Biochemical Pharmacology, Vol. 53, pp. 223–231, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00730-7

pH Dependence of Methotrexate Transport by the Reduced Folate Carrier and the Folate Receptor in L1210 Leukemia Cells

FURTHER EVIDENCE FOR A THIRD ROUTE MEDIATED AT LOW pH

*Esteban E. Sierra, Kevin E. Brigle, Michael J. Spinella and I. David Goldman**

DEPARTMENTS OF MEDICINE AND MOLECULAR PHARMACOLOGY, THE EINSTEIN CANCER CENTER, BRONX, NY 10461, U.S.A., AND MASSEY CANCER CENTER, RICHMOND, VA 23298, U.S.A.

ABSTRACT. F2-MTX^{r}A is an L1210 leukemia cell line with a functional defect in the reduced folate carrier and high level expression of folate receptor β. The pH-dependence of methotrexate (MTX) influx by folate receptor β in F2-MTX^{r}A cells was characterized and compared with that of the reduced folate carrier in parental L1210 cells. MTX influx by folate receptor β had a pH optimum of 6.5, whereas influx mediated by the reduced folate carrier showed a pH optimum of 7.5. Increased folate receptor β-mediated MTX influx at pH 6.5 relative to pH 7.5 was accompanied by a 5-fold increase in binding affinity of the receptor for MTX without a change in the number of binding sites. At pH 6.2, approximately 24% of MTX influx in F2-MTX^{r}A cells proceeded by another mechanism. This transport route became active at pH <7.5, operated optimally at pH 6.0 to 6.5, and, unlike folate receptor β-mediated MTX influx, was insensitive to the presence of low levels of folic acid (100 nM). MTX influx by the low pH system showed saturability, with a K_t of 5.3 μM and a V_{max} of 1.53 nmol/g dry wt/min, was energy dependent, was inhibited by sulfobromophthalein with a K_i of 148 μM, and had similar relative affinities for folic acid, leucovorin, and 5-methyltetrahydrofolate. Influx of 5-methyltetrahydrofolate was also mediated by this route. The data provide further confirmatory evidence for an MTX influx route in F2-MTX^{r}A cells, optimal at low pH and distinct from the reduced folate carrier or the folate receptor. BIOCHEM PHARMACOL 53;2:223–231, 1997.

KEY WORDS. reduced folate carrier; folate receptor; methotrexate; transport; binding

Multiple transport systems mediate the translocation of folates and antifolates into and out of L1210 leukemia cells [1–4]. There is a transport carrier (RFC†) that mediates the bidirectional fluxes of MTX and reduced folates via a process that is not directly coupled to energy metabolism but is capable of sustained uphill transport through co-transport with intracellular anions [1, 5–7]. Recently, murine, hamster, and human cDNAs have been cloned and their products shown to restore MTX transport in RFC-deficient cells [8–11]. Though typically not present in L1210 cells, there are at least two glycosylphosphatidylinositol-linked FRs that transport folates via an endocytotic, energy-requiring process that can be expressed under low folate selective pressure [12, 13]. There is, in addition, evidence for an influx route that operates optimally at low pH [14]. Studies with membrane vesicles from L1210 cells suggest the presence of another transport system with lower affinity but higher capacity for folates than the RFC [15]. Finally, two independent, energy-requiring efflux routes pump MTX out of the cell [16–19].

Folate transport mechanisms with properties distinct from those in L1210 cells have been described in small intestine, liver, and kidney [20–24]. A detailed understanding of the characteristics of MTX transport via all these routes has become increasingly important as the cDNAs encoding the proteins that transport folates in other tissues are cloned and their functional properties determined.

This laboratory recently demonstrated that RFC-mediated MTX influx in L1210 and FR-β-mediated MTX transport in the RFC-defective L1210 derivative F2-MTX^{r}A exhibit different pH dependencies [25]. Lowering the extracellular pH from 7.4 to 6.2 resulted in decreased MTX influx via the RFC but strongly stimulated transport via FR-β. In this paper, we further characterize this pH dependence and report that increased MTX transport in F2-MTX^{r}A cells at low pH is due to both an increased affinity of FR-β for MTX and the appearance of another transport route, distinct from the RFC and FR-β and while

* Corresponding author: Dr. I. David Goldman, Albert Einstein College of Medicine, Belfer Building, Room 1002, 1300 Morris Park Ave., Bronx, NY 10461. Tel. (718) 430-2302; FAX (718) 430-8550; E-mail: igoldman@aecom.yu.edu

† *Abbreviations:* RFC, reduced folate carrier; MTX, methotrexate; FR, folate receptor; MES, 2-[*N*-morpholino]ethanesulfonic acid; and BSP, sulfobromophthalein.

Received 2 April 1996; accepted 20 August 1996.

similar in several respects to an influx route previously reported to operate optimally at low pH in L1210 cells [14] has properties that differ from those described previously.

MATERIALS AND METHODS

Chemicals

[3′,5′,7-^{3}H]MTX and [3′,5′,7′,9′-^{3}H]folic acid were obtained from Moravek Biochemicals (Brea, CA). [^{3}H]-[6S]-5-Methyltetrahydrofolate was the gift of Dr. Barton Kamen (University of Texas Southwestern Medical Center). Folic acid and [6*R*,*S*]-5-methyltetrahydrofolate were from Sigma (St. Louis, MO); [6*R*,*S*]-5-formyltetrahydrofolate was from Lederle (Carolina, Puerto Rico). Folate compounds were purified by high performance liquid chromatography prior to use [26].

Cell Culture

L1210 cells were grown in RPMI 1640 medium containing 2.2 μM folic acid supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL). F2-MTX^{r}A is a clonal line derived from the RFC-defective, MTX-resistant line MTX^{r}A [27]. F2-MTX^{r}A was selected for growth on low levels of folic acid, overexpresses FR-β, and is maintained in folate-free RPMI 1640 medium with 10% dialyzed bovine calf serum supplemented with 0.5 nM folic acid [28].

