J. Membrane Biol. 101, 247–258 (1988)

The Journal of
Membrane Biology

Mediated Uptake of Folate by a High-Affinity Binding Protein in Sublines of L1210 Cells Adapted to Nanomolar Concentrations of Folate

Gary B. Henderson, Judy M. Tsuji, and Harish P. Kumar
Division of Biochemistry, BCR-7, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, California 92037

Summary. An L1210 cell line (JT-1), which can grow in medium supplemented with 1 nM folate, has been isolated. These cells exhibit a slower growth rate than folate-replete parental cells and have a lower ability to transport folate or methotrexate via the reduced folate transport system. Measurements at nanomolar concentrations of folate revealed that the adapted cells have acquired a high-affinity folate-binding protein. Binding to this component at 37°C was rapid and reached a maximum value after 30 min which corresponded in amount to 0.23 ± 0.3 pmol/mg protein, and excess unlabeled folate added 30 min subsequent to the [^{3}H]folate led to a rapid release of the bound substrate. Radioactivity bound to or released from the cells after 30 min at 37°C remained as unmetabolized folic acid. Binding was also rapid at 0°C but uptake at the plateau was only one-half the value obtained at 37°C. Half-maximal saturation of the binding component (K_D) occurred at a folate concentration of 0.065 nM at pH 7.4, while the affinity for folate decreased 30-fold when the pH was reduced to 6.2 (K_D = 2.0 nM). 5- Methyltetrahydrofolate was also bound by this component (K_i = 13 nM at pH 7.4) but with a much lower affinity than for folate, while progressively weaker interactions were observed with 5-formyltetrahydrofolate (K_i = 45 nM) and methotrexate (K_i = 325 nM). When the same adaptation procedure was performed with limiting amounts of 5-formyltetrahydrofolate, two additional cell lines, JT-2 and JT-3, were isolated which expressed elevated levels of the folate-binding protein. The binding activity of the latter cells was 0.46 and 1.4 pmol/mg protein, respectively. When the level of binding protein was compared in cells grown at different concentrations of folate, an increase in medium folate from 1 to 500 nM caused a sevenfold reduction in binding activity in the JT-3 cell line, while these same growth conditions had no effect on binding by the other cells. These results indicate that L1210 cells adapted to low concentrations of folate or 5-formyltetrahydrofolate contain elevated levels of a high-affinity binding protein and that this protein is able to mediate the intracellular accumulation of folate compounds. L1210 cells thus appear to have two potential uptake routes for folate compounds, the previously characterized anion-exchange system and a second route mediated by a high-affinity binding protein. An additional low-affinity, high-capacity transport system for folate that had been proposed previously was not observed under a variety of experimental conditions in either the adapted or parental cells.

Key Words folate · folate transport · high-affinity folate binding proteins · L1210 cells

Introduction

The transport characteristics of folate compounds in L1210 and other leukemic cells have been investigated in various laboratories for nearly two decades, yet a general consensus on the routes of uptake has not yet been achieved. Previous studies [5–7, 18–20, 23–26, 31] and recent work by Sirotnak et al. [27, 32] have supported a model in which folate enters L1210 cells primarily via a low-affinity, high-capacity transport system which is separate from the anion-exchange system for methotrexate and 5-methyltetrahydrofolate. Distinguishing features of this proposed low-affinity system are a lack of inhibition by methotrexate and a rapid initial influx which is followed by a much slower second phase [27]. Other recent studies, however, indicated that folate enters L1210 [11] and CCRF-CEM [12] cells via the same route as methotrexate and 5-methyltetrahydrofolate. Folate uptake in the latter studies exhibited monophasic kinetics and was inhibited by methotrexate, and, moreover, trace amounts of labeled impurities in the [^{3}H]folate were implicated as a possible explanation for previous results [11, 12]. A critical problem has been that interfering decomposition products can rapidly reappear in purified samples of [^{3}H]folate [11], although this problem can be avoided by substrate purification just prior to use.

The identify and characteristics of routes available for the transport of folate compounds into leukemic cells have a potential impact on chemotherapy with methotrexate and other antifolate compounds. Since resistance to methotrexate often arises from a decrease in transport [18, 26], it may be possible to circumvent this defect by employing antifolates which enter cells via alternate routes. In particular, a folate-specific route might be exploited by developing antifolate compounds which can

preferentially utilize this system and not the anion-exchange system for methotrexate and reduced folates. Efforts to develop additional drugs, however, require a clear understanding of the routes available to folate compounds. In the present study, we have adapted parental L1210 cells to grow on nanomolar concentrations of folate by progressive transfer from standard medium containing 2.2 μM folate to medium containing 1 nM folate. It was anticipated that cells able to grow on folate in the nanomolar range would exhibit an increased efficiency in transporting folate and that an evaluation of the basis for improved uptake might lead to insight into the routes and mechanism for folate transport. A similar rationale also led to the isolation of cell lines able to grow at low concentrations of 5-formyltetrahydrofolate (folinate). The adaptation of cells to limiting concentrations of both folate and folinate was found to occur via the overproduction of a high-affinity folate-binding protein. Similar proteins have been detected in other cell lines [1, 21, 22] and in two cases a role in folate or 5-methyltetrahydrofolate transport was proposed [1, 21]. The present report describes the properties of these adapted cells and the characteristics of the observed folate-binding activity. Additional kinetic evidence is also presented which indicates that a high-capacity, low-affinity transport system for folate does not exist in L1210 cells.

Materials and Methods

Chemicals

[3',5',7,9-^{3}H]Folic acid (40 Ci/mmol) and [3',5',7-^{3}H]methotrexate (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, Calif.) and stored at −80°C. Folic acid, [6R,6S]-5-formyltetrahydrofolate (folinate), methotrexate, bromosulfophthalein (sulfobromophthalein), HEPES,[1] and MES were obtained from Sigma Chemical Co. (St. Louis, Mo.). [6R,6S]-5-Methyltetrahydrofolate was a gift of Dr. J.M. Whiteley.

Purification of Labeled Substrates

Thin-layer chromatography was employed as the standard procedure for purification of the [^{3}H]folate and [^{3}H]methotrexate. The [^{3}H]folate was either retained at the initial specific activity (16,000,000 cpm/nmol) or diluted with unlabeled compound to a specific activity of 100,000 cpm/nmol and then purified on Baker-flex cellulose sheets at 23°C in 50 mM sodium HEPES, pH 7.5. Elution from the plates was achieved as described previously [11] with HEPES-buffered saline containing 2% ethanol. [^{3}H]Methotrexate was either retained at the initial specific activity (8,000,000 cpm/nmol) or diluted to a specific activity of 50,000 cpm/nmol and purified by the same procedure as [^{3}H]folate using a solvent of 100 mM sodium HEPES, pH 7.5, and elution with 10% ethanol. The time required for purification of either substrate by TLC was 2 to 3 hr. The [^{3}H]folate was used on the day of purification, while the [^{3}H]methotrexate was stored up to 30 days at −20°C. Purification of the [^{3}H]folate was also achieved (at 23°C) using HPLC on a C18 Altex Ultrasphere-ODS column and solvent systems of acetate, pH 5.1, and acetonitrile [27] or 2% acetic acid and a gradient of methanol (K.S. Vitols, *unpublished results*). In the latter procedure, reservoir A contained 2% acetic acid, while reservoir B contained 2% acetic acid plus 50% methanol. Samples (250 μl) were applied to the column equilibrated with 70% A and 30% B and the same solvent mixture was maintained for 5 min. The percent of solvent B was then increased from 30 to 70% over the next 30 min. Peak fractions containing the [^{3}H]folate were pooled, dried overnight in vacuo, dissolved in the desired buffer, and employed immediately.

