Antiviral Activity of Arabinosylthymine in Herpesviral Replication: Mechanism of Action In Vivo and In Vitro

J. F. ASWELL,¹ G. P. ALLEN, A. T. JAMIESON, D. E. CAMPBELL,² AND G. A. GENTRY*

Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi 39216,* and Medical Research Council Institute of Virology, Glasgow University, Glasgow, Scotland

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1- β -D-Arabinofuranosylthymine (ara-T), a metabolite of the sponge *Tethya crypta*, has shown selective activity against herpes simplex virus (HSV) replication (G. A. Gentry and J. F. Aswell, *Virology* 65:294-296, 1975). Analysis of HSV-infected and uninfected cell lysates by CsCl isopycnic centrifugation showed that ara-T blocked the incorporation of [³H]hypoxanthine into viral deoxyribonucleic acid and, to a large extent, into host deoxyribonucleic acid of infected (but not uninfected) cells. Additional experiments with [γ -³²P]adenosine 5'-triphosphate as a radiophosphate donor demonstrated that ara-T is phosphorylated by extracts of HSV-infected BHK cells and not by those of uninfected cells. At an ara-T concentration that almost completely inhibited the growth of LM cells, which had been transformed to a pyrimidine deoxyribonucleoside kinase⁺ (dPyK⁺) phenotype by ultraviolet-inactivated HSV-1, the growth of uninfected LM cells was not affected. These results indicate that the viral dPyK is responsible for the selective antiviral activity of ara-T. This conclusion was further supported by experiments that showed that the replication of a variety of dPyK⁻ mutants of HSV-1 and HSV-2 were not affected by ara-T and that ara-T inhibited the phosphorylation of deoxycytidine and deoxythymidine by HSV-1 dPyK, but not by host deoxycytidine and deoxythymidine kinases, respectively. Ara-T also selectively inhibited the replication of equine herpesvirus type 1 (EHV-1) in vitro and was effective against EHV-1 infection in vivo in hamsters. Further, EHV-1 was inhibited by ara-T and by bromodeoxyuridine in LM cells lacking a cytosol thymidine kinase, suggesting that EHV-1 induces a dPyK. Finally, spectrophotometric assay for thymine suggested that ara-T is not a substrate for nucleoside phosphorylase of hamster liver, and a microbiological assay indicated that substantial amounts of ara-T were excreted in the urine of uninfected hamsters that had received a single injection of 5 mg of ara-T, the amount given in each injection in the in vivo experiments with EHV-1.

1- β -D-Arabinofuranosylthymine (ara-T; spongothymidine) was first isolated, rather by coincidence, by Bergmann and Feeney (5) in a study of the sterol content (4) of *Tethya crypta*, a sponge first described as growing almost buried in the ocean floor off the Bahamas (16). Subsequently, ara-T was found to have little effect on the multiplication of tumor cells in culture (11, 15) but was later shown to be somewhat inhibitory to herpesvirus replication in vivo (17, 18, 53, 54, 56). The demonstration (26) that herpes simplex virus (HSV)-infected cells contain a pyrimidine deoxyribonucleoside kinase (dPyK) with a broad substrate specificity (29, 30) suggested that ara-T might be phospho-

rylated in HSV-infected cell cultures, but not in uninfected ones, and might therefore show a selectivity that, at that time (1974), was not ordinarily associated with inhibitors of virus replication. A reinvestigation supported this suggestion by showing that ara-T at 2×10^{-4} M completely blocked the replication of HSV type 1 (HSV-1) and HSV type 2 (HSV-2) in BHK cells but had no measurable effect on the multiplication of uninfected BHK cells subcultured at low cell density and followed for several generations (23). It has subsequently been shown that several other deoxythymidine (dT) analogs have similar selectivity against HSV, including 5'-amino-2',5'-dideoxy-5-iodouridine, 5-ethyldeoxyuridine, 5-propyldeoxyuridine, 5-allyldeoxyuridine (8, 10); 5-bromodeoxycytidine, 5-iododeoxycytidine (13, 20, 24, 31); and 5-methoxymethyldeoxyuridine (2, 3). This suggests that the HSV-specified dPyK may be a useful tool in

¹ Present address: The Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060.

² Present address: Department of Veterinary Science, University of Kentucky, Lexington, KY 40506.

designing anti-HSV compounds. Additional studies have shown that the 4-amino derivative of ara-T (4-amino-1- β -D-arabinofuranosylthymine, better known as 5-methyl-1- β -D-arabinofuranosylcytosine or 5-methyl ara-C) also inhibits HSV replication when tested in HEP-2 cells (1), which are known to deaminate dC, but has no such effect on HSV when tested in BHK cells, which lack deoxycytidine (dC) deaminase activity (13). These findings suggested that, in HEP-2 cells, 5-methyl ara-C was being deaminated to ara-T, which was then phosphorylated by the viral by specified enzyme. Finally, Miller and co-workers have shown that ara-T is active against varicella-zoster virus (40). This, together with the data of Dobersen and co-workers (20, 31) and of Ogino et al. (46), suggests that varicella-zoster virus also specifies a dPyK.

These studies have raised numerous questions, of which several have now been answered. In particular we report in this paper evidence that ara-T: (i) is phosphorylated by extracts of HSV-infected BHK cells but not by those of uninfected BHK cells; (ii) blocks deoxyribonucleic acid (DNA) synthesis (host as well as viral) in HSV-infected BHK cells but not in uninfected cells; (iii) does not show activity against dPyK⁻ mutants of HSV; (iv) shows a distinct antiviral effect in vivo in hamsters infected with equine herpesvirus type 1 (equine abortion virus; EAV, EHV-1); (v) is not cleaved to thymine by extracts of hamster liver; and (iv) is excreted in the urine in substantial amounts by hamsters.

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MATERIALS AND METHODS

Cell lines. The LM strain (35) of mouse fibroblasts was routinely grown in suspension in YeLP medium (yeast extract, lactalbumin hydrolysate, peptone) supplemented with 3% fetal bovine serum as described by O'Callaghan et al. (44, 45).

BHK cells (BHK-21, clone 13) and a thymidine kinase-less (5-bromodeoxyuridine [BrdU]-resistant) BHK-C13-derived cell line (C13, dTK⁻) were propagated as described (39) in Eagle minimal essential medium with Hanks balanced salts solution and supplemented with 6% tryptose phosphate broth and 6% calf serum (ETC).

HEP-2 cells (human laryngeal epidermoid carcinoma cells) (41) were also used in some experiments and were cultivated in ETC.

LMTK⁻ cells (clone 171), which had been transformed by HSV-1 to a thymidine kinase (dTK⁺) phenotype (dPyK⁺-transformed cells) (43) were kindly provided by William Munyon and Robert Hughes, Jr. The cells were grown in HAT medium, a dTK⁺-selective medium consisting of ETC containing hy-

poxanthine (10^{-4} M), aminopterin (6×10^{-7} M), and thymidine (2×10^{-5} M).

