Herpes Simplex Virus Type 2 Induced Pyrimidine Nucleoside Kinase: Enzymatic Basis for the Selective Antiherpetic Effect of 5-Halogenated Analogues of Deoxycytidine†

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ABSTRACT: Studies were undertaken to determine the enzymatic basis for the selective inhibition of herpes simplex virus (HSV) by 5-halogenated analogues of deoxycytidine (dCyd). The following results suggest that HSV-2-induced thymidine (dThd), dCyd, and 5-bromodeoxycytidine (5-Br-dCyd) kinase are catalytic activities of the same protein: (a) cofractionation of the virus-induced activities was demonstrated during ammonium sulfate fractionation, Sephadex gel filtration, DEAE ion-exchange chromatography, and polyacrylamide disc gel electrophoresis; (b) the three activities were inactivated at the same rate by heat and were equally protected from thermal inactivation in the presence of dThd, dCyd, or 5-Br-dCyd; (c) the three activities were similarly inhibited by dTTP but were not affected by dCTP; (d) the subunit aggregation of the protein which catalyzes the three reactions was affected identically by $MgCl_2$ and ATP; and (e) viral mutants selected for the absence of one activity (either dThd or dCyd kinase) were deficient in the induction of all three activities. Kinetic studies suggested that phosphorylation of the three nucleosides occurs at a common active site. Substrate specificity studies with the HSV-2 pyrimidine nucleoside kinase indicated that, generally, the 5-substituted analogues of deoxyuridine were more readily phosphorylated than the 5-substituted analogues of dCyd. 5-Methyl and 5-halogenated pyrimidine ribonucleosides are effective substrates for the enzyme. Phosphorylation of purine nucleosides was not detected. The activities of various nucleoside triphosphates as phosphate donor for dThd kinase were in order of CTP > dCTP > dATP > ATP > UTP > GTP; for dCyd kinase, dATP > ATP > CTP > dCTP > UTP; for 5-Br-dCyd kinase, CTP > dATP > ATP > dCTP > UTP. ADP, AMP, dGTP, dTTP, dUTP, dCMP, and dTMP were not active as phosphate donors. Experiments with kinase-deficient mutants suggested that the antiviral activity of 5-Br-dCyd is dependent upon the induction of a functional pyrimidine nucleoside kinase. Furthermore, kinetic studies indicated that in contrast to its relatively poor affinity for the host cell cytosol kinases, 5-Br-dCyd has a relatively high affinity for the HSV-2 induced kinase (K_m = 1.3 μM). Therefore, the basis for the selective inhibition of HSV-2 by 5-halogenated analogues of dCyd is reflected in the induction of a pyrimidine nucleoside kinase with a high affinity for 5-Br-dCyd.

The discovery that extracts of herpes simplex virus (HSV) infected cells could catalyze the phosphorylation of 5-bromodeoxycytidine (5-Br-dCyd)[1] while extracts of uninfected cells could not suggested the possibility of selective chemotherapy of herpetic infections with 5-halogenated analogues of deoxycytidine (dCyd) (Cooper, 1973). This selectivity was subsequently demonstrated in tissue culture in this laboratory by Schildkraut et al. (1975). Further, the halogenated analogues of dCyd have also been shown to be effective in the treatment of experimental herpes keratitis in rabbits (Perkins et al., 1962), experimental herpes encephalitis in the mouse (Greer et al., 1975) and of an experimental cutaneous infection in the hairless mouse (Fox, Dobersen, Jerkofsky, and Greer, unpublished results). Also, 5-iododeoxycytidine has been shown to be more effective in the treatment of herpes keratitis in humans than 5-iododeoxyuridine (Kurimoto et al., 1969).

The induction of an HSV specific pyrimidine nucleoside kinase, represented by thymidine (dThd) and dCyd kinase activity, has been described by Jamieson & Subak-Sharpe (1974), Leung et al. (1975), and Cheng (1976). Subsequent to the studies on 5-halogenated analogues of deoxycytidine by Cooper & Greer (1973a, b) with mammalian dCyd kinase and those of Cooper (1973) with sonicates of uninfected and HSV-infected cells, the effectiveness of a number of other antiherpetic pyrimidine analogues appears to be based on the broad specificity of this enzyme (DeClerq et al., 1975, 1977; Chen et al., 1976; Aswell et al., 1977) The purpose of the research described in this paper was to study the relationship between HSV-induced dThd, dCyd, and 5-Br-dCyd kinase activities with the goal of providing an enzymatic basis for the selective inhibition of this virus by the 5-halogenated analogues of dCyd.

Materials and Methods

Cells and Cell Culture. Baby hamster kidney cells, BHK-21 (C-13), were purchased from the American Type Culture Collection. PyY/TG/CAR/BUdR cells were obtained from Drs. A. T. Jamieson and J. H. Subak-Sharpe of the Institute of Virology, University of Glasgow, Glasgow, Scotland. All cells were grown as previously described (Schildkraut et al., 1975).

Cells were routinely analyzed for PPLO and in all cases found to be free of these agents as determined by the fluo-

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[1] Abbreviations used: HSV-2, herpes simplex virus type 2; DEAE, diethylaminoethylcellulose; dThd, thymidine; dCyd, 2′-deoxycytidine; 5-Br-dCyd, 5-bromo-2′-deoxycytidine; 5-Br-dUrd, 5-bromo-2′-deoxyuridine; PFU, plaque forming units.

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rescent cytochemical method described by Russell et al. (1975).

Virus Strains. HSV-2 strain G was purchased from the American Type Culture Collection. HSV-2 strain HSG 52 and the derived mutant strains, ara C^r and $BUdR^r$, were obtained from Drs. A. T. Jamieson and J. H. Subak-Sharpe of the Institute of Virology, University of Glasgow, Glasgow, Scotland. HSV was propagated and virus titrations carried out as previously described (Schildkraut et al., 1975).

Chemicals. Tetrahydrouridine was a gift of Dr. Harry Wood of the National Cancer Institute. 5-Fluorodeoxyuridine was a gift of Hoffman-La Roche Inc. 5-Fluorodeoxycytidine was a gift of Dr. Jack J. Fox of the Sloan-Kettering Institute for Cancer Research. 5-Trifluoromethyldeoxycytidine was custom synthesized by Peninsular Research Chemicals. Alkaline phosphatase type III and 5′-nucleotidase grade IV from *Crotalus atrox* venom were obtained from Sigma Chemical. The 5′-nucleotidase preparation contained 0.01% 3′-nucleotidase activity (Sigma specifications).

Radiochemicals. [*methyl*-^{3}H]Thymidine, [5-^{3}H]deoxycytidine, [G-^{3}H]5-bromodeoxycytidine, and [γ-^{32}P]ATP were purchased from New England Nuclear Corp. [2-^{14}C]Thymine riboside was purchased from Calatomic (Calbiochem). The radiochemical purity of all compounds was greater than 97%. ^{32}P in the α and β positions of [γ-^{32}P]ATP was less than 0.1% (New England Nuclear specifications).

