We previously described a 1,000-fold pyrimethamine-resistant Chinese hamster ovary cell line (PyrR100) which retains parental dihydrofolate reductase activity and methotrexate (MTX) sensitivity. This study characterizes the basis for the 14-fold decrease in folic acid and leucovorin concentrations required for clonogenic growth of PyrR100 cells relative to parental AA8 cells. Under conditions in which folic acid reduction was blocked by trimetrexate, PyrR100 cells displayed relative to parental AA8 cells a: 1) 17- and 5-fold increase in the net transport of folic acid and MTX, respectively; 2) 23- and 5-fold decrease in the efflux rate constant for folic acid and MTX, respectively; and 3) 2-fold increase in folic acid influx with no significant change in MTX influx. The markedly increased net folic acid transport in PyrR100 cells could not be explained by cellular folic acid binding, mitochondrial sequestration, polyglutamylation, nor by a decreased membrane potential.

The effect of energy deprivation on folic acid and MTX transport in both cell lines was quite different. Glucose and pyruvate deprivation nearly abolished the increase in net folic acid transport in PyrR100 cells. In contrast, energy deprivation increased net MTX transport in AA8 cells, whereas no change was seen with PyrR100 cells. Furthermore, while folic acid influx in PyrR100 and AA8 cells was markedly reduced with energy deprivation, MTX influx was not affected. Provision of glucose and pyruvate to energy-deprived cells resulted in a rapid onset of MTX efflux from parental AA8 cells but not from PyrR100 cells. Taken together these results indicate that the markedly enhanced net transport of folic acid and MTX in PyrR100 cells is largely due to the complete loss of exit pump activity. Furthermore, the energy source that sustains the augmented levels of folic acid appears linked to the influx process and is distinct from the energy source that sustains MTX gradients under these conditions. We conclude that the loss of folic acid efflux is an efficient means of augmenting cellular uptake of folate cofactors and subsequent survival on picomolar folate concentrations. This constitutes the first demonstration of the loss of folic acid exporter activity in mammalian cells as a response to lipophilic antifolate selective pressure.

Folic acid cofactors play a key role in one-carbon metabolism and are essential for the biosynthesis of purine and pyrimidine precursors of nucleic acids, for the metabolism of certain amino acids, as well as for initiation of macromolecular synthesis in mitochondria (1, 2). However, mammalian cells cannot synthesize folic acids and therefore must rely on their uptake from exogenous sources. Several plasma membrane routes have been described in mammalian cells that can accommodate transport of folates and their 4-amino analogs including methotrexate (MTX).1 1) The reduced folate carrier (RFC), a major folate uptake route which is a bidirectional anion exchanger (3, 4) with a high affinity ($K_m = 0.3–5 \mu M$) for reduced folates and MTX, and low affinity ($K_m = 200–400 \mu M$) for folic acid (3–5). 2) Folate receptors, glycosylphosphatidylinositol membrane-anchored proteins that mediate the unidirectional uptake of folates into mammalian cells with a high affinity for folic acid and 5-methyltetrahydrofolate ($K_p = 1–10 \text{ nm}$) but lower affinity ($K_p = 10–300 \text{ nm}$) for other reduced folates and MTX (6–9). 3) An apparently separate transport system with optimal activity at low pH which recognizes folic acid, reduced folates, and MTX with comparable affinities (1–5 \mu M) (10–12).

However, although folates can be taken up efficiently by mammalian cells via these systems (and possibly by additional yet unidentified transport routes), they can be rapidly lost by efflux through RFC as well as ATP-dependent efflux pumps (13) such as the multispecific organic anion transporters (MOATs) (14–17). Thus, intracellular levels of free monoglutamate reflect the net contributions of these multiple bidirectional processes. Furthermore, another element of cellular folate retention is the extent of polyglutamylation (18, 19), as well as compartmentation in organelles including mitochondria (20).

