Similar Characteristics of Folate Analogue Transport in Vitro in Contrast to Varying Dihydrofolate Reductase Levels in Epithelial Cells at Different Stages of Maturation in Mouse Small Intestine

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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 \(^{3}H\) 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 (\(K_{m} = 16 \pm 3 \mu M; V_{\max} = 57 \pm 8 \text{ pmol/min/10}^7 \text{ cells} \)) was delineated, with the same temperature dependence (\(Q_{10} 27-37^\circ = 3.2 \pm 0.4 \); Arrhenius constant = 11.1 \pm 3 kcal/mol) and the same specificity for various folate compounds. Initial efflux of \(^{3}H\) aminopterin was also similar in both cell types. Efflux was first-order (\(t_{1/2} 37^\circ = 1.1 \text{ to } 1.3 \text{ min} ; k_{37} = 0.53 \pm 0.04 \text{ min}^{-1}\)) and equal to the decay-time constant for approach to steady-state during accumulation of \(^{3}H\) aminopterin, but showed higher-temperature dependence (\(Q_{10} 27-37^\circ = 6.7 \pm 0.6 \); Arrhenius constant = 25.3 \pm 4 kcal/mol). Under the conditions used which did not account for polyglutamation of \(^{3}H\) 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 \(^{3}H\) 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 this influx 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. \(^{3}H\) Methotrexate (specific activity, 20 Ci/mmole), \(^{3}H\) aminopterin (specific activity, 12 Ci/mmole), and 10-\(^{3}H\) deazaaminopterin (specific activity, 9 Ci/mmole) 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\%. 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 BDF\(_2\), 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\(^\circ\)) buffered isosoline (0.14 M NaCl ± 0.01 M potassium phosphate, pH 7.3), everted over wooden applicator sticks, and washed thoroughly in cold buffered isosolane plus 0.1% BSA. Stepwise stripping of epithelial cells from everted intestines.

1 The abbreviation used is: BSA, bovine serum albumin.
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 × g, and resuspended (2 to 3 × 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° 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. The 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 [³⁵S]aminopterin. 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 plus 0.1% BSA and 7 mM glucose. The former appears to reduce the osmotic gradient so that originally used (25, 27) for rat small intestine by others. In this study, however, the procedure was modified by using hyaluronidase (Method B). This procedure strips the majority (4) of the epithelial cells from the everted organ. Approximately 20 × 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.

Table 1

| Characteristics of isolated intestinal epithelial cells from mouse small intestine |
|---------------------------------|-----------------|-----------------|-----------------|
| Epithelial cells were stripped from everted small intestine in a stepwise manner by short periods of incubation in Buffer A and Buffer B. Cells were collected by centrifugation after each incubation period and resuspended in either 0.05 mM Tris HCl, 0.14 mM NaCl plus 0.01 mM potassium phosphate, or transport buffer, all at pH 7.4. Cell counting and morphological classification were done microscopically. Other experimental details are given in the text. |

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 agreement 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 × 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 cell) 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 [³²P]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 × 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
also be seen that the variation in thymidine kinase activity among the 4 fractions closely parallels the extent of tritium 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 carried out in a manner described in the text. Average of 4 separate determinations. S.E. of the mean did not exceed ±12%.

**Table 2. Properties of mouse intestinal epithelial cells.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No. of cells/g dry wt</th>
<th>Cell H$_2$O (ml/g dry wt)</th>
<th>Cell chloride (mg/g dry wt)</th>
<th>V, (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.41 ± 0.4 x 10$^6$</td>
<td>3.62 ± 0.4</td>
<td>240 ± 28</td>
<td>-10.5</td>
</tr>
<tr>
<td>IV</td>
<td>3.28 ± 0.4 x 10$^6$</td>
<td>4.29 ± 0.5</td>
<td>285 ± 34</td>
<td>-11.3</td>
</tr>
</tbody>
</table>

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

$$C_{mem} = 138 \text{ mV}, TR/Z_{ref}F = -26.7 \text{ mV}$$

*b* Mean ± S.E. of 3 determinations.

