Quantitative Aspects of Thymidine Uptake into the Acid-soluble Pool of Normal and Polyoma-transformed Hamster Cells

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SUMMARY

The uptake of thymidine into an acid-soluble pool in normal hamster embryo cells and hamster tumor cells transformed by polyoma virus was studied. It was found that tumor cells exhibited a 4-fold higher capacity than normal cells to incorporate thymidine as phosphorylated products into this pool. This was correlated with a 5- to 10-fold higher activity of thymidine kinase in the tumor cell lines. The uptake process was strongly inhibited by the thymidine analog iododeoxyuridine, partially inhibited by deoxyuridine, and not inhibited by uridine.

INTRODUCTION

A comparative study of normal and neoplastic cell membrane functions carried out in this laboratory initially investigated the transport of L-phenylalanine in normal and Py'-transformed hamster cells (10). This amino acid was shown to be actively transported to a similar extent in both cell types by a mechanism with an acceptor site located superficially on the cell membrane. The process behaved as a carrier-mediated system showing a specificity spectrum, saturation kinetics, the phenomenon of exchange diffusion, and a transport rate dependent on substrate concentration. These findings were consistent with the model of amino acid transport in Ehrlich ascites tumor cells developed by Oxender (27) and Oxender and Christensen (28).

An investigation of pyrimidine nucleoside uptake was undertaken when it was found that Py-induced hamster tumor cells showed an enhanced capacity to take up TdR into an acid-soluble pool. This finding has been correlated with an increased TdR kinase activity in tumor cells, thereby suggesting that this enzyme might be involved in the regulation of TdR uptake. In view of studies (3, 8, 12, 13, 15, 29) suggesting the presence of specific pyrimidine transport systems in eukaryotic cells, an investigation of the interrelationship between membrane transport and phosphorylation in the control of pyrimidine uptake has been initiated. A comparison of TdR uptake in normal and neoplastic hamster cells is presented here.

MATERIALS AND METHODS

General. Radioisotope counting technique, extraction procedures, protein determination, cell counts, and measurement of cell diameters were performed as described previously (10).

Radioisotope-labeled Precursors. Tritiated L-phenylalanine (specific activity, 1.5 Ci/mmole) and TdR-3H (specific activity, 6.0 Ci/mmole) were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y.

Media. The minimal essential medium of Eagle supplemented with 10% fetal calf serum with or without 10% Tryptose phosphate broth was used routinely. Tris-buffered 0.9% NaCl solution, pH 7.4 (34), was used as a general solution for washing cells and diluting isotopes.

Chromatography. Descending chromatography on Whatmann No. 3MM paper with an isobutyric acid: concentrated NH₄OH: water (57:38:38) solvent system was used to separate TdR and its phosphorylated products. Five % TCA cell extracts were concentrated by evaporation in a vacuum and redissolved in 5% TCA. Unlabeled reference TMP and TTP were added at a final concentration of 1 mg/ml; the mixture was applied to the paper in 0.1-ml quantities and developed overnight. The strip was scanned in ultraviolet light to locate the reference compounds and then cut into 1- or 2-cm segments, suspended in scintillator, and counted in a spectrometer, Nuclear-Chicago Corporation, Chicago, Ill.

TdR Kinase Assay. The method described by Bresnick and Karjala (4) was used with the following modifications based on the method of Kit and Dubbs (17). Cultured cells were trypsinized, washed in Tris buffer, and resuspended in 4 volumes 0.15 M KCl, 0.03 M 2-mercaptoethanol, and 0.01 M Tris-HCl at pH 8.0. The cell suspension was sonically oscillated for 60 sec on ice and clarified at 30,000 rpm in a No. 40 Spinco rotor for 1 hr. The final volume of the reaction mixture [5 mM ATP, 5 mM MgCl₂, 6 mM 3-phosphoglycerate (cyclohexamine salt), and 40 mM Tris-HCl, pH 8.0] was 1.0 ml containing 0.06 mM TdR-3H (specific activity, 14.4 μCi/μmole). The reaction was carried out at 37°C for 1 hr. Following precipitation of the reaction mixture with 0.1 ml 40% TCA, the extract was separated without concentration by paper chromatography as described above. The labeled peaks
corresponding to phosphorylated products were eluted from the paper in 0.1 N HCl and counted in a scintillation spectrometer. The enzyme activity was expressed as μmoles TdR phosphorylated per μg protein per hr.