In this study we characterized the mechanism(s) underlying the extraordinary decrease in the folate growth requirement of Chinese hamster ovary (CHO) PyrR100 cells that display high level resistance to lipophilic antifolates including 2,4-diamino-pyrimidines (e.g. pyrimethamine and metoprine), trimetrexate (TMQ), and piritrexim (21). We find that these cells have markedly augmented folic acid accumulation and provide evidence that this is due predominantly to the loss of folic acid exporter activity. This constitutes the first demonstration of the loss of folic acid efflux activity in mammalian cells as a mechanism of adaptation to dihydrofolate reductase (DHFR) inhibition with a lipophilic antifolate.

* The abbreviations used are: MTX, methotrexate; RFC, reduced folate carrier; DHFR, dihydrofolate reductase; TMQ, trimetrexate; MOAT, multispecific organic anion transporter; CHO, Chinese hamster ovary; TTP$, tetraphenylphosphonium; HBS, Hepes-buffered saline; HPLC, high performance liquid chromatography.

The JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 28, Issue of July 11, pp. 17460–17466, 1997
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(Received for publication, April 29, 1997)
Deficient Folate Efflux in PyrR<sup>100</sup> Cells

EXPERIMENTAL PROCEDURES

**Chemicals**—[3,5,7,9-<sup>3</sup>H]folic acid, [carboxyl-<sup>14</sup>C]inulin, and [3H]tetraphenylphosphonium (TPP) were obtained from Amersham Corp., and [3,5,7,9-<sup>3</sup>H]MTX was purchased from Moravek Biochemicals (Brea, CA). Radiolabeled and unlabeled folic acid and MTX were purified prior to use by high performance liquid chromatography.

**Trimethazine**—A generous gift from Dr. D. Fry (Warner-Lambert, Parke-Davis, Ann Arbor, MI). Folic acid, leucovorin (calcium salt), MTX, and pyrimethamine were obtained from Sigma.

**Cell Lines and Tissue Cultures**—CHO Pyr<sup>R100</sup> cells were established by stepwise selection of parental AA8 cells in gradually increasing concentrations of pyrimethamine (21). The multiple step selection initiated at 100 nM pyrimethamine (the LD<sub>50</sub>) was terminated at 100 μM, and the 1000-fold pyrimethamine-resistant cells were therefore termed Pyr<sup>R100</sup> parental AA8 cells and their Pyr<sup>R100</sup> parental AA8 cells were maintained in monolayer or suspension culture conditions in RPMI 1640 containing 2.3 μM folic acid (Life Technologies, Inc.), supplemented with 5% dialyzed fetal bovine serum (Gemini Bio-Laboratories Inc.), 1 mM sodium pyruvic acid (Mediatech), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Unless otherwise stated, the growth medium of Pyr<sup>R100</sup> cells was supplemented with 100 μM pyrimethamine. Mono-layer cells were passaged biweekly using a standard trypsination protocol.

**Folate Cofactor Growth Requirement**—Monolayers of AA8 and Pyr<sup>R100</sup> cells grown for at least five doublings in pyrimethamine-free medium were washed with phosphate-buffered saline and detached by trypsination. Cells were then seeded at 500–1,000 cells/60-mm Petri dish in 5 ml of folic acid-free RPMI 1640 medium containing a range of folic acid or leucovorin concentrations. After 7–12 days of incubation at 37 °C when colonies (>50 cells/colony) became visible, cells were washed with phosphate-buffered saline, fixed with methanol, stained with crystal violet, and counted. The control clonogenic growth was determined in medium containing 2.3 μM folic acid or 100 nM leucovorin.

