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# *IN VITRO* METABOLISM OF 1-β-D-ARABINOFURANOSYLCYTOSINE AND 1-β-2′-FLUORO-ARABINO-5-IODOCYTOSINE IN NORMAL AND HERPES SIMPLEX TYPE 1 VIRUS-INFECTED CELLS

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**Abstract**—Phosphorylation of 1-β-D-2′-F-arabino-5-iodocytosine (FIAC), a newly synthesized pyrimidine nucleoside with potent antiherpesvirus activity, was compared with that of its parent compound, 1-β-D-arabinofuranosylcytosine (ara-C). While ara-C was phosphorylated extensively by homogenates of normal, rapidly proliferating mouse tissues, FIAC was a poor substrate for the nucleoside kinase occurring in such normal tissues. With cell homogenates of noninfected Vero cells, thymidine (TdR) was phosphorylated about fifty and twenty times more efficiently than FIAC and ara-C, while infection of Vero cells with Herpes Simplex Virus Type 1 (HSV-1) resulted in a 23-fold increase of TdR- and a 1270-fold increase of FIAC phosphorylation. In contrast, phosphorylation of ara-C was increased only by a factor of 2.6. While the reaction products obtained with homogenates of normal mouse tissues were 5′-mono-, di- and triphosphates of ara-C and FIAC, the reaction products with noninfected and infected Vero cell homogenates were predominantly monophosphates. In contrast, TdR was efficiently phosphorylated to its 5′-mono-, di- and triphosphates by such homogenates. In intact HSV-1-infected Vero cells, FIAC was rapidly taken up and phosphorylated to FIACMP and to an as yet unidentified metabolite. In contrast, TdR was taken up and phosphorylated to 5′-mono-, di- and triphosphates and ara-C was taken up moderately but metabolized poorly to its 5′-mono-, di- and triphosphates. Thus, in normal tissues, FIAC was a poorer substrate than ara-C for nucleoside kinases, but in intact HSV-1-infected Vero cells FIAC was efficiently phosphorylated and thus behaved like a TdR analog, except that it was phosphorylated only to the 5′-monophosphate and a hitherto unidentified metabolite. The greatly increased phosphorylation of FIAC by HSV-1-infected Vero cells probably accounts, at least in part, for its great selectivity of action.

Replication of members of the herpesvirus group of viruses is accompanied by the appearance of at least one, and usually two or more, enzymes coded for by the viral genome [1]. Infection of permissive cells with Herpes Simplex Virus Types 1 and 2 (HSV-1 and HSV-2)† or Herpes Zoster Virus (HZV) results in the induction of a virus-specified thymidine kinase (TK) enzyme activity [2–4], while infection with these or other herpesviruses leads to the expression of a virus-specified DNA polymerase [5–8]. The substrate specificities of the viral enzymes are different than those of the cellular enzymes with similar catalytic responsibilities; thus, the viral enzymes have been proposed as appropriate targets for the development of selective antiherpesvirus chemotherapy [9,10]. In fact, recent studies have shown that the selective antiherpesvirus action of acycloguanosine, a potent inhibitor of several herpesviruses, depends on both of these enzymes [11]. Studies with 1-β-D-2′-F-arabino-5-iodocytosine (FIAC) have demonstrated that it is a potent antiherpesvirus drug, the mechanism of action of which is dependent, at least in part, on the virus-specified TK, because FIAC lacks selective activity against a mutant strain of HSV-1, which lacks the virus-specific TK while it retains the virus-specified DNA polymerase [12]. FIAC, which is active against HSV-1, HSV-2 and HZV and has little toxicity for uninfected cells [12,13], is a structural analog of 1-β-D-arabinofuranosylcytosine (ara-C), a compound that has marked cytotoxic [14–17], and moderate antiviral [18–20], activity. The cytotoxicity and, presumably, the antiviral effects of ara-C appear to be mediated at the DNA polymerase level by competition with the incorporation of dCTP into DNA [21–23].

