Similar Characteristics of Folate Analogue Transport \textit{in Vitro} in Contrast to Varying Dihydrofolate Reductase Levels in Epithelial Cells at Different Stages of Maturation in Mouse Small Intestine$^1$

F. M. Sirotnak,$^2$ D. M. Moccio, and C. H. Yang

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

We describe studies of folate analogue transport in purified epithelial cell fractions isolated from mouse small intestine. Fractionation of these cells into immature proliferative and mature absorptive components and two components representative of intermediate stages of maturation was carried out by stepwise, nonenzymatic stripping of the everted organ. In contrast to the proliferative-specific enzyme markers, thymidine kinase and dihydrofolate reductase, folate analogue transport did not vary with the alteration in proliferative potential of these cells during maturation. The brush-border enzyme, alkaline phosphatase, was used as a positive marker for maturation. Initial influx of $[^3\text{H}]$aminopterin into both mature and immature cell fractions showed the same kinetics and did not exhibit pH dependence within the range of 6.0 to 7.8. A single saturable component ($K_{\text{m}} = 16 \pm 3 \mu\text{M}; V_{\text{max}} = 57 \pm 8 \text{ pmol/min/10}^7 \text{ cells}$) was delineated, with the same temperature dependence ($Q_{10} 27-37^\circ = 3.2 \pm 0.4$; Arrhenius constant = $11.1 \pm 3 \text{ kcal/mol}$) and same specificity for various folate compounds. Initial efflux of $[^3\text{H}]$aminopterin was also similar in both cell types. Efflux was first-order ($t_{1/2} = 1.1$ to $1.3$ min; $K_{\text{eff}} = 0.53 \pm 0.04 \text{ min}^{-1}$) and equal to the decay-time constant for approach to steady-state during accumulation of $[^3\text{H}]$aminopterin, but showed higher-temperature dependence ($Q_{10} 27-37^\circ = 6.7 \pm 0.8$; Arrhenius constant = $25.3 \pm 4 \text{ kcal/mol}$). Under the conditions used which did not allow polyglutamylation of $[^3\text{H}]$aminopterin, steady-state levels of accumulation of exchangeable drug at $37^\circ$ in each cell fraction were accounted for by the various kinetic parameters for each flux. At all concentrations of $[^3\text{H}]$aminopterin examined, both types of epithelial cells appeared to maintain a negative electrochemical gradient under physiological conditions. Overall, the data conform to a two-carrier model for folate analogue transport in which each flux is mediated by a separate system. However, specificity and saturability of influx for folate compounds, and inhibition of thisflux by various agents was markedly different from that reported for various tumor cells.

INTRODUCTION

Studies on the cellular pharmacokinetics of folate analogues, as with other anticancer agents, have for the most part focused (reviewed in Refs. 11 and 18) on target tumor cell populations and less so at the level of the normal proliferative tissues which are drug limiting. It would seem that the reasons for this paucity of studies of these normal tissues relate to their cellular heterogeneity (1, 4, 13, 14, 18) and difficulty in their isolation in a manner suitable for biochemical studies. For folate analogues like methotrexate, the villar crypt epithelium of small intestine is a major site of drug-limiting toxicity in animals and humans (14, 16, 28). The higher intracellular water to plasma ratio of methotrexate or other folate analogues maintained (20, 21) in responsive tumor cell populations compared to intestinal epithelium after therapy appears to reflect, at least to a major extent, the lower potential for inward transport of these agents in this normal cell compartment. We have derived data on folate analogue transport in epithelial cells isolated (4, 13) from the luminal lining of mouse small intestine. These isolated cell fractions were biochemically competent and data derived on folate analogue transport appeared to be consistent (9, 20, 21) with the corresponding pharmacokinetic data. However, only a portion (25 to 75%) of the cells isolated represented the proliferative fraction at which drug-limiting toxicity occurs. For this reason, some limitation must be placed on the interpretation of these results.

We now describe more definitive studies on the membrane transport of folate analogues in various fractions of epithelial cells isolated from mouse small intestine. Fractionation of these cells into immature proliferative and mature absorptive components was carried out by a modification of one (27) of a number of methods (2, 5, 25, 27) available in the literature originally applied to rat or hamster intestine.

