Herpes Simplex Virus Specified Deoxypyrimidine Kinase and the Uptake of Exogenous Nucleosides by Infected Cells

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SUMMARY

Herpes simplex virus can confer to thymidine kinaseless cells the ability to incorporate exogenously supplied thymidine into acid precipitable material. However no incorporation of exogenously supplied deoxycytidine into acid precipitable material can be detected after infection of deoxycytidine kinaseless cells by herpes simplex virus.

This failure to incorporate exogenous deoxycytidine is not due to the failure of the deoxycytidine phosphorylating activity of the virus induced deoxypyrimidine kinase but to a block in the metabolism of deoxycytidine monophosphate in herpes simplex virus infected cells. This block becomes evident with the appearance of the virus induced deoxypyrimidine kinase activity.

INTRODUCTION

Nucleotide precursors of DNA synthesis may be supplied by two alternative pathways in mammalian cells (Kelley, 1972). De novo synthesis of deoxyribonucleotides occurs by reduction of the corresponding ribonucleotide at the diphosphate level except in the case of thymidine nucleotides, where de novo production occurs by deamination and subsequent methylation of cytidine deoxynucleotides at the monophosphate level. The conversion sequence of cytidine deoxyribonucleotides to thymidine monophosphate is inhibited by TTP and dUTP but stimulated by dCTP (Maley & Maley, 1964), control being exerted at the deamination step. The methylation of dUMP by thymidylate synthetase to give TMP is apparently unaffected by deoxypyrimidine triphosphates (Frearson, Kit & Dubbs, 1965). The enzyme ribonucleotide reductase, which reduces all four common ribonucleoside diphosphates (Larsson & Reichard, 1967) requires thioredoxin and thioredoxin reductase (Moore & Reichard, 1964) and is intimately controlled by complex interactions of the various deoxyribonucleoside triphosphates. The allosteric nature of this control has been established for the Escherichia coli enzyme where the triphosphates have been shown to bind to sites distinct from those to which the substrates bind (Brown & Reichard, 1969). More limited studies on the mammalian enzyme indicate that its regulation is essentially similar to that of the E. coli enzyme (Moore & Hurlbert, 1966).

The deoxyribonucleoside diphosphates thus formed are phosphorylated by a nucleoside diphosphate kinase to the triphosphate form, which in mammalian cells acts equally well on both purine and pyrimidine deoxyribonucleoside diphosphates and ribonucleoside diphosphates (Mourard & Parks, 1966).

Deoxyribonucleotides may also be formed from the corresponding pre-existing deoxy-
ribonucleosides by what are generally termed ‘scavenger’ or ‘salvage’ enzymes. One
enzyme, the purine deoxyribonucleoside kinase (Krygier & Momparler, 1970), phospho-
rylates both dG and dA to form dGMP and dAMP respectively. On the other hand
thymidine and deoxycytidine are phosphorylated to their respective monophosphates by
thymidine kinase (TK) and deoxycytidine kinase (dCK). These enzymes in the mammalian
system at least seem to be quite separate (Kit et al. 1963; Durham & Ives, 1970). Once
formed the monophosphates are phosphorylated to the diphosphate by monophosphate
kinases which also phosphorylate the corresponding ribomonophosphates (Kit, 1970). By
means of these ‘salvage enzymes’ it is thus possible to incorporate exogenous radioactively
labelled nucleosides into the cellular DNA. Mutant cells can be selected lacking one or
other, or a combination of these salvage enzymes (Subak-Sharpe, 1968) and they fail to
incorporate the appropriate nucleosides.

After infection of cells by herpes simplex virus (HSV) there is an induction of both
thymidine kinase (Kit & Dubbs, 1963) and deoxycytidine kinase activity (Hay et al. 1970).
These two activities appear to reside in the same virus specified polypeptide (Jamieson &
Subak-Sharpe, 1974). Mutants of HSV can be selected for resistance to either BudR or
araC and all these mutants are found to have simultaneously gained resistance to both
analogues and lost the ability to induce both thymidine and deoxycytidine kinase activities
(Jamieson, Gentry & Subak-Sharpe, 1974).

