Specific Herpes Simplex Virus-induced Incorporation of 5-Iodo-5'-amino-2',5'-dideoxyuridine into Deoxyribonucleic Acid*

(Received for publication, January 26, 1976)

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5-Iodo-5'-amino-2',5'-dideoxyuridine (AIdUrd) is a novel thymidine analog which inhibits herpes simplex virus, type 1 (HS-1 virus) replication in the absence of detectable host toxicity. When murine, simian, or human cells in culture are treated with [3H]AIdUrd for up to 24 hours essentially none of the nucleoside becomes cell-associated. In contrast, upon HS-1 virus infection significant radiolabel is detected in both nucleotide pools and in DNA. The major acid-soluble metabolite has been shown by enzymic and chromatographic analysis to be the 5'-triphosphate of AIdUrd DNA from HS-1 virus-infected Vero cells labeled with [3H]thymidine, 5-[125I]iodo-2'-deoxyuridine (IdUrd), or [125I]AIdUrd was isolated by buoyant density centrifugation and subjected to digestion by pancreatic DNase I, spleen DNase II, micrococcal nuclease, spleen, and venom phosphodiesterases. Analysis of the digestion products clearly indicates that AIdUrd is incorporated internally into the DNA structure. DNA containing AIdUrd therefore contains phosphoramidate (P–N) bonds, known to be extremely acid-labile. The selective HS-1 virus-induced phosphorylation of AIdUrd and its subsequent incorporation into DNA may account for the unique biological activity of the AIdUrd nucleoside.

The search for highly selective antiviral agents has been extensively pursued over the past two decades. While several 5-halo-pyrimidine deoxyribonucleoside analogs, e.g. 5-iodo-, 5-bromo-, and 5-trifluoromethyl-2'-deoxyuridine, have shown significant antiviral activity, they also exhibit cytotoxic or mutagenic effects on host cells (1, 2). We recently described the synthesis and biological properties of a number of pyrimidine nucleosides containing 5'-sugar substituents (3). The 5'-amino analog of 5-iodo-2'-deoxyuridine was a potent and selective inhibitor of herpes simplex virus, type 1, replication in vitro (4). AIdUrd was, however, neither cytotoxic to mammalian cells in culture (4), mutagenic to murine L5178Y cells, nor induced gross or histological toxicity in newborn and 8-day-old mice. In addition, AIdUrd provided effective therapy for experimental herpetic keratitis in rabbits (5).

The present report describes our initial experiments to elucidate the mechanism by which AIdUrd exerts its selective antiviral activity.

EXPERIMENTAL PROCEDURES

Materials—5-[125I]iodo-5'-amino-2',5'-dideoxyuridine was prepared by the procedure of Lin et al. (3). 5-iodo-[2-14C]uracil and arabinosial and pronase (free of nucleases) were purchased from Calbiochem. Na+[125I], 5-[14C]thymidine, and 5-[125I]iodo-2'-deoxyuridine, and 5'[14C]thymidine were from New England Nuclear. Beef pancreas deoxyribonuclease I, bovine spleen phosphodiesterase, and Crotalus adamanteus venom phosphodiesterase were from Worthington Biochemical Co. Porcine spleen acid deoxyribonuclease II and micrococcal nucleases were from E. Merck, Darmstadt. Escherichia coli alkaline phosphatase was generously supplied by Dr. J. Coleman of Yale University. 5-iodo-5'-amino-2',5'-dideoxyuridine-5'-N'-triphosphate was prepared by a modification of the procedure of Letsinger et al. (6). All other common chemicals were reagent grade.

The A-9 strain of L-cells (7), Ehrlich ascites (8), Balb, 3T3, HeLa S, and Vero cells (9) were grown as monolayers in Falcon T-flasks or 90-mm culture dishes using Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (Grand Island Biological Co.).