MATERIALS AND METHODS

All folate compounds were prepared as the sodium salt in aqueous solution. The synthesis of 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin has been described earlier (7). All samples were >98% pure as determined (7) by UV spectroscopy and high-performance liquid chromatography. Samples of methotrexate and aminopterin were provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. $[^3\text{H}]$Methotrexate (specific activity, 20 Ci/mm mol), $[^3\text{H}]$Aminopterin (specific activity, 12 Ci/mm mol), and 10-$[^3\text{H}]$Deazaaminopterin (specific activity, 9 Ci/mm mol) were either purchased, or prepared by custom synthesis, from Moravek Biochemical, City of Industry, CA. These were repurified (3) prior to use and stored at $-70^\circ$. Radiochemical purity was >98%. All other chemicals were reagent grade.

Epithelial cell preparations from mouse small intestine were obtained by a modification of methods (27) originally applied to rat intestine. Male BALB/c mice (20 ± 2 g; S.E.) were obtained from Sprague-Dawley, Madison, WI, and were sacrificed by cervical dislocation and the small intestine surgically removed, cooled in cold (0°) buffered isosolate (0.14 M NaCl ± 0.01 M potassium phosphate, pH 7.3), everted over wooden applicator sticks, and washed thoroughly in cold buffered isosolate plus 0.1% BSA. Stepwise stripping of epithelial cells from everted intestines

$^1$Supported in part by Grants CA 08748, CA 18856, and CA 22764 from the National Cancer Institute, by Grant CH-26 from the American Cancer Society, and by the Elsa U. Pardee Foundation.

$^2$To whom requests for reprints should be addressed, at Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

Received April 10, 1984; accepted August 2, 1984.
was done (Method A) by incubation in Buffer A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, and 7 mM glucose, pH 7.3) plus 0.1% BSA for 10 min at 37° with gentle agitation. The cell suspension (Fraction I) was collected, cooled to 0°, centrifuged at 200 x g, and resuspended (2 to 3 x 10⁷ cells/ml) in cold (0°) transport buffer (107 mM NaCl, 20 mM Tris HCl, 26.2 mM NaHCO₃, 5.3 mM KCl, 1.9 mM CaCl₂, 1 mM MgCl₂, and 7 mM glucose, pH 7.4). The everted intestines were incubated at 37° again in Buffer A with 0.1% BSA for an additional 5 min with gentle agitation. The cells were cooled, centrifuged, and resuspended as before (Fraction II). The everted intestines were incubated at 37° for 10 min in Buffer B (phosphate-buffered saline plus 1 mM EDTA, 0.5 mM dithiothreitol, and 7 mM glucose, pH 7.3) plus 0.1% BSA with gentle agitation. Cells were cooled, centrifuged, and resuspended as before (Fraction III). Finally, everted intestines were incubated for another 10 min at 37° in Buffer B with agitation. These cells were collected and processed as before (Fraction IV). Isolation of epithelial cells was also carried out by enzymatic stripping with hyaluronidase (Method B) of everted intestine following a procedure (13) used during our previous studies (4). These cells were also collected by centrifugation and resuspended in cold (0°) transport buffer. All preparations and centrifugations were carried out in plastic containers. Harvesting of L1210 cell suspensions for limited use in these studies has been described in detail (15, 21, 23–25).

Transport experiments were carried out at varying temperatures by incubating cell suspensions in transport buffer with [³H]thymidine. These methods were described in detail earlier (19). This analogue was chosen as a model substrate for most of these studies, since the results of our earlier work (4) suggested that a much lower concentration range of the analogue would be required compared to methotrexate to demonstrate saturable entry. Even so, because of the high concentrations required in these experiments, and the large surface bound component of drug (18), it was not possible to use rapid sampling procedures (29) followed in our earlier studies (9, 15) of folate analogue transport with tumor cell suspensions. Instead, aliquots of cell suspensions were processed by rapid centrifugation at 0° following 20-fold dilution of the suspension with cold (0°) buffered isotonic saline (0.14 M NaCl plus 0.01 M potassium phosphate, pH 7.3) done twice. The cells were resuspended in buffered isotonic saline and cell number determined by an absorbance (A₄₅₀) measurement. An aliquot of this suspension was added to scintillation fluid and radioactivity counted by scintillation spectrophotometry. Procedures used to derive kinetic constants for influx (Kᵣ, Vₘₐₓ, Kᵢ) and efflux measurements have been described (18).