Here we report the effect of HSV infection with wild type or mutant virus on the in-
corporation of exogenous pyrimidine deoxyribonucleosides by normal cells or cells
lacking in thymidine and/or deoxycytidine kinase activity.

METHODS

Cells. All cell lines were grown in Eagle’s medium containing 10% calf serum (EC10).
BHK C13 cells (Macpherson & Stoker, 1962) were used as the standard cell line having
both thymidine kinase and deoxycytidine kinase activities. PyY/TG/CAR/BudR cells
(Jamieson et al. 1974) lack both thymidine kinase and deoxycytidine kinase activity.
3T3 TK− cells (obtained from Dr J. Pitts of the Department of Biochemistry, Glasgow
University) are a BudR resistant mouse kidney fibroblast cell line lacking thymidine kinase
activity but having deoxycytidine kinase activity. 3T6 dCK− cells (initially selected for
resistance to 200 μg/ml of araC by Dr M. Green) were further selected for resistance to
500 μg/ml of araC, and are devoid of detectable deoxycytidine kinase activity but possess
thymidine kinase activity.

Virus. In all experiments herpes simplex type 1 strain 17 syn (HSV-1, 17 syn) wild type
or mutants selected for either BudR or araC resistance were used as described previously
(Jamieson et al. 1974). The virus was titrated in BHK C13 cells using the plaque assay of
Russell (1962).

Production of resting cells. BHK C13 cells were seeded at a concentration of 10⁶ cells/
50 mm plastic Petri dish in EC10 medium. After 24 h the medium was removed, the cells
washed, and 5 ml Eagle’s medium containing 1% calf serum added. The cells were then
incubated for 5 to 6 days at 37 °C (Burk, 1966).

Incorporation of exogenous labelled nucleosides. Cell monolayers (2 × 10⁶ cells) were
infected at a multiplicity of 10 p.f.u./cell in a total volume of 0.2 ml and the virus allowed
to adsorb at 37 °C for 1 h. Cultures were labelled with ³H-TdR or ³H-CdR (2 to 10 μCi/
plate, in a final concentration of 5 × 10⁻⁸ M) between 4 and 5 h post infection (p.i.). The
medium was then removed and the cell sheet washed twice rapidly with ice-cold phosphate
buffered saline (PBS; Dulbecco & Vogt, 1954). This wash removed >98% of the exogenous thymidine. The cells were then suspended into 5 ml PBS by scraping, followed by brief sonication. Duplicate 10 µl samples were then taken for analysis of acid precipitable counts and spotted on to Whatman No. 1 paper discs, washed twice in ice cold 10% trichloroacetic acid (TCA), rinsed in alcohol, dried and counted in a toluene based scintillant. The remainder of the sonicate was made 10% with TCA and centrifuged at 900 g for 1 min. The supernatant fluid was removed and neutralized with NH₄OH and the TCA removed by shaking with acetone. The samples were concentrated by lyophilization and resuspended in 100 mM-tris, pH 8.2. Samples were either taken directly, spotted on discs and counted or the deoxypyrimidine pools were separated by partition chromatography. 20 µl samples were mixed with non-radioactive deoxypyrimidine nucleotides and applied on Whatman No. 1 paper. The components were separated by a butanol/glacial acetic acid/water (v/v, 2:1:1) solvent in 12 h. The markers were located by u.v. fluorescence and the spots cut out and counted (backgrounds were measured and subtracted). When 14C-cytidine was added as the exogenous nucleoside, the samples were suspended in PBS, and total acid precipitable incorporation measured as described above. The TCA precipitable material was then dissolved in 0.5 M-NaOH and incubated at 60 °C for 1 h. The DNA was then precipitated by acid and incorporation measured as before. By subtraction of non-alkali soluble counts from the total acid precipitable counts the amount of cytidine incorporated into RNA can be determined.