LMTK⁻ cells (which lack cytosol dTK activity) were obtained from Saul Kit and Del Rose Dubbs. These cells were maintained in ETC containing 25 μ g of BrdU per ml.

Cell lines derived from hamster embryo fibroblasts, which have been transformed by ultraviolet (UV)-irradiated HSV-1 (14-012-8-1) and HSV-2 (333-8-9) and from tumors produced in newborn hamsters after injection with 333-8-9 (T1) (22), were provided by Ronald Duff. These cells were maintained in ETC.

All cell lines were subcultured by conventional procedures with 0.05% trypsin plus 0.02% ethylenediaminetetraacetic acid (EDTA). Gentamycin was added at 50 μ g/ml to prevent bacterial growth. In addition, the cells were screened periodically for mycoplasmal contamination.

Propagation of HSV-1 and HSV-2. HSV-1 strain 17, HSV-2 strain 52, and mutants of each lacking dPyK (dPyK⁻) were routinely passaged and titrated in BHK cells as described (23, 55).

Propagation of EHV-1 in LM cells. EHV-1, originally adapted to growth in LM cells from the Syrian hamster by Randall and Lawson (50), was passaged in suspension cultures of LM cells and quantitated as outlined previously (44, 45, 47).

Plaque reduction assays. Plaque reduction assays were performed in 24-well culture trays as described (23). LMTK⁻ cells were passaged once in ETC without BrdU prior to experiments.

Virus yield experiments. BHK-C13 or BHK-C13 dTK⁻ cell monolayers were infected at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU)/cell with either wild-type or dPyK⁻ mutant HSV-1. After adsorption for 1 h at 37°C, the monolayers were overlaid with ETC, with or without ara-T and incubated at 37°C for 24 h. The cells were then scraped into the culture medium, and the virus was released by sonic treatment. The virus was then titrated in BHK-C13 cells to determine the yield.

Drug cytotoxicity assays. The toxic effects of various antiviral agents on growing cells were monitored as described (23). dPyK⁺-transformed cells were passaged once in ETC alone prior to seeding.

Substrate inhibition studies. Enzyme extracts from uninfected and HSV-infected cells were assayed for dT or deoxycytidine kinase (dCK) activity as previously described (30), except that various concentrations of ara-T (from 0 to 5×10^{-4} M) were included in the reaction mixture.

Isopycnic density gradient centrifugation. CsCl density gradient centrifugation was used to separate viral from cellular DNA by minor modifications of the procedure of Halliburton et al. (25). Replicate confluent monolayers in 24-well plates were infected with 50 μ l of HSV-1 (MOI = 1). At 2 h postinfection (p.i.), either 1 ml of ETC alone or ETC containing 50 μ g of ara-T per ml was added to the infected monolayers. At 4 h p.i., 5 μ Ci of [³H]hypoxanthine ([³H]Hx; New England Nuclear Corp., Boston, Mass.; specific activity, 5.0 Ci/mmol) in 50 μ l of Eagle medium was added to each infected well. At 12 h p.i., the cells were harvested by scraping and were mixed with approximately 10^6 uninfected cells,

which had been labeled with 0.14 μCi of [^{14}C]dT per ml (New England Nuclear Corp., Boston, Mass.; specific activity, 50 mCi/mmol) for 8 h. After pelleting by low-speed centrifugation ($300 \times g$ for 10 min), the cells were washed in 1 ml of saline-EDTA (0.154 M NaCl-0.1 M EDTA, pH 8.2), pelleted, and then resuspended in 1 ml of saline-EDTA by sonic treatment (System 40 Sonicator, Ultrasonic Industries, Inc., Albertson, N.Y.). The suspension was transferred to an 8.5-ml polyallomer tube with an additional 1 ml of saline-EDTA. Sarkosyl (Geigy Chemical Corp., Ardsley, N.Y.) was added to a final concentration of 2%, and the mixtures were allowed to lyse in a 60°C water bath for 10 min. After cooling to 37°C, Pronase (Calbiochem, La Jolla, Calif.) was added to a final concentration of 100 $\mu\text{g}/\text{ml}$. One hour of digestion with Pronase at 37°C was followed by the addition of crystalline CsCl (Harshaw Chemical Co., Solon, Ohio) and tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (0.02 M, pH 8.5) to a final density of 1.700 g/cm^3 and a final volume of 8.5 ml. The gradients were formed by centrifugation at $70,000 \times g$ for 72 h at 25°C in a 65 angle rotor in a Beckman model L ultracentrifuge.

After insertion of a probe into the gradient (F. A. Jones, MA thesis, University of Mississippi School of Medicine, Jackson, 1967), 8-drop fractions (ca. 0.12 ml) were collected onto 2.4-cm squares outlined on Whatman no. 3 paper strips. The strips were air-dried and given three 10-min washings in 5% trichloroacetic acid at 0°C, followed by three 10-min washings in 95% ethanol. The strips were air-dried, cut into individual 2.4-cm squares, placed in a toluene base scintillation fluid, and counted in a liquid scintillation counter.

The effect of ara-T on the incorporation of [^3H]Hx into the DNA of uninfected cells was also examined by CsCl density gradient centrifugation. ETC alone (1 ml) or ETC containing 50 μg of ara-T per ml was added to uninfected replicate monolayers of BHK cells. Two hours later, 70 μl of [^3H]Hx (7 μCi) was added to each well. At 12 h, the cells were harvested by scraping and mixed with uninfected cells that had been labeled with [^{14}C]dT. Experimental procedures subsequent to harvesting were the same as those outlined above.

Assay for dPyK. Dobersen and Greer (19) have developed a procedure for the examination of unlabeled pyrimidine nucleoside analogs as substrates for the virally induced dPyK. It is based on the transfer of the γ -phosphate of γ -[^{32}P]adenosine 5'-triphosphate (ATP) to the nucleoside substrate, forming an α - ^{32}P -nucleotide.

BHK monolayers (+/- cells) were infected at an MOI of 2 PFU/cell with HSV-1. At 13 h p.i., the cells were harvested by scraping, pelleted, and washed twice in cold 0.154 M NaCl and once in extraction buffer (25 mM Tris-hydrochloride buffer, [pH 8.0]-10% glycerol-1 mM glutathione, reduced form). The cells were resuspended in extraction buffer (8.5 ml containing approximately 1×10^7 cells/ml) and disrupted by sonic treatment four times for 30 s at 7 A (Branson Instruments, Inc., Stamford, Conn.). The sonic extract was then centrifuged at $105,000 \times g$ for 60 min in a 65 angle rotor in a model L ultracentrifuge.