In this report we present data that indicate clearly that FIAC, like thymidine, is preferentially phosphorylated by viral enzyme and not by normal cellular pyrimidine nucleoside kinases. Furthermore, our studies showed that FIAC was phosphorylated predominantly to the mono- and not di- and tri-

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* Abbreviations: HSV-1 and HSV-2: Herpes Simplex Virus Types 1 and 2; ara-C: 1-β-D-arabinofuranosylcytosine; ara-U: 1-β-D-arabinofuranosyluracil; araCMP, araCDP, araCTP, araUMP, araUDP and araUTP: 5′-mono-, di- and triphosphates of ara-C and araU; FIAC: 1-β-D-2′-F-arabino-5-iodocytosine; FIAU: 1-β-D-2′-F-arabino-5-iodouracil; FIACMP, FIACDP, FIACTP, FIAUMP, FIAUDP and FIAUTP: 5′-mono-, di- and triphosphates of FIAC and FIAU; TdR: thymidine; TMP, TDP and TTP: 5′-mono, di- and triphosphates of TdR; HZV: Herpes Zoster Virus; TK: thymidine kinase; HPLC: high pressure liquid chromatography; THU: tetrahydrouridine; DMEM: Dulbecco's modified Eagle medium; FBS: fetal bovine serum; TCA: trichloroacetic acid; PPO: 2,5-diphenyloxazole; and POPOP: 1,4-bis-[2-(4-5-phenyloxazolyl)]benzene.

phosphates, as was found with thymidine. By comparison, ara-C was phosphorylated by both cellular and viral enzymes and was converted to the triphosphate in significant quantities. A preliminary report was presented elsewhere [24].

## MATERIALS AND METHODS

Tritiated ara-C (sp. act. 11 Ci/mole), labeled predominantly in the 5-position, was provided through the courtesy of Dr. R. R. Engle of the National Cancer Institute. Unlabeled and [2-$^{14}$C]FIAC (sp. act. 31.89 $\mu$Ci/mg) and unlabeled 1-$\beta$-D-2'-F-arabino-5-iodocytosine-5'-monophosphate (FIACMP) were prepared, their structures verified, and supplied by Drs. J. J. Fox and K. Watanabe of this Institute. [2-$^{14}$C]Deoxycytidine (sp. act. 50 mCi/mmole) was purchased from Moravek Biochemicals, City of Industry, CA. [2-$^{14}$C] Thymidine (sp. act. 53 mCi/mmole) was purchased from Schwarz Mann, Orangeburg, NY. The phosphorylated ara-C and ara-U standards were purchased from Terra-Marine Bioresearch, La Jolla, CA, and the Sigma Chemical Co., St. Louis, MO. The radioactive purity was above 98% in all cases. Radiolabeled FIACMP was prepared enzymatically with HSV-1-infected Vero cell cytosol using [2-$^{14}$C]FIAC and separating the reaction products by high pressure liquid chromatography (HPLC). Protein assays were carried out according to the method of Lowry *et al.* [25]. For the separation of nucleosides and nucleotides, a Partisil 10 SAX column (4.6 × 250 mm) (Whatman, Clifton, NJ) was used in conjunction with a Micrometrics model 7000B HPLC apparatus (Norcross, GA). A 20-min linear gradient, 0.005 M to 0.4 M $KH_2PO_4$ (pH 3.0), elutes all the nucleosides and nucleotides of dCR, ara-C, FIAC, thymidine, adenosine and guanosine. The flow rate was 1.5 ml/min and the pressure was about 1200 psi. Absorbance was monitored at 254 nm. The conditions chosen for elution were similar to the ones reported elsewhere [26]. In some instances, in which araCMP did not separate well from ara-C and ara-U, isocratic conditions, using 0.005 M $KH_2PO_4$ (pH 4.0), easily separated nucleosides and monophosphate. The unlabeled 5'-monophosphate of FIAC (FIACMP), contaminated with traces of material considered to be FIAC 5'-di- and triphosphates (FIACDP and FIACTP), was used as a standard for evaluating elution times. The deaminated product, FIAU-5'-monophosphate (FIAUMP), was synthesized from FIACMP by deamination with $HNO_2$. For analytical purposes, a substance considered to be labeled FIAC-5'-triphosphate (FIACTP) was prepared in trace amounts by enzymatic phosphorylation of [2-$^{14}$C]FIAC with a homogenate of mouse thymus. The elution times from the HPLC column of FIAC and FIAU phosphates observed in all experiments were comparable (see Table 1). Radioactive FIAU was prepared from [2'-$^{14}$C]FIAC by enzymatic deamination with mouse kidney homogenate. The reaction products were separated by HPLC, using a Partisil 5 ODS column (4.6 × 250 mm) (Whatman) with a mobile phase of MeOH/0.01 M $NH_4H_2PO_4$ (pH 5.1) (24:76) and a flow rate of 1 ml/min. The pure [$^{14}$C]FIAU was incubated with a homogenate of mouse thymus, producing a substance considered to be predominantly [$^{14}$C]FIAUTP (although its elution time of 28.75 min was slightly different from the one reported in Table 1).