Enzyme Assays. Pyrimidine Nucleoside Kinase. Assays were carried out using a previously described modification of the DEAE disc method (Dobersen et al., 1976) or a method involving the transfer of the γ-phosphare of [γ-^{32}P]ATP to an unlabeled nucleoside substrate (Dobersen & Greer, 1975). For assays utilizing the 20-fold purified enzyme preparation described below, 20 μg of bovine serum albumin (fraction V, Sigma Chemical Co.) was present in the reaction mixture for enzyme stability (Okazaki & Kornberg, 1964).

Cytidine Deaminase. Cytidine deaminase was determined as described by Cooper (1973).

Deoxycytidylate Deaminase. Deoxycytidylate deaminase was determined as described by Maley (1967).

Thymidine Phosphorylase. Thymidine phosphorylase was determined by a modification of the method of Zimmerman & Seidenberg (1964). Reaction mixtures contained 0.1 M potassium phosphate (pH 7.4), 1.2 mM dThd, and 0.1 mL extract in a total volume of 0.5 mL. Reaction mixtures were incubated at 37 °C and terminated by the addition of 0.5 mL of 10% trichloroacetic acid. After removal of the precipitated protein by centrifugation at 5000*g* for 10 min, product formation was determined by assay of deoxyribose 1-phosphate by the diphenylamine reaction (Racker, 1952).

ATP Phosphatase. Reaction mixtures contained 5-mM $MgCl_2$, 10 mM NaF, 50 mM Tris-HCl (pH 7.2), 0.5 mM [γ-^{32}P]ATP (0.4 μCi/μmol), and 0.05 mL of extract in a total volume of 0.1 mL. Control assays were performed which did not contain NaF. Reactions were incubated at 37 °C and terminated by the addition of 0.8 mL of 1 mM K_2HPO_4 and 0.4 mL of a phosphate precipitation reagent (Sugino & Miyoshi, 1964). The precipitate was sedimented by centrifugation at 5000*g* for 10 min. The precipitate was then washed three times with phosphate precipitation reagent (5 mL per tube per wash) and dissolved in 0.6 mL of acetone. An aliquot (0.3 mL) of the solution was then added to 3 mL of Aquasol (New England Nuclear Corp.) and the radioactivity determined by liquid scintillation spectrometry.

Pyrimidine Deoxyribonucleotide Phosphatase. This activity was measured as thymidylate phosphatase. [^{3}H]dTMP was prepared using the HSV-2-induced dThd kinase reaction as described above for the DEAE method. DEAE discs, containing approximately 22 000 cpm (2 nmol) of [^{3}H]dTMP after washing with 1 mM ammonium formate as described above, were rinsed with 95% ethanol and dried and the ^{3}H-labeled nucleotides were eluted into 0.5 M Tris-HCl (pH 8.0) containing 1 M KCl. Reaction mixtures contained 5 mM $MgCl_2$, 10 mM NaF, 50 mM Tris-HCl (pH 7.2), 1.2 μM [^{3}H]dTMP (240 μCi/μmol), and 0.05 mL of extract in a total volume of 0.1 mL. Control assays were performed which did not contain NaF. Reactions were incubated at 37 °C and terminated by placing the tube in boiling water for 1 min. Aliquots (0.05 mL) of each reaction mixture were applied to DE-81 discs which were then washed in 1 mM ammonium formate, rinsed in 95% ethanol, and dried, and the radioactivity was determined by liquid scintillation spectrometry.

Identification of the Nucleotide Product of a Kinase Reaction. For the product identification of the 5-Br-dCyd kinase reaction, the method described by Cooper (1973) was utilized. A modification of this procedure was used for thymine riboside. Alkaline phosphatase treatment was carried out as above. The alkaline phosphatase reaction mixture was chromatographed with carrier thymine riboside and thymidine on Whatman 3MM chromatography paper with the top layer of an ethyl acetate/water/88% formic acid solution (60:35:5). Thymidine and thymine riboside were resolved in this system (R_f = 0.32 and 0.18, respectively).

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951). Also, where noted, the relative protein concentration was measured by OD_{280}.

DEAE-Cellulose Ion-Exchange Chromatography. A crude extract of HSV-2-infected cells in 4 mL, containing 11.2 mg of protein, prepared in buffer A (see Results) was washed onto a preequilibrated 2.5 × 40 cm column of DEAE-cellulose (DE-52, Whatman Biochemicals, Ltd.) with one column volume of buffer A. Protein was eluted with a linear 0.04 M KCl gradient. The flow rate was 23 mL/h and 5-mL fractions were collected. Fractions were assayed for dThd, dCyd, and 5-Br-dCyd kinase activity by the DEAE method as described above. All reaction mixtures were incubated at 37 °C for 15 h. Relative protein concentration was determined by OD_{280}. The molarity of KCl was determined by electrical conductance.

Polyacrylamide Disc Gel Electrophoresis. The gel system described by Davis (1964) was used with the following modification. Riboflavin was substituted for ammonium persulfate as the catalyst in the separating gel and the gels were photopolymerized. Fifty microliters (containing 85 μg of protein) of a crude extract of HSV-2-infected BHK cells was layered onto the stacking gel. Electrophoresis was carried out at 4 °C. The current was 2 mA per gel as the tracking dye (bromophenol blue) moved through the stacking gel and was increased to 5 mA per gel as the tracking dye moved through the separating gel. The gels were fractionated on a lateral gel slicer and each slice was used as the enzyme source for the assay of dThd, dCyd, and 5-Br-dCyd kinase activity. The reaction mixtures were as for the DEAE method described above except that [^{3}H]dThd was present at a concentration of 17 μM (6.7 Ci/mmol), [^{3}H]dCyd was 2.6 μM (38.0 Ci/mmol), and [^{3}H]-5-Br-dCyd was 120 μM (0.21 Ci/mmol).

Results

Upon infection of confluent BHK monolayers with HSV-2, at a multiplicity of infection of 3, dThd, dCyd, and 5-Br-dCyd kinase activities begin to increase concurrently between the second and fourth hour, reaching peak levels at the twelfth hour and gradually decreasing thereafter. Uninfected controls,

TABLE I: Copurification of HSV-2-Induced dThd, dCyd, and 5-Br-dCyd Kinase Activity.