Preparation of Folate-Depleted Fetal Bovine Serum

The folate compounds present in fetal bovine serum were removed by absorption onto Sepharose 4B agarose containing covalently bound rabbit antibodies to methotrexate. In this procedure, fetal bovine serum (in 50-ml amounts) was combined with 10 ml (packed volume) of the methotrexate antibody beads and mixed by slow inversion on a rotating wheel for 16 hr at 4°C. The serum was recovered by filtration through a sintered glass funnel and filter sterilized. To prepare the antibody beads, methotrexate (10 μmol) was attached to bovine serum albumin (10 mg) by incubating the methotrexate for 1 hr at 23°C with a 10-fold molar excess of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide in 2 ml of dimethylsulfoxide [10], adding the reagent to 5 ml of 20 mM sodium HEPES, pH 7.5, containing the bovine serum albumin, and incubating the mixture for 1 hr at 23°C. After dialysis, a spectral analysis revealed that the albumin contained 9 to 12 moles of bound methotrexate per mole of protein. Antibody production was initiated by injecting each of two New Zealand white rabbits with the methotrexate-modified albumin (200 μg in Freund's complete adjuvant). After four weeks, weekly boosts of 200 μg each were given, and the collection of blood from the ear vein was begun the week following the first boost. Pooled rabbit serum containing 3 nmol/ml of methotrexate-binding activity was obtained by this procedure. The pooled serum (in 50-ml portions) was then treated with a sequential addition over a 1-hr period of 5 × 0.2 mg of unmodified bovine serum albumin/ml of serum to precipitate antibodies to the albumin, and the remaining antibodies were separated from other serum proteins by octanoic acid precipitation [30]. After dialysis to remove the octanoic acid, the purified immunoglobulin fraction (250 mg protein) was coupled to cyanogen bromide-activated Sepharose 4B (100 ml packed volume), washed with isotonic saline, and stored at 4°C in 150 mM sodium bicarbonate, pH 7.8.

[1] *Abbreviations:* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate; MES, 2-(N-morpholino)-ethanesulfonate; HBS, HEPES-buffered saline; MHBS, MES/HEPES-buffered saline; MBS, MES-buffered saline; TBBS, Tris/bicarbonate-buffered saline; and HPLC, high-performance liquid chromatography.

Growth of Cells

Parental L1210 mouse cells were grown as described previously [10] in RPMI 1640 medium supplemented with 2.5% fetal bovine serum (Flow Laboratories, Rockville, Md.) and 100 units each of penicillin and streptomycin (ICN Pharmaceuticals, Irvine, Calif.). Folate-depleted cells were obtained by growth of the parental line for six generations on folate-free RPMI 1640 medium (Irvine Scientific) supplemented with 2.5% folate-depleted serum and antibiotics as described above, while growth of the folate-adapted L1210 cells was achieved in the folate-free medium containing folate-depleted serum, antibiotics, and 1 nM folate. Large cultures of parental cells were grown in sealed 1-liter flasks that contained 500 ml of medium and were inoculated with 2×10^5 cells/ml, while the folate-adapted cells were grown similarly from an inoculum of 4×10^5 cells/ml. After 48 hr at 37°C (with gentle shaking), the cells were harvested by centrifugation at $1000 \times g$ (5 min, 4°C), washed with the desired buffer, and suspended to a density of 3×10^7/ml. The suspending buffers were HEPES-buffered saline (HBS, in mM): 20 HEPES, 140 NaCl, 5 KCl, 2 $MgCl_2$, pH 7.4 with NaOH; MES/HEPES-buffered saline (MHBS, in mM): 10 MES, 10 HEPES, 140 NaCl, 5 KCl, 2 $MgCl_2$, pH as desired with NaOH; MES-buffered saline (MBS, in mM): 20 MES, 140 NaCl, 5 KCl, 2 $MgCl_2$, pH 6.2 with NaOH; Mg-HEPES-sucrose (mM): 20 HEPES, 225 sucrose, pH 7.4 with MgO; and Tris/bicarbonate-buffered saline (TBBS, in mM): 20 Tris, 26.2 sodium bicarbonate, 106 NaCl, 5.3 KCl, 1.9 $CaCl_2$, 1.0 $MgCl_2$, 7 glucose, pH 7.4 with HCl.

Isolation of Folate-Adapted Cells

Parental L1210 cells that had been grown in standard RPMI 1640 medium containing 2.2 μM folate were adapted initially to folate-free RPMI medium containing antibiotics, 2.5% folate-depleted fetal bovine serum, and 50 nM folate. Cells that had been transferred at weekly intervals under these conditions for four months grew slowly, showed an irregular morphology, and underwent substantial lysis. After five months, a marked improvement in growth rate occurred and cell morphology normalized. The cells were then transferred sequentially over a two-month interval into media containing 25, 5 and 1 nM folate without an obvious reduction in growth rate or induction of visible stress on the cells. These cells were maintained in medium containing 1 nM folate for three months with no apparent change in growth rate or cell morphology and then cloned in soft agar.

Isolation of Folinate-Adapted Cells

Parental L1210 cells that had been depleted of intracellular folates were transferred into folate-free RPMI 1640 medium containing antibiotics, 2.5% folate-depleted fetal bovine serum, and 5 nM folinate, and transferred at weekly intervals for three months. At the latter time, the cells began to exhibit improved growth. The folinate was then decreased stepwise to 2.5, 1.0 and 0.5 nM over a one-month period and then maintained at 0.5 nM for three additional months.

Uptake Measurements

Uptake at micromolar substrate concentrations was determined in duplicate assay mixtures containing cells (3×10^7), the desired additions, [^{3}H]folate (100,000 cpm/nmol) or [^{3}H]methotrexate (50,000 cpm/nmol), and buffer in a final volume of 1.0 ml. After incubation at 37°C for the desired time, the cells were chilled to 0°C, diluted with 7 ml of ice-cold saline (160 mM NaCl, 1 mM sodium phosphate, pH 7.4), and recovered by centrifugation at $1000 \times g$ (5 min, 4°C). Folate uptake samples were washed once with 4 ml of saline, suspended in 0.5 ml of saline, and analyzed for radioactivity, whereas the wash step was omitted in uptake samples with methotrexate. Uptake at 0°C served as the control. K_t values for half-maximal influx and V_{max} were determined from double-reciprocal plots of influx (determined after 3 min at 37°C) versus substrate concentration and were reported as the average of two separate determinations.

Uptake at nanomolar concentrations of [^{3}H]folate and [^{3}H]methotrexate was measured similarly except that the labeled substrates had an initial specific activity of 16,000,000 and 8,000,000 cpm/nmol, respectively. For measurements at [^{3}H]folate concentrations between 1 and 20 nM, the assay volume was 1.0 ml and the cells were diluted with 7 ml of ice-cold saline, recovered by centrifugation, and analyzed for radioactivity. At [^{3}H]folate concentrations below 1 nM, the assay volume was increased to 10 ml and the cells (without dilution) were recovered by centrifugation and analyzed for radioactivity. Similar procedures were employed to determine folate uptake in cells that had been grown in the presence of excess folate (500 nM) except that a preincubation was performed for 5 min at 37°C in 100 volumes of MBS, pH 6.2, to reduce the amount of bound or loosely associated folate. K_D values for half-maximal uptake (binding) were obtained from double-reciprocal plots of uptake (determined after 10 min at 37°C) versus substrate concentration, while K_i values were determined from Dixon plots of the inverse of uptake versus inhibitor concentration. Calculations of K_i utilized the Dixon equation and a K_D for folate of 0.065 nM.