Cytidine kinase assay. Enzyme extracts were prepared as described previously (Jamieson et al. 1974). The reaction mixture contained in a final volume of 100 µl, 10 mM-ATP, 10 mM-MgCl₂, 30 mM-tris/HCl, pH 7.5, 10 µM-³H-cytidine (10 Ci/mmol) and 100 µg of enzyme. The mixture was incubated at 37 °C for 30 min and the products separated by ion exchange paper chromatography as described previously (Jamieson et al. 1974).

RESULTS

Cell lines which lack the enzyme thymidine kinase, PyY/TG/CAR/BUdR, and 3T3TK⁻ cells, acquire the ability to incorporate exogenous labelled thymidine into acid precipitable material, after infection with wild type herpes simplex virus (HSV-1; Table 1). Cells which possess normal thymidine kinase activity (BHK C13, PyY/TG/CAR, 3T6 dCK⁻) demonstrate increased incorporation after infection. The amount of increase is variable depending on the physiological state of the cells, but in the experiments recorded here it is approximately twofold. Infection with deoxypyrimidine kinaseless mutant virus (dPyK⁻) does not result in increased thymidine incorporation but rather in a substantial decrease in the amount of exogenous thymidine incorporated by the cells although these cells possess their own thymidine activity (Table 1).

In contrast, after infection of three different deoxycytidine kinaseless (dCK⁻) cell lines with either wild type or dPyK⁻ virus, no increase can be demonstrated in the amount of exogenous deoxycytidine incorporated into acid precipitable material. Infection with wild type or with dPyK⁻ virus in fact results in a marked decrease in the incorporation of deoxycytidine into the acid precipitable material of dCK⁺ cell lines (Table 1). dPyK⁻ virus tends to cause a marginally greater decrease in the incorporation (some seven results consistently showed this).

It is conceivable that the deoxycytidine incorporated by the HSV infected cells is metabolized via the de novo thymidine pathway, namely CdR → dCMP → dUMP → dTMP and that the labelled moiety on the 5' position of the pyrimidine ring is replaced by the
addition of a methyl group by the enzyme thymidylate synthetase. However, experiments using uniformly labelled $^{14}$C-CdR demonstrate that there is essentially no difference in the incorporation of $^{3}$H-CdR and $^{14}$C-CdR.

HSV-1 infection results in the induction of a deoxypyrimidine kinase which in vitro phosphorylates both thymidine and deoxycytidine (Jamieson & Subak-Sharpe, 1974). Therefore failure to incorporate deoxycytidine could either be a consequence of the HSV deoxycytidine kinase activity being non-functional in vivo, or of some block subsequent to the CdR $\rightarrow$ dCMP step in the metabolism of this compound.

After infection of both TK$^+$dCK$^+$ cells and TK$^-$dCK$^-$ cells with wild type HSV the increase in the amount of thymidine incorporated into acid precipitable material is accompanied by a concomitant substantial increase in the uptake of thymidine into the acid soluble pools of the cells (Table 2). Moreover, although HSV infection results in a net decrease in the amount of deoxycytidine incorporated into acid precipitable material, the amount of deoxycytidine phosphorylated and taken up into the acid soluble pools is increased nearly three- and 20-fold respectively (Table 2).

Further analysis of the acid soluble fractions demonstrated that in uninfected TK$^+$dCK$^+$ cells 52 % of the thymidine taken up is present as TDP plus TTP, 35 % as TMP, and 13 % as the nucleoside. After infection, only 37 % occurs as TDP plus TTP, 51 % as TMP and 12 % as thymidine, although the total amount incorporated is increased fivefold in these experiments. PyY/TG/CAR/BuDR cells take up very little thymidine as acid soluble material and 100 % of this is present as the nucleoside. After HSV infection the thymidine metabolized is distributed in a similar fashion to that in infected BHK C13 cells (Table 3 A).