Step	Kinase act.	Protein recovered (mg)	Protein recovered (% mg)	Units[a] recovered	% units recovered	Spec. act.[b]	Fold purification	R[c]
Crude extract	dThd			432	100	24.5		
	dCyd	17.0	100	11.0	100	0.65		0.7:0.02:1
	5-Br-dCyd			622	100	36.6		
20–40% ammonium sulfate fraction	dThd			230	53	31.1	1.2	
	dCyd	7.40	44	6.30	57	0.85	1.3	0.6:0.02:1
	5-Br-dCyd			357	57	48.3	1.3	
Sephadex G-100 pooled active fractions	dThd			202	47	430	17	
	dCyd	0.47	2.8	5.35	49	11.4	18	0.6:0.02:1
	5-Br-dCyd			317	51	673	18	

[a] 1 unit = 1 nmol of substrate phosphorylated/min under assay conditions described in Materials and Methods. [b] Specific activity = nmol phosphorylated/min per mg of protein. [c] R = ratio of thymidine kinase:deoxycytidine kinase:bromodeoxycytidine kinase.

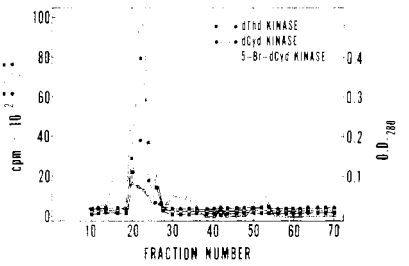

FIGURE 1: Gel filtration of HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase activity on Sephadex G-100. Equilibration and elution were carried out with buffer B. The enzyme extract used was the 20–40% ammonium sulfate fraction. Alternate fractions were assayed for dThd (■), dCyd (●), and 5-Br-dCyd (○) kinase activity by the DEAE method. Relative protein concentration (△) was measured by OD_{280}. Details of procedure are reported in the text.

on the other hand, showed a gradual decrease in these activities. At 12 h after infection, dThd, dCyd, and 5-Br-dCyd kinase activities in HSV-2 infected cells were increased 145-, 80-, and 280-fold, respectively, over uninfected controls. Production of infectious virus, under these conditions, reached a peak in 24 h.

Purification of HSV-2-Induced dThd, dCyd, and 5-Br-dCyd Kinases

HSV-2-infected BHK cells were harvested at 12 h after infection. Infected cell monolayers were washed twice with 0.9% NaCl and suspended ($1–5 \times 10^7$ cells/mL) in a buffer consisting of 25 mM Tris-HCl (pH 8.0), 1 mM glutathione, and 10% glycerol (buffer A), or this buffer with the addition of 5 mM $MgCl_2$ and 0.1 mM ATP (buffer B). The cells were disrupted by sonication for 45 s at 7 A in a Bronson Sonifier Model W140. The sonicate was then centrifuged at 105 000*g* for 60 min in a Beckman Model L ultracentrifuge. The supernatant fluid was termed crude extract. This and all subsequent steps were carried out at 0–4 °C.

1. *Ammonium Sulfate Fractionation.* The crude extracts were further purified by ammonium sulfate fractionation. Crystalline ammonium sulfate was slowly added with constant stirring to the extract to a concentration of 20% saturation. After the salt was completely dissolved, stirring was continued for 20 min. The resultant precipitate was pelleted by centrifugation at 20 000*g* for 10 min in a Sorvall RC2-B refrigerated centrifuge. The precipitate was discarded and the supernatant fluid was treated as above to yield a 20–40% ammonium sulfate precipitate which was dissolved in the appropriate extraction buffer. For some experiments, the 20–40% ammonium sulfate fraction, dissolved in buffer A, was used as the enzyme source. The enzyme activities in this preparation were stable for at least 9 months when stored at −70 °C. Upon further purification of extracts made with buffer A, however, marked instability of the enzyme activities was apparent. Extractions carried out with buffer B were also stable for at least 9 months when stored at −70 °C, but the stability afforded by the addition of $MgCl_2$ and ATP allowed for further purification as described below.

2. *Sephadex Gel Filtration.* A 20–40% ammonium sulfate purified preparation with buffer B (3.6 mL containing 5.0 mg of protein) was applied to a column (2.6 × 40 cm) of Sephadex G-100 which was preequilibrated with buffer B. Elution (upward-flow) was carried out with buffer B. The flow rate was 12 mL/h and 2.5-mL fractions were collected. Fractions were assayed for dThd, dCyd, and 5-Br-dCyd kinase activity by the DEAE method. Reaction mixtures were incubated at 37 °C for 2 min (dThd kinase), 35 min (dCyd kinase), and 1.5 min (5-Br-dCyd kinase). The molecular weight of the enzyme activities was estimated by utilizing the method of Andrews (1964). Blue dextran 2000 (Pharmacia) was used to define the void volume. RNase A, chymotrypsinogen A, ovalbumin, and aldolase (Pharmacia) were used as calibration standards. The fractionation described refers to Figure 1. The active fractions were pooled, divided into small aliquots, and stored at −70 °C. The activities remained stable for at least 9 months under these conditions. A summary of the purification is given in Table I.

The results demonstrate that the activities cofractionated through the two steps described. Each activity was purified approximately 20-fold. The overall yield for each activity was approximately 50% while only 2.8% of the initial protein remained. The ratio of the three activities to each other remained constant throughout the purification (Table I). Marked instability of the activities was observed during attempts at further purification.

The elution pattern of the HSV-2-induced activities on Sephadex G-100 was consistent with that of a protein having a molecular weight of 90 000.

Properties of HSV-2-Induced dThd, dCyd, and 5-Br-dCyd Kinases

Effects of $MgCl_2$ and ATP on the Gel Filtration Pattern of HSV-2-Induced dThd Kinase Activities.[2] Extractions and

[2] Cofractionation of the three HSV-2-induced activities was observed during gel filtration in Buffers A (data not shown) and B (see Figure 1). Therefore, in this experiment, dThd kinase is representative of the virus-induced kinase activities.

TABLE II: Chromatographic Analysis of the HSV-2-Induced dThd and dCyd Kinase Reactions.[a]

	cpm			
dThd kinase, sample	dThd (0.77)[b]	dTMP (0.59)	dTDP (0.38)	dTTP (0.26)
Time zero	13 800	121	17	17
Ammonium sulfate purified	8 280	5850	29	22
20-fold purified (Sephadex)	8 270	5210	19	12
dCyd kinase, sample	dCyd (0.63)	dCMP (0.48)	dCDP (0.36)	dCTP (0.20)
Time zero	18 100	302	29	20
Ammonium sulfate purified	10 700	6460	53	19
20-fold purified (Sephadex)	13 900	3900	45	18

[a] Reaction mixtures were as described for the DEAE method except that the concentrations of [^{3}H]dThd and [^{3}H]dCyd were 21 μM (23.8 Ci/nmol) and 22 μM (22.7 Ci/nmol), respectively. Reaction mixtures with the ammonium sulfate purified enzyme source contained 4 μg of protein; those with the 20-fold purified enzyme source contained 0.56 μg of protein. After terminating the reaction, 5 μL of the assay mixture was applied to Whatman no. 1 chromatography paper along with the appropriate carrier nucleoside and nucleotides and developed with 1-butanol/acetone/formic acid/1 M ammonium formate (35:25:15:25). UV-absorbing areas were cut out and the radioactivity was determined by liquid scintillation spectrometry. [b] Numbers in parentheses denote R_f value.