Solid ammonium sulfate was slowly added to the supernatant, with constant stirring, to a final saturation of 20%. Once dissolved, the solution was allowed to stir for an additional 30 min. The supernatant resulting from centrifugation at $10,000 \times g$ for 20 to 30 min was collected. Ammonium sulfate was then added to 40% saturation, and the precipitate resulting from a second centrifugation at $10,000 \times g$ was suspended in 2 ml of extraction buffer. The suspension was dialyzed against two changes of 1-liter volumes of extraction buffer for 18 h. All procedures described above were performed at 0 to 4°C. Protein was determined by the method of Lowry et al. (38), with bovine serum albumin as the standard. The enzyme was stored at -70°C. Enzyme extracts from uninfected BHK cells were prepared by the same procedure.

The dialyzed extracts were assayed for dPyK activity as described (19). Controls consisted of reaction mixtures that did not contain nucleoside substrate as well as zero-time reaction mixtures containing all ingredients.

Evaluation of the antiviral activity of ara-T in hamsters. The Kentucky A strain of EHV-1 (equine abortion virus; EAV) adapted to hamsters by Doll et al. (21) was used in *in vivo* experiments with ara-T. The procedure for routine passage of EHV-1 was basically that of Darlington and Randall (14). Hamsters (25 to 30 g) were each injected intraperitoneally (i.p.) with 10 mean lethal doses (LD_{50}) of EHV-1 in 1 ml. The LD_{50} titer was calculated by the Reed-Muench method (52). Excluding controls, which received only virus, 1 ml containing 5 mg of ara-T or 10 mg of phosphonoacetic acid (PAA) in distilled water was injected i.p. 4 h after the virus and at 4-h intervals thereafter.

Comparison of the phosphorolysis of ara-T and dT by pyrimidine nucleoside phosphorylase. The method of Hotchkiss (28) was used to determine if ara-T was cleaved by nucleoside phosphorylase of hamster liver. This procedure depends on the observation that, at neutral pH, dT and thymine have an identical λ_{max} (ca. 268 nm), whereas at pH 12, the λ_{max} for thymine shifts to approximately 290 nm, with the λ_{max} for dT remaining at 268 nm. It was first necessary to verify that the λ_{max} of ara-T would be the same at pH 12 as at pH 7. Accordingly, the λ_{max} for ara-T (with those of dT and thymine for comparison) was determined with a McPherson (model EU-721) recording spectrophotometer. It was found that the λ_{max} of ara-T, like that of dT, remained at 268 nm when measured at pH 12, and that of thymine shifted as expected.

Enzyme extract for the assay was prepared from adult male hamster liver. An homogenate was prepared in 0.02 M Tris-hydrochloride buffer, pH 7.8, containing 0.15 M KCl in a Potter-Elvehjem homogenizer. The homogenate was filtered twice through cotton gauze and then centrifuged at $300 \times g$ for 10 min to remove cell debris. The supernatant was centrifuged at $105,000 \times g$ at 2°C for 1 h (model L ultracentrifuge, rotor 65). The supernatant was removed to an ice bath and used immediately. Protein was determined by the method of Lowry et al. (38).

Reaction mixtures consisted of 50 μl of enzyme extract (1 mg of protein), 0.15 ml of 0.1 M dT or ara-

T, and 0.8 ml of 0.1 M potassium phosphate buffer, pH 6.0, the optimum pH. The mixtures were incubated at 37°C, and, at various time intervals, 50- μ l samples were transferred to tubes containing 1 ml of 0.1 N NaOH. Absorbancy was then read at 300 nm.

Urinary excretion of ara-T. Two male Syrian hamsters were each injected with 5 mg of ara-T in 1 ml of water i.p. The hamsters were immediately placed in urine collection chambers. At 4 h postinjection, the urine remaining in the bladder at sacrifice was combined with that voided. Each urine sample was diluted to 5 ml, clarified by low-speed centrifugation, and filter-sterilized through a 0.2- μ m Millex filter (Millipore Corp., Bedford, Mass.). Dilutions of each sample (as well as control urine) were used in a plaque reduction assay (HSV-1) for determination of ara-T content.

Antiviral agents. Ara-T and 5-methyl ara-C were generously provided by Jack Fox. PAA was obtained from Abbot Laboratories. Stock solutions were usually prepared in distilled water and were filter-sterilized with 0.2- μ m, disposable, Millex filters.

RESULTS

Effect of ara-T on HSV and uninfected host cell DNA synthesis. That ara-T might selectively inhibit HSV DNA synthesis was suggested by the experiments of Gentry and Aswell (23). Isopycnic centrifugation in CsCl was used to separate viral and cellular DNA that had been labeled with [3 H]Hx as described above. [3 H]Hx was used as the DNA precursor because ara-T might interfere with the incorporation of [3 H]dT or [3 H]dC, since all three would

presumably be competing for the same sites on the viral dPyK. The DNA peak of cellular DNA was identified by including 14 C-labeled marker. The 3 H-labeled peaks in Fig. 1A represent viral and cellular DNA from lysates of BHK cells infected with HSV-1 in the absence of ara-T. The effect of the addition of 50 μ g of ara-T per ml, at 2 h p.i., on the incorporation of [3 H]Hx into these peaks is shown in Fig. 1B. Comparison of these figures indicates that 50 μ g of ara-T per ml reduced viral DNA synthesis 91% and also inhibited cellular DNA synthesis of the infected cultures to a large extent. The titer of replicate monolayers harvested at 24 h p.i. was reduced 99% by ara-T at the same concentration, as shown by comparison with the titer of monolayers not treated with ara-T.

As controls, [3 H]Hx was added to replicate monolayers of uninfected BHK cells either in the presence or absence of 50 μ g of ara-T per ml (as described above), followed by centrifugation of the respective lysates in CsCl. A comparison of the 3 H-labeled peaks in Fig. 2A and B indicates that ara-T has little, if any, effect on cellular DNA synthesis in uninfected cultures.

Effect of ara-T on the growth of wild-type and dPyK⁻ mutant HSV-1 and HSV-2. The replication of wild-type HSV-1 is drastically inhibited by the presence of ara-T in the culture medium (Fig. 3). The 24-h yield obtained in the presence of 20 μ g of ara-T per ml is 5 logs lower than that obtained in the drug's absence. The

FIG. 1. (A) Analysis of DNA synthesis in HSV-1 infected BHK cells. HSV-1-infected cells (3×10^6 cells, MOI = 1) were pulse-labeled with 5 μ Ci of [3 H]Hx (closed circles, solid line) from 4 to 12 h p.i. The cell lysates, which included [14 C]dT-labeled DNA marker (open circles, dashed line) from uninfected cells, were subjected to CsCl centrifugation. (B) Same as (A) except ara-T (2×10^{-4} M) was added at 2 h p.i.