In uninfected BHK C13 cells, 69 % of the deoxycytidine taken up occurs as the di-plus tri-phosphates with 16 % as dCMP and 15 % as the nucleoside. After infection with wild type HSV only 2 % of the deoxycytidine occurs as dCDP plus dCTP with 83 % as dCMP and 15 % as the nucleoside. PyY/TG/CAR/BuDR contain no detectable deoxycytidine

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### Table 1. Incorporation of exogenous pyrimidine deoxyribonucleosides into the acid precipitable material of HSV infected or uninfected cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Virus at m.o.i. of 10 p.f.u./cell</th>
<th>Acid precipitable incorporation (ct/min/10^6 cells)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK C13 (TK$^+$dCK$^+$)</td>
<td>—</td>
<td>3876±180$^+$ 1900±200</td>
</tr>
<tr>
<td>BHK C13</td>
<td>HSV-1</td>
<td>8204±286 410±75</td>
</tr>
<tr>
<td>BHK C13</td>
<td>dPyK$^-$</td>
<td>2100±123 320±100</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR (TK$^-$dCK$^-$)</td>
<td>—</td>
<td>120±15 95±27</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>HSV-1</td>
<td>3540±195 82±12</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>dPyK$^-$</td>
<td>115±35 110±6</td>
</tr>
<tr>
<td>PyY/TG/CAR (TK$^+$dCK$^+$)</td>
<td>—</td>
<td>2865±93 135±30</td>
</tr>
<tr>
<td>PyY/TG/CAR</td>
<td>HSV-1</td>
<td>5400±120 140±18</td>
</tr>
<tr>
<td>PyY/TG/CAR</td>
<td>dPyK$^-$</td>
<td>1876±84 78±20</td>
</tr>
<tr>
<td>3T6 dCK$^-$ (TK$^-$dCK$^+$)</td>
<td>—</td>
<td>3450±90 100±10</td>
</tr>
<tr>
<td>3T6 dCK$^-$</td>
<td>HSV-1</td>
<td>5876±125 112±14</td>
</tr>
<tr>
<td>3T6 dCK$^-$</td>
<td>dPyK$^-$</td>
<td>890±70 95±22</td>
</tr>
<tr>
<td>3T3 TK$^-$ (TK$^-$dCK$^+$)</td>
<td>—</td>
<td>98±6 2020±100</td>
</tr>
<tr>
<td>3T3 TK$^-$</td>
<td>HSV-1</td>
<td>2500±112 560±85</td>
</tr>
<tr>
<td>3T3 TK$^-$</td>
<td>dPyK$^-$</td>
<td>130±28 426±50</td>
</tr>
</tbody>
</table>

* These values are computed on the basis of four separate experiments here and in subsequent tables.
† Standard deviation of the mean.
Deoxypyrimidine uptake by HSV infected cells

Table 2. Incorporation of thymidine and deoxycytidine into acid precipitable and acid soluble material in HSV-infected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus</th>
<th>Label</th>
<th>Acid soluble</th>
<th>Acid precipitable</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) BHK C13 (TK⁺dCK⁻)</td>
<td>—</td>
<td>$^3$H-TdR</td>
<td>1860 ± 115</td>
<td>5200 ± 175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>420 ± 80</td>
<td>870 ± 135</td>
</tr>
<tr>
<td>BHK C13</td>
<td>HSV</td>
<td>$^3$H-TdR</td>
<td>6756 ± 165</td>
<td>8580 ± 200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>1125 ± 75</td>
<td>621 ± 80</td>
</tr>
<tr>
<td>BHK C13</td>
<td>dPyK⁻</td>
<td>$^3$H-TdR</td>
<td>1220 ± 90</td>
<td>2076 ± 90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>456 ± 100</td>
<td>725 ± 52</td>
</tr>
<tr>
<td>(B) PyY/TG/CAR/BuDR (TK⁻dCK⁻)</td>
<td>—</td>
<td>$^3$H-TdR</td>
<td>125 ± 20</td>
<td>206 ± 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>45 ± 8</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>HSV</td>
<td>$^3$H-TdR</td>
<td>2980 ± 65</td>
<td>3998 ± 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>890 ± 15</td>
<td>59 ± 20</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>dPyK⁻</td>
<td>$^3$H-TdR</td>
<td>150 ± 16</td>
<td>250 ± 43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{14}$C-CdR</td>
<td>82 ± 12</td>
<td>21 ± 3</td>
</tr>
</tbody>
</table>