purification steps carried out in buffer B (25 mM Tris-HCl (pH 8.0), 1 mM glutathione, 10% glycerol, 5 mM $MgCl_2$, and 0.1 mM ATP) resulted in significantly greater enzyme stability than in those carried out in buffer A (25 mM Tris-HCl (pH 8.0), 1 mM glutathione, and 10% glycerol). In an attempt to determine the basis for this enhanced stabilization, the effect of $MgCl_2$ and ATP on the molecular weight was studied. As depicted in Figure 2, the molecular weight of the kinase calculated on the basis of its elution volume in the presence of 5 mM $MgCl_2$ and 0.1 mM ATP (buffer B) was 91 000, whereas the molecular weight was 43 000 in the absence of these factors (buffer A). These results suggest that the stabilization afforded by $MgCl_2$ and ATP is brought about by dimerization.

Interfering Enzyme Activities. Both the ammonium sulfate purified and the 20-fold purified enzyme preparations were used for the experiments to be described. These preparations were analyzed for the presence of various activities which, if present, would interfere with an unambiguous interpretation of the nucleoside kinase reaction. In assays for cytidine deaminase, deoxycytidylate deaminase, thymidine phosphorylase, ATP phosphatase, pyrimidine deoxyribonucleotide phosphatase, and nucleoside phosphotransferase (see Table IV), no significant product formation over background levels was detected. For each enzyme assay either an extract of calf thymus (Durham & Ives, 1970) or rabbit liver (Maley, 1967) was used as a positive control. It should be noted that low levels of pyrimidine deoxyribonucleotide phosphatase were detected in the ammonium sulfate purified preparation in the absence of NaF. However, in the presence of 10 mM NaF (as in the nucleoside kinase reaction), this activity was completely inhibited. This is consistent with the effect of NaF on rat liver deoxyribonucleotide phosphatase (Maley & Maley, 1960).

Although only a partial purification was obtained, the data given above indicate that the enzyme preparations used in the following experiments were sufficiently free of interfering enzyme activities to provide readily interpretable results.

The properties of the pyrimidine nucleoside kinase used in the following experiments differ from the host cell kinases in a number of ways. Mammalian cytosol dCyd kinase is not inhibited by dTTP (Durham & Ives, 1970), while the virus-induced dCyd kinase described here (see Table VI) and elsewhere (Cooper, 1973) is strongly inhibited. Cytosol dCyd kinase and mitochondrial dThd-dCyd kinase are extremely sensitive to feedback inhibition by dCTP, in contrast to the virus-induced dThd and dCyd kinases (see Table VI) which are refractory (Durham & Ives, 1970; Leung et al., 1975; Lee

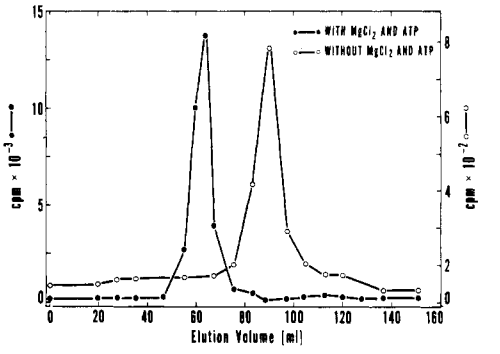

FIGURE 2: Effect of $MgCl_2$ and ATP on the gel-filtration pattern of HSV-2-induced dThd kinase. Crude enzyme extracts of HSV-2-infected BHK cells, prepared with either buffer A (1.6 mL containing 3.8 mg of protein) or buffer B (2.0 mL containing 4.0 mg of protein), were chromatographed on a calibrated Sephadex G-150 column (2.6 × 40 cm) after preequilibration of the column with the appropriate buffer. Calibration was carried out for both buffer systems with identical results. The flow rate was 12 mL/h and 2-mL fractions were collected. Fractions eluted in the absence (○) and presence (●) of $MgCl_2$ and ATP were assayed for dThd kinase activity by the DEAE method. Reaction mixtures were incubated at 37 °C for 15 h (fractions from buffer A elution) and 40 min (fractions from buffer B elution).

& Cheng, 1976). Cytosol dThd kinase does not efficiently use CTP as a phosphate donor; however, it is the preferred phosphate donor for the virus-induced dThd kinase (see Table V) (Kit, 1976; Cheng, 1976). Also, as noted below, differences in pH optima were observed between the major host cell kinase activities and the corresponding virus-induced activities. Kinetic experiments using a cytosol enzyme preparation (post-microsomal supernatant fraction; Kit et al., 1973) gave K_m values identical with those determined with the 20-fold purified enzyme preparation for dThyd, dCyd, and 5-BrdCyd. However, since whole cell extracts were used as the starting material and only a partial purification obtained, the presence of low levels of the host cell kinases cannot be entirely ruled out.

Identification of Reaction Products. To further analyze the HSV-2-induced kinase activities, the reaction products were chromatographically identified. The results for the dThd and dCyd kinase reactions (Table II) demonstrate that the sole reaction products formed by either enzyme preparation are dTMP and dCMP, respectively. In the case of the dCyd kinase reaction, a slight amount of dCDP was detected (approximately 0.4% of the dCMP formed).

Since the phosphorylated derivatives of 5-Br-dCyd were not

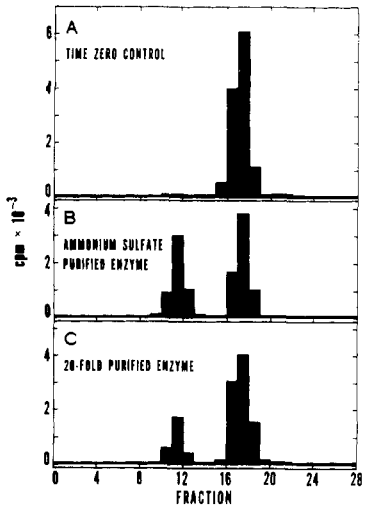

FIGURE 3: Chromatographic analysis of the HSV-2-induced 5-Br-dCyd kinase reaction. The reaction mixtures were as previously described for the DEAE method, except that the concentration of [^{3}H]-5-Br-dCyd was 0.12 mM (0.21 Ci/mmol). After termination of the reaction, 5 μL of the assay mixture was applied to Whatman no. 1 chromatography paper and developed with 1-butanol/acetone/formic acid/1 M ammonium formate (35:25:15:25). The dried chromatogram was cut into 1-cm sections and the radioactivity of each was determined by liquid scintillation spectrometry. Fraction 1 represents the origin. (A) Time zero control. (B) Enzyme source: 20–40% ammonium sulfate fraction. The reaction mixture contained 4 μg of protein. (C) Enzyme source: 20-fold purified preparation. The reaction mixture contained 0.56 μg of protein.