FIG. 2. (A) Analysis of DNA synthesis in uninfected BHK cells. Uninfected BHK cells (1×10^6 cells) were pulse-labeled with 7 μ Ci of [3 H]Hx (closed circles, solid line) 4 to 12 h after mock infection. The cell lysates, which included [14 C]dT-labeled DNA marker (open circles, dashed lines) from uninfected cells, were subjected to CsCl centrifugation. (B) Same as (A) except that ara-T (2×10^{-4} M) was added at 2 h.

FIG. 3. Effect of ara-T on the replication of wild-type and dPyK⁻ HSV-1. Twenty-four-hour yield of virus is plotted against the concentrations of ara-T.

yield of dPyK⁻ mutants of HSV-1, on the other hand, is unaffected even at much higher concentrations of ara-T (e.g., 250 μ g/ml). The yields for several dPyK⁻ mutants of HSV-1 and one of HSV-2 are shown in Table 1. These mutants behaved in an identical fashion. Yield experiments performed in BHK dTK⁻ cells gave identical results, with a 5-log reduction in wild-type virus and no effect with the dPyK⁻ mutants (data not shown). Not only does ara-T prevent the production of progeny virus from wild-type infection, on the basis of yield recovered, but also, as can be seen from Table 2, the presence of ara-T prevents plaque formation, even when there is a high MOI. The plaque-forming ability of dPyK⁻ mutants, in contrast, is unaffected by the presence of ara-T.

Phosphorylation studies of ara-T and dT in

TABLE 1. Effect of ara-T on virus yields

Virus	Yields	
	+ ara-T ^a	Control
HSV-1		
17syn ⁺ (dPyK ⁺)	10^2	5×10^7
17syn (dPyK ⁺)	$<10^2$	6.3×10^6
17dPyK ⁻¹	1×10^7	2×10^7
17dPyK ⁻⁷	2×10^6	5×10^6
17dPyK ⁻⁹	1.5×10^7	1×10^7
34MP (dPyK ⁺)	$<10^2$	5×10^6
34MP dPyK ⁻	7×10^7	5×10^7
HSV-2		
WT (dPyK ⁺)	$<10^2$	7×10^7
dPyK ⁻	2.3×10^6	1.9×10^6

^a Final concentration 50 μ g/ml.

^b Syn⁺ and syn refer to wild type and syncytium-forming mutants of HSV-1, respectively. MP, Macroplaque; WT, wild type.

infected and uninfected cell extracts. The experiments of Kit et al. (32), showing that ara-T had no effect on the phosphorylation of [3 H]deoxyuridine by uninfected cell extracts, and those of Jamieson et al. (29, 30), demonstrating a viral dPyK with a relatively broad substrate specificity, strongly suggested that the basis of ara-T's selective inhibition of viral DNA synthesis was the selective phosphorylation of ara-T by the viral dPyK. To test this possibility, the procedure of Dobersen and Greer (19) was utilized. Extracts from both uninfected and HSV-1-infected BHK cells were examined for 32 P-nucleotide formation in the presence of either dT or ara-T.

As shown in Fig. 4, 32 P-nucleotide was

TABLE 2. Effect of ara-T on plaque formation of wild-type and dPyK⁺ HSV-1

Virus	ara-T ($\mu\text{g/ml}$)	Input virus (PFU/ 4×10^6 cells)	Plaques produced	Titer
17WT(dPyK ⁺)	0	128	128	1.28×10^9
	20	1.28×10^6	2	2.0×10^3
	100	12.8×10^6	0	$< 10^2$
17dPyK ⁻¹	0	60	58	5.8×10^8
	20	60	60	6.0×10^8
	100	60	54	5.4×10^8
17dPyK ⁻¹	0	120	117	1.17×10^8
	20	120	101	1.01×10^8
	100	120	100	1.00×10^8

FIG. 4. Nucleoside kinase activities in extracts of uninfected and HSV-infected cells with dT and ara-T as substrates. Extracts of uninfected BHK cells were prepared 13 h after infection with HSV-1 (2 PFU/cell) or from mock-infected BHK cells. Reaction mixtures contained 50 mM Tris-hydrochloride buffer, pH 8.0, 5 mM MgCl₂, 10 mM NaF, 0.6 mM dT or ara-T, 0.5 mM γ -[³²P]ATP (0.4 $\mu\text{Ci/mol}$), and 50 μl of enzyme extract (60 μg of protein) and were terminated after 60 min of incubation at 37°C as described in the text. Precipitation of inorganic phosphate and determination of radioactivity were performed as described in the text.

formed by uninfected cell extracts only when dT was the nucleoside substrate; ara-T apparently was not phosphorylated. Infected cell extracts, however, catalyzed the phosphorylation of both dT and ara-T. The same relationship between HSV-1 infection and ³²P-nucleotide formation in the presence of ara-T occurred after incubation of reaction mixtures for 45 min. These data implicate the involvement of the virally induced kinase in the selective inhibition of DNA synthesis in virus-infected cells by ara-T.

Substrate inhibition studies. The presence of ara-T in the assay mixture for wild-type HSV-1-induced dTK or dCK activities inhibits the phosphorylation of either deoxyribonucleoside (Fig. 5). At a concentration of 10^{-5} M, ara-T inhibits the phosphorylation of dT (10^{-5} M) by 53% and dC by 60%. Similar studies on uninfected or dPyK⁻ HSV-1-infected cells showed no such inhibition, even when ara-T was present

FIG. 5. Effect of ara-T on the phosphorylation of dT and dC by extracts (prepared 8 h p.i.) of uninfected and HSV-1-infected BHK cells. (A) Phosphorylation of dT. The reaction mixture contained 10^{-5} M [³H]TdR plus varying concentrations of ara-T. The results are given as the percentage of dT phosphorylated in the presence of ara-T as compared with the control. (B) Phosphorylation of dC. The reaction mixture contained 10^{-5} M [³H]CdR plus varying concentration of ara-T. The results are given as the percentage of dC phosphorylated in the presence of ara-T as compared with the control.

at a 50-fold higher concentration than the normal substrate. Kinetic studies show that ara-T acts as a competitive inhibitor of dT and dC, and that the inhibition of the phosphorylation

of dC is greater than that of dT. Similar findings have been reported by Cheng (9).

