH 7.5), and 20–60 units of purified dThd kinase. Product formation was measured as described by Miller and Miller (7).

One unit of enzyme activity catalyzes the formation of 1 pmole of product per minute at 37°. For the virus-coded bifunctional enzyme (dThd-dTMP kinase), 0.1 mM dThd or 0.1 mM dTMP was used as the standard substrate. Protein concentration was estimated by the Coomassie brillant blue dye method (15, 16).

Samples for HPLC analysis were processed at 2–10°. Reaction mixtures (100 μl) were treated with $HClO_4$ (to 4%) to remove protein and then neutralized with KOH. The supernatant was stored at −80°. Portions (100 μl) were analyzed by HPLC (3) with exceptions noted.

The monophosphate of BrVdUrd was synthesized by incubating (37°) a reaction mixture (0.85 ml) containing 4 mM BrVdUrd, 12 mM ATP·Mg, 100 mM Tris·HCl (pH 7.5), and 2.4×10^3 units of dThd phosphorylating activity (0.3 mg of protein) from a desalted cytosol fraction of HS-II virus-infected Vero cells. Formation of product was monitored by HPLC analysis and identified as BrVdUMP (see Results). It was purified by HPLC under the same conditions, neutralized, lyophilized to 0.3 ml, and desalted in 0.1-ml portions through a Waters μ-Bondapack HPLC column (0.39 × 30 cm) equilibrated with H_2O. The ratio of free phosphate to BrVdUMP in the final solution was less than 1:1.

To test for phosphorylation of the BrVdUMP, it was incubated with ATP·Mg and desalted cytosol extracts for 1 hr at 37° and then treated with perchloric acid for HPLC analysis. Alternatively, incubations to quantitate the rate of formation of the diphosphate product utilized purified enzymes and were incubated for up to 6 hr at 30°.

Cytosol fractions of uninfected and HS virus-infected cells were prepared in a manner similar to that described previously (4). Cells infected with VZ virus were dounced in the same manner, and then 0.2 M sucrose and 0.05 M ε-aminocaproic acid were added to the extract. This mixture was centrifuged at 600 × *g* for 20 min, and the supernatant was centrifuged at 27,000 × *g* for 1 hr. The supernatant was removed, and ATP, KCl, Tris·HCl (pH 7.5), and glycerol were added to 2 mM, 130 mM, 20 mM, and 10%, respectively (1.2-fold dilution of protein).

The dThd kinase from the cytosol fraction of LMTK$^-$ mouse cells infected with HS-I virus (Patton) was purified as described by Chen and Prusoff (12), except that a slightly higher ionic strength and a higher concentration of dThd were used to elute the main portion of enzyme (Fig. 1). The enzyme (Fractions 40–42) was stored at −80° in the presence of the eluting solution and was desalted by gel filtration on the day it was used.

The virus-coded enzyme from Vero cells infected with HS-II virus (MS strain) was purified similarly except that the 20–80% ammonium sulfate precipitate and a different elution sequence were used (Fig. 1). Some dThd phosphorylating activity eluted from the column with a high-salt rinse (Peak 1), and a smaller amount of activity eluted under the conditions for host cell dThd kinase elution (Peak 3) (4). The majority of the activity was eluted with 500 μM dThd, and these fractions (Fractions 31–35, Peak 4) were combined as the virus-coded dThd kinase enzyme (17). Fractions containing the host-cell dTMP phosphorylating activity (Peak 2, Fractions 19 and 20) were collected into 0.5 mg of albumin and also saved from this column. Both enzymes were stored and desalted before use as described for the HS-I virus-coded enzyme.

The dThd kinase from a cytosol fraction of WI-38 cells infected with VZ virus was purified (18) as described for the HS-I virus enzyme except that a 0–0.6 mM dThd gradient containing 1 mM ATP and albumin (0.5 mg/ml) was used (Fig. 1). Maximal activity eluted at about 0.2 mM dThd. Fractions 41–47 were combined and stored as above.

RESULTS

Purification of the dThd-dTMP kinases. Each of the three purified dThd kinases was found to have an associated dTMP phosphorylating activity (Fig. 1). The HS-I virus-coded dThd kinase was previously shown to be bifunctional (12), but this has not been reported with the HS-II or VZ virus enzymes.