During transport experiments, cell suspensions were also made in [carboxy-¹⁴C]inulin and centrifuged in order to determine the volume of intracellular water derived in this manner. Values for intracellular water derived in this manner were in agreement with values also determined from wet and dry weights according to the method followed in our earlier (4) studies. These methods were described in detail earlier (18) from this laboratory. The procedure used for determining intracellular water of tumor cell suspensions. Instead, aliquots of cell suspensions were processed by rapid centrifugation at 0° following 20-fold dilution of the suspension with cold (0°) buffered isotonic saline (0.14 M NaCl plus 0.01 M potassium phosphate, pH 7.3) done twice. The cells were resuspended in buffered isotonic saline and cell number determined by an absorbance (A₄₅₀) measurement. An aliquot of this suspension was added to scintillation fluid and radioactivity counted by scintillation spectrophotometry. Procedures used to derive kinetic constants for influx (Kᵣ, Vₘₐₓ, Kᵢ) and efflux measurements have been described (18).

Analysis for polyglutamates of [³H]thymidine in other folate analogues was carried out (3) by reverse-phase, high-performance liquid chromatography. The incorporation of [³H]thymidine into DNA of intestinal epithelial cells was determined by standardized procedures reported earlier (18) from this laboratory. The procedure used for determining intracellular chloride was also described elsewhere (6). The fraction of nonexchangeable chloride (<3%) was determined by prolonged incubation of cells in 20 mM N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid/225 mM sucrose adjusted with KOH to pH 7.4 prior to the chloride measurement.

RESULTS

Separation of Proliferative and Nonproliferative Epithelial Cell Fractions. The method followed in this study for the isolation and fractionation of intestinal epithelial cells is a modification of that originally used (25, 27) for rat small intestine by others. In accordance with the method followed in our earlier (4) studies, we exposed everted intestine to isolation buffer containing both 0.1% BSA and 7 mM glucose. The former appears to reduce the extent of cell disruption, and the latter would be expected to prevent (9) the depletion of cellular ATP pools, which may be important to membrane transport. This sequence of fractionation was derived empirically by a trial-and-error process in which the morphological properties of the isolated cells was monitored microscopically.

The intestinal epithelial cells were isolated in 4 separate fractions using the procedure (Method A) described above. The cellular content of these fractions is summarized in Table 1 for a series of individual separations carried out on different days. A total of approximately 15 x 10⁷ cells were isolated per mouse intestine. In Fraction I, which contained the least number of cells, the proportion of mature (columnar) to proliferative (noncolumnar) cells were approximately 11:1. In Fraction IV, which also contained a relatively small number of cells, the proportion of the same cell types was reversed and was approximately 1:20. Photomicrographs of cells from each fraction are shown in Fig. 1. Most cells in Fraction I have a distinct columnar appearance characteristic of mature absorptive cells. Most cells in Fraction IV are smaller, less dense, spherical, and often appear in aggregates. These are characteristic of the undifferentiated crypt cell (proliferative stem cells) compartment. Both Fractions II and III, which contained most of the cells isolated, showed proportions of each cell type which were intermediate between that obtained in Fractions I and IV. Using mice given injections of [³H]thymidine prior to intestinal epithelial cell isolation and fractionation, it can be seen (Table 1) that the amount of radioactivity recovered in DNA was least in Fraction I cells and increased with the isolation of each additional fraction. Epithelial cells were also isolated using hyaluronidase (Method B). This procedure strips the majority (4) of the epithelial cells from the everted organ. Approximately 20 x 10⁷ cells/intestine were obtained by this procedure, and the proportion of columnar to noncolumnar cells (1:4) obtained was similar to that found (14) in the intact organ. The radioactivity in DNA of this cell population was intermediate to that obtained in Fractions I and II.