Effect of ara-T on dPyK⁺-transformed cells. Since Munyon and co-workers (42, 43) have transformed LMTK⁻ cells to dPyK⁺-LM cells with UV-irradiated HSV, the effect of ara-T on the growth of these cells and uninfected LM cells (the vast majority of which are dTK⁺ as shown by the lethality of BrdU) was also studied. Figure 6 compares the effect of ara-T at various concentrations on the growth of dPyK⁺-LM cells and uninfected LM cells. The experimental procedure is described above. By 96 h, ara-T, even at 4×10^{-5} M ($10 \mu\text{g/ml}$), had killed sufficient dPyK⁺-LM cells to reduce the cell count to less than 10% of the initial concentration, whereas uninfected LM cells were not affected by ara-T at 4×10^{-5} M. Ara-T at 1×10^{-4} M ($25 \mu\text{g/ml}$) and 2×10^{-4} M ($50 \mu\text{g/ml}$) inhibited the growth of LM cells 17 and 34%, respectively. In addition to confirming the results of the previous experiment, which indicate that ara-T is phosphorylated only by the HSV-induced dPyK, this experiment provides further evidence that the dTK of the cells transformed to dTK⁺ by UV-irradiated HSV-1 is coded for by viral DNA.

The addition of ara-T ($50 \mu\text{g/ml}$) to cells transformed by UV-irradiated HSV-1 and HSV-2 (cell types described above) did not appear to affect their growth (data not shown). Cooper (13) found that extracts from two of these lines (335-8-9 and 333-8-9 T1) did not catalyze the phosphorylation of 5-[³H]bromodeoxycytidine (BrdC), a compound which, like ara-T, appears to be a substrate of dPyK induced by HSV but not of the respective enzymes of uninfected cells. Such observations imply that, in these transformed cell lines, the viral dPyK activity is not expressed.

Evidence for an EHV-1 specified dTK. Although Jamieson et al. (29) and Leung et al. (36) had reported that EHV-1 did not induce a dTK or a dPyK, the ability of ara-T (and PAA) to inhibit the replication of EHV-1 was determined by plaque reduction assay. Figure 7 indicates that at 2×10^{-5} M ($5 \mu\text{g/ml}$) ara-T completely blocked plaque formation by EHV-1. PAA was less effective; at 7×10^{-5} M, PAA ($10 \mu\text{g/ml}$) reduced plaque formation 20%. In other studies (data not shown), $100 \mu\text{g}$ of PAA per ml was required to completely suppress plaque formation by HSV-1 and HSV-2 in BHK cells.

Since these findings and those of previous experiments suggested that EHV-1 either alters or induces dTK activity in LM cells, the effect of BrdU and ara-T on EHV-1 replication in LMTK⁻ cells was tested. As indicated in Fig.

FIG. 6. Effect of ara-T on the growth of uninfected LM cells and dPyK⁺-LM cells. Cells were planted in 1 ml of ETC alone (controls) or in 1 ml of ETC containing ara-T at the specified concentrations. At 24-h intervals, replicate monolayers were trypsinized and counted with a hemacytometer.

FIG. 7. Inhibition of EHV by ara-T and PAA. After removal of medium from replicate monolayers of LM cells, $50 \mu\text{l}$ of the appropriate dilution of EHV was added to each well. After 2 h of viral adsorption at 37°C with intermittent agitation, 0.5 ml of methylcellulose overlay containing either ETC alone or ETC plus a specified amount of ara-T or PAA was added to each well. Plaques were counted at 5 days p.i., the control wells yielding 110 and 90 plaques for PAA and ara-T, respectively.

8, both BrdU and ara-T inhibit plaque formation by EHV-1, although BrdU was more effective. Since LMTK⁻ cells lack cytosol dTK activity and are resistant to BrdU (6, 33, 36), the sensitivity of EHV-1 to BrdU and ara-T in

FIG. 8. Inhibition of EHV replication in LMTK⁻ cells by ara-T and BrdU. After removal of medium from replicate monolayers of LMTK⁻ cells, 50 μ l of the appropriate dilution of EHV-1 was added to each well. After 2 h of viral adsorption at 37°C with intermittent agitation, 0.5 ml of methylcellulose overlay containing either ETC alone (controls) or ETC plus a specified amount of ara-T or BrdU was added to each well. Plaques were counted at 5 days p.i., the control well yielding 76 plaques.

these cells is consistent with the induction of a virally specified dTK, possibly a dPyK.

Antih herpes activity of ara-T in vivo. The experiments involving EHV-1 and ara-T in vitro were expanded to an in vivo study, using the EHV-Syrian hamster system. This system has also been used to evaluate the antih herpes activity of 9- β -D-arabinofuranosyladenine (ara-A) (37). As described by Randall and Bracken (49), experimental EHV-1 infection of hamsters is characterized by a viremia and fatal hepatitis. The results of a series of experiments with this system are shown in Fig. 9. Ara-T and PAA were both protective in this system, although ara-T was more effective than PAA when compared under the same injection schedule. A preliminary experiment showed that injection of 5 mg of ara-T every 4 h allowed longer survival than did similar injections every 8 h. This observation suggested that between injections effective levels of ara-T were not being maintained in the animals. Hill et al. (27) have shown that dT (at doses of 0.4 μ mol/kg or larger) is rapidly catabolized to CO₂ as well as excreted in the urine of hamsters. Since there is indirect evidence that ara-T is not catabolized to thymine by pyrimidine nucleoside phosphorylase (12, 34), most of the injected ara-T is probably excreted in the urine.

The possibility that ara-T does undergo phosphorylase was tested with an enzyme extract from hamster liver, as described above, and the results of the experiment are shown in Fig. 10. Spectrophotometric examination of the reaction

mixtures indicates that thymine is released after incubation of dT with the enzyme extract; however, the reaction mixtures containing ara-T failed to show an increase in absorption, indicating that ara-T is not a substrate of the pyrimidine nucleoside phosphorylase of hamster liver.

If, as suggested by the above experiment, ara-T is not catabolized to thymine by the ham-

FIG. 9. Antiherpes activity of ara-T in vivo. Each 25- to 30-g Syrian hamster received 10 LD₅₀ of EHV-1 in 1 ml by i.p. injection at zero hour. Ara-T (5 mg/animal per injection, 5 mg/ml of water) or PAA (10 mg/animal per injection, 10 mg/ml of water) were given i.p. at 4-h intervals, beginning at 4 h postinoculation with EHV. The drugs were continued for the indicated intervals to a total of six injections per 24 h, 12 per 48 h, and 18 per 72 h. The control group contained 15 animals; each of the other groups contained five except for the one receiving ara-T for 48 h, which contained four.

FIG. 10. Assay for phosphorylase of ara-T and dT by pyrimidine nucleoside phosphorylase. Reaction mixtures contained 0.015 M ara-T or dT, 0.08 M potassium phosphate buffer, pH 6.0, and 50 μ l of enzyme extract (1 mg of protein). At specified intervals, 40- μ l samples were transferred to tubes containing 0.1 N NaOH, and optical density (O.D.) was determined at 300 nm.

ster, then most of the ara-T injected must be rapidly excreted in the urine. The results of a plaque reduction assay, using HSV-1 and the urine of hamsters injected with 5 mg of ara-T, show that 1:400 and 1:800 dilutions of the urine result in 87 and 82% reductions in plaque formation, respectively. Control urine did not inhibit plaque formation. These points on a dose-response curve (data not shown; similar to that in reference 23) correspond to 3.5×10^{-6} and 3×10^{-6} M, respectively, which means that approximately half of the ara-T is excreted within 4 h of injection.