**Folic Acid and MTX Transport**—Influx measurements were performed according to a previously described method (22) with some modifications. Exponentially growing cells from suspension cultures in pyrimethamine-free growth medium were collected by centrifugation (750 × g for 2 min), and washed three times with ice-cold HBS (20 mM HEPES pH 7.4, 140 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, and 5 mM glucose). Cells were resuspended to a density of 2–6 × 10<sup>6</sup> cells/ml in 1 mM pyruvate-containing HBS with 5 μM TMQ for 10 min at 37 °C, and then loaded for 1 h with 1 μM [3H]folic acid or [3H]MTX. After 20 min of incubation at 37 °C, transport was terminated by addition of [3H]folic acid or [3H]MTX and 0.5–1-ml portions of the cell suspension were removed at designated times into ice-cold HBS followed by centrifugation and washed three times with ice-cold HBS. After centrifugation (750 × g for 2 min) at 4 °C, cells were resuspended in ice-cold HBS, washed twice in the same buffer, and processed for determination of intracellular uptake, intracellular radioactivity with normalization to dry weight (22). Intracellular water of AA8 and Pyr<sup>R100</sup> cells was determined using the membrane-impermeable-[carboxyl-<sup>14</sup>C]inulin as the extracellular marker as described elsewhere (24). Efflux measurements were performed by first loading cells with [3H]folic acid (in the presence of TMQ) or [3H]MTX for 10 min at 37 °C. Then, a portion was removed for determination of the intracellular level prior to efflux. The remaining radiolabeled cells were sedimented by centrifugation and resuspended in a large volume of prewarmed HBS containing 1 mM pyruvate and 5 μM TMQ. Portions of cell suspension were drawn at given times and processed as detailed above for cellular radiolabel. The DHFR binding capacity was the level of [3H]MTX remaining in cells after efflux for 30–60 min, an incubation time sufficient to allow for the loss of all free drug.

**Measurement of Plasma Membrane Potentials**—We have employed the method of equilibrium distribution of the lipid-soluble cation TPP<sup>+</sup> across the plasma membrane to determine the membrane potential of AA8 and Pyr<sup>R100</sup> cells (25). The uptake of TPP<sup>+</sup> was measured at 2 μM and reached a steady-state after 10 min of incubation at 37 °C. Total cellular TPP<sup>+</sup> was corrected for a bound component that was retained in cells after efflux into a buffer free of TPP<sup>+</sup>. The Nernst equation was employed to calculate the membrane potential using the cell volume of determination (see Table II) and intracellular/extracellular TPP<sup>+</sup> concentrations.

**Analysis of the Polyglutamate Derivatives of Folic Acid and MTX**—[3H]folic acid, [3H]MTX, and their polyglutamate derivatives were analyzed by HPLC (19) using two continuous linear gradients of 0–10% and 10–15% acetonitrile over 35 and 15 min, respectively. Unlabeled MTX-Glu(1–6) standards (Schircks Laboratories, Switzerland) served as internal markers and were monitored by UV absorbance at 280 nm. Typically, MTX-Glu(1–6) eluted during the 0–10% acetone gradient whereas MTX-Glu(2–1) resolved during the 10–15% acetone gradient.

**Data Presentation**—Means presented for the 50% clonogenic viability (CV<sub>50</sub>) values are the means of two experiments.

**RESULTS**

Parental AA8 cells and their Pyr<sup>R100</sup> subline with high level resistance to lipophilic antifolates were examined for their

![FIG. 1. Time course of folic acid uptake in Pyr<sup>R100</sup> and AA8 cells. Exponentially growing suspension cultures of Pyr<sup>R100</sup> (squares) and AA8 cells (triangles) were harvested by centrifugation, washed three times with ice-cold HBS, and suspended to 5 × 10<sup>6</sup> cells/ml in 5 μM TMQ-containing HBS supplemented with 1 mM pyruvic acid. After 20 min of incubation at 37 °C, transport was initiated by addition of [3H]folic acid to a final concentration of 1 μM. Portions (0.5–1 ml) of the cell suspension were removed at designated times into ice-cold HBS following which cells were processed for determination of intracellular radiolabel as described under "Experimental Procedures." Inset, expanded time course of [3H]folic acid accumulation in AA8 cells.](image-url)
Deficient Folate Efflux in Pyr\textsuperscript{R100} Cells

Data presented are the means \( \pm \) S.E. of 4-13 independent experiments.