Kinase assays were done using the disc assay method of Bresnick and Karjala [27], as reported earlier [28,29]; however, higher specific activities of the substrates (80,000–160,000 cpm/assay) were used. All samples were incubated for 30 or 45 min either with or without the cytidine deaminase inhibitor, tetrahydrouridine (THU) (2 mg/assay), for evaluation of the effect of enzymatic deamination upon the activities of the kinases. Substrate conversion for normal tissues and control Vero-cytosol preparations never exceeded 16.4% for ara-C or 1.5% for FIAC. With infected Vero cell cytosol, FIAC conversion amounted to 79%; linearity of the reaction was established for this preparation over 45 min for both FIAC and TdR.

One to two grams of normal tissue or 0.15 ml of bone marrow of $BDF_1$ mice was homogenized with cold 0.25 M sucrose on ice with ten strokes in a Stir-R model S63 homogenizer (Tri-R Instruments, Jamaica, NY). This 20% homogenate was centrifuged at 100,000 *g* in a Beckman (Palo Alto, CA) L265B centrifuge for 60 min and the supernatant fraction was used immediately for kinase assays. Vero cell monolayers were grown to near confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (GIBCO) and 10% fetal bovine serum (FBS). These cells were inoculated with Herpes Simplex Virus Type 1 (HSV-1, strain 2931 [12]) at a multiplicity of infection of about 1 plaque-forming unit of virus per cell. After a 2-hr adsorption period, the virus inoculum was washed off and maintenance medium (DMEM) with 2% FBS was used to overlay the cells. Cytosols of HSV-1-infected Vero cells or uninfected Vero cells were prepared by the methods of Kit *et al.* [3] after 12 hr of incubation at 37° in a humidified $CO_2$ incubator. Uninfected and HSV-1-infected cells also were prepared for the whole cell assay of drug phosphorylation. Infected and uninfected monolayers were incubated for either 2.5 or 5 hr with the various radiolabeled substrates prior to the preparation of extracts.

For the preparation of extracts to be separated by HPLC, the reaction products were precipitated with trichloroacetic acid (TCA) (final conc 3%) and the TCA was extracted with Freon–Amine (Aldrich Chemical Co., Milwaukee, WI), according to the procedure of Khym [30]; the ratio of TCA extract to Freon–Amine was 1:1. The extracts were analyzed by HPLC, either directly or after lyophilization and reconstitution with water for low count samples. Such extracts (50–100 $\mu$l) were injected and sixty to seventy individual fractions were collected directly into scintillation vials over a 30- or 35-min period and a 10-ml aliquot of a mixture of 1000 ml Triton X-100 (Packard, Downers Grove, IL), 16.5 g PPO, 0.3 g POPOP and 2000 ml toluene was added to each vial. The radioactivity was evaluated in a Packard model 3380 liquid scintillation spectrometer. Radioactivity recovered after passage through the HPLC

apparatus amounted to 96.8 ± 4.9% for ara-C and 102.1 ± 3.9% for FIAC reaction products.