Table 3. Analysis of acid soluble pools in HSV infected cells

(A) Thymidine nucleotides

<table>
<thead>
<tr>
<th>Cells</th>
<th>Label as:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TTP + TDP</td>
</tr>
<tr>
<td>BHK C13</td>
<td>—</td>
</tr>
<tr>
<td>BHK C13</td>
<td>HSV</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>—</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>HSV</td>
</tr>
</tbody>
</table>

(B) Deoxycytidine nucleotides

<table>
<thead>
<tr>
<th>Cells</th>
<th>Label as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dCTP + dCDP</td>
</tr>
<tr>
<td>BHK C13</td>
<td>—</td>
</tr>
<tr>
<td>BHK C13</td>
<td>HSV</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>—</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>HSV</td>
</tr>
</tbody>
</table>

(C) AraC nucleotides

<table>
<thead>
<tr>
<th>Cells</th>
<th>Label as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>araCTP + araCDP</td>
</tr>
<tr>
<td>BHK C13</td>
<td>—</td>
</tr>
<tr>
<td>BHK C13</td>
<td>HSV</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>—</td>
</tr>
<tr>
<td>PyY/TG/CAR/BuDR</td>
<td>HSV</td>
</tr>
</tbody>
</table>

except as the nucleoside. After HSV infection 100% of the deoxycytidine present occurs as dCMP or the nucleoside and none as dCDP or dCTP (Table 3B). In these cells there is no significant difference in the metabolism of araC and deoxycytidine, although in the case of araC a trace is detectable as araCDP plus araCTP (Table 3C).

The possibility exists that in HSV infected cells the levels of the deoxypyrmidine triphos-
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Fig. 1. Incorporation of labelled pyrimidine deoxynucleosides into acid precipitable material. $^3$H-thymidine or $^3$H-deoxycytidine (final concentration $10^{-4}$ M and 2 $\mu$Ci/ml) was added at time of infection. Samples were removed and processed at the indicated times as detailed in Methods. (a) $^3$H-TdR (2 $\mu$Ci/ml) incorporation into HSV-1 infected (○ — ○) and uninfected (■—■) exponentially growing BHK C13 cells. (b) $^3$H-TdR (2 $\mu$Ci/ml) incorporation into HSV-1 infected (○ — ○) and uninfected (■—■) resting BHK C13 cells. (c) $^3$H-CdR (2 $\mu$Ci/ml) incorporation into HSV-1 infected (○ — ○) and uninfected (■—■) exponentially growing BHK C13 cells. (d) $^3$H-CdR (2 $\mu$Ci/ml) incorporation into HSV-1 infected (○ — ○) and uninfected (■—■) resting BHK C13 cells.

phates are such as to be inhibitory to the phosphorylation of dCMP. However, even resting cells which have a low rate of basal metabolism and also low levels of all four deoxyribonucleoside triphosphates (Bray & Brent, 1972) exhibit no increase in the amount of exogenous deoxycytidine incorporated into acid precipitable material following HSV infection (Fig. 1).