[^{3}H]MTX Influx

[^{3}H]MTX influx was measured as previously described with minor modifications [29]. Briefly, cells were harvested, and washed with 0° acid HEPES saline (20 mM HEPES, 137 mM NaCl, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, 7 mM glucose, pH 4.5) to release folates bound to FR-β. This was followed by a wash with buffer at the desired pH. Experiments at pH 5.0 to 6.5 were performed in MBS (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose) [14] and those at pH 7.0 to 8.0 in HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose). Prior to influx measurements, cells were resuspended into the desired buffer at 1.5×10^7 cells/mL, equilibrated at 37°, and uptake was initiated by the addition of the radiolabeled compound. Uptake was terminated by injecting 1.0 mL of the cell suspension into 10 mL of 0° acid HEPES saline. Cells were collected by centrifugation, washed twice with 0° HBS (pH 7.4), and processed for determination of intracellular tritium as previously described [29]. All studies were performed with 0.2 μM [^{3}H]MTX unless otherwise stated. MTX influx mediated by the FR-β independent low pH route was measured in the presence of 100 nM folic acid to block FR-β-mediated influx [25]. Influx kinetics were determined over a range of MTX concentrations (0.5 to 7.5 μM) and intervals in which [^{3}H]MTX uptake did not exceed the dihydrofolate reductase binding capacity. Experiments utilizing metabolic poisons were performed in transport buffer without glucose. Cellular viability at the end of the incubations was assessed by trypan blue exclusion and determined to be >90%. MTX accumulation is expressed as nanomoles per gram dry weight. Total cellular protein of L1210 and MTX^{r}A cells per mg dry weight were 0.36 ± 0.01 and 0.37 ± 0.01 mg, respectively.

Analysis of Specific MTX and Folic Acid Binding to the Cell Surface

Specific binding was determined using minor modifications of a previously reported assay [30, 31]. The assay mixture contained 1×10^7 cells in HBS or MBS buffer and [^{3}H]MTX, as indicated in the figures. Following incubation for 5 min at 0°, the samples were centrifuged (12,000 *g*, 0°, 2 min), the supernatant was aspirated, and residual supernatant was removed by a second centrifugation and aspiration step. Cell pellets were processed and radioactivity was determined as previously described [31]. Specific binding was determined as the difference between total radiolabeled folate bound and binding in the presence of 100 μM unlabeled compound. Binding parameters were obtained by nonlinear fitting of the data to a one-site model using the GraphPad PRISM computer software.

MTX Growth Inhibition

Cell cultures (5×10^4 cells/mL, 0.2-mL cultures) in 96-well plates were exposed continuously to increasing concentrations of MTX for 48 hr, following which cell numbers were determined by hemocytometer count, and viability was determined by trypan blue exclusion. The pH of RPMI 1640 was adjusted to 7.4 or 6.2. Experiments were performed in duplicate.

RESULTS

pH-Dependence of MTX Influx in L1210 and F2-MTX^{r}A Cells

This laboratory previously described the L1210 subline F2-MTX^{r}A, which overexpresses FR-β and fails to transport MTX via the RFC due to impaired mobility of the carrier [25, 28]. This cell line transports MTX via FR-β in contrast to L1210 cells, in which MTX influx proceeds via the carrier. Initial observations indicated that decreasing the pH of the transport buffer from 7.4 to 6.2 inhibits RFC-mediated MTX influx but stimulates FR-β-mediated MTX transport [25]. Studies were undertaken to characterize in detail the pH-dependence of FR-β-mediated MTX transport in F2-MTX^{r}A cells and compare it with that of the RFC in L1210 cells. As shown in Fig. 1, MTX influx in L1210 and F2-MTX^{r}A cells showed very different pH profiles. RFC-mediated MTX influx in L1210 cells had a pH optimum of ~7.5, whereas F2-MTX^{r}A cells showed maximal influx at pH ~6.5 (Fig. 1A). MTX binding to FR-β in F2-MTX^{r}A

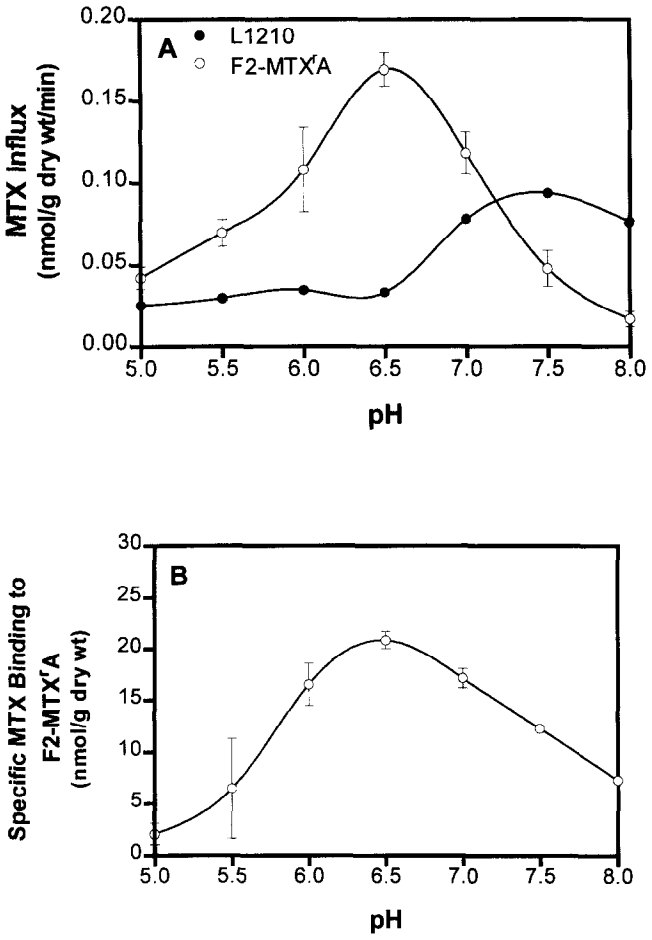


FIG. 1. (A) Effects of pH on MTX influx in L1210 and F2-MTX^{r}A cells. Cells were harvested, washed as described, resuspended in buffer at the indicated pH, and exposed to 0.2 μM [^{3}H]MTX, and influx was determined over 10 min. Results are the averages of two (L1210) or four (F2-MTX^{r}A) experiments. (B) pH-Dependence of specific MTX binding to FR-β. F2-MTX^{r}A cells were incubated with 0.2 μM [^{3}H]MTX for 5 min at 0° in buffer of the desired pH, and specific binding was determined as described in Materials and Methods. Results are the means ± SD of three experiments.