In the case of the experiments with [$^3$H]ara-C, where exchange of tritium is possible, no radioactive peaks other than the ones reported in Tables 3 and 5 were observed. Furthermore, in these experiments no substantial loss of radioactivity upon lyophilization of the reaction products was evident. Since the radioactivity was low in some samples, in all instances a 1:1 mixture of the reaction products derived from samples incubated with and without THU was analyzed by HPLC. Because the deaminated di- and triphosphates of both ara-C and FIAC were obtained in only minor or trace amounts or not at all (Table 3), this procedure could influence the ratios of deaminated to nondeaminated products mainly at the level of the monophosphates. Furthermore, deamination of ara-C and probably also of FIAC can take place not only at the nucleoside, but also at the nucleotide level [28], which cannot be prevented by THU.

Table 2. Apparent phosphorylation rates of thymidine (TdR), deoxycytidine (dCR), ara-C and FIAC by homogenates of normal mouse tissues, and uninfected and HSV-1-infected Vero cells* (disc assay:total phosphates)

| Tissue | TdR | dCR | Ara-C | FIAC |
|---|---|---|---|---|
| | [nmoles·(mg protein)$^{-1}$·hr$^{-1}$] | | | |
| Normal mouse | | | | |
| Liver | 0.10 | 0.18 | 0.09 | 0.12 |
| Spleen | 3.36 | 1.04 | 0.68 | 0.09 |
| Adrenals | 1.51 | 0.46 | 0.12 | 0.40 |
| Thymus | 11.06 | 3.71 | 3.12 | 0.09 |
| Bone marrow | 23.65 | 5.50 | 2.29 | 0.25 |
| Vero cells-Cytosol | | | | |
| Control | 3.68 | 0.77 | 0.30 | 0.07 |
| HSV-1-infected | 84.81 | 5.36 | 0.78 | 88.86 |

* All samples contained no THU in assay. Tissues of fifteen to thirty mice were pooled. Results are mean values of four discs.

## RESULTS

The elution times on HPLC of the five 5′-monophosphates of ara-C, ara-U, TdR, FIAC and FIAU (Table 1) revealed substantial differences in polarity. FIACMP was considerably more polar than araCMP, and the introduction of a hydroxy group instead of an amino group in the 4-position of the pyrimidine ring rendered these two compounds (FIAUMP and araUMP) substantially more polar. Deoxythymidine 5′-monophosphate eluted similarly to araUMP, but FIAUMP was much more polar than the nonhalogenated araUMP. Due to the small amounts of di- and triphosphates of FIAC and FIAU produced, the identities of FIACDP, FIAUDP, FIACTP and FIAUTP must be regarded as only tentative. In the 5′-diphosphate series, the phosphate groups obviously contributed more to the polarity than did the 4-substituent in the pyrimidine group, although the halogens of FIAUDP still had some influence upon the elution time. In the 5′-triphosphate series a difference was still noticeable among araCTP vs FIACTP, but in the UTP series all three elution times for araUTP, FIAUTP and TTP were comparable.

As demonstrated in Table 2, FIAC was phosphorylated less efficiently than ara-C, dCR, or TdR with homogenates of most normal mouse tissues. This was especially true with rapidly poliferating and metabolically active tissues. Nonproliferating tissues, such as liver, demonstrated little phosphorylation of any of the substrates tested (Table 2). Phosphorylation of FIAC (Table 3) by homogenates of normal tissues resulted predominantly in monophosphates (both FIACMP and FIAUMP), but a low, albeit significant, conversion rate to the tentatively identified FIACTP was observed only with thymus homogenate (2.3%). Conversion of FIAC to the triphosphate level was also observed with bone marrow homogenate (0.7%), although this was very low as compared to ara-C (4.3%).

The analysis of phosphorylation products by HPLC (Table 3) revealed a predominance of araCTP when ara-C was incubated with homogenates of normal tissues. The rapidly proliferating tissues, such as bone marrow and thymus, had higher percentages of araCTP production (ranging from 4.3% of total cpm in bone marrow to 24.1% in thymus) than the nonproliferating tissues (range 0.5 to 1.6%).