Figure 1 shows the co-elution of dThd and dTMP phosphorylating activities (Peak 4) from a cytosol fraction of HS-II virus-infected Vero cells. The purification procedure was similar to that used previously (12, 17).

[2] All pH measurements were made at 25°.

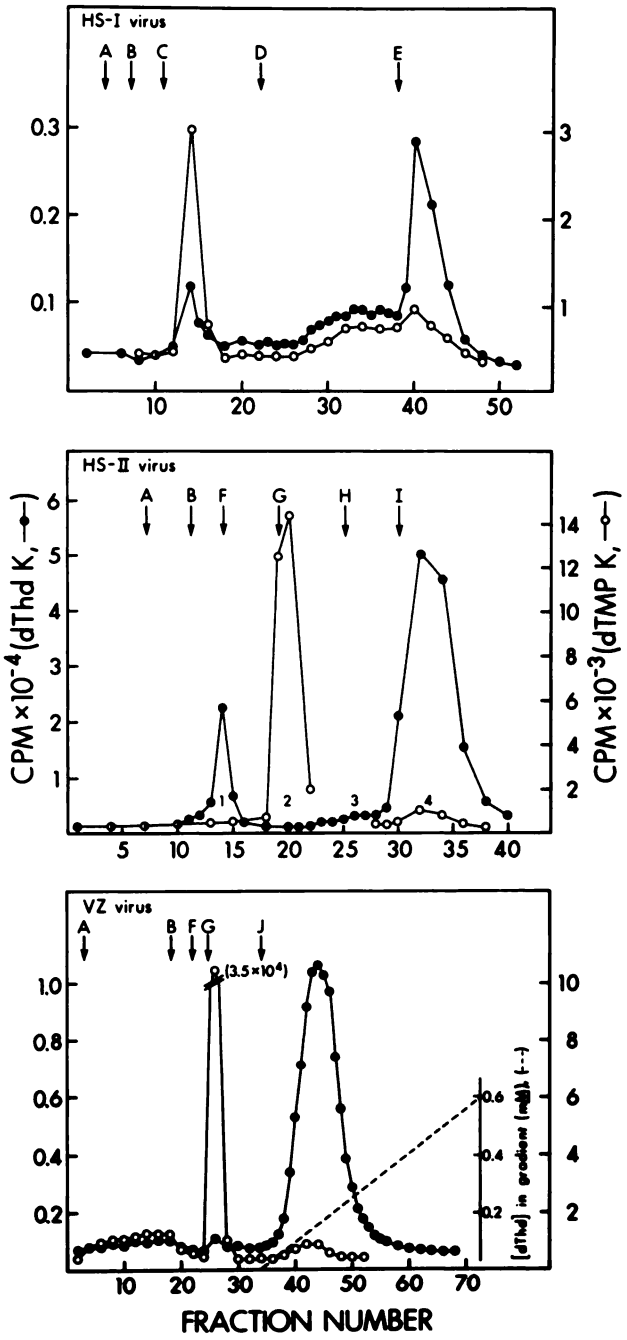

FIG. 1. *Kinase activity profiles of fractions from dThd-agarose affinity columns*