|         | AA8      | Pyr\textsuperscript{R100} | Pyr\textsuperscript{R100}/AA8 |
|---------|----------|---------------------------|-----------------------------|
| Initial uptake rate (nmol/min/g dry wt) | 0.058 \( \pm \) 0.006 | 0.108 \( \pm \) 0.014 | 1.9 |
| Efflux rate constant (min\textsuperscript{-1}) | 0.084 \( \pm \) 0.012 | 0.0036 \( \pm \) 0.0011 | 0.043 |
| Total cell folic acid (nmol/g dry wt) | 0.51 \( \pm \) 0.03 | 7.52 \( \pm \) 1.17 | 14.8 |
| H\textsubscript{2}O/dry wt (\( \mu \)l/mg) | 4.98 | 4.43 | 0.89 |
| Observed [folic acid], (\( \mu \)M) | 0.102 | 1.70 | 16.7 |
| Predicted [folic acid], (\( \mu \)M) | 0.07 | 0.07 | 1 |
| Observed/Predicted | 1.46 | 24.3 | 16.6 |
| Membrane potential (mV) | -35 | -35 | 1.0 |

*Based upon the Nernst equation using the measured membrane potential at an extracellular folic acid level of 1 \( \mu \)M.

clonogenic growth requirements for folic acid and leucovorin. Whereas parental AA8 cells maintained a 50% clonogenic viability at 70 \( \mu \)M folic acid and 0.8 \( \mu \)M leucovorin, Pyr\textsuperscript{R100} cells required 14-fold less folate cofactors and displayed a 50% clonogenic viability at leucovorin concentrations as low as 60 \( \mu \)M (Table I). Consequently, Pyr\textsuperscript{R100} cells could grow solely on the residual (\( \sim \)75 \( \mu \)M) folic acid-free medium supplemented with 5\% dialyzed fetal bovine serum (data not shown). Thus, Pyr\textsuperscript{R100} cells possess a markedly decreased folate growth requirement and picomolar concentrations of leucovorin are sufficient to support clonogenic growth.

To explore the role of membrane transport in the markedly decreased folate growth dependence of Pyr\textsuperscript{R100} cells, \([\mathrm{3H}]\)folic acid transport was measured in the presence of 5 \( \mu \)M TMQ to abolish DHFR activity and subsequent folic acid reduction (23). Net uptake of folic acid into AA8 and Pyr\textsuperscript{R100} cells reached steady-state at 30 min (Fig. 1, inset) and 60 min (Fig. 1) with folic acid levels at 0.51 \( \pm \) 0.03 and 7.52 \( \pm \) 1.17 nmol per g of dry weight, respectively (Table II). Based on the measured intracellular water, AA8 and Pyr\textsuperscript{R100} cells achieved free intracellular folic acid concentrations of 0.1 and 1.7 \( \mu \)M, respectively (Table II). Therefore, Pyr\textsuperscript{R100} cells achieved a 17-fold higher intracellular folic acid concentration than parental AA8 cells.

Net uptake of \([\mathrm{3H}]\)MTX (Fig. 2) was also higher in Pyr\textsuperscript{R100} than in AA8 cells (7.63 \( \pm \) 0.64 versus 3.40 \( \pm \) 0.28 nmol MTX/g of dry weight, respectively, Table III). To assess free MTX, cells loaded with \([\mathrm{3H}]\)MTX were resuspended in MTX-free buffer allowing for discrimination between the free and bound components. While the tightly bound MTX fraction was only slightly increased in Pyr\textsuperscript{R100} cells, the free intracellular MTX concentration of 1.1 \( \mu \)M was increased 4.8-fold in this cell line as compared with 0.23 \( \mu \)M in AA8 cells (Table III). Hence, net accumulation of free folic acid in Pyr\textsuperscript{R100} cells was much greater than for MTX.