Cells. Normal hamster embryo cells were prepared as described previously (10). A line of hamster embryo cells (HEF-SM-1) was derived from normal embryo cells by cultivation in 10% fetal calf serum and 10% Tryptose phosphate broth medium which was noted to favor the selection of cuboid epithelioid cells and suppression of fibroblastic cells. This line was studied in "early" passages, i.e., 83 and 109 days after initiation, and in "late" passages, i.e., after 630 days in continual cultivation.

Tumor cell lines, HTC-3049-3 and HTC-3049-91TC, were derived as described previously (9, 10). The HTC-3049-3 was induced by inoculation of newborn hamsters with the 3049 strain of Py while the HTC-3049-91TC and HTC-3049-176TC lines were induced by the infection of hamster embryo cells with the 3049 virus in suspension. HTC-3049-176TC and HTC-3049-160TC were derived from primary hamster embryo cells infected in suspension and cultivated under conditions identical to those of the HTC-3049-91TC line and assayed for TdR and l-phenylalanine incorporation 106 and 25 days after infection, respectively.

Cell lines used in this study were routinely tested for the presence of Mycoplasma spp., using the BYE medium (1) incubated anaerobically in 95% N₂ and 5% CO₂.

RESULTS

Time Course of TdR Incorporation into an Acid-soluble Fraction. Preliminary experiments revealed that TdR⁻³H was incorporated rapidly at room temperatures into an acid-soluble fraction of hamster embryo and tumor cells which could not be readily removed by repeated washing. An experiment to define the rate of incorporation of metabolite into an acid-soluble fraction was performed. Monolayers of normal and tumor cells were washed with Tris buffer and labeled in the same buffer at room temperature. In tumor cells, 2 tritium-labeled compounds were found. One comprising approximately 90% of the tritiated products was identified as TMP and the other as TTP. TTP was observed in normal cells but not in tumor cells. On this basis, an uptake period of 5 min was chosen for subsequent studies as both convenient and manageable, as well as being long enough to be stable and reproducible.

The results of this experiment also demonstrated that the uptake of TdR⁻³H into hamster tumor cells was significantly greater than the uptake by hamster embryo cells. Other experiments designed to study this phenomenon are discussed below.

Identity of the Intracellular Labeled Compound. For identification of the tritium-labeled compound or compounds in the acid-soluble pool, paper chromatographic separation of the isolated concentrated acid-soluble material was carried out. In neither normal nor Py-transformed tumor cells could TdR⁻³H be found after incubation for either 1, 2, or 4 min with extracellular TdR⁻³H at room temperature. In tumor cells, 2 tritium-labeled compounds were found. One comprising approximately 90% of the tritiated products was identified as TMP and the second was identified as TDP. TTP was observed only under conditions in which DNA synthesis had been inhibited more completely than occurred at 22°, such as in the presence of 20 μg/ml actidione for 4 hr.

Similar phosphorylated compounds were found in normal cells, but the quantities were significantly less than in tumor cells, suggesting that the level of TdR kinase was lower in normal than tumor cells.

This study indicated that there was very little detectable intracellular pool of free TdR and that which was taken up in the acid-soluble pool was rapidly phosphorylated. This finding agrees with work previously reviewed (7).

TdR Kinase Levels in Normal Embryo and Tumor Cells. It is well known that enzymes related to DNA synthesis such as TdR kinase are elevated in cells productively infected with certain DNA viruses such as Py (2, 11, 14, 19, 21, 23, 33), SV40 (6, 18), vaccinia (17, 25), and herpesvirus (16, 26). TdR kinase has been shown to be increased in 3T3 (20) and WI38 cells (6) transformed by SV40 virus, but this phenomenon has not been described for Py-transformed hamster cells. However, it has been shown that Py fails to induce TdR kinase in the 3T3 cell line deficient in this enzyme during a productive cycle (2) as well as in BHK21 cells lacking the enzyme following neoplastic transformation (23). These findings indicate

**Chart 1. Time course of TdR⁻³H incorporation into the acid-soluble fraction of hamster embryo and tumor cells over short labeling period at 24°. TdR⁻³H, 1 μCi/ml with a specific activity of 3.5 μCi/mmole. Uptake expressed as cpm/mg protein.**
that the TdR kinase induced by Py during both the productive and neoplastic cycle is an enzyme coded for by the host genome and that its induction is not a necessary factor in the neoplastic event.