Herpes simplex virus type I, strain CL-101 (10), vaccinia, strain WR (11), and SV40, strain 777 (12), were grown and plaque-assayed on Vero cells. Minute virus of mice, a parvovirus, was propagated and assayed on A-9 cells (13).

Methods—Virus infections for AIdUrd metabolism studies were done at a multiplicity of 10 plaque-forming units/cell. After a 1-hour adsorption at 37°, the viral inoculum was removed, the cells washed once with phosphate-buffered saline (0.9% NaCl solution) (14), and

* This work was supported by United States Public Health Service Grants CA-05262 and CA-16038 from the National Institute of Cancer and by the United States Energy Research and Development Administration Research Contract E(11-1)-2468.

† The abbreviations used are: AIdUrd, 5-iodo-5'-amino-2',5'-dideoxyuridine; IdUrd, 5-iodo-2'-deoxyuridine; AIdUMP, AIdUTP, the 5'-N'-monophosphate and 5'-triphosphate of AIdUrd, respectively; IdUTP, the 5'-triphosphate of IdUrd; 3'-AIdUMP, the 3'-monophosphate of AIdUrd; PBS, phosphate-buffered saline; HS-1 virus, herpes simplex virus, type 1.

‡ R. L. Capizzi, unpublished results.


medium containing either [14C]AldUrd, [125I]AldUrd, or [131I]thymidine (each at a concentration of 30 μM, specific activity of 50 μCi/μmol) was added. After incubation at 37° for 24 hours, the cells were harvested by scraping into PBS, washed twice in PBS, and frozen at -70° until used.

To determine the nature of AldUrd and IdUrd metabolites, labeled cells were suspended in 1 mM triethylammonium bicarbonate buffer, pH 8.1, containing 1 mM EDTA and disrupted by sonication (20-30 s) pulses) using a Bronwill Biosonic sonicator at a setting of 50. After centrifugation at 3,000 x g for 10 min, the supernatant fraction (0.5 ml), which contained ~92% of the radioactivity, was applied directly to a column (1 x 28 cm) of Sephadex G-25 and eluted with the pH 8.1 buffer described above. Fractions of 1.5 ml were collected and their radioactive content determined directly using an Intertechnique CG-30 automatic γ counter. The fractions containing acid-soluble material were pooled, lyophilized, and then analyzed for metabolic products.

DNA from control or HS-1 virus-infected Vero cells, grown in radiolabeled AldUrd, IdUrd, or thymidine (see above), was isolated as follows. Freshly harvested cells were digested for 3 hours at 37° in a solution which contained 0.015 M NaCl/1.5 mM sodium citrate buffer, pH 7.3 (NaC1/citrate), 20 mM EDTA, 0.1% Sarkosyl N-30 (Geigy Chemical Corp.), and 0.1% pronase. After digestion, 0.2 ml of sample was mixed with 4.8 ml of NaC1/citrate-buffered cesium chloride (Harshaw Chemical Co.) at a density of 1.746 g/ml. The solution was centrifuged at 30,000 rpm for 72 hours at 18° in a Beckman L2-65B ultracentrifuge with an SW 65 rotor. Five-drop fractions were collected from the bottom of each gradient, and part of each fraction was spotted onto a disc of Whatman No. 5 paper. The discs were washed twice in 5% trichloroacetic acid for 20 min, once in 50% ethanol for 10 min, and then air-dried. Samples containing carbon-14-labeled DNA were placed into vials with 3 ml of toluene-1,4-bis(2-5-phenyloxazoyl)-benzene (POPOP) and counted in a Packard liquid scintillation counter. Samples labeled with 125I were counted directly in the automatic y counter. The fractions containing acid-insoluble radioactivity were pooled, dialyzed against 1 mM Tris-HCl buffer, pH 8.1, and lyophilized prior to enzyme digestion studies.