Comparative cytotoxicity of antiherpes agents. The cytotoxic effects of ara-C and ara-T on growing LM cells are compared in Fig. 11. At approximately 50 $\mu\text{g/ml}$, ara-C reduced the cell count at 96 h to less than half the initial concentration; ara-T inhibited growth 30%. When tested at 10, 50, and 100 $\mu\text{g/ml}$, PAA (Fig. 11B) inhibited the growth of LM cells 43, 69, and 90%, respectively (96 h).

DISCUSSION

If ara-T's selectivity is dependent upon its initial phosphorylation by the viral dPyK, then ara-T should inhibit DNA synthesis in infected cells but should have no effect on the DNA synthesis of uninfected cells. Results of the studies with CsCl density gradient centrifugation indicate that this is the mechanism of the

selective inhibition of HSV-1 by ara-T. At concentrations that almost completely inhibited HSV-1 replication, ara-T inhibited viral DNA synthesis and, to a large extent, cellular DNA synthesis of infected BHK monolayers. Ara-T at the same concentration that inhibited HSV-1 DNA synthesis had little, if any, effect on the DNA synthesis of uninfected host cells. The inhibitory form of ara-T and the means by which it blocks DNA synthesis of virus-infected cells have not been examined, although either the inhibition of DNA polymerase activity by 1- β -D-arabinofuranosylthymine-5'-triphosphate or its incorporation into the DNA is suspected.

The results of the experiments using γ -[^{32}P]ATP to measure the phosphorylation of unlabeled ara-T showed that ara-T is probably phosphorylated in HSV-1-infected cells but not in uninfected cells. This finding lends further support to the contention that the viral dPyK is responsible for the initial activation of ara-T as well as its selective antiviral activity. Dobersen and Greer (19) based their technique on experiments with BrdC, which is phosphorylated only in extracts of HSV-infected cells. Cooper (13) and Prusoff et al. (48) have reported similar results with labeled BrdC and 5'-amino-2',5'-dideoxy-5-iodouridine, respectively. Dobersen et al. (19, 20) have also reported a high affinity of viral dPyK for BrdC versus a low affinity of the host kinases, indicating further that the se-

FIG. 11. Effect of ara-C, ara-T, and PAA on growth of LM cells. Cells were planted in 1 ml of ETC alone (controls) or in 1 ml of ETC containing a 50- $\mu\text{g/ml}$ amount (2×10^{-4} M) of either ara-C or ara-T (A) or PAA at the specified concentrations (B). At 24-h intervals, replicate monolayers were trypsinized and counted with a hemacytometer.

lective antiherpetic activity of BCdR lies in the induction of the viral dPyK.

Additional support for the role of the viral dPyK in the activation of ara-T is provided by the growth curves of dPyK⁺-LM cells and uninfected LM cells in the presence and absence of ara-T. These data suggest that the expression of the dPyK is necessary for an inhibitory effect of ara-T on LM-cell growth. This is further supported by the fact that while the yield of wild-type HSV-1 is reduced by 5 logs in the presence of ara-T, in both dTK⁺ and dTK⁻ BHK cells in the replication of dPyK⁻ virus is essentially unaffected. Further, ara-T appears to act as a competitive inhibitor of the phosphorylation of both dT and dC by enzyme extracts obtained from wild-type HSV-1-infected cells (Fig. 5).

Such results also indicate that ara-T may be a useful probe for detecting the viral dPyK. Ara-T did not affect the growth of cell lines 333-8-9, 333-8-9 T1, or 14012-8-1 that had been transformed by UV-irradiated HSV in vitro. Cooper (13) also found that extracts from two of the same lines, as well as extracts from two cervical carcinomas, did not catalyze phosphorylation of [³H]BrdC. Rawls et al. (51) have reported that the dTK extracted from cervical cancer cells migrated on polyacrylamide gel electrophoresis in a pattern similar to that of the dPyK induced by HSV, but the enzymes did not appear to be antigenically related. It was suggested that the peak produced by the cervical carcinoma cell extracts might be the "fetal" form of dTK, which is characteristic of many tumor cells. Evidently, the dPyK produced during lytic infection or after the "biochemical" transformation with UV-irradiated HSV is not expressed in cells transformed in vitro by HSV or by cervical carcinoma cells, although there is a possibility that it is expressed in an altered form or at very low levels.

Other results in this study showed that ara-T is an effective inhibitor of EHV-1 replication. In addition, EHV-1 was sensitive to BrdU and ara-T in LMTK⁻ cells. Both of these findings suggest that EHV-1 induces a dTK or possibly a dPyK. Jamieson et al. (29), however, have reported that two different British strains of EHV-1 did not induce dTK or dCK activity in BHK cells. Similar results were reported by Leung et al. (36) for EHV-1 (strain KYD) and EHV-2 (strain LK) when grown in owl monkey kidney (OMK) cells. There are several possible explanations for this discrepancy. First, it is possible that the KYA strain of EHV-1 used in this study differs from other strains in the ability to induce dTK activity. Secondly, EHV-1

may induce dTK activity in LM cells but not in BHK or OMK cells. There is also a remote possibility that the mitochondrial kinase A of the LM cells (which is present in LMTK⁻ cells and which can phosphorylate dC, dT, and BUdR) (6, 7) phosphorylates ara-T and BrdU and is in some way stimulated as a result of infection. In this case, both ara-T and BrdU would be inhibitory to EHV-1 DNA synthesis. Additional experiments are required to determine if any of these explanations are correct.

The in vivo studies presented show that ara-T is protective against an experimental herpesvirus infection. In addition, they indicate that, in the EHV-hamster system, ara-T is even more effective (at least on a molar basis) than PAA, an agent with known in vivo antiherpes activity. The frequency, however, of ara-T injections required to prolong survival time of the hamsters suggested that ara-T was being rapidly excreted. Although Hill et al. (27) found that high doses of dT in the hamster are rapidly catabolized to CO₂ as well as excreted in the urine, the results of the spectrophotometric assay for pyrimidine nucleoside phosphorylase activity indicate that ara-T is not catabolized to thymine. This absence of catabolism suggested that most of the ara-T was being rapidly excreted in the urine of the hamsters between injections. Plaque reduction assays showed that this assumption was probably correct, since approximately half of a 5-mg dose of ara-T was present in the urine of hamsters within 4 h.