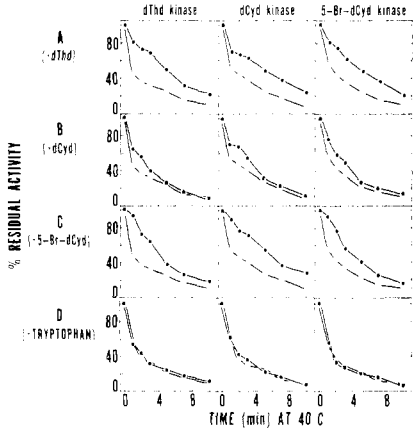

FIGURE 4: Effect of dThd, dCyd, and 5-Br-dCyd on the thermal inactivation of the HSV-2-induced activities. Inactivation mixtures containing 5 mM $MgCl_2$ and either 20 μM dThd (A), 2.5 mM dCyd (B), 20 μM 5-Br-dCyd (C), or 5 mM tryptophan (D) (●) were compared with controls containing 5 mM $MgCl_2$ (○). Assays were carried out using the DEAE method. Reaction mixtures contained 10 μg of protein. Enzyme source was the 20–40% ammonium sulfate fraction prepared in buffer A.

available for use as standards, chromatograms of the 5-Br-dCyd kinase reaction mixtures were analyzed as described in Figure 3. Using the same chromatographic system that separated the phosphorylated derivatives of dThd and dCyd, only a single product species was apparent. Alkaline phosphatase treatment of the phosphorylated reaction product yields 5-Br-dCyd. These results suggest that the sole reaction product formed by either enzyme preparation was 5-Br-dCMP.

Reaction Properties. The pH optima of HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase were 7.2, 8.0, and 7.2, respectively. In contrast, the pH optima of the major dThd and dCyd kinase activities present in uninfected BHK cells were 7.6 and 6.0, respectively.

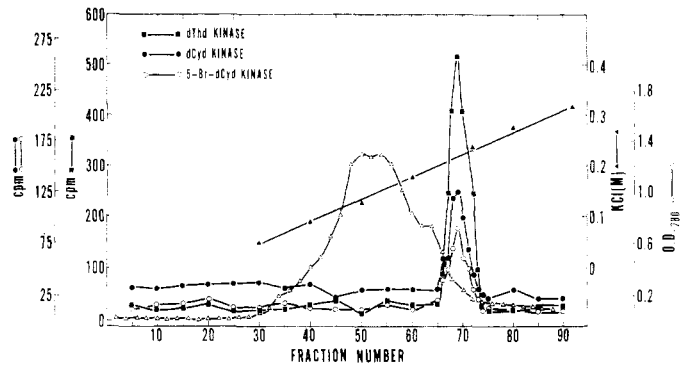

FIGURE 5: DEAE-cellulose ion-exchange chromatography of HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase activities. The enzyme source, a crude extract of HSV-2-infected BHK cells, was applied to a Whatman DE-52 column previously equilibrated with buffer A. Elution was carried out with a linear 0–0.4 M KCl gradient. Fractions were assayed for dThd (■), dCyd (●), and 5-Br-dCyd (○) kinase activity by the DEAE method. Relative protein concentration (△) was measured by the OD_{280}. The molarity of KCl (▲) was determined by electrical conductance.

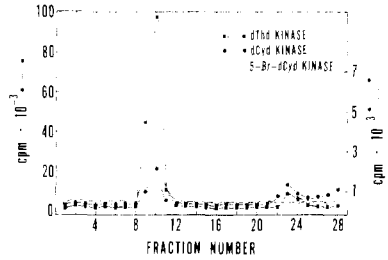

FIGURE 6: Polyacrylamide disc gel electrophoresis of HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase activities. A crude enzyme preparation was electrophoresed as described in Materials and Methods. The gels were fractionated with a lateral gel slicer and each slice was incubated at 37 °C in 0.1 mL of the DEAE assay reaction mixture described in Materials and Methods. The phosphorylation of [^{3}H]dThd (■), [^{3}H]dCyd (●), and [^{3}H]-5-Br-dCyd (○) was then determined. Fraction 28 corresponds to the tracking dye (bromophenol blue).

The virus-induced activities were evident over a broad range of Mg^{2+} concentrations (0.5–50 mM), the optimum being 5 mM. The concentration of ATP was kept constant at 5 mM in this study. The divalent cation requirement was less effectively replaced (approximately 75%) by Mn^{2+}.

Thermal Inactivation. As shown in Figure 4, the virus-induced activities were markedly labile; less than 10% of the original activity remained after 10 min at 40 °C. Also, similar rates of inactivation were seen for the three activities. Panel A indicates that the addition of 20 μM dThd not only protected dThd kinase activity from inactivation at 40 °C but dCyd and 5-Br-dCyd kinase activity as well. Similar results were obtained using 2.5 mM dCyd (panel B) and 20 μM 5-Br-dCyd (panel C). dCyd does not protect the enzyme to the same extent as dThyd or 5-Br-dCyd; this can be explained by the fact that dCyd was present at a concentration (2.5 mM) only four times its K_m concentration, whereas dThyd and 5-Br-dCyd were present at a concentration (20 μM) 50 and 15 times K_m concentrations, respectively. Protection of the activities was not observed in the presence of 5 mM tryptophan which was used as a control (panel D).

Ion-Exchange Chromatography. Figure 5 depicts DEAE-cellulose chromatography of the HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase activities. The three activities were seen to cofractionate, eluting with 0.22 M KCl.

Disc Gel Electrophoresis. Cofractionation of the virus-induced activities was also demonstrated during polyacryl-

TABLE III: Inhibition of the Replication of HSV-2 Mutants by 5-Br-dCyd.[a]

Virus	PFU/mL
HSV-2 WT	
Control	1.2×10^5
5-Br-dCyd	3.0×10^2
HSV-2 BUdR^r	
Control	2.8×10^5
5-Br-dCyd	1.8×10^5
HSV-2 ara C^r	
Control	1.8×10^4
5-Br-dCyd	3.2×10^4

[a] Confluent monolayers of PyY/TG/CAR/BUdR cells were infected with either HSV-2 (strain HSG 52) WT, HSV-2 BUdR^r, or HSV-2 ara C^r at a multiplicity of infection of 0.05. After allowing the virus to adsorb for 90 min, either growth medium containing 100 μg/mL 5-Br-dCyd and 100 μg/mL tetrahydrouridine or analogue-free growth medium was added to the plates. After 24-h incubation at 37 °C, the cells were harvested and titrated for infectious virus yield on BHK cells as previously described (Schildkraut et al., 1975).

amide disc gel electrophoresis (Figure 6). The R_m value calculated on the basis of the electrophoretic mobility of the virus-induced activities was 0.32 (corresponding to fraction 9).

