ABSTRACT

The transport characteristics of folate compounds in a methotrexate-resistant L1210/R81 cell line were compared with parental cells. Concentrations for half-maximal growth of the resistant cells with folate (350 μM) and 5-formyltetrahydrofolate (20 μM) were found to be higher by 2.7-fold and 20-fold, respectively, relative to the parent, suggesting that changes had occurred in the transport of these folate compounds. Transport measurements revealed that the resistant cells have lost the capacity to transport methotrexate and other folate compounds via the reduced-folate transport system but that a second previously undescribed transport system is present. Uptake of folate via this second route is energy dependent, exhibits saturation kinetics, can be inhibited by substrate analogues, and is activated by a reduction in pH. The pH effect is substantial since uptake at 5.0 μM folate can be increased 10-fold by decreasing the pH from 7.4 to 6.2. The observed Kₘ values for methotrexate, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate were 10.2, 3.2, and 2.7 μM, respectively. Transit activity was not affected by depleting internal stores of folate by growth in folate-free medium, and this same transport system was also present in comparable amounts in wild-type cells. In the latter case, interfering uptake via the reduced-folate transport system was blocked by bromosulfophthalein and thiamine pyrophosphate. Methotrexate and 5-formyltetrahydrofolate were also transported via this alternative, pH-dependent route, whereas little or no uptake of 5-methyltetrahydrofolate could be detected. The results indicate that antifolate resistance in L1210/R81 cells was induced by inactivating the reduced-folate transport system and by utilizing a secondary route which has a low capacity for transporting methotrexate but whose activity is sufficient to permit cell growth in medium supplemented with folate or 5-formyltetrahydrofolate.

INTRODUCTION

Methotrexate has been used for several decades in the chemotherapeutic treatment of various types of tumor cells. The effectiveness of methotrexate therapy, however, is often diminished by the development of resistance, and a common mode of resistance found in cultured cells is a reduction in methotrexate transport (1-3). A critical problem for cells in acquiring the ability of cells to obtain sufficient folate for cell growth. In many cells methotrexate and folate compounds share the same transport system for cell entry (4-6). The transport characteristics of folate compounds in L1210 and other leukemic cells have been investigated in various laboratories, and the results support a model in which methotrexate and various reduced-folate compounds gain cell entry via a high-affinity, low-capacity transport system (2, 4, 7, 8). Uptake via this system occurs via an exchange of extracellular folate compounds with intracellular anions, and anion gradients appear to act as a source of energy (4, 9-14). The binding component of this transport system has been labeled with reactive substrate analogues (15, 16), and cell lines which overproduce the binding protein have been isolated and characterized (17). Folate is transported via this system (5), although controversy remains regarding the presence of other possible folate transport routes. Transport measurements from several laboratories (18-23) had suggested that L1210 cells possess a low-affinity, high-capacity system for folate, although the existence of this transport system has been disputed by more recent studies (5, 24). Multiple transport routes for folate compounds and methotrexate have also been suggested from growth studies. Sirotnak et al. (3) showed that a methotrexate-resistant L1210 cell line (L1210/R25) exhibits a substantial decrease in ability to grow at low concentrations of 5-formyltetrahydrofolate, with no concurrent change in growth response for folate, and unlike the parental cells, their ability to grow in implanted mice requires the coadministration of folic acid. Studies by Pincus et al. (25) with another methotrexate-resistant cell line also found a retention of folate growth response and a normal folate transport activity in a cell line which has a marked decrease in growth response and transport activity for 5-formyltetrahydrofolate. L1210/R81 cells isolated by McCormick et al. (26) represent another example of cells which are resistant to methotrexate due to a loss in methotrexate transport via the reduced-folate transport system. These results suggested that either the reduced-folate carrier system can acquire an altered specificity during the development of methotrexate resistance or that other transport routes exist for folate compounds.