The solvent systems used for thin layer chromatography were:
Solvent 1, 0.5 M LiCl; Solvent 2, 0.5 M LiCl/2 N acetic acid; Solvent 3, chloroform/propanol-2, 7/1. MN-polygram ccl 300 PEI/uv 254 plates were developed with Solvents 1 and 2, whereas Eastman silica gel paper chromatography (Solvent F) was propanol-1/NH4OH/H2O, 55/10/35. Paper electrophoresis were run at 2500 volts in 50 mM Tris-HCl, pH 9.6, for 1 hour using a Savant high voltage electrophoresis unit.

RESULTS

Uptake of AldUrd by Control and Virus-infected Cells—The cytotoxic, or mutagenic effects, or both, of most nucleoside analogs arise as a consequence of their phosphorylation and subsequent incorporation into cellular nucleic acids. It was, therefore, of considerable interest to observe that [125I]AldUrd became cell-associated only after HS-1 virus infection (Table I). Human (HeLa), simian (Vero), and murine (A-9, 3T3, and Ehrlich ascites) cell lines were developed with Solvents 1 and 2, whereas Eastman silica gel paper plates were developed with Solvent 3. The solvent system used for paper chromatography (Solvent F) was propanol-1/NH4OH/H2O, 55/10/35. Paper electrophoresis were run at 2000 volts in 50 mM Tris-HCl, pH 9.6, for 1 hour using a Savant high voltage electrophoresis unit.

To determine whether AldUrd and IdUrd were transported, or to metabolize AldUrd, or both. Since similar negative results were obtained upon infection of Vero or A-9 cells with vaccinia, SV40, or MVM viruses, it is apparent that AldUrd uptake is a HS-1 virus-induced phenomenon. In order to determine whether AldUrd was being metabolized intact or was rapidly degraded to iodide, iodouracil, or AldUrd upon HS-1 virus infection, Vero cells, mock infected or HS-1 virus infected, were incubated for 23 hours in the presence of [125I]AldUrd, [14C]IdUrd, or [14C]thymidine. The cells were lysed by sonication, and the cell lysate supernatant fractions chromatographed on columns of Sephadex G-25 (see "Methods"). The chromatographic profiles of the various supernatants are shown in Fig. 1. The void volume of the columns, Fractions 5 and 6, contained radioactive material which was acid-precipitable, whereas radioactivity found in the included volume, Fractions 7 to 16, was acid-soluble.

Analysis of Acid-soluble Fractions—As shown in Table II, AldUrd can be readily distinguished from IdUrd and iodouracil both chromatographically and electrophoretically, superior resolution being obtained by chromatography in Solvent System 3 (CHCl3/propanol-2, 7/1) or by electrophoresis at pH 9.6. The chemically synthesized 5'-triphosphate of AldUrd (AldUTP) also possesses properties which readily distinguish it from IdUTP. For example, AldUTP is extremely acid-labile, hydrolyzing almost instantaneously at pH 1.0 (25°) to form free AldUrd and inorganic phosphates. The acid lability of AldUTP, similar to that reported for the 5'-triphosphate of 5'-aminooxythymidine (6), is a diagnostic characteristic of phosphoramidate (P-N) bonds. 5'-dTPP and IdUTP are stable under the conditions described above.

The nature of the acid-soluble metabolites in [14C]IdUrd and [131I]AldUrd-labeled HS-1 virus-infected Vero cells was determined by comparison with nucleoside and nucleotide reference compounds in the five chromatographic and electrophoretic systems described in Table II. More than 98% of the radioactivity in Fractions 8 to 10 from [125I]IdUrd- and [131I]AldUrd-labeled cells co-migrated with authentic samples of IdUTP and AldUTP, respectively (Fig. 2). Treatment with bacterial alkaline phosphatase converts the radioactive metabolites to compounds which are indistinguishable from the appropriate nucleoside markers, IdUrd and AldUrd. The metabolically phosphorylated AldUrd derivative was also hydrolyzed to AldUrd and inorganic phosphate upon brief exposure to low pH. Although a small amount (<2%) of the 125I radiolabel from AldUrd-treated cells was occasionally found as iodide, no iodouracil or IdUrd could be detected. It is apparent, therefore, that AldUrd is metabolized intact and does not undergo detectable deamination or dephosphorylation. While Fractions 13 and 14 (Fig. 1, a and c) contained mainly unmodified nucleoside (IdUrd or AldUrd) with a small amount of the corresponding nucleoside triphosphate, no mono- and diphosphate derivatives were detected in any of the acid-soluble samples.
Similarly, when [14C]thymidine was incubated with uninfected Vero cells for 24 hours no significant amount of acid-soluble nucleotides was found.