Other properties of these epithelial cell fractions are shown in Chart 1. Here, it can be seen that activity of the absorptive "brush-border" marker, alkaline phosphatase, is highest in Fraction I cells and lowest in Fraction IV cells, while the activity in the case of the "proliferation" marker, thymidine kinase, is lowest in Fraction I cells and highest in Fraction IV cells. Both enzyme activities occur at intermediate levels in Fractions II and III. It can be observed (Table 1) that the fraction of mature cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells and the percentage of noncolumnar cells were intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II and III. The fraction of noncolumnar cells was highest in Fraction I and lowest in Fraction IV. The fraction of noncolumnar cells was intermediate for Fractions II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mean ± S.E.</th>
<th>% of morphology</th>
<th>[³H]Thymidine incorporation (cpm x 10⁷)</th>
<th>Total no. of cells/ intestine (x 10⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.34 ± 0.6*</td>
<td>89-93</td>
<td>7-11</td>
<td>5.82 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>4.42 ± 0.9</td>
<td>75-81</td>
<td>19-25</td>
<td>14.7 ± 3</td>
</tr>
<tr>
<td>II</td>
<td>5.88 ± 1.8</td>
<td>34-42</td>
<td>58-66</td>
<td>34.6 ± 5</td>
</tr>
<tr>
<td>IV</td>
<td>3.12 ± 0.8</td>
<td>3-8</td>
<td>92-97</td>
<td>100.4 ± 8</td>
</tr>
<tr>
<td>B</td>
<td>19.8 ± 3.8</td>
<td>77-85</td>
<td>15-23</td>
<td>8.4 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± S.E.; average of 5 individual determinations.
also be seen that the variation in thymidine kinase activity among the 4 fractions closely parallels the extent of tautomeric incorporation into intestinal DNA found in each cellular fraction obtained from mice pretreated with $^3H$thymidine. Although the variation in the 3 markers examined among these 4 fractions almost certainly reflects the proportion of columnar versus noncolumnar cells present, it probably also reflects the relative proliferative potential of the noncolumnar cells present in each fraction. The increase observed in thymidine kinase activity and $^3H$thymidine incorporation into DNA between Fractions III and IV exceeds substantially the differential in noncolumnar cells present in each fraction. However, the sequence of fractions as obtained by the procedure followed here probably does not define a precise gradient of cells from villus tip, to lower villus, and crypt cell areas.

**Measurements of Membrane Potential in Isolated Intestinal Epithelial Cells.** Since folate compounds are divalent anions, a meaningful interpretation of data on their transport in epithelial cell fractions requires (8-11, 18) information on membrane potential. These measurements were made for cells in each of the 4 fractions and found to be the same. Data obtained for Fractions I and IV are shown in Table 2. Parallel measurements of intracellular water and chloride were made on each group of cells. These data were then used to calculate the Nernst potential (8-11, 18) of the cell membrane, making the assumption that most, if not all, of the chloride is freely exchangeable. The values derived for membrane potential varied less than 10% (-10.5 to -11.3 mV) and were comparable to those we and others derived earlier (8-11, 18) for L1210 cells by the same procedure.

**Measurement of Folate Analogue Influx and Accumulation in Isolated Intestinal Epithelial Cells.** Time courses for intracellular accumulation of $^3H$aminopterin by Fraction I and Fraction IV intestinal epithelial cells. Cells were incubated in transport buffer (2 x 10⁸ cells/ml) with 20 μM $^3H$aminopterin and aliquots removed at varying intervals. The data shown are total accumulation corrected for a surface bound component delineated by a 10-sec incubation at 0° or by back-extrapolation of the time courses for total accumulation to the vertical axis. Other details are given in the text. FAH, dihydrofolate; arrow, dihydrofolate reductase content. Average of 4 separate experiments. S.E. of the mean did not exceed ±15%.

**Table 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No. of cells/g dry wt</th>
<th>Cell H₂O (ml H₂O/g dry wt)</th>
<th>Cell chloride (mg/g dry wt)</th>
<th>$V_m$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.41 ± 0.4 x 10⁶</td>
<td>3.62 ± 0.4</td>
<td>240 ± 28</td>
<td>-10.5</td>
</tr>
<tr>
<td>IV</td>
<td>3.26 ± 0.4 x 10⁶</td>
<td>4.29 ± 0.5</td>
<td>285 ± 34</td>
<td>-11.3</td>
</tr>
</tbody>
</table>

*Calculated from the Nernst equation (9, 18):

$$\frac{V_m}{nF} = \frac{RT}{Z_nF} = -26.7 \text{ mV}$$

b Mean ± S.E. of 3 determinations.

Chart 2. Time course for intracellular $^3H$aminopterin accumulation at 0° and 37° by Fraction I and Fraction IV intestinal epithelial cells. Cells were incubated in transport buffer (2 x 10⁸ cells/ml) with 20 μM $^3H$aminopterin and aliquots removed at varying intervals. The data shown are total accumulation corrected for a surface bound component delineated by a 10-sec incubation at 0° or by back-extrapolation of the time courses for total accumulation to the vertical axis. Other details are given in the text. FAH, dihydrofolate; arrow, dihydrofolate reductase content. Average of 4 separate experiments. S.E. of the mean did not exceed ±15%.