L1210/R81 cells were examined in the present study to determine the mechanism by which methotrexate-resistant cells with a defect in methotrexate transport can achieve folate sufficiency. The results show that these cells lack the reduced-folate transport system when either methotrexate or other folate compounds are used as substrates. Intracellular folates in L1210/R81 cells are also not gained via a high-affinity folate binding protein system which has been described in L1210 (24) and other (27, 28) cells. Uptake occurs, instead, via a system whose properties have not been described previously. This alternative route transports folate and 5-formyltetrahydrofolate more efficiently than methotrexate, but surprisingly it binds but does not transport 5-methyltetrahydrofolate. The same transport system is also present in comparable amounts in the parental cells, but its low activity and similar specificity for reduced folate compounds and methotrexate relative to the reduced-folate carrier system have hindered its detection in prior studies.

MATERIALS AND METHODS

Chemicals. [3',5',7,9-3H]Folic acid (40 Ci/mmol), [3',5',9-3H]methotrexate (20 Ci/mmol), [3',5',7,9-3H]6S5-methyltetrahydrofolate (40 Ci/mmol), and [3',5',7,9-3H]6S5-formyltetrahydrofolate (40 Ci/mmol) were obtained from Moravek Biochemicals. The [3H]folate and [3H]methotrexate were diluted with unlabeled substrate to a specific activity of 100,000 cpm/mm and purified by thin-layer chromatography on Baker-flex cellulose sheets (J. T. Baker) using 100 mM sodium-
TRANSPORT OF FOLATE COMPOUNDS IN L1210 CELLS

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NHS-methotrexate, N-hydroxysuccinimide ester of methotrexate; BSP, bromosulfophthalein; CCCP, carbonylcyanide m-chlorophenylhydrazone; HBS, HEPES-buffered saline; MBS, MES-buffered saline; MHBS, MES/HEPES-buffered saline; MHS, magnesium-HEPES-sucrose; MES, 2-(N-morpholino)ethanesulfonate.

RESULTS

Growth Response of the Resistant Cells to Folate Compounds. Methotrexate-resistant L1210/R81 cells grow at about the same rate in RPMI 1640 medium supplemented with 2.2 μM folate as the parental cells (26). However, a difference in growth response was observed when the concentration of folate was varied. Growth of the resistant cells occurred half-maximally at a folate concentration of 350 nM, whereas the parental cells showed half-maximal growth at 130 nM folate. A more pronounced difference between these two cell lines was observed when growth was measured at varying concentrations of 5-formyltetrahydrofolate. The resistant cells grew half-maximally at 20 nM 5-formyltetrahydrofolate, whereas the parental cells required only 1 nM 5-formyltetrahydrofolate to achieve half-maximal growth. The failure of L1210/R81 cells to grow at folate concentrations in the 1 to 10 nM range also indicated that cell entry of folate compounds was not occurring via the high-affinity folate-binding protein system that is overproduced in folate-adapted L1210 cells (24). Direct measurements of high-affinity folate binding protein in folate-depleted L1210/R81 cells also failed to detect an increase in binding activity in the mutant cells (0.007 pmol/mg of protein) relative to the parent (0.010 pmol/mg of protein).

Comparative Transport of Folate Compounds by L1210 and L1210/R81 Cells. Previous studies had provided evidence that L1210/R81 cells have a greatly reduced capacity for transporting methotrexate (26), and this same result was also obtained in the present study (Fig. 1A). The initial rate of methotrexate transport by the mutant cells was reduced 98% relative to the parental cells. A reduction in the uptake of 5-methyltetrahydrofolate (Fig. 1B) and folate (Fig. 1C) was also observed. The extent of the loss in influx activity with these latter compounds was 98% and 89%, respectively.

The slow transport of folate compounds in the mutant cells was also examined for its response to changes in buffer composition. Since transport via the reduced-folate/methotrexate carrier system in the parental cells is increased by removing competing extracellular anions (9–13), it was reasoned that, if the remaining uptake activity in the mutant proceeds via a severely inhibited but partially functional reduced-folate system, then a corresponding response to changes in buffer composition should be observed. Consistent with previous results (5), folate influx in the parental cells (Fig. 2A) increased substantially (nearly 10-fold) upon transfer from a saline solution (HBS) to an anion-deficient buffer (MHS). However, buffer composition had no stimulatory effect on the transport of folate in L1210/R81 cells (Fig. 2B). A similar lack of an effect by buffer composition was observed for the transport of methotrexate and 5-methyltetrahydrofolate, indicating that the reduced folate carrier system was not operative in L1210/R81 cells. Folate

Fig. 1. Time-dependent uptake of [3H]methotrexate (A), 5-[3H]methyltetrahydrofolate (B), and [3H]folate (C) by L1210 and L1210/R81 cells in HBS, pH 7.4. Substrate concentrations: [3H]methotrexate (MTX), 2.0 μM; 5-[3H]methyltetrahydrofolate (MeFH), 2.0 μM; and [3H]folate, 5.0 μM.