**Analysis of Acid-insoluble Fractions**—Radioactivity associated with the macromolecular fractions (tubes 5 and 6, Fig. 1, a to c) was acid-precipitable and remained at the origin when subjected to chromatography in basic, neutral, or acidic Solvent Systems F, 1, and 2. Treatment of the macromolecules from either [14C]IdUrd or [12SI]AldUrd-labeled cells with deoxyribonuclease I led to essentially total solubilization of the radiolabel, in sharp contrast to the results obtained after incubation with either pronase or ribonuclease A (Table III). Thus the radioactivity appears to be associated with DNA rather than either protein or RNA. The failure to release free nucleoside from [12SI]AldUrd-labeled DNA under acidic chromatography conditions also suggested that AldUrd, if incorporated intact, was located internally in the DNA structure. Had AldUrd lead to termination of the growing DNA chain, acid treatment should have released AldUrd as the nucleoside.

To further substantiate the incorporation of AldUrd into DNA and to determine the nature of the internucleotide linkage, [14C]AldUrd, [12SI]IdUrd, and [14C]thymidine-labeled DNA was isolated from HS-1 virus-infected cells and purified by equilibrium centrifugation in CsCl (see "Methods"). The sedimentation profiles of these DNA preparations are shown in Fig. 3. Fractions containing radioactive DNA were pooled, dialyzed, lyophilized, and incubated sequentially with DNase I, venom phosphodiesterase, and alkaline phosphatase. The

![Graph](https://example.com/graph1.png)

**TABLE II**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R₅-Paper Solvent F</th>
<th>R₅-TLCSolvent 1</th>
<th>R₅-TLCSolvent 2</th>
<th>R₅-TLCSolvent 3</th>
<th>Rₑ electrophoresis</th>
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</thead>
<tbody>
<tr>
<td>I⁻</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>°</td>
<td>±</td>
</tr>
<tr>
<td>5-Iodoauracil</td>
<td>0.73</td>
<td>±</td>
<td>±</td>
<td>°</td>
<td>±</td>
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<tr>
<td>5-Iodo-5'-amino-2',5'-dideoxyuridine</td>
<td>0.67</td>
<td>0.78</td>
<td>0.85</td>
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<td>±</td>
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<tr>
<td>5-Iododeoxyuridine</td>
<td>0.72</td>
<td>0.71</td>
<td>0.75</td>
<td>0.50</td>
<td>±</td>
</tr>
<tr>
<td>5-Iodo-5'-amino-2',5'-dideoxyuridine 5'-N'-triphosphate</td>
<td>0.41</td>
<td>0.07</td>
<td>0.00</td>
<td>°</td>
<td>±</td>
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<tr>
<td>5-Iododeoxyuridine triphosphate</td>
<td>0.49</td>
<td>0.02</td>
<td>0.05</td>
<td>0.0</td>
<td>±</td>
</tr>
<tr>
<td>Thymidine 3'-phosphate</td>
<td>0.56</td>
<td>0.41</td>
<td>0.64</td>
<td>°</td>
<td>±</td>
</tr>
<tr>
<td>5-Iododeoxyuridine 5'-monophosphate</td>
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<td>0.26</td>
<td>0.50</td>
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<td>±</td>
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<tr>
<td>Thymidine 5'-phosphate</td>
<td>0.57</td>
<td>0.60</td>
<td>0.71</td>
<td>°</td>
<td>±</td>
</tr>
<tr>
<td>Thymidine 5'-triphosphate</td>
<td>0.40</td>
<td>0.05</td>
<td>0.00</td>
<td>0.0</td>
<td>±</td>
</tr>
</tbody>
</table>

* TLC, thin layer chromatography.
  ° Not determined.
  ± Sample decomposed immediately to AldUrd.