To assess the role that changes in influx and efflux parameters contribute to the alterations in steady-state (anti)folate levels, bidirectional fluxes were measured. While folic acid influx was increased 2-fold in Pyr\textsuperscript{R100} cells (Table II), no significant difference in the influx of MTX was observed between Pyr\textsuperscript{R100} and AA8 cells (Table III). Analysis of efflux revealed that parental AA8 cells had a very rapid loss of folic acid \( t_{1/2} = 4 \) min), whereas the efflux \( t_{1/2} \) in Pyr\textsuperscript{R100} cells was 80 min (Fig. 3A). While folic acid efflux in Pyr\textsuperscript{R100} cells was not complete even after 3 h of incubation at 37 °C, the process could be described by a single exponential in both cell lines (Fig. 3B). The folic acid efflux rate constant \( k\) was decreased by a factor of 23 in Pyr\textsuperscript{R100} relative to AA8 cells \( k = 0.0036 \) min\textsuperscript{-1} and 0.084 min\textsuperscript{-1}, respectively, Table II). MTX efflux was also decreased in Pyr\textsuperscript{R100} cells (Fig. 4A), albeit to a lesser extent than observed for folic acid with a 5-fold fall in the MTX efflux rate constant in Pyr\textsuperscript{R100} relative to AA8 cells (0.041 min\textsuperscript{-1} and 0.22 min\textsuperscript{-1}), respectively (Fig. 4B and Table III). Hence, the large increase in the steady-state folic acid levels in Pyr\textsuperscript{R100} cells is associated with a two fold increase in influx but more than an order of magnitude greater decline in efflux. The smaller increase in MTX uptake in Pyr\textsuperscript{R100} cells is due entirely to a decline in efflux and to a much smaller extent than observed for folic acid in Pyr\textsuperscript{R100} cells. A variety of studies were undertaken to confirm that the markedly diminished efflux of folic acid and MTX in Pyr\textsuperscript{R100} cells was due entirely to a change in membrane transport and not due to: 1) increased (anti)folylpolyglutamylation, 2) increased compartmentation of folate in mitochondria, 3) sequestration by a highly overexpressed (anti)folate-binding protein, or 4) decreased plasma membrane potential.

![Fig. 2. Time course of MTX accumulation in Pyr\textsuperscript{R100} and AA8 cells.](image)

Following 20 min incubation of Pyr\textsuperscript{R100} (solid squares) and AA8 (solid triangles) cell suspensions (3 \( \times \) 10\textsuperscript{6} cells/ml) in HBS containing 1% pyruvate, transport was initiated by addition of \([\mathrm{3H}]\)MTX to a final concentration of 1 \( \mu \)M. Following 30 min of loading with 1 \( \mu \)M MTX, the cells were resuspended into a large volume of drug-free buffer for determination of the loss of free drug (indicated by the double-headed arrows) and the tightly bound component (open symbols).
conditions, mitochondria-associated folic acid in both PyrR100 and AA8 cells did not exceed 0.5% of cellular radiolabel. To explore the possibility of intracellular folic acid sequestration by a potentially overexpressed folate binding protein, cells were loaded with 1 mM [3H]folic acid for 1 h after which G-25 gel filtration of total cellular lysate was performed. All the radio-label (>99.9%) was retained on the column indicating that all the [3H]folic acid was free and not protein-associated. The possibility of a decline in the membrane potential in PyrR100 cells that would result in an increase in the levels of folates was explored by the equilibrium distribution of the lipid-soluble cation TPP⁺ across the plasma membrane. Based on the Nernst equation the mean membrane potential value for both PyrR100 and AA8 cells was calculated to be −35 mV (Table II). Hence, a decline in the membrane potential did not play a role in the marked accumulation of folic acid in PyrR100 cells.