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TRANSPORT OF FOLATE COMPOUNDS IN L1210 CELLS

Fig. 2. Effect of buffer composition on the influx of [3H]folate by L1210 and L1210/R81 cells. Influx was measured in L1210 (A) and L1210/R81 (B) cells after incubation at 37°C with the indicated concentrations of [3H]folate for 3 min and 10 min, respectively.

Fig. 3. Effect of pH on the uptake of [3H]folate by L1210/R81 cells. The buffers used were HBS, pH 7.4 (A), HBS, pH 6.8 (B), and MBS, pH 6.2 (C), containing 5 mM glucose and 100 μM BSP. [3H]Folate concentration, 5.0 μM.

Table 1. Effect of various additions on folate influx in L1210/R81 cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Folate influx (pmol/min/mg of protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.22</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.34</td>
<td>155</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.06</td>
<td>27</td>
</tr>
<tr>
<td>-Na⁺</td>
<td>0.16</td>
<td>73</td>
</tr>
<tr>
<td>-BSP</td>
<td>0.23</td>
<td>105</td>
</tr>
<tr>
<td>NHS-MTX*</td>
<td>0.23</td>
<td>105</td>
</tr>
</tbody>
</table>

* NHS-MTX, NHS-methotrexate.

transport (at 5 μM) was also not reduced by 100 μM BSP or by 2 mM thiamine pyrophosphate, potent inhibitors of the reduced-folate carrier system in wild-type cells (11), or by 100 μM adenine (5). But inhibition of folate transport by 80 to 90% could be achieved by the addition of a 40-fold excess of unlabeled folate.

Characteristics of Folate Transport in L1210/R81 Cells. The above inhibitor studies suggested that L1210/R81 cells acquire folate compounds via a folate-sensitive carrier-mediated process whose specificity differed from that of the reduced-folate carrier system. To probe this possibility further, transport studies were performed using [3H]folate as the substrate and with excess unlabeled folate (200 μM) in the control to block nonspecific uptake. BSP (100 μM) was also added to ensure the absence of any residual uptake activity via the reduced-folate transport system and to enhance the linear phase of folate influx by retarding folate efflux. Initial experiments showed that a slow rate of folate uptake by L1210/R81 cells could be sustained at pH 7.4 for at least 30 min at 37°C but that a much higher rate and extent of uptake could be achieved by decreasing the pH (Fig. 3). The extent of this increase was 5-fold when the pH was reduced from 7.4 (Fig. 3A) to 6.8 (Fig. 3B), and an additional 2-fold stimulation was observed upon further reduction to pH 6.2 (Fig. 3C). The linear phase of uptake also decreased from 30 min at pH 7.4 to 15 and 10 min at pH 6.8 and 6.2, respectively. In contrast, folate transport via the reduced-folate carrier system of wild-type L1210 cells did not vary between pH 7.4 and pH 6.8 (0.4 ± 0.1 pmol/min/mg of protein at 5.0 μM [3H]folate).

Folate transport by L1210/R81 cells was dependent on energy (Table 1). Glucose stimulated initial rates of folate uptake at pH 6.8 by 55%, whereas the metabolic inhibitor, CCCP, reduced uptake by 73%. Influx decreased slightly upon removal of sodium ions and replacement with choline, but no effect was observed in cells treated with an N-hydroxysuccinimide ester of methotrexate, which reacts covalently with and inactivates the reduced-folate carrier system of the parental cells (15). BSP also had no effect on folate influx under these conditions.