Concentrations of protein were determined by the biuret reaction [8] using bovine serum albumin as the standard.

Identification of Folate Metabolites

Analyses for folate metabolites were performed with cells (6×10^7) that had been incubated for 30 min at 37°C with 10 nM [^{3}H]folate in 2 ml HBS, pH 7.4, washed twice with saline, and resuspended in 1.0 ml 0.1 N HCl. After 30 min at −20°C, the samples were thawed and centrifuged for 5 min at $30,000 \times g$. The supernatant fraction was retained, dried in vacuo over NaOH pellets, and analyzed by HPLC for folate metabolites using the acetic acid/methanol gradient system described above. Folate, folinate and methotrexate were employed as standards. Radioactivity released from cells was determined similarly except that 10 μM unlabeled folate was added 30 min subsequent to the [^{3}H]folate and the incubation was continued an additional 30 min at 37°C. The cells were then removed by centrifugation and the supernatant fraction was analyzed directly by the same HPLC procedure.

Results

Isolation and Growth Properties of Folate-Adapted L1210 Cells

A stable L1210 cell line (JT-1) has been isolated which possesses a substantially increased ability to

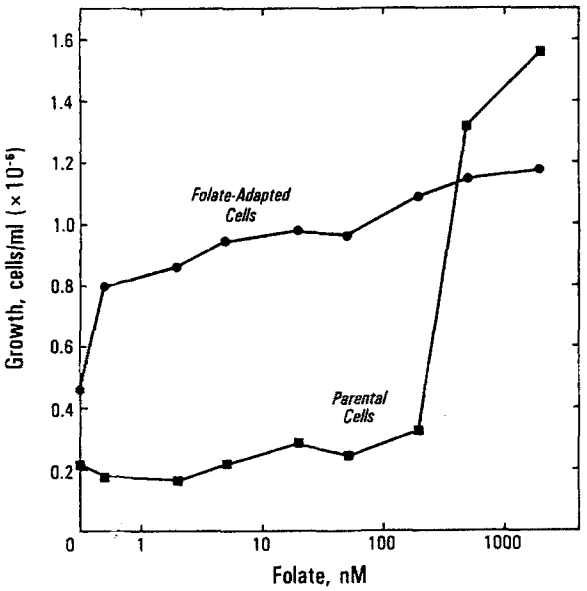

Fig. 1. Comparison of the folate dependence for growth by folate-adapted cells and by folate-depleted parental cells. Folate-adapted cells grown at 1 nM folate were diluted to 100,000/ml in folate-free medium containing the indicated concentrations of folate, incubated for 48 hr at 37°C, and analyzed for cell number in a Coulter counter. Parental cells were inoculated and grown similarly after depletion of intracellular folate by prior growth in folate-free medium for six generations (*see* Materials and Methods)

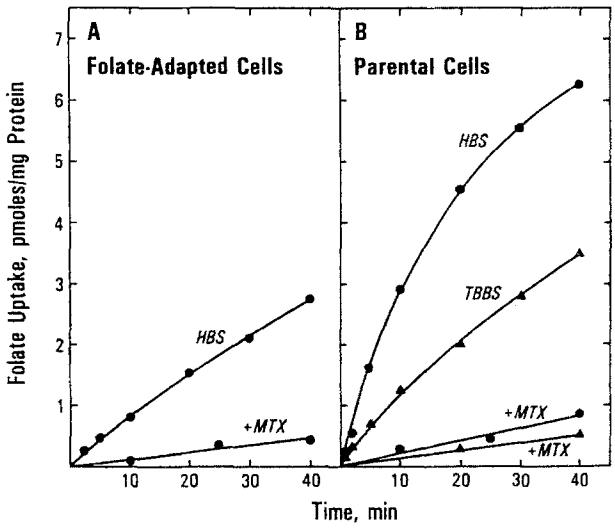

Fig. 2. Transport properties of folate-adapted and parental L1210 cells at 5 μM [^{3}H]folate. Uptake at 5 μM [^{3}H]folate (at 37°C) was determined at the indicated times with cells suspended in HEPES-buffered saline (HBS) (●—●), or Tris/–bicarbonate-buffered saline (TBBS) (▲—▲), with and without the addition of 200 μM methotrexate (MTX). (*A*) Folate-adapted cells; (*B*) parental cells

grow at low levels of folic acid, an unnatural source of intracellular folate compounds in mammalian cells. The method of isolation involved the growth of parental L1210 cells in media containing progressively lower concentrations of folic acid (*see* Materials and Methods). Cells obtained by this procedure were able to grow at 1 nM folate and the growth rate was 60% that of parental cells grown at the standard level of 2.2 μM folate. An increase in the folate concentration to 500 nM increased the growth rate of the adapted cells to the same value as observed for parental cells. The growth response of the adapted cells to increasing concentrations of folate is shown in Fig. 1. An increase in cell growth was apparent at folate concentrations below 1 nM, although a maximum was not reached and total cell number increased steadily as the folate concentration was raised to 2.2 μM. Some growth by the adapted cells occurred in medium lacking added folate (*see* Fig. 1), which appeared to result either from a trace of residual folate in the medium or from stored folate within the cell. These cells died after transfer into a second portion of medium lacking added folate. In contrast, folate-depleted parental cells grew only at folate concentrations above 200 nM (Fig. 1).

Transport Properties of the Adapted Cells at High Concentrations of Folate

The ability of the adapted cells to grow at much lower concentrations of folate than the parental cells suggested that an increase in folate transport efficiency had occurred. Since folate can be transported in L1210 cells by the same anion-exchange system as 5-methyltetrahydrofolate and methotrexate [11], although at a much lower efficiency, folate uptake via this system in the adapted cells was evaluated. In these measurements, HEPES-buffered saline (HBS) was employed as the suspending medium and uptake was compared for both the adapted (Fig. 2*A*) the parental cells (Fig. 2*B*). Uptake by each cell line exhibited the same linear response during the initial 5 min and then gradually decreased thereafter, and methotrexate reduced this uptake by more than 85%. However, in the adapted cells uptake of [^{3}H]folate via the exchange system proceeded at only 50% the rate observed in the parental cell line. Influx measurements with [^{3}H]methotrexate (*data not shown*) revealed further that the K_t for half-maximal influx of methotrexate (4.5 ± 0.5 μM) was the same in both cells lines, whereas the V_{max} for methotrexate in the adapted cells (8.5 pmol/min/mg protein) had declined to 50% of the value observed in the parental cells (18 pmol/min/mg protein). The uptake of folate by the parental cells is also shown in Fig. 2(*B*) for an alternative