The conditions of chromatography and electrophoresis are given under "Methods." The Rₑ value is relative motility with respect to 5-iodo-5'-amino-2',5'-dideoxyuridine (AldUrd).
were used to determine the distribution of acid-insoluble radioactivity.

Virus-infected, [\textsuperscript{3}H]thymidine-labeled (Z'dr). The conditions of radiolabeling, isolation, and centrifugation are described under "Methods." DNA was extracted from either 1.0 x 10\textsuperscript{5} cells (a and c) or 3.5 x 10\textsuperscript{5} cells (b and d). Thirty-microliter samples of each fraction derived from 3 x 10\textsuperscript{5} cells were then digested with either 25 units of bovine pancreas deoxyribonuclease I in 50 mM sodium acetate buffer, pH 5.0, containing 8.2 mM MgCl\textsubscript{2}, 46 units of bovine pancreatic ribonuclease A in 50 mM sodium acetate buffer, pH 5.0, containing 7 mM EDTA, or 45 units of pronase in 50 mM Tris buffer, pH 8.0, 7 mM EDTA, at 37° for 1 hour. After incubation, 0.5 mg of salmon sperm DNA was added to each sample. The samples were made 5% in trichloroacetic acid and, after 30 min on ice, were filtered and washed three times with 3 ml 5% trichloroacetic acid. The radioactivity on the filters was counted in a \gamma counter. Untreated control samples contained 1100 to 1200 cpm of acid-precipitable material.

The macromolecular fractions from Fig. 1 (tubes 5 and 6) were heat-denatured in a boiling water bath for 5 min, then dialyzed against 9 liters of H\textsubscript{2}O for 24 hours with one change. The macromolecular fraction derived from 3 x 10\textsuperscript{5} cells were then digested with either 25 units of bovine pancreas deoxyribonuclease I in 50 mM sodium acetate buffer, pH 5.0, containing 8.2 mM MgCl\textsubscript{2}, 46 units of bovine pancreatic ribonuclease A in 50 mM sodium acetate buffer, pH 5.0, containing 7 mM EDTA, or 45 units of pronase in 50 mM Tris buffer, pH 8.0, 7 mM EDTA, at 37° for 1 hour. After incubation, 0.5 mg of salmon sperm DNA was added to each sample. The samples were made 5% in trichloroacetic acid and, after 30 min on ice, were filtered and washed three times with 3 ml 5% trichloroacetic acid. The radioactivity on the filters was counted in a \gamma counter. Untreated control samples contained 1100 to 1200 cpm of acid-precipitable material.

Table III

<table>
<thead>
<tr>
<th>Source of macromolecular fraction</th>
<th>Per cent of radioactivity made acid-soluble by enzymic hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNase I</strong></td>
<td><strong>RNase</strong></td>
</tr>
<tr>
<td>Herpes simplex virus-infected cells labeled with [\textsuperscript{3}H]AldUrd</td>
<td>98</td>
</tr>
<tr>
<td>Herpes simplex virus-infected cells labeled with [\textsuperscript{3}H]dUrd</td>
<td>100</td>
</tr>
<tr>
<td>Mock infected cells labeled with [\textsuperscript{125}I]dUrd</td>
<td>96</td>
</tr>
</tbody>
</table>