Measurements of the concentration dependence of folate influx at pH 6.8 showed that the uptake process is saturable (Fig. 4). Calculations from a double-reciprocal plot of these data (Fig. 4, inset) gave a Kᵣ for half-maximal influx of 5.2 μM and a Vₒ for 0.55 pmol/min/mg of cellular protein. The same measurements at pH 7.4 (see Table 2) revealed a higher Kᵣ for folate of 10.0 μM and a lower Vₒ for 0.24 pmol/min/mg of protein. At pH 6.2, the Kᵣ for folate (4.8 μM) was essentially the same as the value obtained at pH 6.8 (5.2 μM), whereas the Vₒ increased about 2-fold to 1.15 pmol/min/mg of protein.
Additional measurements at pH 6.8 showed that the $K_i$ and $V_{max}$ for folate did not vary when the 100 mM BSP was omitted from assay mixtures or when both 100 mM BSP and 2 mM thiamine pyrophosphate were present. The $V_{max}/K_i$ ratio for folate influx, which approximates the relative efficiency of transport, shows a 10-fold improvement in transport when the pH is decreased from 7.4 to 6.2 (Table 2). Cells grown for 2 transfers (5 generations) in the absence of added folate to deplete internal folate reserves exhibited the same $K_i$ and $V_{max}$ for folate transport as cells grown in the standard medium containing 2.2 $\mu$M folate.

Folate Transport in Wild-Type L1210 Cells. The system which mediates folate uptake in L1210/R81 cells is also present in the parental cells. Measurements in the presence of 100 mM BSP (to block folate uptake via the reduced-folate carrier) revealed that an uptake component for folate was present which exhibited essentially the same kinetic parameters as the system observed in L1210/R81 cells. Comparable values were observed for both the $V_{max}$ and $K_i$ for folate at each of three pH values (Table 2), and moreover, folate uptake by the wild-type cells was (a) stimulated 1.5-fold by the presence of glucose, (b) inhibited 75% by CCCP, (c) affected minimally by the removal of sodium ions, and (d) not inhibited by NHS-methotrexate treatment (data not shown). In contrast, measurements in parental CCRF-CEM cells, a human lymphoblastic cell line of T-cell origin, failed to detect measurable levels of a pH-dependent folate transport system.

Specificity of the Uptake System for Folate Compounds. Folate influx in L1210/R81 cells was measured at pH 6.8 in the presence of varying concentrations of folate compounds to determine the binding specificity of this transport system. Dixon plots of the data combined with a $K_i$ for each folate compound. 5-Formyltetrahydrofolate (F-FH4) (A).

Table 2 Variation with pH of the kinetic parameters for folate influx in L1210/R81 and L1210 cells via the transport system sensitive to folate. $K_i$ and $V_{max}$ values at the indicated pH were determined from double-reciprocal plots of influx versus [3H]folate concentration (see Fig. 4). Assay mixtures consisted of cells, 5 mM glucose, 100 $\mu$M BSP, and the following buffers: HBS, pH 7.4; MHBS, pH 6.8; and MBS, pH 6.2. Influx was measured at 37°C for 30, 15, and 10 min at pH 7.4, 6.8, and 6.2, respectively, and was corrected for control samples containing 500 $\mu$M unlabeled folate.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pH</th>
<th>$K_i$ (mM)</th>
<th>$V_{max}$ (pmol/min/mg of protein)</th>
<th>$V_{max}/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210/R81</td>
<td>7.4</td>
<td>10.0</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>5.2</td>
<td>0.55</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>4.8</td>
<td>1.15</td>
<td>0.24</td>
</tr>
<tr>
<td>L1210</td>
<td>7.4</td>
<td>8.3</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>5.0</td>
<td>0.63</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>4.3</td>
<td>0.98</td>
<td>0.23</td>
</tr>
</tbody>
</table>

![Fig. 5. Effect of pH on the uptake of [3H]methotrexate, 5-formyltetrahydrofolate, and 5-[3H]formyltetrahydrofolate by L1210/R81 cells. Measurements were performed in HBS, pH 7.4 (A), MHBS, pH 6.8 (B), and MBS, pH 6.2 (C), containing 5 mM glucose, 100 mM BSP, and either 5.0 mM [3H]methotrexate (MTX) (●), 5.0 mM 5-[3H]methyltetrahydrofolate (Me-FH4) (○), or 5.0 mM 5-[3H]formyltetrahydrofolate (F-FH4) (△).](image)