Cytosol preparations of uninfected Vero cells showed moderate phosphorylation of TdR [sp. act. 3.68 nmoles·(mg protein)$^{-1}$·hr$^{-1}$] and even less of dCR, ara-C and FIAC [sp. act. 0.77, 0.30 and 0.07 nmole·(mg protein)$^{-1}$·hr$^{-1}$ respectively]. Homogenates of HSV-1-infected Vero cells exhibited a marked increase in phosphorylation of TdR and FIAC, but much less impressive increases in

Table 1. Elution times of 5′-mono-, di- and triphosphates of ara-C, FIAC and TdR from Partisil 10 SAX under standard conditions of HPLC*

| 5′-Nucleotide | Elution times in min ± S.D. | | | | |
|---|---|---|---|---|---|
| | Ara-C | FIAC† | Ara-U | FIAU† | TdR |
| Nucleoside | | | | | |
| 5′-Monophosphate | 2.98 ± 0.18‡ | 7.65 ± 0.61 | 6.66 ± 0.25 | 9.15 ± 0.39 | 6.75 ± 0.19 |
| 5′-Diphosphate | 12.34 ± 0.32 | 12.45 ± 0.30 | 14.77 ± 0.14 | 15.25 ± 0.56 | 14.78 ± 0.36 |
| 5′-Triphosphate | 23.16 ± 0.20 | 24.40 ± 0.33 | 27.52 ± 0.34 | 27.45 ± 1.17 | 27.85 ± 0.46 |

* For technique, see Materials and Methods.

† Due to the small amounts of labeled FIAC available, the values for and identity of 5′-di- and triphosphates are tentative only.

‡ Mean ± S.D. of eight to twenty-seven HPLC runs.

Table 3. Phosphorylation of ara-C and FIAC by homogenates of normal mouse tissues and uninfected and HSV-1-infected Vero cells*

| Tissue | Total ara-C phosphates (nmoles mg/hr) | Ara-C phosphates in % of total cpm eluted from HPLC | | | | | | Total FIAC phosphates (nmoles mg/hr) | FIAC phosphates in % of total cpm eluted from HPLC | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ara-CMP | Ara-UMP | Ara-CDP | Ara-UDP | Ara-CTP | Ara-UTP | | FIA-CMP | FIA-UMP | FIA-CDP | FIA-UDP | FIA-CTP | FIA-UTP |
| Normal mouse | | | | | | | | | | | | | | |
| Liver | 0.09 | 0.4 | 0.3 | 0.2 | T† | 1.6 | T | 0.12 | 0.7 | 1.0 | ND‡ | ND | 0.1 | ND |
| Spleen | 0.68 | 0.4 | T | 0.9 | ND | 8.0 | ND | 0.09 | 0.5 | 0.9 | ND | ND | 0.3 | T |
| Adrenals | 0.12 | 0.5 | 0.3 | 0.1 | T | 0.5 | T | 0.04 | 1.2 | 0.9 | ND | ND | 0.1 | ND |
| Thymus | 3.12 | 0.5 | 0.3 | 2.3 | ND | 24.1 | T | 0.09 | 0.3 | 0.3 | 0.2 | ND | 2.3 | 0.1 |
| Bone marrow | 2.29 | 1.5 | 0.3 | 1.3 | ND | 4.3 | ND | 0.25 | 0.3 | 0.2 | 0.3 | ND | 0.7 | 0.1 |
| Vero cells-Cytosol | | | | | | | | | | | | | | |
| Control | 0.30 | 0.4 | 0.3 | T | T | T | ND | 0.07 | 0.3 | ND | ND | ND | T | T |
| HSV-1-infected | 0.78 | 0.9 | 0.4 | T | T | T | T | 88.86 | 88.4 | ND | ND | ND | ND | ND |

* For conditions of experiment, see Table 2. Results are mean values of four discs.
† Trace amount (<0.1% of total cpm).
‡ None detectable.

phosphorylation of dCR and especially ara-C [sp. act. 84.81, 88.86, 5.36 and 0.78 nmoles·(mg protein)$^{-1}$·hr$^{-1}$ respectively]. In the cytosol derived from control Vero cells, a doubling of the specific activity of ara-C and FIAC phosphorylation was observed in samples incubated with the cytidine deaminase inhibitor, tetrahydrouridine (THU).