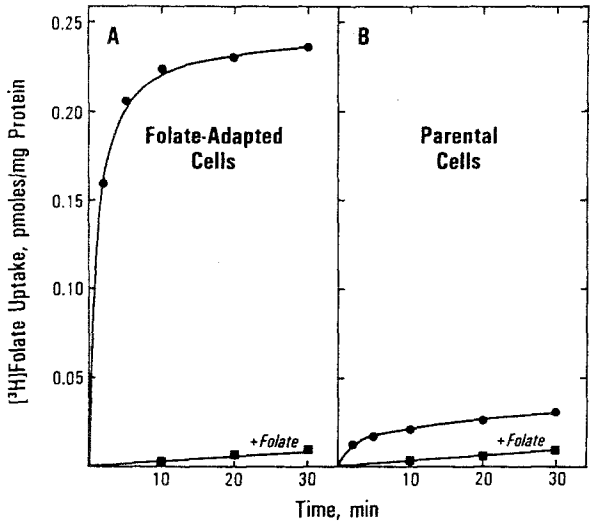

Fig. 3. Transport properties of folate-adapted and parental L1210 cells at 5 nM [³H]folate. Uptake at 5 nM [³H]folate (at 37°C) was determined at the indicated times with cells suspended in HBS with and without the addition of 10 μM unlabeled folate. (*A*) Folate-adapted cells; (*B*) parental cells

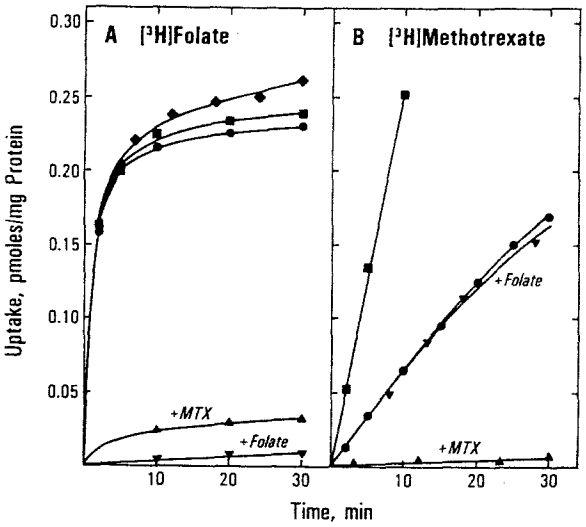

Fig. 4. Comparative effect of buffer composition and glucose on uptake at 5 nM [³H]folate and 5 nM [³H]methotrexate. (●) HBS; (■) MHS; (♦) HBS plus 5 mM glucose; (▼) HBS plus 10 μM folate; (▲) HBS plus 200 μM methotrexate (MTX). (*A*) [³H]Folate; (*B*) [³H]methotrexate

saline buffer (TBBS) containing glucose and bicarbonate [27]. Uptake in this buffer was inhibited by methotrexate and proceeded with the same uptake profile as in the HBS, except that initial influx and total uptake were each reduced by 50%. It was thus apparent that the ability of the adapted cells to grow at low concentrations of folate was not due to an increase in folate transport via the anion-exchange system for folate compounds or to the induction of an alternative high-capacity, low-affinity transport system for folate [27].

Transport Properties of the Adapted Cells at Low Concentrations of Folate

The inability to observe an improvement in folate transport characteristics at micromolar concentrations of folate led to an examination of transport at much lower substrate levels. When the [³H]folate concentration was reduced to 5 nM, the adapted cells were observed to take up substantial amounts of folate via a process which had the characteristics of a specific binding component. Uptake was rapid initially and then decreased to an apparent steady state by 30 min, and both phases were inhibited by 10 μM unlabeled folate (Fig. 3*A*). In various measurements, the extent of uptake after 30 min at 37°C was 0.23 ± 0.03 pmol/mg protein, and the bound radioactivity was confirmed to be greater than 98% unmetabolized folic acid by HPLC analysis (*data not shown*). The parental cells (Fig. 3*B*) analyzed under the same conditions exhibited a similar uptake profile for folate, although the extent of uptake (0.025 pmol/mg protein) was only 10% that of the adapted cells. Further analysis with the adapted cells (Fig. 4*A*) showed that total uptake increased to only a small extent upon the addition of glucose, whereas no change was observed upon transfer of the cells to an anion-deficient buffer, and methotrexate added at 10 times higher levels than unlabeled folate was less effective than unlabeled folate in blocking [³H]folate uptake. Corresponding uptake measurements at 5 nM [³H]methotrexate (Fig. 4*B*) revealed that the uptake of this folate analog was approximately linear for 30 min at 37°C, anion-deficient buffers increased its uptake by threefold, and a marked inhibition was observed with 100 μM methotrexate, but not with 10 μM folate. These latter findings indicate that uptake at 5 nM methotrexate was primarily transport and was occurring via the anion-exchange system for folate compounds [9, 13–15].

The [³H]folate that had been taken up by cells after 30 min at 37°C could be released into the medium by the addition of 10 μM unlabeled folate (Fig. 5). This release occurred at a rate which was relatively rapid, although somewhat slower than the rate of uptake, and a nearly complete loss of cellular radioactivity was observed after 30 min. The radioactivity released from the cells was greater than 98% folic acid as determined by HPLC analysis. At 0°C (Fig. 5), uptake of [³H]folate was also rapid initially but reached a plateau which was only 50%

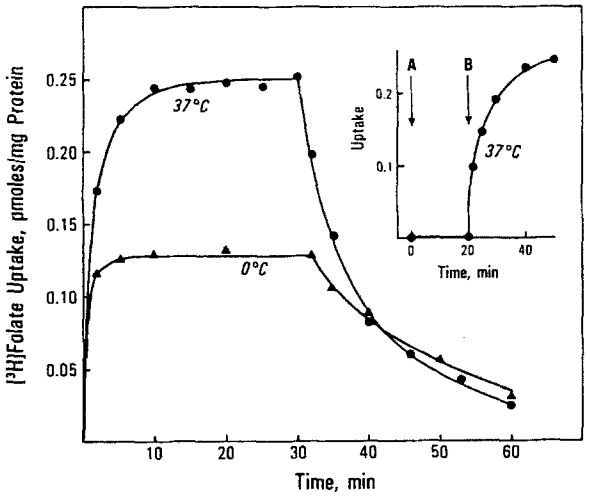

Fig. 5. Temperature dependence for the uptake and release of [³H]folate by folate-adapted L1210 cells. Uptake was measured in cells that had been combined at zero time with 5 nM [³H]folate and then incubated at either 0 or 37°C. After 30 min, 10 μM unlabeled folate were added and the incubation was continued for an additional 30 min. *Inset:* uptake at 20 nM [³H]folate by cells preincubated for 20 min at 37°C with 20 nM unlabeled folate and then washed twice to remove free folate. Arrow *A*, time of addition of 20 nM unlabeled folate; arrow *B*, time of buffer washes and addition of 20 nM [³H]folate