Table 3 Kinetic parameters in L1210/R81 for the influx of methotrexate and 5-formyltetrahydrofolate via the transport component sensitive to folate. $K_i$ and $V_{max}$ measurements for [3H]methotrexate and 5-[3H]formyltetrahydrofolate were performed in MHBS, pH 6.8, containing 5 mM glucose and 100 mM BSP as described in the legend to Table 2 for [3H]folate. $K_i$ values for folate were calculated from Dixon plots of influx at 5.0 mM [3H]methotrexate or 5.0 mM 5-[3H]formyltetrahydrofolate versus folate concentration.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_i$ (mM)</th>
<th>$V_{max}$ (pmol/min/mg of protein)</th>
<th>$V_{max}/K_i$</th>
<th>$K_i$ (folate) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>9.8</td>
<td>0.52</td>
<td>0.05</td>
<td>4.9</td>
</tr>
<tr>
<td>5-Formyl FH4*</td>
<td>3.3</td>
<td>0.35</td>
<td>0.11</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* 5-Formyl FH4, 5-formyltetrahydrofolate.

at pH 6.8 (15 min) or pH 6.2 (10 min). Competition studies showed that folate inhibited this uptake component for methotrexate. The $K_i$ for folate at pH 6.8 was 4.9 $\mu$M (Table 3), which is in good agreement with the $K_i$ for folate influx (5.2 $\mu$M). Double-reciprocal plots of methotrexate influx versus methotrexate concentration (at pH 6.8) yielded a $K_i$ for half-maximal influx of 9.8 $\mu$M and a $V_{max}$ of 0.52 pmol/min/mg of protein (Table 3). Corresponding measurements in wild-type L1210 cells performed in the presence of both 100 mM BSP and 2 mM thiamine pyrophosphate (to block the more active reduced-folate carrier system of parental cells) showed that the same uptake component could be detected. In the parental cells the $K_i$ for half-maximal methotrexate influx was 8.6 $\mu$M, the $V_{max}$ was 0.67 pmol/min/mg of protein, and the $K_i$ for inhibition of methotrexate influx by folate was 5.5 $\mu$M.

Evidence for the transport of 5-formyltetrahydrofolate by the alternative folate uptake route was also obtained using L1210/R81 cells. An uptake component for 5-[3H]formyltetrahydrofolate was observed which was sensitive to low concentrations of folate and could be increased with decreasing pH (Fig. 5), but the uptake profile was different from that of folate and methotrexate. Initial rates of 5-formyltetrahydrofolate uptake showed a strong pH dependence, but the linear phase of uptake decreased rapidly with decreasing pH. Linear uptake occurred for 30 min at pH 7.4, but it decreased to 10 min at pH 6.8 and to only 1 to 2 min at pH 6.2. The observed $K_i$ for half-maximal influx of 5-formyltetrahydrofolate was 3.3 $\mu$M at pH 6.8, the $V_{max}$ was 0.35 pmol/min/mg of protein, and folate inhibited 5-formyltetrahydrofolate influx with a $K_i$ of 5.4 $\mu$M (Table 3). 5-Methyltetrahydrofolate, which is a good inhibitor of folate...
influx, was accumulated to only a minimal extent (Fig. 5) under assay conditions which permitted a substantial uptake of the other folate compounds. \( V_{\text{max}}/K_e \) values for the influx of methotrexate and 5-formyltetrahydrofolate via the alternative folate-preferring transport system (Table 3) are similar to the corresponding values for folate transport via this same system (Table 2), but much higher values for \( V_{\text{max}}/K_e \) are observed for transport of these substrates via the reduced-folate transport system of parental L1210 cells (Table 4).

Changes in the Medium pH during Growth of L1210 Cells. The enhancement of folate transport that occurred in L1210/R81 with decreasing pH prompted measurements of the pH of the culture medium during cell growth. Complete medium without cells that had been incubated for 48 h in a humidified CO₂/air incubator had a pH of 7.32, whereas medium containing cells decreased in pH with increasing cell growth. A medium pH of 6.6 ± 0.1 was observed for cells which had reached a late log phase of growth (1.2 to 1.5 × 10⁶ cells/ml) where cultures are routinely harvested for transport measurements. The same changes in medium pH were observed during growth of parental L1210 cells.