Also apparent in Figure 6 are two minor kinase species: a dCyd kinase, R_m = 1.0 (fraction 28) and a dThd-dCyd kinase, R_m = 0.78 (fraction 23). Further experiments with uninfected cell cytosol and mitochondrial preparations identified these species as cytosol dCyd kinase and mitochondrial dThd-dCyd kinase, respectively. Uninfected cell cytosol dThd kinase, which was found to migrate at an R_m of 0.15, was not detectable in virus-infected cell extracts (Figure 6).

It should be noted that the virus-induced activities were markedly unstable during ion-exchange chromatography and disc gel electrophoresis, requiring assay modifications such as lengthy incubation times (Figure 5) and high specific activity ^{3}H-labeled nucleoside substrates (Figure 6) for their detection. These factors account for the discrepancies between the observed activity ratios and that described in Table I. The host cell kinases, particularly the mitochondrial enzyme, were relatively stable during electrophoresis in contrast to the virus-induced activities.

Viral Mutant Studies. Two HSV-2 mutants, one selected for the absence of dThd kinase (5-bromodeoxyuridine resistant) and one for the absence of dCyd kinase (cytosine arabinoside resistant), were compared with the wild-type strain in their ability to induce dThd, dCyd, and 5-Br-dCyd kinase activities. Cells deficient in dThd and dCyd kinase (PyY/TG/CAR/BUdR cells) were used. It was determined that each mutant was unable to induce not only dThd and dCyd kinase (which confirms previous experiments by Jamieson et al., 1974) but 5-Br-dCyd kinase as well (<0.01 nmol phosphorylated per min per mg of protein, data not shown).

The mutant viruses were also compared with the wild-type strain for their sensitivity to 5-Br-dCyd. Table III shows that, while 5-Br-dCyd (in the presence of tetrahydrouridine, a potent inhibitor of the enzymatic deamination of 5-Br-dCyd to 5-Br-dUrd; Schildkraut et al., 1975) resulted in a 400-fold decrease in the titer of wild-type HSV-2, no significant inhibition was observed with the two kinase-deficient mutants.

Phosphate Acceptor Specificity. A wide variety of nucleosides were examined for possible substrate activity. An assay method was utilized which measures the phosphorylation of unlabeled nucleoside substrate through the transfer of γ-phosphate of [γ-^{32}P]ATP (Dobersen & Greer, 1975). The results depicted in Table IV indicate that a 4-amino substitution on the various 2′-deoxyuridine (and uridine) derivatives decreases the apparent substrate activity. An exception to this is 5-methyldeoxycytidine (or 4-aminothymidine). Additions to the 5 position of deoxyuridine (methyl-, iodo-, bromo-, chloro-, fluoro-, or trifluoromethyl-) resulted in a slight increase in substrate activity. Phosphorylation of uridine was not

TABLE IV: Phosphate Acceptor Specificity.[a]

Substrate (60 μM)	nmol phosphorylated/60 min
Deoxyuridine	0.55
Thymidine	0.64
5-Iododeoxyuridine	0.63
5-Bromodeoxyuridine	0.74
5-Chlorodeoxyuridine	0.86
5-Fluorodeoxyuridine	0.77
5-Trifluoromethyldeoxyuridine	0.56
Uridine	<0.02
Thymine riboside	0.36
5-Iodouridine	0.42
5-Bromouridine	0.44
Deoxycytidine	0.22
5-Methyldeoxycytidine	0.89
5-Iododeoxycytidine	0.45
5-Bromodeoxycytidine	0.30
5-Chlorodeoxycytidine	0.40
5-Fluorodeoxycytidine	0.40
5-Trifluoromethyldeoxycytidine	0.19
Cytidine	<0.02
5-Iodocytidine	0.11
Adenosine	<0.02
Deoxyadenosine	<0.02[b]
Guanosine	<0.02
Deoxyguanosine	<0.02[b]

[a] Phosphorylation of the various nucleosides was determined by the ^{32}P-transfer method. Reaction mixtures contained 0.56 μg of protein. Enzyme source was the 20-fold purified preparation. The values given represent the average of two separate determinations. [b] Determined by the DEAE method.

TABLE V: Phosphate Donor Specificity.[a]

Phosphate donor (5 mM)	% activity		
	dThd	dCyd	5-Br-dCyd
None	<1.0	<1.0	<1.0
ATP	100	100	100
ADP	<1.0	<1.0	<1.0
AMP	<1.0	<1.0	<1.0
dATP	162	106	119
dGTP	<1.0	<1.0	<1.0
dCTP	173	10.2	93.0
dTTP	<1.0	<1.0	<1.0
dUTP	<1.0	<1.0	<1.0
GTP	6.1	<1.0	<1.0
CTP	192	38.8	133
UTP	84.6	6.1	27.9
dCMP	<1.0	<1.0	<1.0
dTMP	<1.0	<1.0	<1.0

[a] Phosphorylation of [^{3}H]dThd, [^{3}H]dCyd, and [^{3}H]-5-Br-dCyd in the presence of various phosphate donors (5 mM) was determined by the DEAE method. Reaction mixtures contained 26 μg of protein (for dCyd kinase) and 13 μg of protein (for dThd and 5-Br-dCyd kinase). Enzyme source was the 20–40% ammonium sulfate fraction. The values given represent the average of two separate determinations.

TABLE VI: Effects of dCTP and dTTP on the HSV-2-Induced Kinase.[a]

	% activity		
	dThd	dCyd	5-Br-dCyd
(A) dCTP (μM)			
0	100	100	100
25	99.1	98.4	97.3
50	98.4	99.5	98.0
100	100	102	99.6
200	99.4	98.0	97.8
1000	95.0	92.1	98.8
2000	98.0	90.1	100
4000	86.6	78.2	87.3
(B) dTTP (μM)			
0	100	100	100
25	60.7	16.0	56.9
50	47.3	6.3	36.4
100	32.4	1.4	22.3
200	18.2	1.0	12.3

[a] Phosphorylation of [^{3}H]dThd, [^{3}H]dCyd, and [^{3}H]5Br-dCyd in the presence of various concentrations of dCTP (A) and dTTP (B) was determined by the DEAE method. Reaction mixtures contained 0.56 μg of protein. Enzyme source was the 20-fold purified preparation.

TABLE VII: HSV-2-Induced dThd, dCyd, and 5-Br-dCyd Kinase; Kinetic Parameters.

Kinase act.	K_m	K_i	v_{max}[a]
dThd	0.40 μM	0.54 μM	40
dCyd	0.63 mM	0.50 mM	90
5-Br-dCyd	1.3 μM	1.8 μM	90

[a] Nanomoles of substrate phosphorylated per min per mg of protein.