With both uninfected and HSV-1-infected Vero cell homogenates, monophosphates represented the bulk of the phosphorylation products with ara-C and FIAC substrates (Table 3). Although the phosphorylation of FIAC by homogenates of uninfected Vero cells was one-fourth that observed with ara-C, phosphorylation of FIAC by homogenates of *infected* Vero cells resulted in much greater conversion of FIAC (88.86%) than of ara-C (0.78%). Di- and triphosphates of FIAC were observed in only trace amounts with control cytosol and, unlike the situation with ara-C, triphosphates of FIAC or FIAU were not observed with the homogenates of infected Vero cells.

When labeled TdR, ara-C or FIAC was added to intact uninfected or HSV-1-infected Vero cells and the cells were harvested and extracted with TCA, analysis by HPLC of the acid-soluble material revealed an impressive increase of total acid-soluble material with TdR and FIAC, but not with ara-C, in infected vs uninfected cells (Tables 4–6). The increase of TTP due to HSV-1 infection amounted to a 16.8-fold increase for the group incubated for 2.5 hr and a 8.3-fold increase for the cells incubated for 5 hr (Table 4). Substantial increases due to infection were also observed for TMP and TDP (Table 4). Two minor, unidentified peaks were also seen. Only a small increase in total acid-soluble material was observed in cells incubated with ara-C (Table 5). The amounts of araCTP found were small and showed little change during the 2.5- to 5-hr periods of incubation.

The most striking changes found were in the cells incubated with FIAC. Total acid-soluble material increased 18.9- and 28.6-fold in infected vs uninfected groups in the 2.5-hr and 5-hr incubated cells respectively (Table 6). Analysis for 5′-nucleotides by HPLC revealed the formation of the 5′-monophosphate if FIAC (FIACMP), with no radioactive material located in areas where the tentatively identified FIAUMP or 5′-di- or 5′-triphosphates of FIAC and FIAU were eluted. Besides FIACMP, a hitherto undetected FIAC-derived peak was observed (elution time, 17 min). This material did not co-elute with any of the commercially available pyrimidine 5′-mono-, di- or triphosphates. Although this unknown compound was detected in small amounts in the uninfected cells, it was found in a much larger concentration in the infected cells, reaching levels comparable to those of FIACMP after 2.5 hr of incubation (Table 6). After 5 hr of incubation, the levels of the unknown material were twice those of FIACMP (Table 6).

Since TdR, ara-C and FIAC were added to the cell samples at the same concentrations (0.0125 mM), it is of special interest that the total acid-soluble material in the uninfected cells was highest for the cells incubated with ara-C (0.84 and 1.02 nmoles for the 2.5- and 5-hr incubated cells),

Table 4. TdR metabolism in uninfected and HSV-1-infected Vero cells in tissue culture*

| Cell culture | Time of exposure to TdR (hr) | Total acid-soluble material | TdR | TMP | TDP | TTP | Unknown 1 | Unknown 2 |
|---|---|---|---|---|---|---|---|---|
| Vero uninfected | 2.5 | 0.65 | 0.23 | ND† | 0.09 | 0.04 | 0.06 | 0.05 |
| Vero uninfected | 5.0 | 0.56 | 0.33 | 0.01 | 0.08 | 0.08 | 0.01 | 0.02 |
| Vero HSV-1-infected | 2.5 | 2.52 | 0.99 | 0.23 | 0.55 | 0.67 | ND | ND |
| Vero HSV-1-infected | 5.0 | 3.66 | 1.98 | 0.41 | 0.51 | 0.66 | 0.02 | 0.02 |

* Representative experiment of two studies with comparable results. All values are in nmoles per $6.7 \times 10^6$ cells in assay.
† None detectable.