Two hours after infection of exponentially growing BHK C13 cells with wild type HSV the incorporation of exogenous thymidine into acid precipitable material is already twofold
Deoxypyrimidine uptake by HSV infected cells

Fig. 2. Uptake of pyrimidine deoxynucleosides into acid soluble material. ³H-deoxycytidine (final concentration $10^{-4}$ M, 2$\mu$Ci/ml, was added at time of infection. Samples were removed and processed at the indicated times as described in the Methods. (a) ³H-TdR (2$\mu$Ci/ml) incorporation into HSV-1 infected (○—○) and uninfected (●—●) exponentially growing BHK C13 cells. (b) ³H-TdR (2$\mu$Ci/ml) incorporation into HSV-1 infected (○—○) and uninfected (●—●) resting BHK C13 cells. (c) ³H-CdR (2$\mu$Ci/ml) incorporation into HSV-1 infected (○—○) and uninfected (●—●) exponentially growing BHK C13 cells. (d) ³H-CdR (2$\mu$Ci/ml) incorporation into HSV-1 infected (○—○) and uninfected (●—●) resting BHK C13 cells.

greater than that found in uninfected cells. The amount of thymidine incorporated increases linearly until at least 8 h p.i. When resting cells are infected with wild type HSV the incorporation does not differ significantly from the control until 4 h p.i. after which time it changes to a rate similar to that found in infected exponentially growing cells (Fig. 1a, b).

For the first 2 h after infection with HSV the incorporation of deoxycytidine into acid precipitable material is similar to that found in uninfected exponentially growing BHK C13 cells, but at all later times the rate of incorporation into infected cells is subsequently less
Table 4. Incorporation of cytidine into DNA, RNA and the acid soluble pools of HSV infected and uninfected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus</th>
<th>Acid soluble pools</th>
<th>Acid precipitable</th>
<th>Acidity</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK C13</td>
<td>—</td>
<td>334 ± 50</td>
<td>487 ± 38</td>
<td>2206 ± 120</td>
<td></td>
</tr>
<tr>
<td>BHK C13 HSV</td>
<td>931 ± 80</td>
<td>940 ± 57</td>
<td>1436 ± 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PyY/TG/CAR/BUDR</td>
<td>438 ± 45</td>
<td>822 ± 62</td>
<td>1950 ± 150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PyY/TG/CAR/BUDR HSV</td>
<td>636 ± 60</td>
<td>1040 ± 35</td>
<td>588 ± 42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Activity of cytidine kinase in wild type HSV infected, dPyK- HSV infected and uninfected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus</th>
<th>Cytidine kinase activity (nmol cytidine phosphorylated/150 μg protein/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK HSV</td>
<td>6.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>BHK dPyK-</td>
<td>5.4 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

than is found in the uninfected controls (Fig. 1c). After infection of resting BHK C13 cells incorporation of deoxycytidine into acid precipitable material is always less than in the uninfected cells except at 2 h p.i. (Fig. 1d).

In contrast, the uptake of both thymidine and deoxycytidine into acid soluble material of both exponentially growing and resting BHK C13 cells was greater at all times after infection than that observed in the uninfected controls (Fig. 2).

Although the incorporation of deoxycytidine into DNA is impaired after HSV infection, there is an increase in the amount of cytidine incorporated into DNA (Table 4). After infection of both BHK C13 and PyY/TG/CAR/BUDR cells with wild type HSV there is an increase in the total amount of cytidine incorporated into acid soluble material, although the total amount incorporated into acid precipitable material is decreased. The decrease is due to the large reduction in RNA synthesis found after infection, despite the significant increase in the incorporation into DNA (Table 5). This increase in the incorporation of deoxycytidilate may be due to three changes: (a) an increase in the amount of all ribonucleotides reduced to the corresponding deoxyribonucleoside diphosphate, (b) a specific increase in the amount of CDP reduction to dCDP and (c) an increase in the amount of pre-formed cytidine incorporated by the infected cell. Table 5 shows that after infection with wild type HSV there is a twofold increase in the activity of the enzyme cytidine kinase and that this induction does not occur after infection with dPyK- virus.

DISCUSSION

Radioactively labelled thymidine can be shown to be incorporated into the acid precipitable material of HSV-infected cells, while no incorporation of exogenously supplied deoxycytidine into the DNA can be detected. Both the thymidine kinase and deoxycytidine kinase activities of the virus induced deoxypyrimidine kinase function after infection; but whereas the product of the thymidine kinase activity is metabolized further to the triphos-
Deoxypyrimidine uptake by HSV infected cells

phate and then incorporated into DNA, the product of the deoxycytidine kinase activity does not appear to be metabolized in any identified way.