**Chart 1**

Biochemical properties of various fractions of epithelial cells isolated from everted mouse small intestine. Details of the cell isolation procedure and enzyme determinations are given in the text. Average of 4 separate determinations. S.E. of the mean did not exceed ±12%.

**Properties of mouse intestinal epithelial cells**

Epithelial cells were resuspended in transport buffer, and determinations for wet and dry weights and intracellular and chloride were carried out in a manner described in the text. Cell number was determined microscopically.

**Cancer Research**

5206

CANCER RESEARCH VOL. 44

F. M. Sirotnak et al.

**Chart 1**

Biochemical properties of various fractions of epithelial cells isolated from everted mouse small intestine. Details of the cell isolation procedure and enzyme determinations are given in the text. Average of 4 separate determinations. S.E. of the mean did not exceed ±12%.
Folate Analogue Transport in Epithelial Cells

Table 3
Dihydrofolate reductase levels and folate analogue transport in epithelial cell fractions from mouse small intestine

For determinations of dihydrofolate reductase levels, a known number of cells was resuspended in 0.05 M Tris HCI (pH 7.4), disrupted by sonicatkxi and supernatant collected after centrifugation. Extracts were treated with [3H]aminopterin at pH 6.2 and dialyzed for 24 hr. Retained radioactivity was a measure of enzyme content. For transport experiments, cells were resuspended in transport buffer (pH 7.4) and incubated at 37° or 0° with varying [3H]aminopterin concentrations. Data on initial velocity of influx were analyzed by conventional kinetic methods (18). Additional details are given in the text for the experiment and the processing of samples.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dihydrofolate reductase (pmol/10⁷ cells)</th>
<th>Vmax (pmol/min/10⁷ cells)</th>
<th>Km (µM)</th>
<th>Vmax (pmol/min/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.41 ± 0.9 ^a</td>
<td>34.2 ± 4</td>
<td>15.8 ± 3</td>
<td>58.5 ± 7</td>
</tr>
<tr>
<td>II</td>
<td>5.45 ± 1.4</td>
<td>31.8 ± 5</td>
<td>14.9 ± 2</td>
<td>3.65 ± 0.6</td>
</tr>
<tr>
<td>III</td>
<td>7.54 ± 1.3</td>
<td>36.5 ± 6</td>
<td>15.2 ± 3</td>
<td>3.97 ± 0.7</td>
</tr>
<tr>
<td>IV</td>
<td>12.3 ± 2.5</td>
<td>58.5 ± 7</td>
<td>15.6 ± 3</td>
<td>6.35 ± 0.6</td>
</tr>
</tbody>
</table>

^a [3H]Aminopterin = 20 µM.

Mean ± S.E.; average of 5 individual determinations.

Data are also shown in Table 3 for the various kinetic parameters of [3H]aminopterin influx measured during incubation of various cell fractions with this analogue. These data were derived from the results of experiments measuring intracellular accumulation of drug like that shown for Fraction IV cells in Chart 3. Influx of drug was measured at various intervals with the first min of incubation at 37° with varying concentrations of [3H]aminopterin. Since uptake is constant with time during this period, a valid kinetic analysis of initial influx was possible. A double-reciprocal (velocity/concentration) plot of these data is presented in Chart 4. A similar plot of the measurements made at 0° with Fraction IV cells are also shown in this chart. In each case, only a single saturable component of influx could be delineated. The values for influx Km derived at each temperature (Table 3) were the same, while values for influx Vmax were about 15-fold lower at 0°. The same results were also derived from the same experiments (data not shown) done with Fraction I cells (Table 3). Although kinetic analysis was not carried out with cells from Fractions II and III, the same values would be expected by inference from the values for initial influx velocities obtained. Also, additional components of mediated influx with low saturability may contribute to total influx. However, at much higher concentrations of [3H]aminopterin, which would be required to delineate these components, passive diffusion of this analogue becomes (18) an appreciable complication and would prevent this determination of multiplicity. In contrast to the temperature dependence observed, influx of [3H]aminopterin did not vary with pH over a range of 6.0 to 7.8.