cells showed a pH profile similar to that of MTX influx, with a pH optimum of ~6.5 (Fig. 1B). Specific MTX binding to the RFC in L1210 cells was barely detectable so that a reliable pH profile could not be obtained. Hence, in F2-MTX^{r}A cells MTX influx paralleled MTX binding to FR-β, and both parameters fell as the pH increased to the physiological range.

pH-Dependence of the K_d and B_{max} for MTX Binding to FR-β

Increased binding of MTX at pH 6.5 could result from an increase in the number of ligand binding sites at the cell surface, increased affinity of FR-β for MTX, or both. Equilibrium binding of MTX to FR-β was measured at pH 6.5 and 7.5 over a range of MTX concentrations. Binding saturation at pH 6.5 occurred at a lower MTX concentration than at pH 7.5 (Fig. 2). Scatchard analysis (Fig. 2, inset; Table 1) indicated an approximately 5-fold decrease in K_d at pH 6.5 relative to pH 7.5 with no change in B_{max}. MTX binding to F2-MTX^{r}A cells was abolished completely by the addition of 100 nM folic acid at both pHs, confirming that all measurable MTX associated with the cells was bound to FR-β. Hence, at a pH of 6.5 there was a large increase in the affinity of FR-β for MTX without an increase in the number of MTX binding sites at the cell surface. Again, MTX binding to L1210 cells was barely detectable over the range of concentrations used in this study (Fig. 2).

Presence of an FR-β-Independent Influx Component in F2-MTX^{r}A Cells

Increased MTX transport at pH 6.5 could be accounted for partially by increased drug binding to FR-β in F2-MTX^{r}A cells. However, in the presence of 100 nM folic acid, MTX binding to F2-MTX^{r}A cells was abolished, but 24 ± 8% (N = 10) of influx remained in contrast to the complete abolition of transport by FR-β at pH 7.4 (Fig. 3), suggesting the presence of a second low pH transport route independent of FR-β. This was further quantitated by assessing the effect of 100 nM folic acid on MTX influx over a broad pH range, as shown in Fig. 4. An MTX transport route insensitive to 100 nM folic acid became evident at pH levels below 7.5, showed optimal activity at pH 6.0, and accounted for the majority of MTX influx below this pH. The observed increase in MTX influx at low pH in the presence of 100 nM folic acid was not due to decreased binding affinity of FR-β for folic acid. Hence, folic acid binding to FR-β on F2-MTX^{r}A cells at pH 6.2 and 7.4 over a range of concentrations (0.01 to 50 nM) indicated identical near-stoichi-

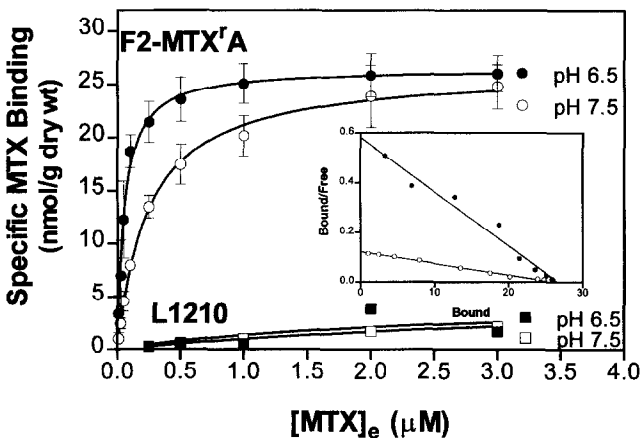


FIG. 2. Equilibrium binding of MTX to F2-MTX^{r}A (circles) and L1210 (squares) cells. Cells were incubated with increasing MTX concentrations at pH 6.5 (closed symbols) or 7.5 (open symbols). Specific binding was determined as described in Materials and Methods. Results are the means ± SD of four experiments. Inset: Scatchard representation of the binding data.

TABLE 1. Parameters of MTX binding to FR-β at pH 6.5 and 7.5

pH	K_d (μM)	B_{max} (nmol/g dry wt)
6.5	0.057 ± 0.010	26.64 ± 0.08
7.5	0.267 ± 0.043	26.56 ± 1.16

Specific MTX binding was determined over a broad range of concentrations (0.01 to 3.0 μM), and the binding parameters were obtained as described in Materials and Methods. Results are the means ± SD of four experiments.

ometric association under these conditions, and 100 nM folic acid completely abolished binding of 0.2 μM MTX to FR-β at pH 6.2 and 7.4 (data not shown). Finally, higher MTX concentrations were required to compete for folic acid binding to FR-β at pH 6.2 than at 7.4 (data not shown). Taken together, these results indicate equal or higher affinity of FR-β for folic acid at pH 6.2 than at 7.4 and further substantiate the existence of a second MTX influx component, independent of FR-β, that operates most efficiently at low pH.

The presence and magnitude of an FR-β-independent MTX transport route are further illustrated from an analysis of the effect of increasing folic acid concentrations on MTX influx in F2-MTX^{r}A cells at pH 7.4 (Fig. 5A) and 6.2 (Fig. 5B). While MTX influx was essentially abolished by 50–100 nM folic acid at pH 7.4, a component much less sensitive to folic acid was seen at pH 6.2. Dixon plots clearly demonstrated that inhibition of MTX influx by folic acid was monophasic at pH 7.4 but biphasic at pH 6.2 (Fig. 5, insets), consistent with heterogeneity of transport at the lower pH. When the less sensitive component of MTX influx at pH 6.2 was subtracted from total influx (Fig. 5B, dashed line), the IC_{50} values for folic acid inhibition of MTX influx were virtually the same at pH 6.2 and 7.4 (19 and 14 nM, respectively), and the Dixon plot was monophasic (not shown), corroborating that the folic acid sensitive component at pH 6.2 represents FR-β.