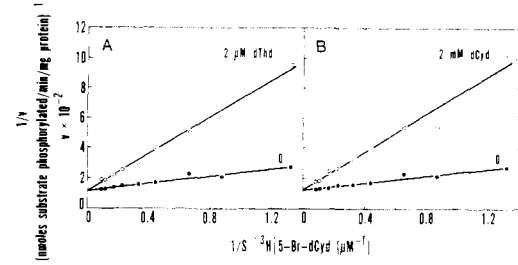

FIGURE 7: Inhibition of [^{3}H]-5-Br-dCyd phosphorylation by dThd (A) and dCyd (B). Phosphorylation of [^{3}H]-5-Br-dCyd was measured by the DEAE method. Reaction mixtures contained 0.13 μg of protein. Enzyme source was the 20-fold purified preparation. (A) Reaction mixtures contained varying concentrations of [^{3}H]-5-Br-dCyd and either no inhibitor (●) or 2 μM dThd (○). (B) Reaction mixtures contained varying concentrations of [^{3}H]-5-Br-dCyd and either no inhibitor (●) or 2 mM dCyd (○). The specific activity of [^{3}H]-5-Br-dCyd at all concentrations was 0.21 Ci/mmol.

detected unless a 5 substitution (methyl-, iodo-, or bromo-) was present. Halogen substitutions onto the 5 position of deoxycytidine (except trifluoromethyl-, which showed no increase) resulted in an approximate twofold increase in substrate activity. Addition of a methyl group gave a fourfold increase. As in the case of uridine, phosphorylation of cytidine occurred when a 5-iodo substitution was present. Phosphorylation of purine ribonucleosides and deoxyribonucleosides was not detected.

An unexpected finding in the studies on substrate specificity (Table V) was the efficient phosphorylation of thymine riboside and its analogues by the HSV-2-induced kinase. In confirmation, 98% of the ^{14}C-labeled nucleotide formed from [^{14}C]thymine riboside by reaction with the viral-induced kinase chromatographed with thymine riboside after alkaline phosphatase treatment. Kinetic experiments demonstrated that this compound competitively inhibits the phosphorylation of [^{3}H]-5-Br-dCyd (K_i = 32.3 μM, data not shown).

Since thymine riboside was not phosphorylated by extracts of uninfected BHK cells (<0.01 nmol phosphorylated per min per mg of protein) and had been reported to interact poorly, if at all, with host cell dThd kinase (Cheng & Prusoff, 1974), the antiviral properties of 5-iodo- and 5-bromouridine were investigated. While 5-iodo and 5-bromodeoxyuridine significantly inhibited the replication of HSV-2 (5000- and greater than 20 000-fold, respectively, at concentrations of 100 μg/mL), no significant inhibition was seen with the uridine analogues at the same concentrations (data not shown). Herrmann (1961) had previously obtained similar results with 5-bromouridine as determined by the lack of its effect on the diameters of HSV plaques. It would appear that the monophosphate derivatives of these analogues formed as a result of the virus-induced kinase are not substrates for the enzymes leading to their further phosphorylation and incorporation into DNA. Either nucleotide kinases or ribonucleotide reductase may be the limiting step.

Phosphate Donor Specificity. The virus-induced activities were found to differ with respect to phosphate donor requirements (Table V). For dThd kinase, the activities of nucleoside triphosphates were in the order of CTP > dCTP > dATP > ATP > UTP. A low but reproducible level of activity was seen with GTP. These results are consistent with those of Cheng (1976) except that phosphorylation of dThd was not detected in the presence of dUTP or dGTP. For dCyd kinase the order was dATP > ATP > CTP > dCTP. A low but reproducible level of activity was seen with UTP. For 5-Br-dCyd kinase the order was CTP > dATP > ATP > dCTP > UTP. ADP, AMP, dGTP, dTTP, dUTP, dCMP, and dTMP were not active as phosphate donors. GTP was not utilized in the dCyd and 5-Br-dCyd kinase reaction but displayed a low activity in the dThd kinase reaction.

Effect of dCTP and dTTP on the HSV-2-Induced Kinases. As expected from the above data, all three activities were refractory to inhibition by dCTP at concentrations as high as 4 mM (Table VI). In contrast, marked inhibition was seen with dTTP. At a concentration of 200 μM, dTTP inhibited the dThd kinase and 5-Br-dCyd kinase reactions by 82 and 88%, respectively. At the same concentration, dTTP exhibited significantly greater inhibition (>98%) of dCyd kinase activity. The effects of dTTP and dCTP on HSV-2-induced dThd and dCyd kinase observed above are consistent with other studies (Cooper, 1973; Ogino et al., 1973; Leung et al., 1975).

Kinetic Studies. To investigate further the relationship between HSV-2-induced dThd, dCyd, and 5-Br-dCyd kinase activities, kinetic studies were undertaken. Figure 7 demonstrates the effect of dThd and dCyd on the phosphorylation of [^{3}H]-5-Br-dCyd. The inhibition is seen to be competitive in each case. Similar results were obtained for the inhibition of [^{3}H]dThd phosphorylation with dCyd and 5-Br-dCyd as well as for the inhibition of [^{3}H]dCyd phosphorylation by dThd and 5-Br-dCyd (data not shown).

The kinetic parameters determined by the above experiments are given in Table VII. In the case of all three nucleoside substrates, the K_m and K_i values are in close agreement, indicating that the nucleosides had the same affinity for the

enzyme whether they were tested as substrates or inhibitors. dThd (K_m = 0.40 μM) and 5-Br-dCyd (K_m = 1.3 μM) are seen to have relatively high affinities for the enzyme while dCyd has a significantly lower affinity (K_m = 0.63 mM).

Discussion

Previous studies have suggested that dThd and dCyd are phosphorylated by a single viral-induced enzyme at a common active site (Hay et al., 1971; Jamieson & Subak-Sharpe, 1974; Leung et al., 1975; Cheng, 1976). Evidence presented in this paper from cofractionation studies, competition kinetics, heat protection studies and experiments with virus mutants is consistent with these findings and further indicates that 5-Br-dCyd shares the same active site.

Studies with various phosphate acceptors illustrate the broad substrate specificity of the virus-induced kinase. The presence of a 5 substitution and 2′-deoxyribose appears to be critical in determining substrate activity. Ribonucleosides with substitutions in the 5 position (e.g., thymine riboside and 5-iodocytidine) were active; ribonucleosides without a 5 substitution (e.g., uridine and cytidine) were not active. The presence of a deoxyribose will impart substrate activity to compounds without 5 substitutions (deoxyuridine and deoxycytidine); all pyrimidine 2′-deoxyribonucleosides examined displayed substrate activity. Generally, substrate activity was not greatly altered by differences in the size or electronegativity of the 5 substitution.