Another analysis of the data in Chart 3 also show that intracellular accumulation of [3H]aminopterin in Fraction IV cells at each concentration is characterized by the same exponent. From a decay time analysis (8) of these data and other data (not shown) for Fraction I cells, we derived values for t½ varying only between 1.1 and 1.3 min. From these data, values for the corresponding rate constant were calculated and found to vary between a range of 0.46 to 0.53 min⁻¹ for each cell fraction incubated at these extracellular [3H]aminopterin concentrations.

Measurements of Folate Analogue Efflux. Epithelial cells from Fractions I and IV were loaded with [3H]aminopterin by 20-min incubation at 0° with 20 µM of the drug. The data in Chart 5 show the efflux time-course following washing and resuspension of cells in drug-free transport buffer. Efflux of drug from cells of each fraction at 37° was rapid and showed the same time course.
inset), we show that efflux at 37° was first order (f% = 1.1 to 1.4 for a period of 10 sec, showing that drug associating with cells was also of interest to note that numerical values for the efflux rate constant (0.49 to 0.58 min⁻¹) derived at 37° with each cell fraction was the same as that derived for the time constant for intracellular [3H]aminopterin was delineated in each cell which is saturable. Saturability was in the decreasing order [3H]aminopterin, 10-[3H]deazaaminopterin, [3H]methotrexate, and 10-[3H]-ethyl-10-deazaaminopterin. [3H]Aminopterin influx was competively inhibited by each of these analogues and by the naturally occurring folate compounds, folic acid, and 5-formyltetrahydrofolate. However, in the case of the natural folates, inhibition was much weaker, and values for Ks derived were substantially greater. Similar results (data not shown) were obtained in other experiments done with Fraction I cells.

Temperature Dependence for [3H]Aminopterin Influx and Efflux. In view of the difference seen in [3H]aminopterin influx and efflux at 37°, it was of interest to obtain more quantitative information on the dependence of each flux on temperature. The extent of initial flux in each direction was determined in Fraction I and IV cells at a variety of temperatures. These results showed that efflux was more sensitive to change in temperature than was influx. This differential in sensitivity was expressed as a 2-fold difference above the liquid-gel transition temperature in values derived for each flux for temperature coefficients (Q10) of 3.2 ± 0.4 for influx and 6.7 ± 0.8 for efflux) and activation energy (Arrhenius constant = 11.3 ± 3 kcal/mol for influx and 25.3 ± 4 kcal/mol for efflux). Similar data were also derived for Fraction I cells.

Steady-State Kinetics for Intracellular [3H]Aminopterin Accumulation. Experimental values for steady-state levels of intracellular exchangeable [3H]aminopterin for Fraction IV cells were obtained from data of the sort shown in Chart 5. By subtracting the value for enzyme bound drug (nonexchangeable component delineated from the time courses shown in Chart 5) from the value for total intracellular drug, values for exchangeable level of drug at steady-state were obtained. These are shown in Chart 6. The plot (solid line) drawn in this chart was not derived from the data points themselves, but was obtained from the calculated values for steady state derived for each external concentration by means of the following empirical equation:

\[
[dru g]_{sw} = \frac{V_{max} ([drug]_e)}{(K_m + [drug]_e)K_B}
\]

Having used values for influx Km and Vmax and efflux constant (K_B) obtained independently during the experiments described above, we note that this equation provides a good description of the data for [drug]sw which vary over a 20-fold range of [3H]aminopterin concentrations; i.e., no new parameters ap-
the results of these experiments given in Table 5, we note
analogue influx in various mammalian cells (10,11,18, 24). For
the effect of various agents found to be inhibitors of folate
transport in the pharmacokinetic behavior of folate analogues (18, 20,
21) in this drug-limiting compartment following therapy with these
agents. However, there were some differences between the
numerical values for the various transport parameters derived in
these studies compared to our earlier studies (4) which now
appear to be required to account for the experimental results.
It is of additional interest from the aspect of the energetics,
which might be involved, to observe that a large negative gradient
of [3H]aminopterin occurred across the cell membrane. This
differs appreciably from that expected for a passively distributed
ion of similar charge. Assuming that this folate analogue behaves
as a negatively charged divalent ion, the expected distribution
ratio can be calculated from the Nernst equation (8-11, 18).
Values obtained using measured values for chloride distribution
ratio and intracellular water in Fraction IV cells (Table 2) are
represented by the dashed line in Chart 6. Similar results (data
not shown) were derived for epithelial cells from Fraction I.