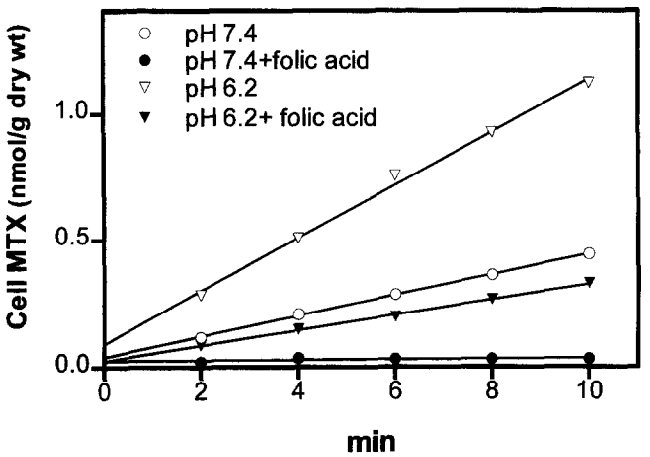


FIG. 3. Effects of folic acid on MTX influx in F2-MTX^{r}A cells at pH 6.2 and 7.4. Cells were harvested, washed, and resuspended in either HBS (pH 7.4) or MBS (pH 6.2) at 37°. Folic acid (100 nM) was added 5 min prior to addition of [^{3}H]MTX. At time zero, [^{3}H]MTX was added to achieve a concentration of 0.2 μM. Results are the averages of two experiments.

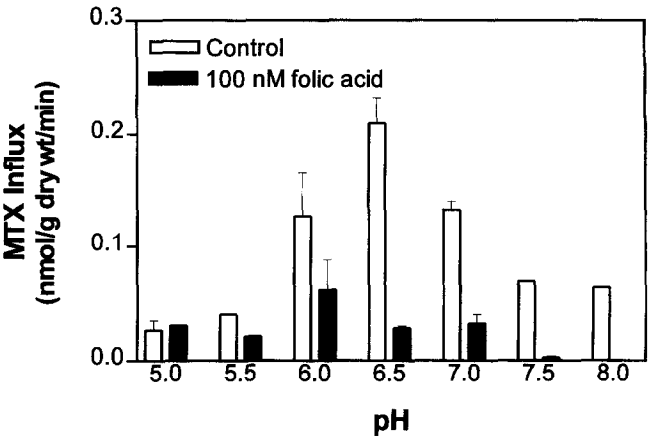


FIG. 4. Effects of 100 nM folic acid on MTX influx in F2-MTX^{r}A cells as a function of pH. Influx of 0.2 μM [^{3}H]MTX was measured at the indicated pH in the absence (open bars) or presence (closed bars) of folic acid. Results are the means ± SD of three experiments.

Kinetics of MTX Influx and the Effects of Other Folates on Transport Mediated via the FR-β-Independent Low pH Route; Transport of 5-Methyltetrahydrofolate at pH 7.4 and 6.2

MTX influx kinetics mediated by the FR-β-independent low pH system were measured in the presence of 100 nM folic acid at pH 6.2 as a function of the extracellular MTX concentration (Fig. 6). Saturability was apparent, and a Lineweaver–Burk plot indicated a K_t of 5.3 ± 0.9 μM with a V_{max} of 1.53 ± 0.16 nmol/g dry wt/min (N = 5, Fig. 6).

Influx of folates via the RFC and FRs shows very distinct structural specificities. While the RFC prefers reduced folates, FRs have very high affinity for folic acid. The inhibitory effects of folates on MTX influx by the low pH system were compared. Inhibition by folic acid, 5-methyltetrahydrofolate, and leucovorin (5-formyltetrahydrofolate) was comparable at pH 6.2 (Fig. 7). Consistent with its inhibition of MTX transport at low pH, influx of 5-methyltetrahydrofolate in F2-MTX^{r}A cells was 3.9-fold higher at pH 6.2 than 7.4 (Fig. 8). MTX influx at pH 6.2 (Fig. 3) was ~50% that of 5-methyltetrahydrofolate.

Energy Dependence and BSP Sensitivity of MTX Transport in F2-MTX^{r}A Cells via the Low pH Route

While MTX influx via the RFC is unaffected by metabolic inhibitors, influx mediated by FRs is virtually abolished by these agents [25, 32]. Sodium azide (10 mM) and dinitrophenol (0.1 mM) inhibited MTX influx in F2-MTX^{r}A cells at pH 6.2 by 74 and 69%, respectively, in the presence of 100 nM folic acid.

BSP is a potent inhibitor of MTX influx via the RFC and

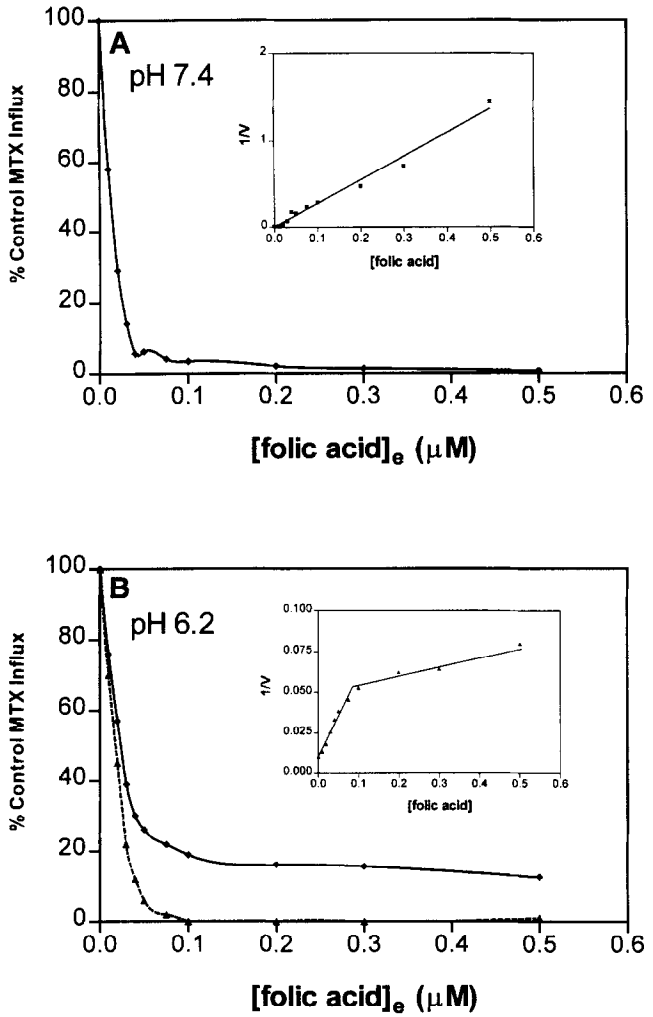


FIG. 5. Effects of folic acid on MTX influx at pH 7.4 (panel A; control: 0.74 nmol/g dry wt/10 min) and 6.2 (panel B; control: 1.82 nmol/g dry wt/10 min). Influx of 0.2 μM MTX in F2-MTX^{r}A cells was measured in the presence of increasing concentrations of folic acid. MTX and folic acid were added simultaneously. Panel B, dashed line: calculated influx after subtraction of the component less sensitive to folic acid. Results are the averages of two experiments. Insets: Dixon plot of the inhibition data.