A similar time course is shown in Chart 2. Time courses 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 10$^7$ cells/ml) with 20 mU $[^3H]$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 reductase. Arrow, dihydrofolate reductase content. Average of 4 separate experiments. S.E. of the mean did not exceed ±12%.
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 sonication 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>Influx (37°)</th>
<th>Influx (0°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vmax (pmol/min/10⁷ cells)</td>
<td>Km (µM)</td>
</tr>
<tr>
<td>I</td>
<td>3.41 ± 0.9*</td>
<td>15.8 ± 3</td>
<td>14.9 ± 2</td>
</tr>
<tr>
<td>II</td>
<td>5.45 ± 1.1</td>
<td>31.8 ± 5</td>
<td>3.65 ± 0.6</td>
</tr>
<tr>
<td>III</td>
<td>7.54 ± 1.3</td>
<td>36.5 ± 6</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12.3 ± 2.5</td>
<td>16.3 ± 4</td>
<td>15.2 ± 3</td>
</tr>
</tbody>
</table>

*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 is 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 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 satura- bility 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.

Other 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.

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.
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Inset), we show that efflux at 37° was first order (r^2 = 1.1 to 1.4 for a period of 10 sec, showing that drug associating with cells for 4 separate experiments. S.E. of the mean did not exceed ±14%.

Inset, decay-time analysis of the efflux time course for Fraction I and IV cells. FAH2, fractions were the same as that derived for the time constant for [3H]aminopterin from these cells could be measured (data not shown). However, following prolonged incubation at 0°, loss of exchangeable drug (see Chart 5, panel 1), 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 coefficient (Q10). The data for values of [drug]ext which vary over a 20-fold range was obtained from the calculated drug at steady-state were obtained. These are shown in Chart 5. 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:

\[ \frac{[\text{drug}]_{\text{ext}}}{[\text{drug}]_{\text{in}}} = \frac{V_{\text{max}}}{K_m + [\text{drug}]_{\text{e}}} \]

Having used values for influx K_m and V_max and efflux constant K_e obtained independently during the experiments described above, we note that this equation provides a good description of the data for values of [drug]_{\text{e}} which vary over a 20-fold range of [H]aminopterin concentrations; i.e., no new parameters ap-
the results of these experiments given in Table 5, we note these agents on influx of $[^{3}H]$aminopterin in L1210 cells. From analogue influx in various mammalian cells (10,11,18, 24). For the effect of various agents found to be inhibitors of folate not shown) were derived for epithelial cells from Fraction I. represented by the dashed line in Chart 6. Similar results (data ratio and intracellular water in Fraction IV cells (Table 2) are Values obtained using measured values for chloride distribution as a negatively charged divalent ion, the expected distribution differs appreciably from that expected for a passively distributed potential for $[^{3}H]$aminopterin. Average of 4 separate experiments. S.E. of the mean did not exceed ±15%.

It is of additional interest from the aspect of the energetics, which might be involved, to observe that a large negative gradient of $[^{3}H]$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 $[^{3}H]$Aminopterin Influx in Fraction IV Epithelial Cells and L1210 Cells. We examined initial influx of $[^{3}H]$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 $[^{3}H]$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 chloromercuribenzoate was less and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic 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 remained 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 phosphatase, 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 (proliferative) 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 measurements of accumulation and loss of these substrates necessary 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. However, 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. Therefore, the differential in these levels and of the other enzymes examined between Fraction I and Fraction IV cells is probably understated by as much as 2-fold, and the pattern in the shift of this enzyme activity during development can only be approximated. 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 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 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 incubation at 37° with a hydrolytic enzyme in an isolation buffer.
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 recorded (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 [14C]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 equivalence 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 [14C]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


Fig. 1. Phase-contrast photomicrographs of Fraction I and Fraction IV intestinal epithelial cells, x 400.
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