The results show that there is effective uptake of both thymidine and deoxycytidine into acid-soluble material mediated by the virus induced enzyme, and that this effect is demonstrable from 2 to 6 h p.i. When resting cells – which contain low levels of DNA precursors (Bray & Brent, 1972) – are infected with wild type virus, both deoxynucleosides are phosphorylated; however, there is a more rapid uptake of thymidine than deoxycytidine into the soluble pools. The uptake of nucleosides into cells is not increased following infection by a dPyK⁻ mutant of HSV.

Not only is no deoxycytidine incorporated into DNA when dCK⁻ cells are infected with wild type HSV, but the actual amount of deoxycytidine incorporated by dCK⁺ cells is drastically reduced after infection. The inhibition of incorporation begins at about 2 h p.i., the time at which the virus induced deoxypyrimidine kinase activity begins to appear (Jamieson & Subak-Sharpe, 1974).

This failure of herpes virus infected cells to incorporate exogenous deoxycytidine implicates a block, which prevents further metabolism of deoxycytidine monophosphate and which occurs irrespective of the cell type infected.

Lindberg et al. (1969) demonstrated a large increase in the thymidine triphosphate pool, after infection of 3T3 cells with polyoma virus. Our data shows a similar increase after infection of BHK cells with wild type HSV, as measured by the amount of exogenous thymidine metabolized to the triphosphate. This effect is not observed after infection by dPyK⁻ mutant HSV. If this increased amount of label in the thymidine triphosphate pool reflects a real increase of pool size, then the pool size per se may regulate the metabolism of dCMP. The size of the thymidine triphosphate pool is known to regulate the size of the dCTP pool (Reichard, 1968); when large, the dTTP pool inhibits the enzymes deoxycytidine monophosphate deaminase and CDP reductase. It is not known whether dTTP also regulates the enzyme deoxycytidine monophosphate kinase.

Herpes simplex virus has been reported to induce a modified cytidine diphosphate reductase after infection which is resistant to thymidine triphosphate inhibition (Cohen, 1972). This altered reductase may boost the deoxycytidine triphosphate pool to a level which either exerts a feedback inhibition on the enzyme dCMP kinase, or alternatively dilutes the incorporated label to such an extent that it is no longer detectable in our analysis. Thus the dTTP and dCTP pools may act in concert, to prevent the further metabolism of dCMP.

However, there are a number of observations which are difficult to reconcile with this hypothesis. In vitro inhibition studies (Jamieson & Subak-Sharpe, 1974) reveal that while the viral thymidine kinase activity is stimulated by dCTP, the viral deoxycytidine kinase activity is inhibited by both dTTP and dCTP. In the situation described above the high levels of dTTP and dCTP expected after HSV infection should increase the thymidine kinase activity but decrease the deoxycytidine kinase activity. In fact, the results show that the deoxycytidine kinase is functioning normally in vivo (Table 4). The dCMP kinase and dCMP deaminase activities could, however, be more sensitive to feed-back inhibition by deoxypyrimidine triphosphates than the virus induced deoxycytidine kinase activity. After infection of serum-starved BHK C13 cells (where de novo deoxynucleotide synthesis and cellular scavenger enzyme levels are low) there is still no detectable incorporation of deoxycytidine into acid precipitable material. Here it is difficult to envisage the triphosphate pools reaching an inhibitory level rapidly enough to prevent all incorporation of extracellular deoxycytidine into the triphosphate and thus into DNA.

Alternatively, the nucleoside kinases may form part of an enzyme complex which is
involved in the sequential synthesis of triphosphates. Electrophoresis of herpes simplex soluble proteins on non-SDS polyacrylamide gels has shown that the band of virus induced thymidine kinase activity also contains enzyme activities which will phosphorylate dTMP to the di- and triphosphates (Munyon et al. 1972). These results suggest some association between enzymes which act sequentially along a particular pathway.