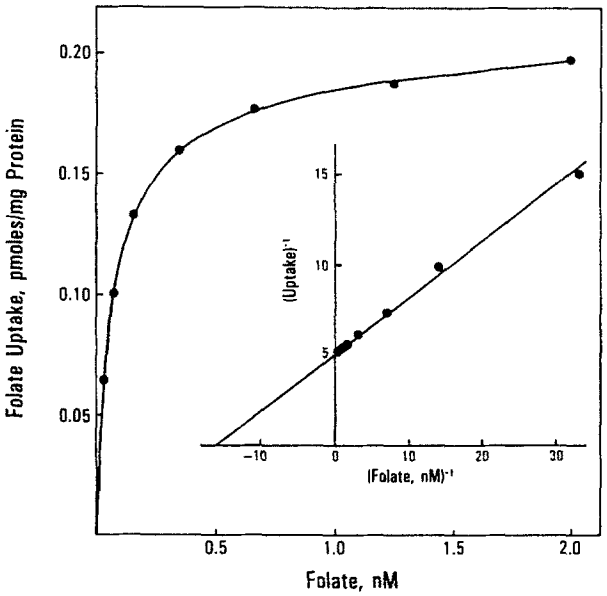

Fig. 6. Concentration dependence for [³H]folate uptake by the folate-adapted cells. Uptake was measured after 10 min at 37°C in samples containing the indicated nanomolar concentrations of [³H]folate in 10 ml of HBS (*see* Materials and Methods). *Inset:* double-reciprocal plot of uptake versus the free [³H]folate concentration

that at 37°C. The subsequent addition of unlabeled folate after 30 min at 0°C led to the release of associated [³H]folate, although at a slower rate than at 37°C. Cells that had been exposed to unlabeled folate for 20 min at 37°C and then washed to remove the remaining extracellular folate also exhibited the ability to subsequently take up [³H]folate (Inset, Fig. 5). The rate of folate uptake in these pretreated cells was about one-half that of cells not exposed to unlabeled folate, although the extent of uptake after 30 min at 37°C (0.24 pmol/mg protein) was the same for both treated and untreated cells. When the adapted cells were transferred to medium containing 500 nM folate and allowed to grow for six generations, no reduction was noted in the steady-state level of folate uptake (0.27 pmol/mg protein). In addition, an active binding protein for folate was not released during cell growth. When expended growth medium from cells grown at 1 nM folate was concentrated 10-fold and examined for folate binding activity, none could be detected by equilibrium dialysis (24 hr at 4°C) in HBS containing 5 nM [³H] folate.

Affinity Determinations and Substrate Specificity of the Binding Component

Additional measurements were performed with the adapted cell line, which were based on the assumption that uptake at nanomolar concentrations of folate occurred primarily via a high-affinity binding protein. The concentration dependence for [³H]folate uptake after 10 min at 37°C is shown in Fig. 6. This uptake exhibited saturation kinetics and reached a maximum value at substrate concentrations above 2 nM. A double-reciprocal plot of uptake versus free [³H]folate concentration (Inset, Fig. 6) indicated that half-maximal uptake (binding) occurred at a folate concentration of 0.065 nM. An increase in the time of incubation with [³H]folate from 10 min to 60 min did not alter the folate concentration required for half-maximal uptake (*data not shown*), indicating that a steady state had been achieved within the initial 10-min incubation period. Similar measurements at lower pH values revealed that the affinity of the binding component for folate decreases with decreasing pH. Half-maximal uptake at pH 6.8 and 6.2 occurred at 0.105 and 2.0 nM, respectively. Competition experiments (Fig. 7) showed that uptake at 5.0 nM [³H]folate could be inhibited by more than 98% at micromolar concentrations of unlabeled folate and that half-maximal inhibition occurred at 6.8 nM unlabeled folate. The K_i for folate calculated from a Dixon plot of these data was 0.088 nM. Similar measurements with 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and methotrexate (Fig. 7) showed that these folate compounds have substantially lower affinity for the

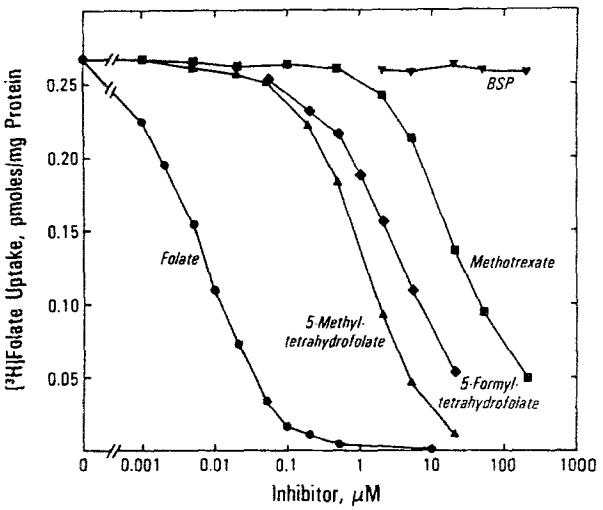

Fig. 7. Concentration dependence for the inhibition of [³H]folate uptake by unlabeled folate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, methotrexate, and bromosulfophthalein (BSP). Uptake at 5 nM [³H]folate was measured in HBS after incubation for 10 min at 37°C in the presence of the indicated concentrations of each inhibitor

Table. Comparative properties of the folate-binding protein of parental L1210 cells and L1210 cells adapted to low concentrations of folate or folinate[a]

Adaptation conditions	Cell line	Folate-sufficient cells		Folate-replete cells
		K_D (folate) (nM)	Folate bound (pmol/mg protein)	Folate bound (pmol/mg protein)
None	Parent	ND	0.025	0.015
Folate	JT-1	0.065	0.23	0.27
Folinate	JT-2	0.060	0.46	0.43
Folinate	JT-3	0.080	1.4	0.16

[a] Measurements were performed as described in Materials and Methods employing HBS, pH 7.4, as the suspending buffer. Folate-replete wild-type cells were obtained by growth in standard medium containing 2.2 μM folate, while folate-replete adapted cells were grown for six generations at 500 nM folate. Folate-sufficient wild-type cells were grown for six generations at 500 nM, while folate-sufficient adapted cells were grown at 1 nM folate.

binding component than folate. Half-maximal inhibition occurred at 1.03 μM 5-methyltetrahydrofolate, 3.5 μM 5-formyltetrahydrofolate, and 25 μM methotrexate, which correspond to K_i values of 13, 45 and 325 nM, respectively. Bromosulfophthalein, a large aromatic polyvalent anion which inhibits the folate exchange carrier of these cells [14], as well as a methotrexate efflux component [15], did not compete for folate uptake via the high-affinity binding component.

Characteristics of L1210 Cell Lines Adapted to Low Concentrations of Folinate

During the course of isolating an L1210 cell line adapted to nanomolar concentrations of folate, a parallel set of cultures were prepared which were exposed to limiting concentrations of folinate. Analysis of two separate cultures isolated under these conditions revealed that these cell lines also had acquired elevated amounts of a high-affinity binding protein. Binding studies showed further that the two folinate-adapted cell lines (JT-2 and JT-3) exhibited similarities to the folate-adapted cells (JT-1) in uptake kinetics (*data not shown*) and affinity for folate, but they differed in amount of binding activity (*see* Table). The levels of binder in the JT-2 and JT-3 cell lines were two- and sixfold higher than in the folate-adapted cells. Further analyses indicated that JT-2 and JT-3 each grew in medium containing 0.5 nM folinate at about 75% the rate of folate-replete wild-type cells, while maximal growth could be achieved at 5 nM folinate. Growth of these cell lines also occurred in medium supplemented with low concentrations of folate. At 1 nM folate, JT-2 and JT-3 exhibited a growth rate which was 75 and 50%, respectively, of the wild-type control. When folate binding was compared in cells grown at different concentrations of folate (Table), an increase in medium folate from 1 to 500 nM caused a sevenfold decrease in binding activity in JT-3 cells, while these same growth conditions had no effect on binding in the JT-1 and JT-2 cell lines. Transport measurements at micromolar concentrations of methotrexate showed that the V_{max} of the folate-exchange system of each adapted cell line (9 ± 2 pmol/min/mg protein) had decreased twofold relative to the parental cells (18 pmol/min/mg protein) but that no change had occurred in the K_t for methotrexate influx (4.5 ± 0.5 μM).