TdR kinase was assayed in nonconfluent hamster embryo cells and in several Py-transformed hamster cell lines. Table 1 shows that the level of TdR kinase was 5- to 10-fold higher in 3 tumor cell lines than in normal embryo cells, providing an explanation for the enhanced incorporation of TdR-1H into acid-soluble phosphorylated products of tumor cells.

**The Concentration Dependence of TdR Uptake.** The relationship between extracellular concentrations of TdR and the 5-min uptake into the acid-soluble fraction at room temperature (22° ± 1) in normal and tumor cells was then studied. Labeled 1 mM solutions of TdR were prepared by mixing TdR-1H of known specific activity with unlabeled TdR, and the mixture was further diluted to give appropriate concentrations of TdR of constant specific activity for uptake studies. The specific activity of each stock solution was measured in every experiment as well as the radioactivity in the acid-soluble fractions. The uptake (V) was expressed as μmoles of substrate/g protein/5 min.

In Chart 2, the uptake (V) of TdR is plotted against extracellular substrate concentrations (S) in normal embryo and tumor cells. The uptake of TdR in tumor cells shows a definite deviation from a straight line at low substrate concentrations with a nonsaturable portion at higher concentrations. The slope of the linear portion of the curve was measured, and this value was subtracted from the observed data to give a second curve which reached a plateau at an extracellular TdR concentration of 0.13 mM.

The uptake of TdR in normal embryo cells showed a slight deviation from the straight line at low substrate concentrations in a number of experiments, but was of a much different order of magnitude than that seen in tumor cells.

From the data presented in Chart 2, a partition coefficient for TdR was calculated based on known extracellular concentrations and the intracellular concentration calculated from the uptake data and cellular water volume. The latter was estimated by assuming that this was 0.8 of the total cell volume, which in turn was derived from observed cell diameters and total cell numbers. The partition ratio (internal:external) increased from approximately 1.5 at an extracellular concentration of 1 mM to 3.7 at 0.015 mM. At very low extracellular concentrations, i.e., 0.083 μM, the partition reached 30- to 50-fold, indicating that a labeled compound was being concentrated intracellularly.

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme activity (μmoles dTMP/μg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell lines</td>
<td></td>
</tr>
<tr>
<td>HTC-3049-3</td>
<td>6.70</td>
</tr>
<tr>
<td>HTC-3049-91TC</td>
<td>5.17</td>
</tr>
<tr>
<td>HTC-3049-176TC</td>
<td>9.50</td>
</tr>
<tr>
<td>Embryo cells</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Embryo cells were nonconfluent monolayers growing on plastic in order to be representative of actively proliferating log phase cells.
Table 2

<table>
<thead>
<tr>
<th>Specific TdR-'H uptake (cpm/mg protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>967</td>
</tr>
<tr>
<td>Thymine, 5 μM</td>
<td>856</td>
</tr>
<tr>
<td>TdR, 5 μM</td>
<td>210</td>
</tr>
<tr>
<td>TMP, 5 μM</td>
<td>248</td>
</tr>
<tr>
<td>Iododeoxyuridine, 5 μM</td>
<td>242</td>
</tr>
<tr>
<td>Deoxyuridine, 5 μM</td>
<td>853</td>
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<tr>
<td>Uridine, 5 μM</td>
<td>1023</td>
</tr>
<tr>
<td>dCMP, 5 μM</td>
<td>816</td>
</tr>
<tr>
<td>Control</td>
<td>—*</td>
</tr>
<tr>
<td>TdR, 10 μM</td>
<td>—</td>
</tr>
<tr>
<td>TdR, 1 μM</td>
<td>—</td>
</tr>
<tr>
<td>Iododeoxyuridine, 10 μM</td>
<td>—</td>
</tr>
<tr>
<td>Iododeoxyuridine, 1 μM</td>
<td>—</td>
</tr>
</tbody>
</table>

* Not done.