Some Comparative Studies of [3H]Aminopterin Influx in
Fraction IV Epithelial Cells and L1210 Cells. We examined
initial influx of [3H]aminopterin in Fraction IV epithelial cells for
the effect of various agents found to be inhibitors of folate
analogue influx in various mammalian cells (10, 11, 18, 24). For
the purposes of comparison, we also examined the effect of
these agents on influx of [3H]aminopterin in L1210 cells. From
the results of these experiments given in Table 5, we note
substantial differences in the pattern of sensitivity to these
agents in each cell type. Inhibition of influx of this folate analogue
in epithelial cells by chloromercuribenzene-sulphonate was less and
by 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid,
and probenecid was substantially less than of influx in L1210 cells.
However, influx of this analogue in each cell type was inhibited
by a similar extent by 4,4'-diisothiocyanato-2,2'-disulphonic acid
stilbene.

DISCUSSION

The procedure followed during these studies for isolating
mouse intestinal epithelial cells appears to be highly effective for
the separation of the mature absorptive fraction and the highly
proliferative, immature crypt-cell fraction. Both cell types re-
mained intact, were biochemically active, and were competent
for folate analogue transport. Each fraction of cells showed the
morphological characteristics expected for mature and immature
intestinal epithelium. We also found that dihydrofolate reductase,
the intracellular target for folate analogues, was proliferation-
specific and, as in the case of thymidine kinase, was a negative
marker for intestinal epithelial cell maturation. Alkaline phospha-
tase, on the other hand, was a positive marker for maturation
similar to that shown for rat intestine (27). Of interest was the
finding that none of the parameters examined for mediated folate
analogue transport varied between mature and immature (prolif-
erative) cell fractions, suggesting equal potential for accumulation
of these compounds in all of the cells which comprise the
epithelial cell compartment in mouse small intestine. Valid mea-
surements of accumulation and loss of these substrates neces-
sary for adequate kinetic analysis of data on flux and steady-
state were possible, since the transport buffer did not allow
conversion to a polyglutamate.

Actual levels of dihydrofolate reductase measured in the
various epithelial cell fractions would, to some extent, be expected
to reflect different stages in the maturation process itself. How-
ever, one must also expect that these levels reflect the cross-
contamination of mature and immature cellular fractions with cell
types at opposite ends of the developmental spectrum. There-
fore, the differential in these levels and of the other enzymes
examined between Fraction I and Fraction IV cells is probably
underestimated by as much as 2-fold, and the pattern in the shift
of this enzyme activity during development can only be approxi-
mated. Still, these appear to be similar to those reported in earlier
studies (2, 25, 27) in other rodents.

The results of our present studies would appear to provide a
less ambiguous basis for evaluating the role of membrane trans-
port in the pharmacokinetic behavior of folate analogues (18, 20,
21) in this drug-limiting compartment following therapy with these
agents. However, there were some differences between the
numerical values for the various transport parameters derived in
these studies compared to our earlier studies (4) which now
appear to be unrelated to the heterogeneous nature of the
epithelial cell suspensions used at that time. Values for influx
V_{max} and efflux reported for folate analogues in our earlier studies
(4) were substantially lower (8- to 10-fold lower on a dry weight
basis) than those derived in the current studies. Although these
differences are still under investigation, it is possible that the
cells isolated in our earlier studies (4) were energetically depleted,
since the isolation of these cells was done by prolonged incu-
bation at 37° with a hydrolytic enzyme in an isolation buffer

---

The text continues with further details and results, including a table summarizing the effect of various agents on the influx of [3H]aminopterin into intestinal epithelial cells and L1210 cells.

Table 5

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K_{in} of epithelial cells (uM)</th>
<th>K_{in} of L1210 cells (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCMBSS</td>
<td>108 ± 18</td>
<td>42.2 ± 8</td>
</tr>
<tr>
<td>DIDS</td>
<td>23 ± 4</td>
<td>20.8 ± 2.3</td>
</tr>
<tr>
<td>SITS</td>
<td>&gt;500</td>
<td>24.3 ± 3.6</td>
</tr>
<tr>
<td>Probenecid</td>
<td>&gt;20,000</td>
<td>1,350 ± 270</td>
</tr>
</tbody>
</table>

* PCMBSS, parachloromercuribenzoate sulfonate; DIDS, 4,4'-disothio-cyano-2,2'-disulphonic acid stilbene; SITS, 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulphonic acid.