Table 5. Ara-C metabolism in uninfected and HSV-1-infected Vero cells in tissue culture*

| Cell culture | Time of exposure to ara-C (hr) | Total acid-soluble material | Ara-C and Ara-U | Ara-CMP | Ara-UMP | Ara-CDP | Ara-UDP | Ara-CTP | Ara-UTP |
|---|---|---|---|---|---|---|---|---|---|
| Vero uninfected | 2.5 | 0.84 | 0.65 | ND† | 0.03 | 0.06 | ND | 0.09 | ND |
| Vero uninfected | 5.0 | 1.02 | 0.80 | ND | 0.05 | 0.07 | ND | 0.09 | ND |
| Vero HSV-1-infected | 2.5 | 0.98 | 0.69 | 0.04 | 0.08 | 0.05 | ND | 0.10 | ND |
| Vero HSV-1-infected | 5.0 | 1.15 | 0.85 | 0.05 | 0.10 | 0.05 | ND | 0.10 | ND |

* Representative experiment of two studies with comparable results. All values are in nmoles per $6.7 \times 10^6$ cells in assay.
† None detectable.

Table 6. FIAC metabolism in uninfected and HSV-1-infected Vero cells in tissue culture*

| Cell culture | Time of exposure to FIAC (hr) | Total acid-soluble material | FIAC and FIAU | FIA-CMP† | Unknown |
|---|---|---|---|---|---|
| Vero uninfected | 2.5 | 0.25 | 0.20 | 0 | 0.01 |
| Vero uninfected | 5.0 | 0.24 | 0.19 | 0.01 | 0.01 |
| Vero HSV-1-infected | 2.5 | 4.72 | 2.29 | 1.19 | 1.11 |
| Vero HSV-1-infected | 5.0 | 6.87 | 3.39 | 1.12 | 2.32 |

* Representative experiment of two studies with comparable results. All values are in nmoles per $6.7 \times 10^6$ cells in assay.

† No other phosphates, such as radioactive peaks with elution times comparable to the tentatively identified FIAUMP, FIACDP, FIAUDP, FIACTP and FIAUTP, were observed.

followed, in order, by TdR (0.65 and 0.56 nmole) and FIAC (0.25 and 0.24 nmole). By contrast, in the acid-soluble pools of HSV-1-infected cells the highest radioactivity was observed in the experiments with FIAC (4.72 and 6.87 nmoles for $6.7 \times 10^6$ cells incubated 2.5 and 5 hr, respectively), followed by TdR 2.52 and 3.66 nmoles) and ara-C (0.98 and 1.15 nmoles).

## DISCUSSION

A comparison of the specific activities of kinases involved in the phosphorylation of TdR, dCR, ara-C and FIAC in homogenates of normal mouse tissue and uninfected and HSV-1-infected Vero cells demonstrates striking differences in the rates of phosphorylation of these related nucleosides. The specific activities for TdR phosphorylation were highest in rapidly proliferating tissues, such as thymus, spleen and bone marrow. Deoxycytidine and ara-C require the same enzymes for phosphorylation [29] and were found to be far better substrates than FIAC for phosphorylation by homogenates of spleen, adrenals, thymus and bone marrow. While these crude extracts do not allow any conclusions as to which nucleoside kinase is responsible for the initial phosphorylation of FIAC, it is obviously a poor substrate for all the nucleoside kinases found in normal tissue homogenates. Of special interest was the low phosphorylation rate of FIAC with bone marrow homogenate, which might be predictive of low toxicity of this compound for the bone marrow.

With cell homogenates of uninfected Vero cells, TdR was phosphorylated about fifty times more efficiently than was FIAC; however, infection with HSV-1 dramatically changed FIAC phosphorylation to the same high level found with TdR phosphorylation. This resulted in a 1270-fold increase of FIAC phosphorylation with uninfected vs infected cell homogenates. For TdR, this increase was only twenty-three times, due mainly to relatively active TdR kinase in uninfected cells. The comparable absolute values for TdR and FIAC phosphorylation with cytosol of HSV-1-infected cells indicated that both substrates were likely to be phosphorylated by the same virus-induced kinase. In contrast, phosphorylation of ara-C was increased by a factor of only 2.6 after infection of the Vero cells, and the total amounts of ara-C-derived phosphates were less than 1/100 the amount accruing with FIAC. The greatly increased phosphorylation of FIAC by HSV-1-infected vs uninfected Vero cell homogenate probably accounts, at least in part for the great selectivity of its action [12, 13]. Thus FIAC suppressed HSV-1 replication at concentrations 1/600 those needed to suppress replication of normal cells. By comparison, the concentration of ara-C that inhibited HSV-1 replication was very similar to that which inhibited normal cell replication [31, 32]. The results of these phosphorylation experiments thus corroborate data obtained with a thymidine kinase-negative ($TK^-$) strain of HSV-1: FIAC was found to inhibit the wild strain of HSV-1 at concentrations 1/8000 those required to inhibit the $TK^-$ mutant [12], whereas ara-C demonstrated little or no selective activity (C. Lopez, unpublished observation).