less so of transport by FRs [25, 32]. BSP inhibited MTX influx by the FR-β-independent low pH route in F2-MTX^{r}A cells with a K_i of 148 ± 22 μM as determined by Dixon analysis (data not shown).

Effect of pH on MTX Inhibition of Cell Growth

Growth inhibition studies of F2-MTX^{r}A cells at pH 6.2 under conditions where FR-β is blocked by folic acid and all MTX transport is mediated by the low pH transporter indicated a 2-fold increase in MTX sensitivity relative to pH 7.4 (IC_{50} = 260 vs 520 nM, respectively, Fig. 9). In contrast, growth inhibition by MTX in L1210 cells at pH 6.2 decreased by 50% compared with pH 7.4 (IC_{50} = 13 vs

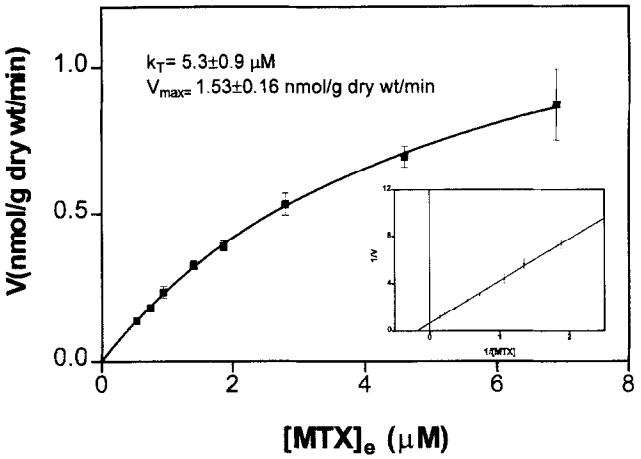


FIG. 6. MTX influx as a function of $[MTX]_e$. F2-MTX^{r}A cells were harvested, washed, and resuspended in MBS buffer, pH 6.2. [^{3}H]MTX was added simultaneously with 100 nM folic acid, to block FR-β-mediated transport, over a concentration range of 0.5 to 7.5 μM. Samples were taken over an interval during which intracellular MTX accumulation did not exceed the dihydrofolate reductase binding capacity. Results are the means ± SD of five different experiments. Inset: Lineweaver–Burk representation of the data.

6.5 nM). These changes in drug sensitivity are consistent with the observed differences in MTX transport.

DISCUSSION

These results characterize the pH-dependence of MTX transport mediated by the RFC and FR-β and establish the presence of another influx route that is functional only at low pH in F2-MTX^{r}A cells. As the pH decreases from neutral to slightly acidic, MTX transport shifts from that me-

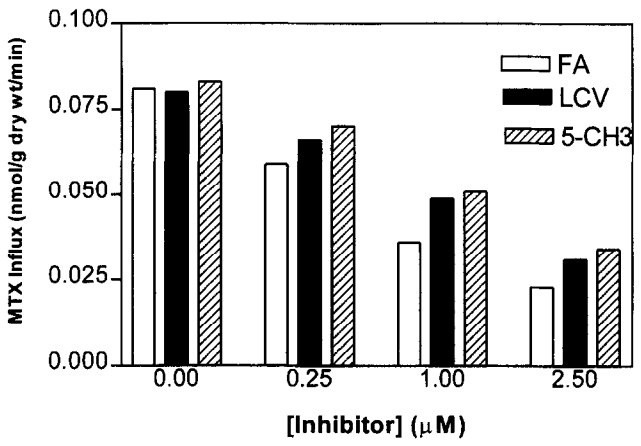


FIG. 7. Inhibition of MTX influx by different folates. F2-MTX^{r}A cells were harvested, washed as described, and resuspended in MBS (pH 6.2). At time zero, cells were exposed to 0.2 μM [^{3}H]MTX simultaneously with 100 nM folic acid, to block FR-β-mediated transport, and the indicated concentrations of folic acid (FA), leucovorin (LCV), or 5-methyltetrahydrofolate (5-CH_3). Influx was measured over 10 min. Results are the averages of two experiments.

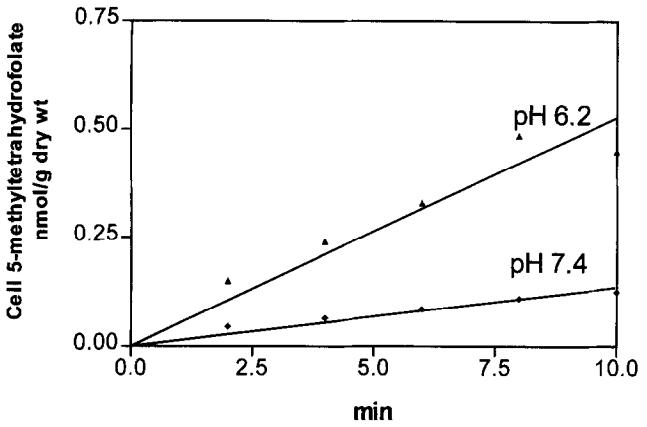
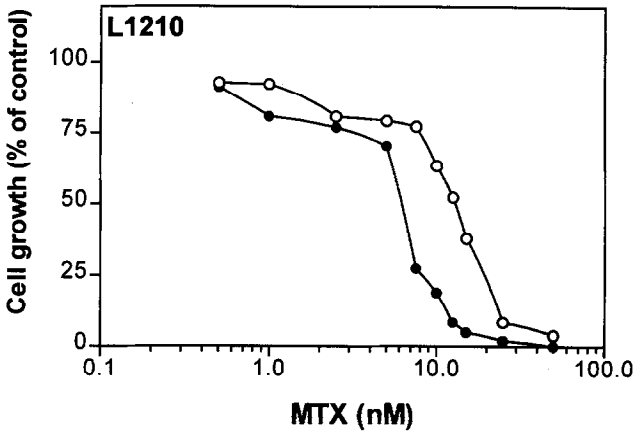