Fig. 3. Isopycnic centrifugation in CsCl of DNA from Vero cells. a, HS-1 virus-infected, [\textsuperscript{14}C]dUrd labeled; b, HS-1 virus-infected, [\textsuperscript{3}H]AldUrd labeled; c, uninfected, [\textsuperscript{125}I]dUrd labeled; d, HS-1 virus-infected, [\textsuperscript{3}H]dUrd labeled. The conditions of radiolabeling, isolation, and centrifugation are described under "Methods." DNA was extracted from either 1.0 x 10\textsuperscript{5} cells (a and c) or 3.5 x 10\textsuperscript{5} cells (b and d). Thirty-microliter samples of each fraction were used to determine the distribution of acid-insoluble radioactivity.

Fig. 4. Chromatographic analysis of [\textsuperscript{3}H]dUrd-labeled DNA (A) and [\textsuperscript{14}C]AldUrd-labeled DNA (B) after sequential treatment with DNase I, venom phosphodiesterase, and alkaline phosphatase. DNA from HS-1 virus-infected cells was isolated and purified as described under "Methods." After enzymic incubation samples were subjected, with appropriate reference compounds, to thin layer chromatography using 0.5 M LiCl as the solvent. DNA was incubated with DNase I (10 units) in 50 mM Tris-HCl/27 mM MgCl\textsubscript{2}, pH 8.1 for 30 min at 37°; DNA incubated with DNase I (as above) and then with venom phosphodiesterase (0.5 unit) for 20 min at 37°; DNA incubated with DNase I and venom phosphodiesterase (as above) and then alkaline phosphatase (0.06 unit) for an additional 30 min at 37°.

Fig. 5. Susceptibility of macromolecular fractions to enzymic hydrolysis.

The above enzymic studies were designed to produce 5',N'-phosphorylated derivatives of AldUrd. Because of the acid lability of such compounds we attempted to produce stable 3'-AldUMP by hydrolyzing AldUrd-substituted DNA with DNase II and spleen phosphodiesterase, using a modification of the procedure of Manteuil et al. (15). As shown in Fig. 5, the oligonucleotides produced by the action of spleen DNase II were not subsequently attacked by spleen phosphodiesterase, although addition of venom phosphodiesterase did produce free AldUrd. The inability of spleen phosphodiesterase to degrade AldUrd-labeled DNA was not entirely unexpected since Letsinger and Mungall (16) had previously shown that dinucleotides containing 5'-aminohydridine were resistant to hydrolysis by this enzyme. In a separate experiment (not shown) combined treatment with alkaline phosphatase and venom phosphodiesterase following DNase II produced a rapid and quantitative conversion to AldUrd. AldUrd-labeled DNA was also found to be resistant to digestion by micrococcal

products of nuclease digestion were then analyzed by chromatography and electrophoresis. Typical results obtained from AldUrd and dUrd-labeled DNAs are shown in Fig. 4. Treatment with DNase I caused both DNAs to become acid-soluble, however, no IdUMP, IdUrd, AldUMP, or AldUrd was released. Upon subsequent treatment with venom phosphodiesterase all the [\textsuperscript{14}C]dUrd migrated as 5'-IdUMP; conversion to IdUrd finally occurred upon addition of alkaline phosphatase. Surprisingly, [\textsuperscript{14}C]AldUrd-labeled DNA yielded exclusively AldUrd after only venom phosphodiesterase digestion. The failure to detect the expected 5'-N'-AldUMP was not due to the presence of phosphomonoesterase or phosphatase contaminants in the DNase I or venom phosphodiesterase preparations. It is most likely that the phosphoramidate bonds in AldUrd-substituted DNA, because of their extreme lability, had been ruptured during the process of DNA purification. Identification of AldUrd as the nuclease digestion product was also established by analysis in several additional chromatographic systems.