Since the data indicated that changes in net folic acid and MTX transport were associated with a large increase in the transmembrane electrochemical potential difference for these folates in PyrR100 cells, studies were focused on the bioenergetic characteristics of these processes in the two cell lines. It has been well established that at least one component of MTX efflux in various mammalian cells is mediated by energy requiring exit pump(s) (14–17), while uphill transport into cells appears to be mediated by RFC, through an anion exchange mechanism with intracellular organic phosphates (3, 4). Hence

### Table III
Comparison of MTX transport parameters between AA8 and PyrR100 cells

|                  | AA8          | PyrR100      | PyrR100/AA8 |
|------------------|--------------|--------------|-------------|
| Initial uptake rate (nmol/min/g dry wt) | 0.366 ± 0.036 | 0.421 ± 0.030 | 1.15⁺        |
| Efflux rate constant (min⁻¹)              | 0.22 ± 0.06  | 0.041 ± 0.012 | 0.186        |
| Total cell MTX (nmol/g dry wt)            | 3.396 ± 0.276 | 7.63 ± 0.64  | 2.25         |
| Bound MTX (nmol/g dry wt)                 | 2.25 ± 0.17  | 2.70 ± 0.24  | 1.20         |
| Free MTX (nmol/g dry wt)                  | 1.14         | 4.93         | 4.30         |
| Observed [MTX], (µM)                      | 0.23         | 0.041 ± 0.012 | 0.07         |
| Predicted [MTX], (µM)                      | 0.07         | 1.11         | 4.83         |
| Observed/Predicted                          | 3.29         | 15.9         | 4.8          |

* The difference is not statistically significant (p = 0.1805).

* Based upon the Nernst equation using the measured membrane potential of −35 mV and an extracellular MTX level of 1 µM.

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**FIG. 3.** Time course of folic acid efflux in PyrR100 and AA8 cells. Following 30 min of loading of PyrR100 (squares) and AA8 cells (triangles) with 1 µM [3H]folic acid in 10 µM TMQ-containing HBS at 37 °C, cells were rapidly sedimented by centrifugation and resuspended in TMQ-containing folic acid-free HBS at 37 °C, and efflux was followed for up to 3 h (A). To determine the folic acid efflux rate constants, the log value of the percentage of initial free folic acid level at each time point was plotted as a function of time (B).

**FIG. 4.** Time course of MTX efflux in PyrR100 and AA8 cells. After 30 min loading of PyrR100 (squares) and AA8 cells (triangles) with [3H]MTX at 1 µM and 5 µM (to compensate for the small exchangeable pool of MTX in AA8 cells, see Table III), respectively, cells were rapidly sedimented by centrifugation and suspended in drug-free HBS (A). To determine the MTX efflux rate constants, the log value of the percentage of the initial free MTX (corrected for tightly bound MTX, see Table III) was plotted as a function of time (B).
studies were undertaken to assess the energy requirement for (anti)folate transport in PyrR100 and AA8 cells. Energy deple-
tion achieved by incubating cells for 30 min in glucose- and 
pyruvate-deficient transport buffer (27) decreased the aug-
mented net folic acid transport in PyrR100 cells by 85%, while 
net folic acid uptake in AA8 cells at steady-state was essen-
tially unchanged (Fig. 5). In contrast, glucose and pyruvate 
deprivation increased net MTX transport in AA8 (Fig. 6A) 
but not in PyrR100 cells (Fig. 6B). Provision of glucose and pyruvate 
after energy depletion resulted in an immediate fall in the 
free MTX in AA8 (Fig. 6A, arrow) but no change in PyrR100 cells 
(Fig. 6B, arrow). Folic acid influx in PyrR100 and AA8 cells was 
decreased by 80 and 60%, respectively, in the absence of glu-
cose and pyruvate and under these conditions was comparable 
in the two cell lines (Fig. 7). Energy deprivation did not alter 
MTX influx in either AA8 or PyrR100 cells (based upon four 
experiments).

**DISCUSSION**

This study demonstrates that the underlying basis for the 
markedly decreased folate growth requirement of PyrR100 cells 
is due to a major decline in folic acid efflux and a small increase 
in folic acid influx. This resulted in a markedly augmented folic 
acid accumulation. Net uptake of MTX was also increased in 
PyrR100 cells but to a lesser extent; this was due entirely to a 
fall in efflux. Studies excluded the possibility that augmented 
folic acid uptake was related to: 1) increased expression of a 
cellular folate-binding protein, 2) increased sequestration of 
folates in mitochondria, 3) increased polyglutamylation, or 4) a 
fall in the membrane potential of PyrR100 cells. This constitutes 
the first demonstration of the loss of folic acid exporter activity 
in mammalian cells selected for lipophilic antifolate resistance.