FIG. 8. Effect of pH on [^{3}H]5-methyltetrahydrofolate influx in F2-MTX^{r}A cells. Cells were harvested, washed, and resuspended in either HBS buffer, pH 7.4, or in MBS buffer, pH 6.2, as described. At time zero, [^{3}H]5-methyltetrahydrofolate was added to a final concentration of 0.2 μM. Results are the averages of two experiments.



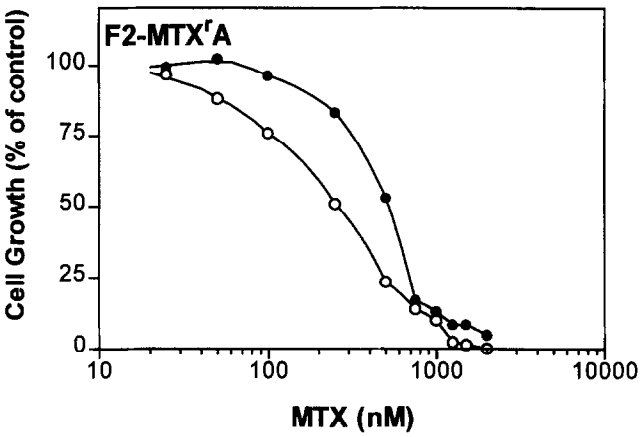


FIG. 9. Effect of pH on growth inhibition by MTX in L1210 and F2-MTX^{r}A cells. Cells were incubated for 48 hr in RPMI 1640 in the presence of the indicated MTX concentrations, and growth was determined as described under Materials and Methods. Key: (○), pH 6.2; and (●), pH 7.4. Viability of control cultures was >98% at pH 7.4 and >90% at pH 6.2. Control cultures (cells/mL): L1210, pH 6.2, 1.1 × 10^6; L1210, pH 7.4, 2.5 × 10^6; F2-MTX^{r}A, pH 6.2, 4.8 × 10^5; F2-MTX^{r}A, pH 7.4, 1.13 × 10^6; results are the averages of two experiments performed in duplicate.

diated by the RFC to transport mediated by both FR-β and the third transport route described in this paper. Hence, the extracellular pH as well as the level of expression of these MTX transport systems will determine the magnitude of the influx mediated by these three processes.

Although a low pH (<5.0) has been shown to completely dissociate folates from FRs [33], a detailed analysis of the pH-dependence of folic acid binding has been reported only for kidney [23, 24, 33]. Folic acid binding to FR isolated from porcine kidney exhibits a broad pH optimum of 5.5 to 7.6 [33]. Folic acid binding by rat kidney brush border membrane vesicles showed a sharp pH optimum of 5.6; however, the basis of increased binding at low pH was not investigated [24]. This report indicates that MTX binding to FR-β in an L1210 subline shows a pH optimum of 6.5 due to a 5-fold increase in binding affinity. Interestingly, this increased MTX binding at low pH was not FR isoform specific: preliminary experiments also showed increased MTX binding to FR-α at pH 6.5.

Since the pK_a values estimated for the MTX α and γ carboxyl groups are 3.8 and 4.8, respectively [34], the effects of the decreased pH over the range reported on MTX binding to FR-β cannot be explained by changes in the ionization of these groups. The pK_a of the 1-nitrogen position of the pteridine ring of MTX is higher than that of folic acid (5.7 vs 2.8, respectively; [34]). While this group may play a role in determining the affinity of FR-β for MTX, the increased concentrations of MTX required to compete for folic acid binding to FR-β at 6.2 vs 7.4, as well as the marked decrease in affinity for MTX at pH >6.5, cannot be explained solely by the ionization state of this group. Increased binding of MTX to FR-β at pH 6.5 could reflect specific requirements of deprotonation and protonation of amino acids residues, such as histidine (pK 6.0) [35], causing a conformational change in FR-β that results in increased affinity for MTX. It is of interest that pH and ligand binding were reported to affect the state of aggregation of FRs [36].

As observed for MTX transport mediated by FR-β, the increase in folic acid transport in rat kidney brush border vesicles showed the same pH optimum as binding [24]. This appears to be due to the presence of a transmembrane pH gradient rather than the acidic pH itself or electrical coupling caused by the inwardly directed positive gradient [37]. FR-β-mediated folate transport has been suggested to occur by a multi-step endocytic process in which folate bound to FRs are internalized within a membrane vesicle and, after acidification, the folates dissociate from the FR and enter the cell either directly or by some other, as yet undefined,

mechanism [12, 13]. Further studies are required to determine if, in addition to increasing the affinity of MTX for FR-β, the extracellular acidic pH affects any of the other steps in this complex process. The observation that the reduction in pH from 7.5 to 6.5 has a greater effect on MTX influx than binding to FR-β (3.5- and 1.7-fold, respectively, Fig. 1) suggests an additional stimulating effect on the translocation of drug into the cells.

The defect in RFC-mediated MTX transport in F2-MTX^{r}A cells is due to a single mutation that replaces a proline for an alanine in a predicted transmembrane domain [10]. While this mutation alters the functional properties of the RFC, the data collectively indicate that it is unlikely that the transport component active at low pH represents reactivation of the mutant RFC. First, MTX influx at pH 6.2 in F2-MTX^{r}A cells is sensitive to metabolic poisons in contrast to RFC-mediated MTX influx. Second, the affinity of the low pH route for folic acid is much higher than that of the RFC. Based on the kinetic parameters for MTX transport via this component (Fig. 6) and the degree of inhibition of folic acid (Fig. 7), the K_i for folic acid inhibition of MTX influx via this route is 1 μM, two orders of magnitude lower than that of the RFC [32]. Finally, while 100 μM BSP abolishes RFC-mediated MTX influx at pH 7.4 in L1210 cells, it only partially inhibits MTX transport via the low pH route. Likewise, evidence that this third transport component is distinct from FR-mediated transport includes: (1) relatively low affinity for folic acid (Fig. 5), (2) different pH profiles for MTX influx (Fig. 4), and (3) a different spectrum of apparent affinities for other folates. On the other hand, MTX influx mediated by FR-β and the low pH route showed similar sensitivities to BSP and metabolic poisons.