The samples added onto the columns (7.8×10^3, 1.4×10^5, and 1.2×10^4 units, respectively, of dThd phosphorylating activity) were from the cytosol fractions of cells infected with HS-I, HS-II, and VZ viruses. Fractions assayed for dThd kinase (●) were diluted into the reaction mixtures (1:2, 1:4, or 1:2 for HS-I, HS-II, or VZ virus) and incubated for 10, 10, or 60 min (HS-I, HS-II, or VZ virus); those for dTMP kinase (○) were diluted 1:2 and incubated for 45, 45, or 120 min. The elution solutions were as follows: *arrow A*, sample addition; *arrow B*, 20 mM Tris (pH 7.5), 2 mM dithiothreitol, and 10% glycerol; *arrow C*, solution at *arrow B* with 1 mM dTMP; *arrow D*, 300 mM Tris (pH 7.5), 2 mM dithiothreitol, and 0.2 mM dThd; *arrow E*, 400 mM Tris (pH 7.5), 2 mM dithiothreitol, and 0.4 mM dThd; *arrow F*, 800 mM Tris (pH 6.8), 2 mM dithiothreitol, and 10% glycerol; *arrow G*, solution at *arrow* F with 1 mM dTMP; *arrow H*, solution at *arrow F* with 1 mM ATP, 0.2 mM dThd, and albumin (0.5 mg/ml); *arrow I*, same as *arrow H* except with 0.5 mM dThd; *arrow J*, same as *arrow H* except with a gradient of dThd of 0–0.6 mM. Fractions were 0.5, 0.5, or 1 ml from the three columns (HS-I, HS-II, or VZ virus). The activities shown for dTMP phosphorylation were adjusted for isotope dilution from solution *C* or *G* but not for inhibition from dThd (solutions *D*, *E*, or *H–J*). Other details are described under Materials and Methods.

The activities were eluted from an affinity column, biospecifically, with 500 μM dThd. From Fractions 30–36, the ratio of the product radioactivities with dTMP versus dThd as substrates was constant (0.014 ± 0.0013 SD). The possibility that the dTMP phosphorylating activity was a contaminant host-cell activity was excluded, since the activity could be inhibited >95% by 100 μM dThd in the standard assay. The host-cell dTMP kinase (Fractions 19 and 20) was inhibited <5% under the same conditions.

Similar results were obtained with the purified enzyme from VZ virus-infected cells (Fig. 1). Again, the ratio of product radioactivities with dTMP versus dThd as substrates was constant (after extensive dialysis of the fractions to remove dThd) across the active fractions (Fractions 39–49). Also, the dTMP phosphorylating activity was inhibited by dThd. Other workers did not find the dTMP phosphorylating activity with the VZ virus enzyme (19). Perhaps this was because phosphorylation of dTMP by the virus-coded dThd kinase is inhibited by the high concentration of dThd used to elute the enzyme from the affinity column. By using 50 μM dTMP and 120-min incubation times, this problem was partially avoided in the present study. For further studies, dThd was removed from each of the purified enzymes by gel filtration before use.

Although dTMP phosphorylating activities were associated with each of the purified virus-coded dThd kinases, the ratio of activities (the rate with dTMP/rate with dThd, standard assay conditions) from HS-I was significantly greater than that from HS-II virus, but about the same as that from VZ virus. The ratios were, respectively, 1.3, 0.016, and 1.1 for the purified enzymes.

Relative amounts of extractable virus-coded dTMP phosphorylating activities could be estimated, then, from the ratios of activities (dTMP/dThd) and the levels of virus-coded dThd kinases extracted from cells infected with the three virus strains. About 0.6 as much dThd kinase was extracted from cells infected with HS-I virus as from cells infected with HS-II virus (17), whereas extracts of cells infected with HS-I virus were estimated to have about twice as much virus-coded dThd kinase as those with VZ virus (data not shown). Thus, the relative extractable amounts of virus-coded dTMP phosphorylating activity should be about 100:2:40 (HS-I:HS-II:VZ virus).

The dTMP kinase (EC 2.7.4.9) from Vero cells (Fig. 1, *middle panel*, Peak 2) was purified at the same time as the HS-II virus dThd kinase. This enzyme preparation did not catalyze the phosphorylation of dThd (<0.3% the rate with dTMP).

Phosphorylation of BrVdUrd. All of the purified virus-coded dThd kinases phosphorylated BrVdUrd. At 1 mM nucleoside, the relative phosphorylation rates (BrVdUrd/dThd) were 1.1, 1.8, and 0.95 with the HS-I, HS-II, and VZ virus enzymes. These rates are in contrast to the sensitivities of the three strains to BrVdUrd treat-

ment in cell culture. The minimal inhibitory doses have been reported to be 0.024, 3, and 0.09 μM, respectively (5).

Attempts to detect the phosphorylation of BrVdUrd to its diphosphate with purified HS-I virus dThd-dTMP kinase were unsuccessful, possibly because BrVdUrd strongly inhibited the dTMP kinase. Therefore, BrVdUMP was prepared to test its phosphorylation directly to the diphosphate.