The above enzymic studies were designed to produce 5',N'-phosphorylated derivatives of AldUrd. Because of the acid lability of such compounds we attempted to produce stable 3'-AldUMP by hydrolyzing AldUrd-substituted DNA with DNase II and spleen phosphodiesterase, using a modification of the procedure of Manteuil et al. (15). As shown in Fig. 5, the oligonucleotides produced by the action of spleen DNase II were not subsequently attacked by spleen phosphodiesterase, although addition of venom phosphodiesterase did produce free AldUrd. The inability of spleen phosphodiesterase to degrade AldUrd-labeled DNA was not entirely unexpected since Letsinger and Mungall (16) had previously shown that dinucleotides containing 5'-aminohydridine were resistant to hydrolysis by this enzyme. In a separate experiment (not shown) combined treatment with alkaline phosphatase and venom phosphodiesterase following DNase II produced a rapid and quantitative conversion to AldUrd. AldUrd-labeled DNA was also found to be resistant to digestion by micrococcal
nuclease when assayed according to method of Heins et al. (17). In contrast, [134]IdUrd-labeled DNA was completely digested by DNase II and spleen phosphodiesterase (Fig. 5) as well as by micrococcal nuclease.

As seen in Fig. 3, DNA derived from infected IdUrd-treated cells exhibited a significant increase in the buoyant density of both host (p increase 1.700 to 1.760 g/cm³) and viral DNA (p increase 1.725 to 1.790). In contrast, no detectable shift was observed in the buoyant density of IdUrd-substituted DNA, even though a substantial amount of radioactivity was incorporated. Based on the observed density shifts one can calculate that the host DNA was 72% substituted with IdUrd (100% = p of 1.897 g/cm³) while viral DNA was substituted to ≈30% (100% = p of 1.887 g/cm³). The extent of IdUrd substitution in mock infected cells was considerably lower (9.5%).

**Discussion**

Since neither iodide, iodouracil, nor IdUrd were detected in virus-infected Vero cells after treatment with [134]IdUrd, it is apparent that catalytic enzymes, such as nucleoside phosphorylase or nucleosidase, do not play a significant role in IdUrd metabolism under the incubation conditions tested. Indeed, the major acid-soluble metabolic product was clearly shown to be the 5'-N-triphosphate of IdUrd. Although attempts to identify IdUMP as a degradation product of IdUrd labeled DNA after treatment with DNase I and venom phosphodiesterase were unsuccessful, presumably because of the acid lability of the phosphoramidate linkage, it was also clear that IdUrd was incorporated into both host and viral DNA (see Figs. 3 and 4). While the resistance of IdUrd-labeled DNA to spleen phosphodiesterase and micrococcal nuclease also prevented the isolation of 3'-IdUrd, several lines of evidence demonstrate that IdUrd is incorporated internally in the DNA structure. If incorporation of IdUrd had been restricted to the 3' terminus of the growing DNA chain, free IdUrd should have been detected: (a) after acid hydrolysis of intact IdUrd-labeled DNA or (b) under the acidic chromatographic conditions used to analyze the DNase II digestion products (Fig. 5). No free IdUrd was observed under these conditions. In addition, the release of free IdUrd from DNase I- and DNase II-treated IdUrd-DNA only after subsequent digestion with venom phosphodiesterase shows that the 3'-OH group of IdUrd is involved in a standard phosphodiester linkage. Although the treatment of the DNase II hydrolysate with venom phosphodiesterase released free IdUrd, a small amount of undigestable oligonucleotides remained. Oligonucleotides with 3'-phosphate moieties are known to be more resistant than non-3'-phosphorylated oligonucleotides to the action of venom phosphodiesterase (18).