The marked decrease in folic acid and MTX export activities 
in PyrR100 cells resulted in a marked increase in the transmem-
brane gradients for these folates. Hence, the chemical gradi-
ents for folic acid and MTX increased by factors of 17 and 5 in 
PyrR100 as compared with AA8 cells. When the membrane 
potential, measured at −35 mV in both cell lines is considered, 
the expected ratio of intracellular to extracellular concentra-
tions of both folates should be 0.07 when the extracellular level 
is 1 μM, if transport were passive or equilibrating, i.e. no energy 
consumed in the transport process. In fact, the intracellular 
level of folic acid is only 50% greater, and MTX is three times 
higher than this predicted value in AA8 cells, representing only 
a small electrochemical potential difference across the cell 
membrane. On the other hand, net transport is markedly in-
increased in PyrR<sub>100</sub> cells, the folic acid level exceeds the predicted equilibrium value by a factor of 24, and MTX exceeds this value by a factor of 16. The maintenance of these high electrochemical potential differences across the cell membrane must require a substantial energy source.

Uphill transport of MTX into mammalian cells (most thoroughly characterized in mouse L1210 leukemia cells) is dependent upon an RFC-mediated anion exchange with intracellular organic anions. In this model, the downhill flow of these organic anions out of the cell via the carrier results in the uphill flow of MTX into the cell by the same mechanism. This process is opposed by independent MTX exit pump(s) that are tightly coupled to ATP hydrolysis (14–16). When the exit pump is inhibited under conditions of energy deprivation, influx of MTX is unchanged or only slightly increased, efflux is markedly decreased, and the net uptake of MTX is consequently rapidly and substantially increased.

This was the behavior observed for MTX in AA8 cells. There was essentially no change in influx with deprivation of energy substrates, and net uptake was increased, consistent with a reduction in efflux due to cessation of efflux pump activity. When energy substrates were added back to the cells, there was an immediate and rapid net loss of MTX from the cells consistent with the energization of the efflux pump, the pattern that has been described for L1210 mouse leukemia cells (13, 14, 28–30). The pattern in PyrR<sub>100</sub> cells was fundamentally different; while net MTX uptake was markedly greater than in AA8 cells this process was not affected by the absence of energy substrates, consistent with a loss of exporter function but no change in energy-coupling to the RFC system.

The energetics of folic acid uptake in AA8 cells was different from that observed with MTX. Influx was decreased, net transport essentially unchanged with energy deprivation. The affinity of folic acid for RFC is one or two orders of magnitude lower than that for MTX (3–5). However, the export pump(s) have a potent effect on folic acid efflux. Hence the exit pump dominates, and only very low steady-state levels of folic acid are achieved in AA8 cells. In PyrR<sub>100</sub> cells the lack of energy substrates markedly decreased both influx and net transport of folic acid. Thus, folic acid influx in these cells appears to be linked to metabolic energy in a process that accounts for sustained uphill transport under conditions in which the exporter is not functional. This appears to be a mechanism that is specific for folic acid with little or no impact on the transport of MTX.