Additional Transport Routes for Folate in L1210 Cells

The present results indicate that a high-affinity folate-binding protein can be induced in L1210 cells and that folate bound to this protein can be internalized at a sufficient rate to fulfill the folate requirements of these cells. This latter route thus compliments the anion-exchange system for folate compounds which can also transport folate into

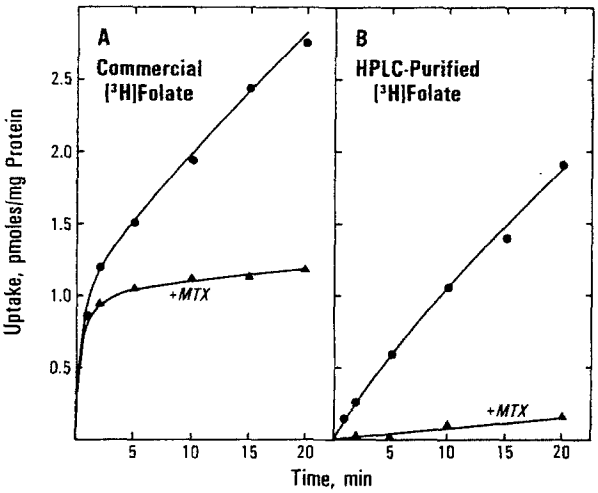

Fig. 8. Comparative uptake of radioactivity by parental L1210 cells using an impure commercial sample of [^{3}H]folate and [^{3}H] folate purified by HPLC. Uptake was measured at 37°C in cells suspended in TBBS containing 5 μM [^{3}H]folate or TBBS containing labeled substrate plus 200 μM methotrexate (MTX). The HPLC procedure of Sirotnak et al. [27] was employed to purify the [^{3}H]folate. (*A*) Commercial [^{3}H]folate; (*B*) purified [^{3}H]folate

L1210 cells [11]. A third potential route has also been described which has a very low-affinity for folate but proceeds with a high V_{max} [27, 32]. The distinguishing properties of folate uptake via this system are a rapid initial uptake (over a 1- to 2-min interval) which is followed by a second slower phase, and a lack of inhibition of the initial phase by methotrexate. It was suggested further [27] that previous investigations [11] had not observed this component since measurements had been performed under different assay conditions. An opposing viewpoint has been that this rapid initial uptake component does not represent the uptake of [^{3}H] folate but instead the uptake of ^{3}H-labeled pterin impurities in the [^{3}H]folate [11, 12]. In an attempt to resolve this controversy, we have measured the uptake kinetics for [^{3}H]folate under different buffer conditions and in the presence and absence of glucose and/or methotrexate and have used [^{3}H]folate that had been purified by procedures described previously by ourselves [11, 12] and by others [27]. To illustrate the problems which arise with impure [^{3}H] folate, the uptake profile of a commercial sample of [^{3}H]folate that had not undergone purification is shown in Fig. 8(*A*). In these measurements, the cells were suspended in the same saline medium (TBBS) containing glucose in which the alternate influx route had been observed previously by others [27]. The uptake profile under these conditions was biphasic and was comprised of a rapid initial component which was followed by a much slower phase, and methotrexate inhibited only the second slower portion of uptake. Total impurities in this sample were about 7%. When the [^{3}H]folate was purified by HPLC [27] and then employed without storage, the corresponding uptake profile shown in Fig. 8(*B*) was obtained. In contrast to the impure sample, an initial rapid uptake component was not observed and inhibition by methotrexate was greater than 90%. [^{3}H]Folate that had been purified by our standard thin-layer chromatography procedure and employed in HBS (*see* Fig. 2*B*) also gave the same uptake profile as the HPLC-purified sample, with no indication of an initial, rapid uptake component. Uptake in HBS was about twofold higher than in TBBS but could be reduced to nearly the same level as in TBBS by the addition of 5 mM glucose and 10 mM sodium bicarbonate (*data not shown*). The latter additions, however, had no effect on the shape of the uptake profile or on the extent of inhibition by methotrexate. Purification of the [^{3}H]folate by a second HPLC procedure employing 2% acetic acid and a methanol gradient (*see* Materials and Methods) also removed the interfering impurities and resulted in uptake profiles and sensitivities to methotrexate which were similar to those shown in Figs. 2(*B*) and 8(*B*) (*data not shown*). The same results were also obtained using [^{3}H]folate that had been purified by either HPLC procedure, dried in vacuo and stored at −80°C for seven days.

Discussion

A subline of L1210 mouse leukemia cells has been isolated which grows at a concentration of folate (1 nM) which is 200-fold lower than is required for growth of the parental cells (Fig. 1). This improved growth efficiency on folate coincided with the overproduction of a high-affinity folate-binding protein. The amount of high-affinity binder in this cell line was 0.23 pmol/mg protein, which represents a 10-fold increase relative to the parental cells. Growth of the adapted cells at 1 nM folate proceeded at 60% the rate of the parental cells in folate-replete medium, although it could be increased progressively and to the parental level by increasing the folate in the growth medium from 1 to 1000 nM (Fig. 1). This result indicates that the amount of high-affinity binder in the folate-adapted cells is insufficient to fully satisfy the folate requirements of these cells and that additional uptake via the anion-exchange system [9, 11, 14, 15] is necessary to achieve maximum growth. Elevated levels of binding protein were also observed in two additional L1210 cell lines which were adapted to low concentrations (0.5

nM) of folinate (Table), and in one of these cell lines (JT-3), the amount of binding activity was 60-fold higher than the parental cells. When examined for growth characteristics, the JT-3 cell line grew at less than the control rate, indicating that the level of binder in these cells remained below the level required to produce folate-replete cells. Parental cells did not grow at folate concentrations below 200 nM folate (Fig. 1), indicating that their inherently low level of the high-affinity binder is below a critical amount required for cell viability. An alternative mechanism for improved growth efficiency at low concentrations of folate or folinate could have involved changes in the folate exchange carrier, as had been reported previously [29], although an increase in either the amount of carrier activity or its affinity for folate or methotrexate was not observed in the present study (Fig. 2).