Inhibition of Thymidine Uptake by Structurally Related Compounds. The ability of structurally related compounds to inhibit the incorporation of TdR-'H was studied. Table 2 summarizes the inhibition data from several experiments on normal and tumor cells. It is apparent that in both cell types the uptake of TdR into the acid-soluble fraction could be inhibited only by structurally analogous compounds containing both the deoxyribose as well as the pyrimidine rings with either a methyl group, as in TdR, or a substitution, as in 1UdR, in the 5' position. These data provided evidence that, at low levels of TdR, the uptake mechanism was selective. The fact that concentrations of TdR or its analogs required to inhibit transport were more than 10-fold greater than the extracellular concentration of the TdR-'H presented to the cell for uptake would indicate that there was a significant contribution from a noninhibitable process such as passive diffusion.

Enhanced Uptake of Thymidine in Py-transformed Cells. The evidence presented thus far would indicate that the uptake of TdR into the acid-soluble pool of several tumor cell lines is quantitatively greater than in normal embryo cells and that TdR kinase activity was higher in tumor than normal cells. The characteristics of TdR uptake were then studied in a number of normal embryo cell preparations after short or prolonged periods of cultivation in vitro as well as repeatedly in several different Py-induced tumor cell lines.

In order to provide a basis on which the uptake of TdR by normal and tumor cells could be compared, advantage was taken of the fact that the uptake of 1-phenylalanine-'H in normal and tumor cells was similar (10). As shown in Chart 3, when the uptake of 1-phenylalanine-'H was plotted against total protein, the regression line for normal and tumor cells was identical, indicating that 1-phenylalanine uptake was directly proportional to the mass of cells tested, irrespective of cell type. In comparison, the relationship between total protein and TdR uptake is shown in Chart 4. It is apparent that the specific uptake of TdR in tumor cells was greater than normal cells, with that in an embryo cell line falling between the 2 cell groups. The line drawn in this figure merely indicates the separation between the normal and tumor cells. On this basis, it was then possible to normalize the uptake of TdR-'H in the 2 cell types to the uptake of 1-phenylalanine-'H performed on replicate cell preparations.

The observed distinction between 1-phenylalanine and
TdR uptake is further illustrated in Chart 5. While specific uptake of amino acid in normal and tumor cells was quantitatively similar, specific uptake of the nucleoside was significantly greater in tumor cells ($p < 0.01$).

Table 3 summarizes these results on several tumor cell lines, an uninfected continuous epithelioid cell line derived from normal hamster embryo cells and early passage hamster embryo cells. In this case, the specific uptake is calculated on the basis of both umoles substrate per mg protein and per $10^6$ cells. A ratio of specific TdR to L-phenylalanine uptake has also been calculated, providing a third basis on which to compare uptake rates in normal and tumor cells, since L-phenylalanine uptake was comparable in the 2 cell types. From these results, it is clear that the TdR to L-phenylalanine ratio is approximately 4-fold greater in tumor than in normal cells.

Enhanced TdR Uptake in Newly Transformed Hamster Embryo Cells. TdR uptake was also studied in embryo cells which had been infected by Py 25 days previously and had shown a rapid morphological transformation which was observed to begin 7 days after infection. Uninfected control cells carried in parallel appeared normal throughout. Control and infected cultures were heavy enough at 17 days postinfection to be passed to Petri dishes for assay of TdR-$^3$H uptake 25 days postinfection. At this early stage following infection and transformation by Py, the transformed cell cultures demonstrated an enhanced TdR uptake to a level characteristic of established tumor cell lines, while the uptake in uninfected control cultures remained low (Table 3).

A second line of Py-transformed hamster embryo cells, tested 106 days after infection, at a time when it was highly transplantable, demonstrated an enhanced TdR uptake (Table 3).

DISCUSSION

The experiments reported here examined initial aspects of the TdR uptake and utilization pathway, namely, membrane penetration and phosphorylation. As shown in Chart 1, the appearance of labeled substrate in a cell-associated acid-soluble fraction was rapid, the extent of accumulation being greater in tumor than in normal cells. Chromatography of the isotopically labeled compounds in the acid-soluble pool revealed the presence of phosphorylated nucleosides with no detectable free TdR as early as 1 min after exposure. Furthermore, TdR kinase levels were found to be significantly higher in tumor than in normal cells.

These studies document the rapidity and efficiency with which TdR enters the normal and neoplastic hamster cell through the membrane and is phosphorylated.
by TdR kinase, aspects of TdR metabolism which have
been reviewed extensively by Cleaver (7). Several find-
ings described here are of interest in relationship to
the fine details of control over TdR uptake. The first was
the correlation of TdR kinase levels in normal and Py-
transformed tumor cells with the rate and extent of TdR
incorporation into an acid-soluble fraction. This would
suggest that TdR kinase played a rate-determining role
in this salvage pathway.