Rapidly growing cells, particularly those of the intestinal tract, bone marrow, and lymphoid tissues, appear to be most susceptible to the adverse effects of antiviral chemotherapeutic agents (3). An adverse effect on lymphoid tissue is even more serious in immunosuppressed and immunodeficient patients, because the agent may destroy any remaining immune response, which is needed for eventual recovery from the viral infection. In this respect, it is hoped that the absence of toxicity of an agent to dividing cells in vitro is indicative of fewer side effects during actual clinical use. The results of the studies comparing the cytotoxicity of several antiviral agents demonstrated that ara-T is much less toxic than ara-T or PAA to growing LM cells. However, ara-T, PAA, and 5-methyl ara-C were all slightly toxic to growing human (HEp-2) cells, whereas ara-A was considerably more toxic at the same concentration (1). More importantly, as summarized in Table 3, ara-T does not inhibit the growth of uninfected host cells at concentrations that were completely inhibitory to plaque formation by HSV and EHV-1. The same was not true for PAA.

TABLE 3. *Antiviral activity versus cytotoxicity of ara-T and PAA*

Drug	Virus/host cell	Minimal effective concn tested ($\mu\text{g/ml}$)	Uninfected cell growth; % of control at +72 h
araT	EHV/LM cell	5	100
	HSV-1/BHK cell	15	100 ^a
	HSV-2/BHK cell ^a	50	100
PAA	EHV/LM cell	50	38
	HSV-1,-2/BHK cell ^b	100	22

^a From Gentry and Aswell (23).^b Data not shown.

As shown from this study, ara-T is an effective inhibitor of herpesvirus replication, both in vitro and in vivo. A possible disadvantage of ara-T, its rapid urinary excretion, can probably be circumvented by the use of 5-methyl ara-C, an apparent intracellular donor of ara-T (1). The absence of significant concomitant toxicity, as shown by in vitro tissue culture growth curves, suggests that these two drugs may provide effective chemotherapy for herpesvirus infections, especially in the immunosuppressed or immunodeficient patient, with few detrimental side effects.

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LITERATURE CITED

- Aswell, J. F., and G. A. Gentry. 1977. Cell dependent antiherpesviral activity of 5-methylarabinofuranosylcytosine. *Ann. N.Y. Acad. Sci.* 284:342-350.
- Babiuk, L. A., B. Meldrum, V. S. Gupta, and B. T. Rouse. 1975. Comparison of the antiviral effects of 5-methoxymethyldeoxyuridine with 5-iododeoxyuridine, cytosine arabinoside, and adenine arabinoside. *Antimicrob. Agents Chemother.* 8:643-650.
- Babiuk, L. A., and B. T. Rouse. 1975. Effect of anti-herpesvirus drugs on human and bovine lymphoid function in vitro. *Infect. Immun.* 12:1281-1289.
- Bergmann, W. 1949. Marine products. XXV. Comparative biochemical studies on the lipides and sterols of marine invertebrates. *J. Mar. Res.* 8:137-176.
- Bergman, W., and R. J. Feeney. 1950. The isolation of a new thymine pentoside from sponges. *J. Am. Chem. Soc.* 72:2809-2810.
- Berk, A. J., and D. A. Clayton. 1973. A genetically distinct thymidine kinase in mammalian mitochondria. *J. Biol. Chem.* 248:2722-2729.
- Bogenhagen, D., and D. A. Clayton. 1976. Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. *J. Biol. Chem.* 251:2938-2944.
- Cheng, Y. C. 1976. Specific alternative substrates of herpes simplex type 1 (HSV-1) and type II (HSV-2) thymidine kinase (TK); a rational approach for development of antiviral chemotherapy. *Ann. N.Y. Acad. Sci.* 284:594-598.
- Cheng, Y. C. 1976. Deoxythymidine kinase induced in HeLaTK⁻ cells by herpes simplex virus type I and type II. *Biochem. Biophys. Acta* 452:370-381.
- Cheng, Y. C., B. Goz, J. P. Neenan, D. C. Ward, and W. H. Prusoff. 1975. Selective inhibition of herpes simplex virus by 5'-amino-2',5'-dideoxy-5-iodouridine. *J. Virol.* 15:1284-1285.
- Chu, M. Y., and G. A. Fischer. 1962. A proposed mechanism of action of 1- β -D-arabinosylcytosine as an inhibitor of the growth of leukemic cells. *Biochem. Pharmacol.* 11:423-430.
- Cohen, S. S. 1966. Introduction to the biochemistry of D-arabinosyl nucleosides. *Prog. Nucleic Acid Res. Mol. Biol.* 5:1-88.
- Cooper, G. M. 1973. Phosphorylation of 5-bromodeoxycytidine in cells infected with herpes simplex virus. *Proc. Natl. Acad. Sci. U.S.A.* 70:3788-3792.
- Darlington, R. W., and C. C. Randall. 1963. The nucleic acid content of equine abortion virus. *Virology.* 19:322-327.
- De Garihe, M. P., and J. De Rudder. 1970. Activité de certains arabinosides et de leurs associations sur le développement de la tumeur ascitique d'Ehrlich chez la souris. *Prog. Antimicrob. Anticancer Chemother.* 2:180-184.
- DeLaubenfels, M. W. 1949. Sponges of the Western Bahamas. *Am. Mus. Novit.* 1431:1-25.
- De Rudder, J., and M. P. De Garihe. 1966. Inhibitory effect of some nucleosides on the growth of various human viruses in tissue culture, p. 578-583. *Antimicrob. Agents Chemother.* 1965.
- De Rudder, J., and M. P. De Garihe. 1967. Etude comparative de l'action de certains nucléosides sur la croissance virale p. 29-35. *In* K. H. Spitzky (ed.), Fifth International Congress on Chemotherapy, vol. 2, Verlag Wiener Medizinisch. Akad. Vienna.
- Dobersen, M. J., and S. Greer. 1975. An assay for pyrimidine deoxyribonucleoside kinase using γ -³²P labelled ATP. *Anal. Biochem.* 67:602-610.
- Dobersen, M. J., M. Jerkofsky, and S. Greer. 1976. Enzymatic basis for the selective inhibition of varicella-zoster virus by 5-halogenated analogues of deoxycytidine. *J. Virol.* 20:478-486.
- Doll, E. R., M. G. Richards, and M. E. Wallace. 1953. Adaptation of the equine abortion virus to suckling Syrian hamsters. *Cornell Vet.* 43:551-558.
- Duff, R., and F. Rapp. 1973. Induction of oncogenic potential by herpes simplex. *Perspect. Virol.* 8:189-210.
- Gentry, G. A., and J. F. Aswell. 1975. Inhibition of herpes simplex virus replication by araT. *Virology* 65:294-296.
- Greer, S., and I. Schildkraut. 1975. 5-Halogenated analogs of deoxycytidine as selective inhibitors of the replication of herpes simplex virus in cell culture and