Approximate extinction coefficients of BrVdUrd in aqueous solution (pH 7.0) were determined to be about 14 and 10 mM^{-1} cm^{-1} at 250 and 293 nm (λ_{max}). When this material was incubated with a desalted cytosol fraction of HS-II virus-infected cells, most of it was converted in a time-dependent manner to an anionic species with the same UV absorption spectrum. This appeared to be the 5'-monophosphate of BrVdUrd. It could be converted back to the nucleoside with *Crotalus atrox* 5'-nucleotidase (EC 3.1.3.5) as determined by HPLC analysis. The RT of the BrVdUMP was 9.2–9.5 min; that of AMP was 5.8–6.2 min under the same conditions (40 mM KPi, pH 3.5). The purified material co-chromatographed with the chemically synthesized BrVdUMP obtained from Dr. Erik De Clercq and was >97% homogeneous as judged from HPLC analysis with absorbance monitored at either 254 or 310 nm.

Phosphorylation of BrVdUMP. The BrVdUMP was incubated with ATP·Mg and cytosol fractions of HS-I and HS-II virus-infected cells. Samples were chromatographed in 200 mM KPi (pH 3.5) by HPLC. Several peaks were observed from incubation mixtures with cytosol fractions of HS-I virus-infected cells (Fig. 2A). However,

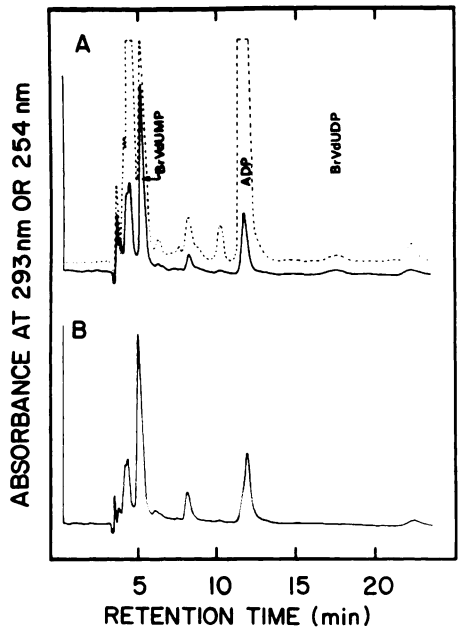

FIG. 2. *Chromatogram from HPLC analysis of a reaction mixture with BrVdUMP and cytosol fractions of HS-I and HS-II virus-infected cells*

The incubation mixtures (37° for 1 hr) contained 30 μM BrVdUMP, 0.5 mM ATP·Mg, 100 mM Tris·HCl (pH 7.5), albumin (1 mg/ml), and about 10^4 units of dThd phosphorylating activity per milliliter. The eluant was 200 mM KPi (pH 3.5). Absorbances at 254 nm (- - -) and 293 nm (——) were monitored with full-scale absorbances of 0.05 and 0.04 *A*, respectively. *Panel A*, HS-I virus; *Panel B*, HS-II virus.