This low pH route can represent a significant pathway for the transport of folates and antifolates at its optimum pH. Hence, the V_{max} for this system (Table 2) at pH 6.2 is only one-half that of the RFC at pH 7.4 and the K_m values are comparable. In contrast, transport mediated by FR-β has a K_m (1.3 μM) one-third to one-fourth that of the RFC (4.2 μM) and the low pH route (5.3 μM), respectively, with a V_{max} one-fourth to one-half of these respective routes. This is under conditions in which FR-β is highly expressed while binding to the surface component of the low pH route is not detectable, consistent with a high turnover number for the latter, in the range of the RFC.

TABLE 2. Parameters of MTX influx via the RFC, FR-β, and the low pH route

Cell line	Transport route	pH	K_m (μM)	V_{max} (nmol/g dry wt/min)
L1210*	RFC	7.4	4.2	2.9
F2-MTX^{r}A*	FR-β	7.4	1.3	0.7
F2-MTX^{r}A†‡	Low pH transporter	6.2	5.3	1.5

MTX influx was measured as described in Materials and Methods.
* Ref. 24.
† Fig. 6.
‡ Influx was measured in the presence of 100 nM folic acid.

Studies are currently underway in this laboratory to define the role of the low pH route in MTX cytotoxicity. The effects of MTX on inhibition of cell growth at pH 6.2 and 7.4 were consistent with the observed pH-mediated differences in MTX transport in L1210 and F2-MTX^{r}A cells. However, extended culture at pH 6.2 resulted in decreased cellular viability and increased doubling times, complicating the interpretation of these results.

Henderson and Strauss [14] first described a transport system activated by a pH reduction in L1210 leukemia cell lines which transports folic acid, MTX, and 5-formyltetrahydrofolate. Like the route described above, this system is energy dependent, shows saturation kinetics, has a similar K_t for MTX, and shows comparable inhibition by folic acid and reduced folates. However, unlike the low pH route in F2-MTX^{r}A cells, that system was not inhibited by 100 μM BSP and did not transport 5-methyltetrahydrofolate efficiently. The discrepancy in BSP inhibition could be related to the lower ratio of BSP:MTX employed in the previous report. The reasons for the observed differences in 5-methyltetrahydrofolate transport are not clear. MTX influx at pH 6.2 in F2-MTX^{r}A cells was approximately twice that of 5-methyltetrahydrofolate at a comparable concentration. Furthermore, MTX transport at pH 6.2 was 3.1 times that at pH 7.4 while 5-methyltetrahydrofolate transport increased 3.9 times, suggesting that influx of both compounds increases proportionally and that both compounds are transported by the same pH-sensitive system. Because of the similarities in the properties of the low pH transport route described in this paper and those previously reported by Henderson and Strauss, it is likely that they represent the same process.

In addition to kidney, physiologically important pH-dependent systems for folate transport have been described in liver [22] and small intestine [20, 38], but their molecular characteristics are unknown. In liver, transport of folate and antifolate compounds appears to occur via different mechanisms [22]. Transport of 5-methyltetrahydrofolate is stimulated by a transmembrane hydrogen ion gradient and is inhibited by other folates and metabolic poisons. The stimulation by low extracellular pH has been attributed to a folate/H^+ cotransport mechanism. In contrast, MTX transport in liver is not inhibited by structural analogues and displays a broad pH optimum [22, 39]. A saturable folate transport route with a pH optimum of 5.0 to 6.0 has also been described in small intestine [20, 38]. Folate transport via this route is energy dependent and competitively inhibited by various folate compounds, including MTX. The low pH optimum of this system has been suggested to be due to both increased affinity of the transporter for folates [40] and a folate/H^+ cotransport or folate/OH^- exchange mechanism [21, 41]. Further studies are required to

elucidate the possible relationship between the FR-β-independent transport process with a low pH optimum in F2-MTX^{r}A cells and other pH-dependent folate transport systems in other tissues.

This work was supported by NCI Grants CA-39807, CA-09340, and CA-09394.