- related studies of intracranial herpes simplex virus infection of mice. *Ann. N.Y. Acad. Sci.* 255:359-365.
25. Halliburton, I. W., E. A. Hill, and G. J. Russell. 1975. Identification of strains of herpes simplex virus by comparison of the density of their DNA using the preparative ultracentrifuge. *Arch. Virol.* 48:157-168.
 26. Hay, J., P. A. J. Perera, J. M. Morrison, G. A. Gentry and J. H. Subak-Sharpe. 1971. Herpes virus-specified proteins, p. 335-376. In G. E. W. Wolstonholme and M. O'Connor (ed.), *CIBA Foundation Symposium on strategy of the viral genome*. Longman, Inc., New York.
 27. Hill, J. M., P. A. Morse, and G. A. Gentry. 1975. Metabolism of deoxycytidine, thymine, and deoxythymidine in the hamster. *Cancer Res.* 35:1314-1319.
 28. Hotchkiss, R. D. 1948. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J. Biol. Chem.* 175:315-332.
 29. Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe. 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. *J. Gen. Virol.* 24:465-480.
 30. Jamieson, A. T., and J. H. Subak-Sharpe. 1974. Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. *J. Gen. Virol.* 24:481-492.
 31. Jerkofsky, M., Dobersen, M. J., and S. Greer. 1976. Selective inhibition of the replication of varicella-zoster virus by 5-halogenated analogs of deoxycytidine. *Ann. N.Y. Acad. Sci.* 284:389-395.
 32. Kit, S., R. A. deTorres, and D. R. Dubbs. 1966. Arabinofuranosyl-cytosine-induced stimulation of thymidine kinase and deoxycytidylic deaminase activities of mammalian cultures. *Cancer Res.* 26:1859-1866.
 33. Kit, S., D. R. Dubbs, L. T. Piekarski, and T. C. Hsu. 1963. Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. *Exp. Cell Res.* 31:297-312.
 34. Kriss, J. P., and S. B. Bond. 1964. The effect of natural and unnatural pyrimidines and pyrimidine nucleosides on the phosphorolysis of 5-iododeoxyuridine by mouse liver extract. *Biochem. Pharmacol.* 13:365-370.
 35. Kuchler, R. J., and D. J. Merchant. 1956. Propagation of strain L (Earle) cells in agitated fluid suspension cultures. *Proc. Soc. Exp. Biol. Med.* 92:803-806.
 36. Leung, W.-C., D. R. Dubbs, D. Trkula, and S. Kit. 1975. Mitochondrial and herpesvirus-specific deoxypyrimidine kinases. *J. Virol.* 16:486-497.
 37. Lieberman, M., A. Pascale, T. W. Schafer, and P. E. Came. 1972. Effect of antiviral agents in equine abortion virus-infected hamsters. *Antimicrob. Agents Chemother.* 1:143-147.
 38. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
 39. Macpherson, I., and M. Stoker. 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology* 16:147-151.
 40. Miller, R., J. P. Iltis, and F. Rapp. 1977. Differential effect of arabinofuranosylthymine on the replication of human herpesviruses. *J. Virol.* 23:679-684.
 41. Moore, A. E., L. Sabachewsky, and H. W. Toolan. 1955. Culture characteristics of four permanent lines of human cancer lines. *Cancer Res.* 15:598-602.
 42. Munyon, W., R. Buchsbaum, E. Paoletti, J. Mann, E. Kraiselburd, and D. Davis. 1972. Electrophoresis of thymidine kinase activity synthesized by cells transformed by herpes simplex virus. *Virology* 49:683-689.
 43. Munyon, W., E. Kraiselburd, D. Davis, and J. Mann. 1971. Transfer of thymidine kinase to thymidine kinaseless L cells by infection with ultraviolet-irradiated herpes simplex virus. *J. Virol.* 7:813-820.
 44. O'Callaghan, D. J., W. P. Cheevers, G. A. Gentry, and C. C. Randall. 1968. Kinetics of cellular and viral DNA synthesis in equine abortion (herpes) virus infection in L-M cells. *Virology* 36:104-114.
 45. O'Callaghan, D. J., J. M. Hyde, G. A. Gentry, and C. C. Randall. 1968. Kinetics of viral deoxyribonucleic acid, protein, and infectious particle production and alterations in host macromolecular synthesis in equine abortion (herpes) virus-infected cells. *J. Virol.* 2:793-804.
 46. Ogino, T., T. Otsuka, and M. Takahashi. 1977. Induction of deoxypyrimidine kinase activity in human embryonic lung cells infected with varicella-zoster virus. *J. Virol.* 21:1232-1235.
 47. Perdue, M. L., M. C. Kemp, C. C. Randall, and D. J. O'Callaghan. 1974. Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1. Proteins of the nucleocapsid and intact virion. *Virology* 54:201-216.
 48. Prusoff, W. H., D. C. Ward, T. S. Lin, M. S. Chen, G. T. Shaiu, C. Chai, E. Lentz, R. Capizzi, J. Indriss, N. H. Ruddie, F. L. Black, H. L. Kumari, D. Albert, P. N. Bhatt, G. D. Hsiung, S. Strickland, and Y. C. Cheng. 1977. Recent studies on the antiviral activity and biochemical properties of 5-halo-5'-amino deoxyribonucleosides. *Ann. N.Y. Acad. Sci.* 284:335-341.
 49. Randall, C. C., and E. C. Bracken. 1957. Studies on hepatitis in hamsters infected with equine abortion virus. I. Sequential development of inclusions and the growth cycle. *Am. J. Pathol.* 33:709-727.
 50. Randall, C. C., and L. A. Lawson. 1962. Adaptation of equine abortion virus to Earle's L-cells in serum-free medium with plaque formation. *Proc. Soc. Exp. Biol. Med.* 110:487-489.
 51. Rawls, W. E., G. Cashon, E. Adam, T. Ogino, R. Duff, and F. Rapp. 1974. Herpesvirus type 2-induced thymidine kinase and carcinoma of the cervix. *Cancer Res.* 34:362-366.
 52. Reed, L., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493-497.
 53. Renis, H. E., and D. A. Buthala. 1965. Development of resistance to antiviral drugs. *Ann. N.Y. Acad. Sci.* 130:345-354.
 54. Renis, H. E., G. E. Underwood, and J. H. Hunter. 1968. Antiviral properties of nucleosides structurally related to 1- β -D-arabinosylcytosine, p. 675-679. *Antimicrob. Agents Chemother.* 1976.
 55. Russell, W. C. 1962. A sensitive and precise plaque assay for herpes virus. *Nature (London)* 198:1029-1029.
 56. Underwood, G. E., C. A. Wisner, and S. D. Weed. 1964. Cytosine arabinoside (CA) and other nucleosides in herpes virus infections. *Arch. Ophthalmol.* 72:505-512.