Deamination of ara-C and FIAC can take place at the nucleoside and probably at the nucleotide level [28]. The difference in deamination of ara-C (at the nucleoside or nucleotide level) by homogenates of HSV-1 vs uninfected Vero cells indicates an increase of such a deaminase caused by the virus infection. According to Kit [1], it is likely that the increased deamination is attributable to an increased alteration at the nucleoside level, rather than on the nucleotide. The observed formation of araUMP and lack of FIAUMP formation in the cytosols of uninfected and infected Vero cells might be due to the differences in the substrate specificities of these two compounds.

The lack of significant further phosphorylation of FIACMP to FIACTP or araCMP to araCTP was in marked contrast to the efficient phosphorylation of TdR to the 5′-mono-, di- and triphosphates by HSV-1-infected cells. Furthermore, in preliminary experiments TMP, but not FIACMP or araCMP, was easily phosphorylated further by the cytosol derived from Vero cells infected with HSV-1. Since [2-$^{14}$C]FIAC is incorporated into DNA and RNA of human leukemia cells and into DNA of HSV-1-infected Vero cells [33], the question arose whether FIACTP and araCTP could be rapidly hydrolyzed to the monophosphates. Preliminary experiments indicate, however, that TTP and araCTP were not hydrolyzed significantly by homogenates of HSV-1-infected Vero cells (W. Kreis, unpublished observation). Our finding of little, if any, detectable

FIACTP or FIAUTP is not consistent with the observation that these compounds become incorporated into DNA of HSV-1-infected Vero cells [33]. It is possible, however, that some FIAC was rapidly converted to the triphosphate and that this material was also rapidly incorporated into DNA. If this was the case, then di- and triphosphates may not have accumulated in sufficient quantities to be detected.

Experiments with intact noninfected and HSV-1-infected Vero cells corroborated, for the most part, our observations with the homogenates. HSV-1 infection of Vero cells resulted in striking increases in the uptake of FIAC-derived radioactivity when compared to uninfected cells. The metabolites were FIACMP and an as yet unidentified metabolite. In uninfected cells the unknown material occurred in much smaller amounts, but in infected cells it equalled (after 2.5 hr) or exceeded (after 5 hr) the concentration of FIACMP. No such unknown FIAC-derived material was observed in experiments *in vitro* with cytosol preparations of either uninfected or infected cells. Of special interest was the consistent lack, in experiments with both intact cells and cell homogenates of infected and noninfected Vero cells, of 5′-di- and triphosphates of FIAC and FIAU. As with FIAC, incubation of TdR with HSV-1-infected vs uninfected cells resulted in substantial increases of uptake and phosphorylation. Unlike the experiment with FIAC, however, the major products of phosphorylation were 5′-triphosphates and di- and monophosphates. Similarly, arabinofuranosylthymine (ara-T) has been shown to be phosphorylated predominantly to the 5′-triphosphates in HSV-1-infected baby hamster kidney cells [34]. Obviously, small changes in the structures of these compounds resulted in marked differences in their metabolism; thus, although FIAC was, like TdR and ara-T, preferentially phosphorylated by viral TK, there was a marked difference in how FIAC was subsequently metabolized.

It appears likely that FIAC was phosphorylated in normal tissues by a dCR kinase but that it was a substrate substantially inferior to ara-C with enzymes from normal tissues. In HSV-1-infected Vero cells, however, FIAC behaved like an analog of TdR, although it was phosphorylated only to the 5′-monophosphate and, in addition, was converted to a hitherto unidentified metabolite. Further studies of this unknown metabolite are required in order to determine its structure, its probable metabolic pathway, and its possible role in the antiviral effect of FIAC.

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