Differences between the ability of any single triphosphate to act as a donor for the dThd, dCyd, or 5-Br-dCyd kinase reaction were observed, indicating a change of donor specificity with respect to the acceptor nucleoside (or vice versa). This interaction between the two substrates suggests a sequential rather than a ping-pong reaction mechanism. Kinetic studies by Cheng (1976) indicate such a mechanism.

The observation that nucleoside monophosphates were not phosphate donors is consistent with the studies of Kit et al. (1975) but contrary to the recent finding of Jamieson & Subak-Sharpe (1976) that nucleoside phosphotransferase activity is associated with HSV-induced kinase activity. The reason for this discrepancy is unknown; however, Jamieson and Subak-Sharpe indicate that host factors may be involved in their finding since the phosphotransferase activity is not induced during infection of cells lacking both dThd and dCyd kinase.

Experiments with kinase-deficient mutants of HSV-2 have demonstrated that the antiviral activity of 5-Br-dCyd is dependent upon the induction of a functional pyrimidine nucleoside kinase. Furthermore, the results of kinetic studies have indicated that, in contrast to its relatively poor affinity for mammalian cytosol dCyd kinase (Cooper & Greer, 1973a) and for mammaliam cytosol dThd kinase (Bresnick & Thompson, 1965; Lee & Cheng, 1976), 5-Br-dCyd has a relatively high affinity for the virus-induced kinase. It is therefore suggested that the selective antiherpetic activity of the 5-halogenated analogues of dCyd results from the induction of a pyrimidine nucleoside kinase with a high affinity for 5-Br-dCyd.

Recent experiments (Fox, Dobersen, & Greer, in preparation) indicate that 5-Br-dCyd is incorporated into DNA of HSV-infected cells *as such* when the deamination of 5-BrdCMP is inhibited in cell culture. This suggests that the phosphorylated derivatives of 5-Br-dCyd may serve as substrates for the deoxyribonucleotide kinases and DNA polymerases of HSV-infected cells.

Although phosphorylation of 5-Br-dCyd by mitochondrial dThd-dCyd kinase was not detected (see Figure 6), studies by Lee & Cheng (1976) indicate a high affinity of 5-Br-dCyd for this enzyme. If mitochondrial dThd-dCyd kinase catalyzes the phosphorylation of 5-Br-dCyd, a priori one would expect little selectivity in the use of 5-halogenated analogues of dCyd as antiherpetic agents. Recent studies with LMTK$^-$ cells may provide an explanation for this discrepancy.

The LMTK$^-$ mouse cell line, isolated by 5-Br-dUrd selection, has been shown to be deficient in cytosol dThd kinase while retaining normal levels of mitochondrial dThd kinase activity (Attardi & Attardi, 1972). As a result, these cells are not able to incorporate 5-Br-dUrd into nuclear DNA but maintain the capacity to incorporate this analogue into mitochondrial DNA with no apparent toxic effects (Lansman & Clayton, 1975). These results indicate that the mutagenic effect of incorporation into DNA does not totally account for the lethality of 5-Br-dUrd. Consistent with this are studies by Meuth & Green (1974) suggesting that the major toxic effect of 5-Br-dUrd is the allosteric inhibition of ribonucleotide reductase by 5-BrdUTP. The resulting decrease in the formation of deoxyribonucleotides (chiefly dCDP and dUDP) impairs the synthesis of DNA.

In view of these findings, the survival of LMTK$^-$ cells containing highly substituted mitochondrial DNA may be explained by proposing that the lethal effects of 5-BrdUTP inhibition of ribonucleotide reductase did not occur in these cells. This hypothesis is strengthened by the findings of Berk & Clayton (1973) that intramitochondrial thymidylate pools are maintained, not only by mitochondrial thymidine kinase and nucleotide kinases but by transport of extramitochondrial thymidylate derived from de novo synthesis (as well as cytosol dThd kinase in normal cells) and that this nucleotide transport system is strongly unidirectional. Therefore, 5-Br-dUrd, once phosphorylated in the mitochondria, is not able to inhibit ribonucleotide reductase in the cytoplasm. 5-Br-dUrd utilization in LMTK$^-$ cells may be analogous to the utilization of 5-Br-dCyd in wild type cells in which cytidine deaminase is inhibited by tetrahydrouridine. That is, 5-Br-dUrd cannot be metabolized in the cytoplasm of LMTK$^-$ cells but can be used for DNA synthesis in the mitochondria and, as discussed above, does not exert significant cytotoxic effects. Similarly, 5-Br-dCyd (in the absence of cytidine deaminase activity) cannot be metabolized in the cytoplasm but can presumably be metabolized at least to the nucleotide level in mitochondria.

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Conformation-Dependent Nitration of the Protein Activator of Cyclic Adenosine 3′,5′-Monophosphate Phosphodiesterase†

Paul G. Richman* and Claude B. Klee

ABSTRACT: The conformational transition observed upon binding of Ca^{2+} to the Ca^{2+}-dependent activator protein of cyclic adenosine 3′,5′-monophosphate phosphodiesterase is reflected in a change in reactivity of its two tyrosine residues toward chemical modification by tetranitromethane (TNM). In the presence of Ca^{2+} both tyrosine residues (99 and 138) are nitrated by TNM, although at different rates, while in the presence of [ethylenebis(oxoethylenenitrilo)]tetraacetic acid (EGTA), only one tyrosine residue (99) is nitrated. The nitrotyrosines produced in the presence of Ca^{2+} exhibit apparent pKs of 7.3 and 8.6 by spectrophotometric titration. The tyrosine nitrated in the presence of EGTA only exhibited the lower pK. The rate of nitration of activator protein by TNM is enhanced by increasing the ionic strength (maximum employed was 1.0). Increasing ionic strength to 1.0 (0.33 M Na_2SO_4) does not lead to any significant change in secondary structure as measured by circular dichroic studies. The apparent rate of reaction of *N*-acetyltyrosinamide with TNM is also enhanced by increased ionic strength but to a much smaller extent than is observed with the protein. The results presented here together with other data recently reported from this laboratory indicate that tyrosine-138 (the one nitrated only in the presence of Ca^{2+}) is in an unusual microenvironment reflected by the high apparent pK of its phenol hydroxyl group (11.9 unmodified and 8.6 as the nitrophenol). The apparent K_m of activator protein for phosphodiesterase is unchanged upon nitration of both tyrosine residues.

The Ca^{2+}-dependent activator protein (also called modulator protein) of cyclic adenosine 3′,5′-monophosphate phosphodiesterase was first detected by Cheung (1967) and later characterized by Cheung (1970, 1971) and Kakiuchi et al. (1970). It was subsequently purified in several laboratories from a number of sources (Teo et al., 1973; Lin et al., 1974a,b; Watterson et al., 1976; Klee, 1977; Beale et al., 1977). The experimental evidence indicates that the mechanism by which the protein activates the enzyme requires an initial binding of Ca^{2+}

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