References

1. Goldman ID, The characteristics of the membrane transport of amethopterin and the naturally occurring folates. *Ann NY Acad Sci* **186:** 400–422, 1971.
2. Fan J, Vitols KS and Huennekens FM, Multiple folate transport systems in L1210 cells. *Adv Enzyme Regul* **32:** 3–15, 1992.
3. Henderson GB, Folate-binding proteins. *Annu Rev Nutr* **10:** 319–335, 1990.
4. Sirotnak FM, Correlates of folate analog transport, pharmacokinetics and selective antitumor action. In: *Membrane Transport of Antineoplastic Agents* (Ed. Goldman ID), pp. 241–281. Pergamon Press, Oxford, 1986.
5. Goldman ID, Lichtenstein NS and Oliverio VT, Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. *J Biol Chem* **243:** 5007–5017, 1968.
6. Goldman ID and Matherly LH, The cellular pharmacology of methotrexate. *Pharmacol Ther* **28:** 77–102, 1985.
7. Sirotnak FM, Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: Biological significance and implications for improved therapy of human cancer. *Cancer Res* **45:** 3992–4000, 1985.
8. Dixon KH, Lanpher BC, Chiu J, Kelley K and Cowan KH, A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J Biol Chem* **269:** 17–20, 1994.
9. Williams FMR, Murray RC, Underhill TM and Flintoff WF, Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. *J Biol Chem* **269:** 5810–5816, 1994.
10. Brigle KE, Spinella MJ, Sierra EE and Goldman ID, Characterization of a mutation in the reduced folate carrier in a transport defective L1210 murine leukemia cell line. *J Biol Chem* **270:** 22974–22979, 1995.
11. Wong SC, Proefke SA, Bhushan A and Matherly LH, Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells. *J Biol Chem* **270:** 17468–17475, 1995.
12. Kamen BA, Wang M, Streckfuss AJ, Peryea X and Anderson RGW, Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J Biol Chem* **263:** 13602–13609, 1988.
13. Anderson RGW, Kamen BA, Rothberg KG and Lacey SW, Potocytosis: Sequestration and transport of small molecules by caveolae. *Science* **255:** 410–411, 1992.
14. Henderson GB and Strauss BP, Characteristics of a novel transport system for folate compounds in wild-type and methotrexate-resistant L1210 cells. *Cancer Res* **50:** 1709–1714, 1990.
15. Yang C, Dembo M and Sirotnak FM, Relationships between carrier-mediated transport of folate compounds by L1210 leukemia cells: Evidence for multiplicity of entry routes with different kinetic properties expressed in plasma membrane vesicles. *J Membr Biol* **75:** 11–20, 1983.
16. Goldman ID, Transport energetics of the folic acid analogue, methotrexate, in L1210 cells: Enhanced accumulation by metabolic inhibitors. *J Biol Chem* **244:** 3779–3785, 1969.
17. Henderson GB and Zevely EM, Transport routes utilized by L1210 cells for the influx and efflux of methotrexate. *J Biol Chem* **259:** 1526–1531, 1984.
18. Henderson GB and Hughes TR, Altered expression of unidirectional extrusion routes for methotrexate and cholate in an efflux variant of L1210 cells. *Biochim Biophys Acta* **1152:** 91–98, 1993.
19. Saxena M and Henderson GB, Identification of efflux systems for large anions and anionic conjugates as the mediators of methotrexate efflux in L1210 cells. *Biochem Pharmacol* **51:** 975–982, 1996.
20. Selhub J and Rosenberg IH, Folate transport in isolated brush border membrane vesicles from rat intestine. *J Biol Chem* **256:** 4489–4493, 1981.
21. Schron CM, Washington C and Blitzer BL, The transmembrane pH gradient drives uphill folate transport in rabbit jejenum. *J Clin Invest* **76:** 2030–2033, 1985.
22. Horne DW, Transport of folates and antifolates in liver. *Proc Soc Exp Biol Med* **202:** 385–391, 1993.
23. McMartin KE, Morshed KM, Hazen-Martin DJ and Sens DA, Folate transport and binding by cultured human proximal tubule cells. *Am J Physiol* **263:** F841–F848, 1992.
24. Bhandari SD, Joshi SK and McMartin KE, Folate binding and transport by rat kidney brush-border membrane vesicles. *Biochem Biophys Acta* **937:** 211–218, 1988.
25. Sierra EE, Brigle KE, Spinella MJ and Goldman ID, Comparison of transport properties of the reduced folate carrier and folate receptor in murine L1210 leukemia cells. *Biochem Pharmacol* **50:** 1287–1294, 1995.
26. Matherly LH, Barlowe CK, Phillips VM and Goldman ID, The effects of 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells. *J Biol Chem* **262:** 710–717, 1987.
27. Schuetz JD, Westin EH, Matherly LH, Pincus R, Swerdlow PS and Goldman ID, Membrane protein changes in an L1210 leukemia cell line with a translocation defect in the methotrexate-tetrahydrofolate cofactor transport carrier. *J Biol Chem* **264:** 16261–16267, 1989.
28. Brigle KE, Seither RL, Westin EH and Goldman ID, Increased expression and genomic organization of a folate-binding protein homologous to the human placental isoform in L1210 murine leukemia cell lines with a defective reduced folate carrier. *J Biol Chem* **269:** 4267–4272, 1994.
29. Fry DW and Goldman ID, Further studies on the charge-related alterations of methotrexate transport in Ehrlich ascites tumor cells by ionic liposomes: Correlation with liposome-cell association. *J Membr Biol* **66:** 87–95, 1982.
30. Henderson GB, Grzelakowska-Sztabert B, Zevely EM and Huennekens FM, Binding properties of the 5-methyltetrahydrofolate/methotrexate transport system in L1210 cells. *Arch Biochem Biophys* **202:** 144–149, 1980.
31. Brigle KE, Spinella MJ, Westin EH and Goldman ID, Increased expression and characterization of two distinct folate binding proteins in murine erythroleukemia cells. *Biochem Pharmacol* **47:** 337–345, 1994.
32. Spinella MJ, Brigle KE, Sierra EE and Goldman ID, Distinguishing between folate receptor-α-mediated transport and reduced folate carrier-mediated transport in L1210 leukemia cells. *J Biol Chem* **270:** 7842–7849, 1995.
33. Kamen BA and Caston JD, Properties of a folate binding protein (FBP) isolated from porcine kidney. *Biochem Pharmacol* **35:** 2323–2329, 1986.
34. Poe M, Acidic dissociation constants of folic acid, dihydrofolic acid, and methotrexate. *J Biol Chem* **252:** 3724–3728, 1977.
35. Schulz GE and Schirmer RH, *Principles of Protein Structure*. 1979.

36. Kaarsholm NC, Kolstrup A-M, Danielsen SE, Holm J and Hansen SI, Ligand-induced conformation change in folate-binding protein. *Biochem J* **292:** 921–925, 1993.
37. Bhandari SD, Fortney T and McMartin KE, Analysis of the pH dependence of folate binding and transport by rat kidney brush border membrane vesicles. *Proc Soc Exp Biol Med* **196:** 451–456, 1991.
38. Smith ME, Matty AJ and Blair JA, The transport of pteroylglutamic acid across the small intestine of the rat. *Biochim Biophys Acta* **219:** 37–46, 1970.
39. Horne DW, Na^+ and pH dependence of 5-methyltetrahydrofolic acid and methotrexate transport in freshly isolated hepatocytes. *Biochim Biophys Acta* **1023:** 47–55, 1990.
40. Mason JB, Shoda R, Haskell M, Selhub J and Rosenberg IH, Carrier affinity as a mechanism for the pH-dependence of folate transport in the small intestine. *Biochim Biophys Acta* **1024:** 331–335, 1990.
41. Schron CM, pH Modulation of the kinetics of rabbit jejunal, brush-border folate transport. *J Membr Biol* **120:** 192–200, 1990.