DISCUSSION

Methotrexate-resistant L1210/R81 cells have been examined for their growth requirements and transport characteristics for folate compounds in an effort to establish the mechanism by which folate coenzymes are acquired for growth. Resistance to methotrexate in L1210/R81 cells has been correlated with a loss in methotrexate transport via the reduced-folate transport system (26), but the cells continue to grow in the presence of folate in spite of the previous finding that the reduced-folate transport system is the principal uptake route for both methotrexate and folate (5). The latter observation could have been explained if the reduced-folate carrier system had acquired an altered substrate specificity which increased folate transport relative to methotrexate transport, but inhibitor and buffer studies (cf. Figs. 1 and 2) indicated that the reduced-folate transport system is not operative in L1210/R81 cells for either substrate. Similarly, the results could not be explained by an over-production of a high-affinity folate binding protein system (24, 27, 28), since growth of L1210/R81 cells did not occur at nanomolar concentrations of folate, and an elevated high-affinity folate binding activity could not be detected by direct measurements. A low-affinity, high \( V_{\text{max}} \) transport system which had been reported for folate in previous studies (18–23) was also not observed with either L1210 or L1210/R81 cells. A possible relation between the existence of the latter route and impurities in [3H]folate has been discussed previously (5, 24).

Folate enters L1210/R81 cells via a previously undetected carrier-mediated transport system which is distinct from the reduced-folate carrier system. Uptake by this alternative route is not affected by several inhibitors of the reduced-folate carrier, but it can be blocked by micromolar concentrations of folate. A distinct feature of this uptake system is its strong dependence on pH. Influx and total uptake of folate both increased 5-fold when the pH was decreased from 7.4 to 6.8, and a further 2-fold increase was obtained by reducing the pH to 6.2. The \( K_e \) for half-maximal influx at pH 6.8 was 5.2 μM, and the \( V_{\text{max}} \) was 0.55 pmol/min/mg of protein (Table 2). The lower transport of folate at pH 7.4 (relative to pH 6.8) was due to both a higher \( K_e \) and a lower \( V_{\text{max}} \), whereas at pH 6.2, an increase in \( V_{\text{max}} \) alone (relative to pH 6.8) accounted for the 2-fold higher transport (Table 2). Kinetic measurements showed further that the folate transport system of L1210/R81 cells is energy dependent (Table 1). CCRF-CEM cells, which have a similar reduced-folate carrier system (6), do not possess measurable levels of this second transport system for folate, whereas a similar pH- and energy-dependent transport system has been reported in intestinal epithelial cells. In the latter case, a proton/folate cotransport mechanism has been proposed (31, 32).

The folate transport system of L1210/R81 cells also mediates the uptake of methotrexate and 5-formyltetrahydrofolate. Transport of the latter compounds was increased 5- to 10-fold by decreasing the pH from 7.4 to 6.2 (Fig. 5), and \( K_e \) values (at pH 6.8) for inhibition of methotrexate and 5-formyltetrahydrofolate influx by folate (Table 3) were comparable to the \( K_e \) value for folate influx (Table 2). Similarly, folate transport was inhibited by methotrexate and 5-formyltetrahydrofolate with \( K_e \) values which were comparable to the \( K_e \) values for the transport of methotrexate and 5-formyltetrahydrofolate (Table 3). Methotrexate influx also exhibited the same lack of inhibition by NHS-methotrexate and BSP as folate influx, and the influxes of both folate and methotrexate responded similarly to effectors of energy metabolism. 5-Formyltetrahydrofolate transport via this route was also consistent with the utilization of this folate compound as a source of intracellular folates in growth experiments. Interestingly, 5-methyltetrahydrofolate was an inhibitor of folate transport (\( K_e = 3.7 \) μM) but was not a substrate for this transport system as determined by direct uptake measurements (Fig. 5). The inability to transport 5-methyltetrahydrofolate is an unexpected observation, since the latter is the most abundant physiological form of folate found in the circulation of mammals.