Of note is that the MTX efflux rate constant (\( k = 0.22 \) min<sup>-1</sup>) obtained for CHO AA8 cells was identical to that observed by the Sirotnak group (28, 29) with mouse leukemia L1210 cells (\( k = 0.21 \) min<sup>-1</sup>). Moreover, the latter group reported that under energy deprivation this MTX efflux rate constant was decreased by 83% (\( k = 0.036 \) min<sup>-1</sup>); this value is again almost identical to the MTX efflux rate constant obtained with PyrR<sub>100</sub> cells (\( k = 0.041 \) min<sup>-1</sup>). In contrast, the folic acid efflux rate constant in parental AA8 cells was 0.084 min<sup>-1</sup> but was profoundly decreased in PyrR<sub>100</sub> cells (\( k = 0.0036 \) min<sup>-1</sup>). Taken together these data suggest that, as with mouse L1210 cells (30), ~80% of the MTX efflux appears to be mediated via a route distinct from RFC, such as the MOATs (14–16), most likely through MOAT 3 (16), whereas the residual MTX efflux ostensibly occurs via RFC (30). In contrast, since RFC has a very much lower affinity for folic acid than MTX, the vast majority of the efflux of this folate in CHO AA8 cells presumably exits via a MOAT. Hence, when transport by the MOAT is abolished, as presumably occurs in PyrR<sub>100</sub> efflux is markedly reduced, and high levels of folic acid accumulate.

Recent studies have shown that some MOATs particularly MOAT 3 and to some degree MOAT 4 can mediate an ATP-mediated efflux of variety of organic anions including MTX (16). A canalicular rat liver organic anion transporter cDNA (termed cMOAT) has been recently cloned which mediates the ATP-driven hepatocellular excretion of numerous toxic organic anions (31). This cMOAT which contains the ATP-binding domains (Walker A and B) was shown to be a member of the well established ABC superfamily of transporters (32). It is possible that like MTX (14, 15), the efflux of folic acid is also mediated by a related ATP-driven exit pump.

PyrR<sub>100</sub> cells exhibit a genetically stable resistance to lipid-soluble antifolates that is fully retained even after long term growth (1,300 cell doublings) in pyrimethamine-free medium (21, 27). Furthermore, the markedly augmented folic acid accumulation as well as the loss of folate efflux activity are stable in PyrR<sub>100</sub> cells grown in the continuous presence of pyrimethamine and in its absence even after 1,300 cell doublings. These characteristics strongly suggest that the putative genetic alteration responsible for the loss of folic acid efflux activity in PyrR<sub>100</sub> cells is stable. It is of interest that the in vivo loss of organic anion transporter activity in canalicular liver cells was associated with a single nucleotide deletion in the cMOAT gene leading to congenital jaundice in these rats (31).

PyrR<sub>100</sub> cells display over 1,000-fold resistance to pyrimethamine and a 30-fold cross-resistance to trimetrexate and piritrexim, all of which are lipid-soluble inhibitors of mammalian DHFR (21, 23). The ability of PyrR<sub>100</sub> cells to accumulate and retain high levels of folic acid is a very useful adaptation to lipophilic antifolates in particular to 2,4-diaminopyrimidines including pyrimethamine which have relatively low affinity for DHFR (21). Augmented net transport and free intracellular folic acid levels results in increased formation of reduced folates and retention of their polyglutamyl derivatives. This, in turn, results in the generation of high dihydrofolate levels as antifolate associates with DHFR, reduced folates are oxidized, and dihydrofolate then effectively competes with the antifolate for the small percentage of enzyme binding sites (<5%) that are sufficient to sustain tetrahydrofolate cofactor pools (33).

The potential loss of folic acid and MTX exporter activity in human tumors due to treatment with, for example, lipophilic antifolates may have potentially important implications for selective chemotherapy. The transporter that mediates the efflux of MTX and folic acid has a broad specificity for various organic anions some of which are cytotoxic including cholate and taurocholate (14–16). Thus, PyrR<sub>100</sub> cells that have lost folic acid efflux activity are likely to accumulate high levels of such toxic organic anions and possess a prominent hypersensitivity to these compounds. Hence, it may be possible to exploit this property to selectively kill these tumor cells with low concentrations of cytotoxic agents that are converted intracellularly to amphiphilic anion conjugates. Thus, the loss of folic acid exporter activity may provide a novel approach for selective elimination of drug-resistant tumor cells with this phenotype.

Acknowledgment—We thank Pi-Jun Wang for expert technical assistance.

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