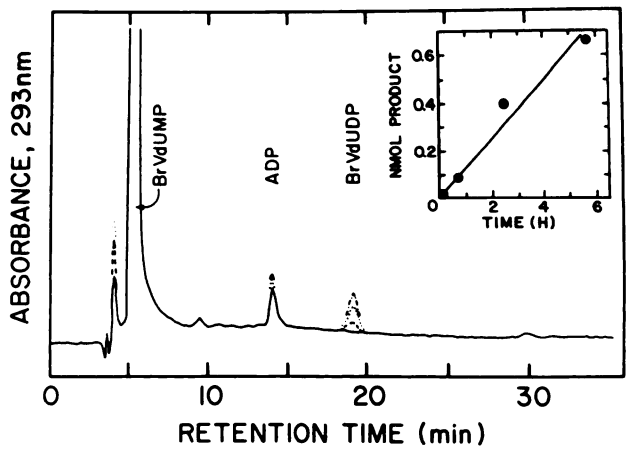

FIG. 3. *Quantitation of product formation with purified HS-I virus enzyme*

The reaction mixture (30°) contained 0.5 mM BrVdUMP, 10 mM ATP·Mg, 75 mM Tris·HCl (pH 7.5), albumin (0.5 mg/ml), and 280 units of purified HS-I virus dTMP kinase per milliliter. Aliquots were analyzed for BrVdUDP formation by HPLC as in Fig. 2. Chromatograms are shown for aliquots of the reaction mixture after 0.03 hr (——), 0.67 hr (- - -), 2.5 hr (· · · ·), and 5.75 hr (-·-·). *Inset*, Time course of formation of BrVdUDP.

only one peak had an appropriate RT for BrVdUDP (16–17 min)[3] and had a ratio of peak heights that was equivalent to that for BrVdUrd. The peak corresponded to about 5% conversion of BrVdUMP to BrVdUDP as calculated from the absorbance and volume of the material in the peak and the total amount of BrVdUrd derivatives chromatographed. The peaks at 5 and 11.3 min were identified as BrVdUMP and ADP by their RTs and absorbance characteristics. Formation of the peak identified as BrVdUDP was enzyme- and ATP·Mg-dependent. This product was sensitive to alkaline phosphatase treatment (EC 3.1.3.1, 1 IU for 10 min at 37°). An incubation mixture with a cytosol fraction of HS-II virus-infected cells did not produce a peak that corresponded to the BrVdUDP (<1%, Fig. 2B). This was not due to an endogenous inhibitor in the HS-II fraction, since an incubation mixture containing one-half as much of both HS-I and HS-II fractions still produced one-half as much of the peak.

Purified enzymes were used to quantitate the formation of BrVdUDP. The purified dThd-dTMP kinase from HS-I virus (280 units of dTMP kinase per milliliter of reaction mixture) catalyzed product formation at a rate of 0.09 pmole/min per dTMP kinase unit (Fig. 3) with 0.5 mM BrVdUMP. This rate could account for all of the activity observed with the cytosol fractions. The only peaks on the chromatograms that increased with time were the "breakthrough" peak (4 min) and the peaks identified as ADP and BrVdUDP. Substitution of the dThd-dTMP kinase from HS-I virus with that from HS-II virus or with the dTMP kinase from Vero cells resulted in no detectable formation of BrVdUDP. About 310 and 4300 units/ml (both expressed as dTMP kinase) of these two enzymes were used. In control reactions without

[3] The ratio of RTs for BrVdUMP versus AMP was 1.5 when 40 mM KPi was used as the eluant. The RT for ADP with 200 mM KPi was about 11.3 min, so it was anticipated that BrVdUDP would have an RT of about 17 min.

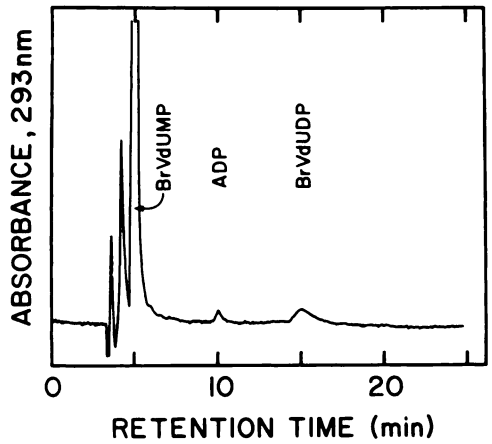

FIG. 4. *Product formation with purified VZ virus enzyme*
The reaction mixture (37° for 1 hr) contained 50 μM BrVdUMP, 0.5 mM ATP·Mg, 100 mM Tris·HCl (pH 7.5), albumin (1 mg/ml), and 870 units of purified VZ virus dTMP kinase per milliliter. An aliquot was analyzed for BrVdUDP formation by HPLC as in Fig. 2 except that 250 mM KPi (pH 3.5) was used as the eluant.

BrVdUMP, no loss of enzyme activity was observed over the 6-hr incubation period. Estimated maximal rates of BrVdUMP phosphorylation by these two enzymes were $<2 \times 10^{-3}$ and $<2 \times 10^{-4}$ pmoles/min per dTMP kinase unit.