If such a scheme operates here the uninfected cells would have two association complexes for deoxypyrimidine metabolism, one for thymidine and one for deoxycytidine, each containing a nucleoside, a monophosphate and a diphosphate kinase. The enzymes in such a complex need not be physically associated, but arranged in some compartmentalized manner so that the product of reaction (1) would be made available as the substrate for reaction (2). After HSV infection, the pre-existing host cells deoxyribonucleoside kinases would be displaced by the virus induced deoxypyrimidine kinase in the association complex. The biochemical studies so far demonstrate that the virus enzyme has an intrinsic strong preference for thymidine as a substrate (Jamieson & Subak-Sharpe, 1974). Thus it might be expected that HSV deoxypyrimidine kinase should preferentially become associated with the ‘thymidine complex’, although still phosphorylating deoxycytidine. The dCMP formed by this complex would be channelled into the dTMP environment, and thus not be available as a substrate for dCMP kinase. Also TMP would be formed in the ‘deoxycytidine complex’ and compete with dCMP for further phosphorylation. This model could explain not only the failure of HSV infected resting BHK C13 cells to incorporate deoxycytidine, but also why in exponentially growing BHK C13 cells, the incorporation of deoxycytidine into DNA occurs normally for the first 2 h of infection (before the virus specified enzyme activity is in evidence) but thereafter no more deoxycytidine is incorporated into acid precipitable material. The ‘enzyme complex’ is visualized as enzymes compartmentalized in close association with one another, the compartmentalization being disrupted on destroying the cells integrity and thus allowing phosphorylation of dCMP, as has indeed been found by *in vitro* assay (Jamieson & Subak-Sharpe, 1974).

Regardless of what mechanism is responsible for the prevention of dCMP metabolism, it appears anomalous that cytosine arabinoside (araC) inhibits the replication of HSV in dCK- cells. The believed mode of action of araC in inhibition DNA synthesis is by specific interaction of araCTP with the DNA polymerase (Furth & Cohen, 1967; Moore & Cohen, 1967; Momparler, 1969; Kaplan, Brown & Ben Porat, 1968), although interaction with the ribonucleotidase reductase has also been postulated (Chu & Fischer, 1962; Kim & Eidinoff, 1965). AraC is metabolized in a similar fashion to deoxycytidine, the bulk of the intracellular araC being in the monophosphate form which should have no effect on DNA replication. AraC, however, is also an analogue of cytidine and therefore once incorporated by the virus specified deoxycytidine kinase the monophosphate can be metabolized via the ribonucleotide pathway to araCDP and araCTP. Cytidine monophosphate kinase is apparently the same enzyme as deoxycytidine monophosphate kinase (Kit, 1970) but the reaction involving cytidine monophosphate may be regulated differently, since HSV-infected cells have an increased ability to incorporate exogenous cytidine into DNA (Table 5). Analysis of the acid soluble pools of HSV infected cells labelled with 3H-araC does indicate trace amounts of araCTP. It may be that only minute intranuclear concentrations of araCTP are required *in vivo* to inhibit DNA polymerase or that phosphorylated araC inhibits HSV replication in some other manner. No distinction can at present be made between these possibilities, although araCTP has been reported not to inhibit HSV specified DNA polymerase (Muller et al. 1973).

A consequence of the blocked dCMP phosphorylation in HSV infected cells is that
cytidine residues must be obtained from a source other than the salvage pathways, to allow viral DNA replication. After HSV infection an altered CDP reductase has been reported (Cohen, 1972) which is resistant to dTTP inhibition and this may account for the observed increase in the incorporation of exogenous cytidine into DNA.

It is unclear why HSV codes for a deoxycytidine kinase activity when the product of this activity is not utilized during replication. It may be that under conditions occurring during natural infections when this virus specified activity becomes essential, the metabolic block is removed, or that the deoxycytidine activity may merely be an accidental by-product of the thymidine kinase activity and thus never essential for virus replication.

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REFERENCES


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