** Mean ± S.E.

---

The text further discusses the implications of these findings for understanding folate transport in the intestinal tract and the role of dihydrofolate reductase as a marker for cell maturation.
without D-glucose. Although values for influx $K_m$ derived with various folate analogues here were somewhat different than those derived in some (19), but not all (4), of our earlier studies, the relative magnitude of these values was always in the same order, i.e., aminopterin < 10-deaza-aminopterin < methotrexate < 10-ethyl-10-deaza-aminopterin. Finally, it should also be mentioned, that the substantially higher values for influx $V_{max}$ measured with epithelial cell preparations in the present study compared to those values reported for tumor cells (4, 11, 15, 21, 24) are more consistent with pharmacokinetic data derived from our in vivo studies (18, 20, 21) with the corresponding tumor model showing extremely rapid entry of folate analogues, initially, into mouse small intestine following therapy.

Folate analogue transport in these epithelial cell fractions shows some similarity in properties to those which (reviewed in Refs. 10, 15, and 18) have been observed in murine and human tumor cells. However, although initial influx was saturable, values for both influx $K_m$ and influx $V_{max}$ were markedly higher than those reported (reviewed in Refs. 10, 15, and 18) for tumor cells. Also, substantial differences compared to tumor cells (10, 11, 18, 19, 30) were seen in the effect on folate analogue transport of natural folate compounds and various chemical and pharmacological agents. Despite the rapidity with which folate analogues are accumulated intracellularly, net accumulation was extremely low. This can be attributed to the efflux of these analogues which is even more rapid than that documented for tumor cells (9) under physiological conditions. At all of the concentrations of $[\text{H}]$aminopterin used, a pronounced negative electrochemical gradient was maintained. By contrast, in L1210 leukemia cells, a positive electrochemical gradient is maintained (8, 9) at low concentrations of folate analogue, and it is only at higher concentrations that a negative gradient is observed.

As in the case of L1210 cells, it appears that net accumulation of folate analogues in these epithelial cell fractions conforms to a model (8, 9) in which influx and efflux are mediated by entirely different systems. This serves to explain the equivalences of the influx and efflux rate constants and the independence of the former on external concentration. Both properties are among the criteria for the 2-carrier model proposed for L1210 cells (8, 9). It is interesting to note that efflux of these analogues, but not influx, in epithelial cells shows the same temperature-dependence of this flux in L1210 cells (10, 11, 22). Because of the lower temperature-dependence of influx, a condition of prolonged unidirectional influx was obtained by incubation of epithelial cells with $[\text{H}]$aminopterin at 0°C. This is consistent with the idea of a separate energy-driven efflux pump which was proposed earlier (9) for L1210 cells.

Other questions which arise pertain to the possible relationship between the system mediating folate analogue influx and the absorptive mechanism for folate compounds in intestinal epithelial cells. In studies in rats with everted duodenal sacs, a saturable system, which was both pH- and sodium-dependent, was demonstrated (17, 26) with $K_m$ values at pH 6.0 for methotrexate and 5-methyltetrahydrofolate in the range of 2 to 5 μM. However, at pH 7.4, transport of methotrexate occurred at a very reduced rate and was nonsaturating. These results contrast with the lack of pH dependence shown in the present studies. Assuming that the properties of the absorptive mechanism would be similar in mice and rats, this result and the poorly saturable nature of influx in our studies would argue against the notion that the absorptive process was involved in influx of these folate analogues. Also, it would be difficult to explain the similarity of influx in mature and immature cell fractions, since the specialized absorptive mechanism (brush border) exists only in mature cells. Further work is continuing in our laboratory addressing these questions.

REFERENCES

Folate Analogue Transport in Epithelial Cells


Fig. 1. Phase-contrast photomicrographs of Fraction I and Fraction IV intestinal epithelial cells. × 400.
Similar Characteristics of Folate Analogue Transport \textit{in Vitro} in Contrast to Varying Dihydrofolate Reductase Levels in Epithelial Cells at Different Stages of Maturation in Mouse Small Intestine

F. M. Sirotnak, D. M. Moccio and C. H. Yang


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/44/11/5204