High-affinity folate-binding proteins have been described previously in various cell lines of human origin [22]. The levels of these binders vary considerably and, moreover, the amounts of binding activity are influenced by the presence of bound folate. The latter conclusion was deduced from the observation that brief treatment of various cell lines with dilute acetic acid improved their subsequent ability to bind [^{3}H]folate. Of various cell lines tested, human KB cells were found to contain the highest amount of total folate-binding activity (936 pmol/mg protein) [22], while no detectable binding activity was observed in CCRF-CEM human lymphoblasts [12]. The high-affinity binder of KB cells shows a preference for the binding of folate, relative to 5-methyltetrahydrofolate and methotrexate, and saturation of the binding site with folate at neutral pH occurs at substrate concentrations below 1 nM [1, 22]. Similar binding properties were observed in the present study for the folate-binding protein of folate-adapted mouse L1210 cells, although distinct differences were also apparent. The L1210 protein binds folate (K_D = 0.065 nM), 5-methyltetrahydrofolate (K_D = 13 nM) and methotrexate (K_D = 325 nM) with the same order of affinity, but the difference in affinity between folate and methotrexate in L1210 cells (4500-fold) is much greater than with other binding proteins [21, 22]. The L1210 protein also appears to release its bound folate with a higher efficiency than other proteins. Folate bound to cells after 30 min at 37°C readily exchanged with excess unlabeled folate (Fig. 5), and, similarly, exposure of the cells first to unlabeled folate and then to labeled folate did not reduce the amount of bound [^{3}H]folate relative to untreated cells (Inset, Fig. 5). The latter results indicate that folate taken up by the cells after 30 min at 37°C remains bound to this protein and is not released into the cell and that the binding sites remain oriented towards or readily accessible to the cell exterior. Tightly bound metabolized forms of folate or inaccessible binding sites also did not appear during longer incubation intervals since a reduction in binding activity was not observed in folate-adapted cells grown in medium containing a high concentration (500 nM) of folate (Table). [^{3}H]Folate binding and exchange with unlabeled folate also occurred at 0°C, but the amount of binding activity was only 50% that observed at 37°C (Fig. 5). The latter finding suggests that the folate binder exhibits a random orientation in which approximately one-half of the binding sites face either the inner or outer cell surface at any given time and that the interconversion of these two states is inhibited by reducing the temperature. A regulatory mechanism for decreased synthesis or increased degradation of the binding protein was also indicated since the binding activity of one of the cell lines adapted to low concentrations of folinate (JT-3) could be reduced sevenfold by growth in medium containing excess folate (Table). The other adapted cell lines, however, retained the same level of binding protein regardless of the level of folate in the medium (Table), indicating that a mechanism for down-regulating the binding protein was absent in the latter cells. Fluctuations in the level of the folate binding protein by the amount of folate in the growth medium have been observed previously in a monkey kidney cell line [21].

An involvement of the high-affinity folate-binding protein in the transport of folate is supported by a direct relationship between the ability of the folate-adapted L1210 cells to grow at low concentrations of folate (Fig. 1) and the appearance of elevated amounts of the binding protein (Fig. 3). The biphasic growth response to folate (Fig. 1) also indicates that the adapted cells contain a distinct high-affinity transport system which can facilitate uptake at 1 nM folate. Optimal cell growth, however, did not occur until the folate concentration was increased to levels needed to enter the cells via the exchange system, indicating that the rate of substrate internalization via the binding-protein system must be relatively slow. A slow rate of substrate internalization via this protein was also apparent from short-term uptake experiments. In either saline or nonanionic buffers, uptake was shown to proceed by a saturable process in which the initial binding phase was not followed by a clearly evident internalization component (Fig. 4). Slow substrate internalization was also evident from the inability to observe metabolites of folate after a 30-min incubation period and by the nearly complete release of unmetabolized bound [^{3}H]folate by excess unlabeled folate (Fig. 5). Glucose, however, increased

[^{3}H]folate uptake beyond the initial binding phase (Fig. 4), suggesting that folate internalization via this protein might be significantly improved by the more favorable energetic conditions present during cell growth. Folate transport via comparable high-affinity binding proteins in other cells is also very slow. In KB cells, labeled substrate remained tightly bound to the membrane for as long as one week after exposure of cells in culture to a pulse of [^{3}H]folate [22].

The primary function and mechanism of action of high-affinity folate binding proteins have not been clearly established. Some of these proteins are hydrophobic and appear in the plasma membrane fraction of cells [1, 3, 21, 22] but they can also be found associated with intracellular membrane structures [22]. Water-soluble forms have also been described which occur in serum [4] or in milk [2, 4]. Antibody studies have shown that the various forms of high-affinity binders from different sources are immunologically related [1, 4]. A role in transport has been indicated for the plasma membrane form of the binding protein in KB cells [1], monkey kidney cells [21], and L1210 cells (present study), although it is unclear how these membrane-associated binders might deliver folate to the cell interior. One possibility is that the binding site internalizes and then releases the bound folate directly into the cytoplasm. Metabolism to other folate forms would then serve to trap the folate in impermeant forms. The turnover rate of this process, however, would be greatly hindered by the slow rate of release of bound folate that would be expected for a binding protein with such a high affinity for folate. The transport of 5-methyltetrahydrofolate, however, might occur at a much higher rate since it is bound less tightly by this protein and hence could be released with greater facility into the cell. Alternatively, Kamen and Capdevila [21] have suggested that the folate/binding-protein complex may be internalized by a process involving endocytosis coupled to the release of bound folate by the acidic environment of the lysozome. This latter process would also be very slow and, in addition, would require a moderately high consumption of cellular energy per molecule of folate transported.

The present study indicates that L1210 cells have the potential for transporting folate via two routes. Parental cells accumulate folate from the growth medium only via the anion-exchange system which transports 5-methyltetrahydrofolate and methotrexate [11], while cells adapted to low concentrations of folate or folinate employ this same exchange system at high concentrations of folate but can also utilize a second process at low substrate levels that appears to involve a high-affinity binding protein. A combination of low binding affinity and moderate turnover capacity would allow the exchange system to support cell growth at folate concentrations above 200 nM (*see* Fig. 1), while a high affinity and low turnover would combine to enable the high-affinity system to support growth at much lower concentrations of folate. The high-affinity binding protein also appears to transport folinate, in spite of a much lower affinity for folinate (K_D = 45 nM) than folate (K_D = 0.065 nM), since two of the L1210 cell lines which overproduce the binding protein were also shown to grow well at reduced concentrations of folinate. The growth characteristics of KB cells have also implicated the high-affinity binding protein in the transport of 5-methyltetrahydrofolate [1].

An additional uptake system that could transport folate, but not methotrexate or other folate compounds, had been proposed from a decade of work in several laboratories [5–7, 18–20, 23–26, 31], but the existence of this route was subsequently questioned by findings that small amounts of labeled impurities in the [^{3}H]folate can interfere with transport measurements and that this folate-specific uptake component could be eliminated by purifying the [^{3}H]folate just prior to use [11]. The latter work, however, has been disputed by recent studies [27, 32] which have provided additional data for a folate-specific route. The distinguishing features of this alternative route are a rapid initial uptake and insensitivity to inhibition by methotrexate. It was suggested further that this folate-specific system may have been suppressed in previous studies by the use of nonphysiological buffer conditions or by the lack of glucose. Findings in the present study, however, do not support the existence of this alternate route. Uptake was compared using the same buffer conditions and purification procedures for [^{3}H]folate that had been employed in the two conflicting studies and in no case was a rapid initial uptake component observed (*see* Figs. 2 and 8). Similarly, variations in buffer composition and the addition of glucose did not affect the influx pattern or the extent of inhibition by methotrexate.

The ability of folate-adapted L1210 cells to acquire folate via a high-affinity binding protein provides a possible explanation for the retention of growth on folate by cells which are resistant to methotrexate due to defects in drug transport [17, 28]. Since this binding protein has a relatively poor affinity for methotrexate (Fig. 7), cells resistant to methotrexate due to a loss in activity in the folate exchange system might have been able to grow on folate (or 5-methyltetrahydrofolate) by utilizing this high-affinity system. The development of resistance to methotrexate by this mechanism would presum-

ably require two cellular changes, one which raises the level of high-affinity binding protein and another which reduces the activity or amount of the methotrexate exchange carrier. A possible means for overcoming this type of resistance might then be to employ methotrexate in combination with a second antifolate compound whose structure more closely resembles folate than methotrexate. A second possible explanation for the growth of methotrexate-resistant cell lines which exhibit reduced methotrexate transport is that these cells may have preferentially retained a normal ability to transport folate by an altered anion-exchange system. This latter possibility has been documented previously for a bacterial folate transport system which was found to retain the ability to transport folate, but had a reduced capacity for the transport of methotrexate, after acquiring cellular resistance to methotrexate [16].