The second was the characteristics of the uptake curve
in response to extracellular substrate concentration and,
finally, the ability of structurally related compounds to
inhibit TdR uptake. At low extracellular concentrations
in the case of tumor cells, small increments of TdR con-
centration resulted in proportionally larger quantities
entering the acid-soluble fraction, producing a nonlinear
uptake curve. At concentrations above 0.1 mM, a linear
portion of the curve from the experimental points, thus
mitigating subtraction of the uptake represented by the linear
relationship between uptake and extracellular concentra-
tion was found. In contrast, normal cells showed only a
slight shoulder at low concentrations associated with a
more shallow slope in the linear portion of the curve.

Shown in Chart 2 is a curve resulting from the arith-
metic subtraction of the uptake represented by the linear
portion of the curve from the experimental points, thus
describing a second curve which reached a maximum at
a concentration of 0.13 mM. This type of curve has been
found in other systems in which purine and pyrimidine
uptake has been studied (24, 30). This has been inter-
preted as representing facilitated diffusion at low concen-
trations and simple diffusion at higher concentrations.
Since there is evidence that the quantitative distinction
between TdR uptake in normal and tumor cells is due to
higher levels of TdR kinase in tumor cells, then the non-
linear portion of the uptake process cannot be interpreted
simply as being due to a facilitated diffusion process.

The role of TdR kinase in this complex phenomenon is
currently being investigated.

That a facilitated diffusion process for pyrimidines
exists in mammalian cells has been shown by studies of
deoxyctydine and cytosine arabinoside uptake in L1210
leukemia cells by Kessel and Shurin (15) and of TdR up-
take in Chinese hamster cells by Breslow and Goldsby
(3). The compounds studied by Kessel and Shurin were
not phosphorylated in the leukemia cell, thus avoiding
problems presented by the phosphorylation reaction.
They found that this facilitated process demonstrated a
rather low degree of stereospecificity in comparison with
that exhibited by TdR kinase (5). The spectrum of py-
rimidines and pyrimidine analogs which have been
shown in the present study to inhibit the uptake of TdR
in both normal and neoplastic hamster cells is quite nar-
row and is thus more similar to that of TdR kinase than
to that of the facilitated system in L1210 cells.

The ability of TMP to inhibit TdR-3H uptake in nor-
mal hamster cells but to be noninhibitory in tumor cells
(Table 2) is best explained by a deficiency of 5'-nucleo-
tidase in tumor cells, an enzyme which hydrolyzes TMP
to TdR, the active inhibitor. Studies to assay the content
of this enzyme as well as TdR phosphorylase in a variety
of hamster cells in culture are currently underway.

The evidence for a small but measurable ability to
concentrate isotopically labeled phosphorylated TdR
compounds in cells which increased as extracellular con-
centration decreased suggested that the phosphorylation
step could be considered a coupled reaction providing
the energy to convert the uptake to an active process.
The coupling of 2 reactions, 1 of which metabolizes the
transported substrate to a second compound, is rem-
imisent of sugar transport models in yeast (31, 35),
Escherichia coli (22), and Ehrlich ascites cells (32).
The findings reported here are of interest in view of the frequency with which DNA viruses either induce host cells to synthesize more TdR kinase (2) or to code for their own enzyme (16). Furthermore, as shown here and in previous work (6), tumor cells induced by both Py and SV40 viruses have increased TdR kinase levels. This enhanced enzyme activity in tumor cells is correlated with an increased capacity to take up TdR from the extracellular environment. From a teleological point of view, if TdR kinase played a direct controlling role in the uptake of TdR, this would give a selective advantage to both DNA viruses and neoplastic cells by providing a direct mechanism to ensure a supply of TdR via the salvage pathway at the low substrate concentrations which ordinarily exist in vivo and in vitro (7) and at which level the uptake process described here is most active. The interrelationship between the membrane transport system described by Breslow and Goldsby (3) and TdR kinase in the uptake of TdR into mammalian cells remains to be determined.

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REFERENCES

31. Rothstein, A., and Van Steveninck, J. Phosphate and Carboxyl...


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