It has been reported that the infection of a cell with herpes simplex virus results in an increase in thymidine kinase (19-21) and DNA polymerase activities (22, 23). These virus-induced enzymes possess properties which distinguish them from the comparable enzymes present in uninfected host cells. The increase in these virus-induced enzymic activities may account in part for the observation that virus-infected cell DNA contained significantly higher levels of IdUrd substitution than the DNA from mock infected cells. The substitution of IdUrd for thymidine in both viral and host DNA was to a much smaller extent than that of IdUrd since no apparent increase in the buoyant density of IdUrd-substituted DNA was found. However, the amount of IdUrd substitution in DNA may be larger than that calculated from the buoyant density of the IdUrd-substituted DNA. Since IdUrd-DNA contains 14 times the radiolabel of IdUrd-DNA, approximately 4 to 5% of the thymidine residues in host DNA should have been replaced with IdUrd. However, the CsCl profiles of IdUrd-DNA exhibited a very high background of 131I throughout the gradient prior to (trichloroacetic acid) precipitation. The lability of the phosphoramidate bond may have lead to extensive fragmentation of the IdUrd-DNA during its isolation and purification; the greater the extent of IdUrd substitution the greater the degree of fragmentation expected.

We have shown conclusively that a 3'-amino analog of a nucleoside can be incorporated into DNA although, in this instance, it is accomplished by an HS-1 virus-induced phenomenon. The enzyme (or enzymes) in the herpes simplex virus-infected cells responsible for initiating the phosphorylation of IdUrd is yet to be identified. The fact that both HS-1 virus and HS-2 virus induce a viral specific thymidine kinase in infected cells (19-21) immediately suggests that this viral
Herpes Virus-induced Incorporation of AldUrd into DNA

TABLE IV
Extent of phosphorylation of 5-ido-5'-amino-2',5'-dideoxyuridine by HS-1 virus-infected and uninfected cell lysate in vitro

<table>
<thead>
<tr>
<th>Source of lysate</th>
<th>Per cent AldUrd phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus-infected cells</td>
<td>7.1</td>
</tr>
<tr>
<td>Mock infected cells</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Virus-infected Vero cells (1 x 10⁹) or mock infected cells (1 x 10⁹) were harvested 8 hours postinfection. Tris-HCl (0.15 ml, 50 mM, pH 7.8) was added to the cell pellet which was then frozen and thawed five times. After centrifugation at 1,500 x g for 10 min, the supernatant fraction was removed and made up to 2 mM ATP, 2 mM MgCl₂, 16 mM creatine phosphate, 0.008 mg of creatine kinase, and 0.08 mM AldUrd (specific activity = 100 µCi/mmol). After incubation at 37°C for 2 hours, portions were taken for thin layer chromatography in 0.5 N LiCl and paper chromatography in Solvent F as described under “Methods.”

enzyme may selectively phosphorylate AldUrd. However, AldUrd is a potent inhibitor of only HS-1 virus replication (4). If viral thymidine kinase is the responsible enzyme and AldUrd phosphorylation is a prerequisite to virus inhibition, then this would indicate a basic difference in the substrate specificity of the two viral enzymes. Since preliminary attempts to phosphorylate AldUrd with purified HS-1 virus thymidine kinase have been unsuccessful it is possible that phosphorylation of AldUrd (a) requires a host factor in addition to the viral enzyme, (b) utilizes a previously undetected viral or host-modified enzyme, or (c) is unstable and hence, although formed, under the conditions of incubation or analysis was not detected. While the failure of AldUrd to be phosphorylated in uninfected cells could also reflect a simple inability to be transported into the cell, we have not been able to detect AldUrd phosphorylation by uninfected cell lysates under conditions where HS-1 virus-infected cell lysates give extensive phosphorylation (Table IV). While considerably more work must be done to elucidate the mechanism by which AldUrd exerts its anti-herpetic activity, it is apparent that the inability of uninfected cells to metabolize AldUrd could account for its lack of cellular toxicity.

Acknowledgments—We wish to thank Mrs. C. Chai and Mrs. E. Lentz for their excellent technical assistance, and we are grateful to Mr. Steve Hill for his collaboration in obtaining the data presented in Table IV.

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