The authors thank K.S. Vitols for helpful advice and Y. Montejano for technical assistance in performing the HPLC of [^{3}H]folate. This work was supported by grants CA23970 from the National Cancer Institute and CH-229 from the American Cancer Society. Research Institute manuscript 4708-BCR.

References

1. Antony, A.C., Kane, M.A., Portillo, R.M., Elwood, P.C., Kolhouse, J.F. 1985. Studies of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. *J. Biol. Chem.* **260:**14911–14917
2. Antony, A.C., Utley, C.S., Marcell, P.D., Kolhouse, J.F. 1982. Isolation, characterization, and comparison of the solubilized particulate and soluble folate-binding protein. *J. Biol. Chem.* **257:**10081–10089
3. Antony, A.C., Utley, C., VanHorne, K.C., Kolhouse, J.F. 1981. Isolation and characterization of a folate receptor from human placenta. *J. Biol. Chem.* **256:**9684–9692
4. Colman, N., Herbert, V. 1980. Folate-binding proteins. *Annu. Rev. Med.* **31:**433–439
5. Goldman, I.D. 1975. Membrane transport of methotrexate (NSC-740) and other folate compounds: Relevance to rescue protocols. *Cancer Chemother. Rep.* **6:**63–72
6. Goldman, I.D. 1977. Membrane transport of antifolates as a critical determinant of drug cytotoxicity. *Adv. Exp. Med. Biol.* **84:**85–111
7. Goldman, I.D., Snow, R., White, J.C. 1975. The effect of methotrexate on the cellular accumulation of ^{3}H after incubation of Ehrlich ascites tumor with ^{3}H-folic acid. *Fed. Proc.* **34:**807
8. Gornall, A.G., Bardawill, C.S., David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:**751–766
9. Henderson, G.B. 1986. Folates and Pterins, Volume 3: Nutritional, Pharmacological, and Physiological Aspects. R.L. Blakley and M. Whitehead, editors. pp. 207–250. John Wiley & Sons, New York
10. Henderson, G.B., Montague-Wilkie, B. 1983. Irreversible inhibitors of methotrexate transport in L1210 cells: Characteristics of inhibition by an *N*-hydroxysuccinimide ester of methotrexate. *Biochim. Biophys. Acta* **735:**123–130
11. Henderson, G.B., Suresh, M.R., Vitols, K.S., Huennekens, F.M. 1986. Transport of folate compounds in L1210 cells: Kinetic evidence that folate influx proceeds via the high-affinity transport system for 5-methyltetrahydrofolate and methotrexate. *Cancer Res.* **46:**1639–1643
12. Henderson, G.B., Tsuji, M., Kumar, H.P. 1987. Transport of folate compounds by leukemic cells: Evidence for a single influx carrier for methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymphoblasts. *Biochem. Pharmacol.* **36:**3007–3014
13. Henderson, G.B., Zevely, E.M. 1983. Use of non-physiological buffers in the analysis of methotrexate transport in L1210 cells. *Biochem. Int.* **6:**507–515
14. Henderson, G.B., Zevely, E.M. 1983. Structural requirements for anion substrates of the methotrexate transport system in L1210 cells. *Arch. Biochem. Biophys.* **221:**438–446
15. Henderson, G.B., Zevely, E.M. 1984. Transport routes utilized by L1210 cells for the influx and efflux of methotrexate. *J. Biol. Chem.* **259:**1526–1531
16. Henderson, G.B., Zevely, E.M., Kadner, R.J., Huennekens, F.M. 1977. The folate and thiamine transport proteins of *Lactobacillus casei*. *J. Supramol. Struct.* **6:**239–247
17. Hill, B.T., Bailey, B.D., White, J.C., Goldman, I.D. 1979. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphocytes, one with impaired transport of methotrexate. *Cancer Res.* **39:**2440–2446
18. Huennekens, F.M., Vitols, K.S., Henderson, G.B. 1978. Transport of folate compounds in bacterial and mammalian cells. *Adv. Enzymol.* **47:**313–346
19. Jackson, R.C., Niethammer, D., Huennekens, F.M. 1975. Enzymic and transport mechanisms of amethopterin resistance in L1210 mouse leukemia cells. *Cancer Biochem. Biophys.* **1:**151–155
20. Kamen, B.A., Bertino, J.R. 1980. Folate and antifolate transport in mammalian cells. *Antibiot. Chemother.* **28:**62–67
21. Kamen, B.A., Capdevila, A. 1986. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc. Natl. Acad. Sci. USA* **83:**5983–5987
22. McHugh, M., Cheng, Y.C. 1979. Demonstration of a high-affinity folate binder in human cell membranes and its characterization in cultured human KB cells. *J. Biol. Chem.* **254:**11312–11318
23. Niethammer, D., Jackson, R.C. 1975. Changes of molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cells. *Eur. J. Cancer* **11:**845–854
24. Niethammer, D., Jackson, R.C. 1976. Transport of folate compounds through the membrane of human lymphoblastoid cells. *In:* Chemistry and Biology of Pteridines. W. Pfeiderer, editor. pp. 197–206. Walter de Gruyter, Berlin
25. Rader, J.I., Niethammer, D., Huennekens, F.M. 1974. Effects of sulfhydryl inhibitors upon transport of folate compounds in L1210 cells. *Biochem. Pharmacol.* **23:**2057–2059
26. Sirotnak, F.M. 1980. Correlates of folate analog transport, pharmacokinetics, and selective antitumor action. *Pharmacol. Ther.* **8:**71–103
27. Sirotnak, F.M., Goutas, L.J., Jacobsen, D.M., Mines, L.S., Barrueco, J.R., Gaumont, Y., Kisliuk, R.L. 1987. Carrier mediated transport of folate compounds in L1210 cells: Ini-

tial rate kinetics and extent of duality of entry routes for folic acid and diasteriomers of 5-methyltetrahydrohomofolate in the presence of physiological anions. *Biochem. Pharmacol.* **36:**1659–1667

28. Sirotnak, F.M., Goutas, L.J., Mines, L.S. 1985. Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis *in vivo*. *Cancer Res.* **45:**4732–4734
29. Sirotnak, F.M., Moccio, D.M., Yang, C.H. 1984. A novel class of genetic variants of the L1210 cell up-regulated for folate analogue transport inward: Isolation, characterization, and degree of metabolic instability of the system. *J. Biol. Chem.* **259:**13139–13144
30. Steinbuch, M., Audran, R. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* **134:**279–284
31. Suresh, M.R., Henderson, G.B., Huennekens, F.M. 1979. Folate uptake in L1210 cells: Mediation by an adenine transport system. *Biochem. Biophys. Res. Commun.* **87:**135–139
32. Yang, C.H., Dembo, M., Sirotnak, F.M. 1983. 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. Membrane Biol.* **75:**11–20

Received 16 September 1987; revised 13 November 1987