The folate transport route in L1210/R81 cells is present at the same level in the parental L1210 cell line. Kinetic measurements performed in the presence of BSP (to block folate uptake by the reduced-folate carrier) showed that a transport system was present in wild-type L1210 cells which exhibited the same pH dependence as folate transport in L1210/R81 cells. In addition, the same \( K_e \) and \( V_{\text{max}} \) values for folate influx were observed in wild-type cells that had been observed in L1210/R81 cells at each of three pH values (Table 2). The transport of methotrexate by this same system could also be demonstrated in the parental cells, although in this case both BSP and thiamine pyrophosphate were added to reduce interference from the high rate of methotrexate uptake via the reduced-folate carrier system. Half-maximal inhibition of methotrexate influx by micromolar concentrations of folate clearly distinguished methotrexate uptake via the alternative folate transport system and the reduced-folate carrier. Prior studies of methotrexate transport routes would not have easily detected this uptake system in the parental cells, since only 1 to 2% of uptake at 5 μM methotrexate and pH 7.4 proceeds via this second route. Likewise, compounds including methotrexate, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate inhibit both systems similarly, which further diminishes the likelihood of separating

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Table 4 Kinetic parameters for the influx of folate compounds in L1210 cells via the reduced-folate transport system

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_e ) (μM)</th>
<th>( V_{\text{max}} ) (pmol/min/mg of protein)</th>
<th>( V_{\text{max}}/K_e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>4.2</td>
<td>18</td>
<td>4.3</td>
</tr>
<tr>
<td>5-Methyl FH₄</td>
<td>1.5</td>
<td>24</td>
<td>16.0</td>
</tr>
<tr>
<td>5-Formyl FH₄</td>
<td>2.5</td>
<td>20</td>
<td>8.0</td>
</tr>
<tr>
<td>Folate</td>
<td>120</td>
<td>14</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* 5-Methyl FH₄, 5-methyltetrahydrofolate; 5-formyl FH₄, 5-formyltetrahydrofolate.
these uptake routes by inhibitor studies, although a clear separation can be achieved by reducing the pH to 6.8 and by using anionic inhibitors of the reduced-folate system which do not interfere with the alternative folate-prefering transport system. The relative transport efficiencies ($V_{max}/K_m$) of methotrexate and reduced-folate compounds via the reduced-folate carrier in parental cells (Table 4) are about 100-fold higher than transport via the alternative system (Tables 2 and 3), but differences in folate transport via these two systems are much less. When a comparison of transport efficiency is made at pH 6.8 (an average pH for the medium of cells grown in culture), both transport systems contribute about equally to folate uptake (cf. Tables 2 and 4). Hence, loss of the reduced-folate carrier system in L1210/R81 cells decreases its growth response to folate relative to parental cells by only 2- or 3-fold, whereas a much greater reduction is observed in the transport of methotrexate (50-fold) and in the growth response of L1210/R81 cells to 5-formyltetrahydrofolate (20-fold).

The different specificities of the folate and reduced-folate transport systems of L1210 cells have provided a facile mechanism for gaining resistance to methotrexate via alterations in transport without compromising growth on folate. Whereas both systems have a similar ability to transport folate (cf. Table 2 and 4), the transport efficiency for methotrexate is about 50-fold higher via the reduced-folate carrier system. L1210/R81 cells have taken advantage of this difference in substrate specificity and have selectively down-regulated the reduced-folate carrier. A similar resistance to methotrexate might have arisen if 5-formyltetrahydrofolate had been used in the selection medium as the folate source, since the alternative folate transport system can also transport this folate compound. Another potential mechanism for developing methotrexate resistance would have been to down-regulate the reduced-folate carrier system and induce a high-affinity folate binding protein system (24, 27, 28). A system of the latter type, which has been identified in L1210 sublines adapted to low concentrations of folate compounds (24), accommodates folate and reduced-folate compounds with a much higher efficiency than methotrexate, and hence cells with an increased reliance upon this system for acquiring folate compounds should also have an increased resistance to methotrexate. An increased level of high-affinity folate binding protein has been implicated in folate utilization in a subline of CCRF-CEM cells which is resistant to methotrexate due to a loss in the reduced-folate transport system (33).

**REFERENCES**


Characteristics of a Novel Transport System for Folate Compounds in Wild-Type and Methotrexate-resistant L1210 Cells

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