The product formed with the HS-I virus enzyme was characterized as described with the cytosol extracts as well as with nucleotide pyrophosphatase (EC 3.6.1.9) from *C. adamanteus* (Sigma, Type II). One unit of this enzyme incubated with 0.1 ml of the reaction mixture for 60 min at 30° converted >90% of the BrVdUDP to BrVdUMP (HPLC analysis). Additionally, the diphosphate product co-chromatographed with chemically synthesized BrVdUDP (see Materials and Methods).

Chromatograms from incubations of the purified VZ virus dThd-dTMP kinase with ATP·Mg and BrVdUMP (Fig. 4) were similar to the chromatograms from HS-I virus. One difference was that only one peak formed (other than that from ADP) in the diphosphate region of the chromatogram. This peak (at 15 min) had the same ratio of absorbances as BrVdUrd. The rate of product formation was 0.03 pmole/min per dTMP kinase unit.

DISCUSSION

BrVdUMP was phosphorylated in reaction mixtures with either cytosol extracts or with purified virus-associated dTMP kinases from cells infected with HS-I virus. No phosphorylation was detected with the corresponding catalysts from HS-II virus or from host cells. Quantitatively, the amount of extractable HS-I virus-associated dTMP kinase was about 50-fold that extractable from HS-II virus-infected cells. In addition, the specificities of the two enzymes were different. The dTMP kinase from HS-I virus phosphorylated BrVdUMP at 9% the rate of dTMP phosphorylation as compared with less than 0.2% for the enzyme from HS-II virus. Anabolism studies with cell cultures are consistent with these results. A close analogue of BrVdUrd, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine, was efficiently phosphorylated to its monophosphate in cells infected with both HS-I and HS-II virus, but a higher phosphate(s) was detected only with HS-I virus-infected cells (20). The inefficient phosphorylation of BrVdUMP with HS-II virus correlates well with the observation that replication of HS-I virus is 100-fold more sensitive to BrVdUrd than is that of HS-II virus (5), and suggests a cause-and-effect relationship.

It has recently been found (21) that for the initial phosphorylation the apparent K_m value for BrVdUrd was substantially higher with the HS-II virus-coded dThd kinase than that with the HS-I virus enzyme (4.2 versus 0.24 μM). This suggests that BrVdUMP could be more readily formed in cells infected with HS-I virus. On the other hand, the maximal rates of phosphorylation of BrVdUrd by the virus-coded dThd kinase (see Results) and the amount of extractable enzyme (17) were greater for cells infected with HS-II virus than for HS-I virus. Thus, the rate of BrVdUMP formation[4] may be even greater with HS-II virus infection than HS-I virus infection at concentrations of BrVdUrd attainable with treatment. Serum levels of BrVdUrd from 6–90 μM have been sustained in mice for up to 80 min after a 100 mg/kg i.p. dose (22). At even higher doses (about 250 mg/kg), BrVdUrd had no effect on HS-II virus infections in mice while it gave complete protection against HS-I infections.[5] These results suggest that the difference in K_m values for the dThd kinases does not explain the antiviral difference.

In conclusion, the second step of BrVdUrd phosphorylation appears to be more selective than the first step and may help determine the effectiveness of the analogue to inhibit virus replication. The possible dependence of some pyrimidine derivatives on the virus-associated dThd-dTMP kinase for *both* phosphorylation steps may be a potentially crucial difference between these compounds and acyclovir. It has been shown that a monophosphate kinase (GMP kinase, EC 2.7.4.8) of the host cell can readily catalyze the phosphorylation of acyclovir monophosphate (7), and sensitivity of HS-I and HS-II viruses to the drug are about equal (9–11).

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[4] The calculation assumes that $v = V_m S/[K_m (1 + I/K_i) + S]$, where V_m (1) = 1, V_m (2) = 2.7, K_m (1) = 0.24, K_m (2) = 4.2, I = 0.5 μM dThd (as an alternate substrate inhibitor), and K_i = 0.5 μM. At 4.2 μM substrate (S), the rates (v) for Case 1 and Case 2 are equal.

[5] E. De Clercq, personal communication.

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