Biochemical Pharmacology, Vol. 36, No. 18, pp. 3007–3014, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

TRANSPORT OF FOLATE COMPOUNDS BY LEUKEMIC CELLS

EVIDENCE FOR A SINGLE INFLUX CARRIER FOR METHOTREXATE, 5-METHYLTETRAHYDROFOLATE, AND FOLATE IN CCRF-CEM HUMAN LYMPHOBLASTS*

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(*Received* 16 *December* 1985; *accepted* 9 *February* 1987)

Abstract— Influx kinetics and inhibitor specificity have been compared for the transport of methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymphoblastoid cells. Influx of each folate compound proceeded with approximately the same V_{max}, fluctuated in the same fashion with the ionic composition of the medium, and was blocked by low concentrations of an *N*-hydroxysuccinimide ester of methotrexate in both an anion-deficient buffer and in a buffered saline medium containing physiological concentrations of glucose and bicarbonate. Moreover, methotrexate influx was inhibited by 5-methyltetrahydrofolate and folate, and the inhibition constants (K_i) of the latter compounds were equivalent to their K_t values for half-maximal influx. Folate influx was likewise inhibited by methotrexate. The K_i for methotrexate was equivalent to its K_t for influx, and *o*-phthalate and phosphate each inhibited folate and methotrexate with the same degree of effectiveness. Various reversible and irreversible inhibitors reduced the influx of each folate substrate by >90%, and the progression of inhibition in each case was indicative of a single uptake component. Folate influx exhibited the same high sensitivity to inhibitors of methotrexate influx when measurements were performed at folate concentrations near the K_t for influx (10–50 μM) or at concentrations approximating physiological conditions (5–20 nM). These results indicate that CCRF-CEM cells possess a single shared transport system for the uptake of methotrexate, 5-methyltetrahydrofolate, and folate and that other high- or low-affinity uptake processes are not present in these cells.

Considerable controversy has arisen regarding the number and identity of influx routes for folate compounds in L1210 and other tumor cell lines. While it has been generally accepted that a high affinity influx carrier mediates the uptake of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and methotrexate [1–5], conflicting results have been obtained on whether this carrier can also transport folate. The presence of a separate influx route for folate had been supported by the observations that folate influx was relatively insensitive to potent inhibitors of the high-affinity system, such as methotrexate [2, 6, 7] and *p*-chloromercuriphenylsulfonate [8, 9], and could be inhibited by adenine [10], a compound which has no effect on the influx of methotrexate. Results of transport studies in plasma membrane vesicles also led to the conclusion that the high-affinity system for methotrexate does not have the capacity to transport folate and that folate uptake occurs via a totally separate route [11]. Other findings, however, provided evidence that folate may be transported by the methotrexate carrier system. In L1210 cells, a major portion of folate influx was found to be inhibited by methotrexate [1, 12], while the efflux of methotrexate was shown to increase upon the addition of folate [13]. Similarities in inhibitor sensitivity also supported a common influx route for folate and methotrexate in Chinese hamster ovary cells [14].

Additional influx routes for folate compounds have also been proposed. A low-affinity influx system for methotrexate had been indicated in leukemic cells by the inability to saturate influx at high concentrations of [^{3}H]methotrexate [2, 15, 16] and from a low sensitivity of methotrexate influx in transport mutants to inhibitors of the high-affinity system [17], whereas a high-affinity system had been observed in various cultured human cells for the cellular uptake of folate and 5-methyltetrahydrofolate [18–20]. Multiple influx routes for folate compounds are of interest since the efficacy of high-dose methotrexate chemotherapy in killing tumor cells might be enhanced if a low-affinity, high-capacity transport system were available for methotrexate. Similarly, if folate and methotrexate enter cells via different routes, then resistance to methotrexate due to a reduction in transport might be overcome by the use of other antifolate compounds that can gain cell entry via a folate-specific route.

Recent studies in L1210 mouse leukemia cells have provided a possible explanation for previous conflicting results on the number and specificity of folate transport routes in tumor cells. It was observed that measurements of folate [21, 22] and methotrexate [23, 24] transport can be complicated by the presence of trace amounts of labeled pteridines [21,22] or *p*-

* This work was supported by Grants CH-229 from the American Cancer Society and CA-23970 from the National Cancer Insititute, DHHS. Manuscript number 4104-BCR from the Research Institute of Scripps Clinic.

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aminobenzoylglutamate [24] that arise during storage of ^{3}H-labeled folate and methotrexate. Subsequent use of highly purified substrates indicated that folate and methotrexate share a common transport system in L1210 cells and that no other route is available for the uptake of these compounds [21, 23]. It thus appeared that the alternative influx routes observed previously for folate and methotrexate may have been the direct result of interference by impurities in the labeled substrate.

The present study was undertaken to characterize the transport routes utilized for the influx of methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymphoblasts. Influx kinetics and inhibitor specificity were compared for each folate substrate in an effort to separate and quantitate common or separate influx systems. The results indicate that CCRF-CEM cells contain only a single shared transport system for methotrexate, 5-methyltetrahydrofolate, and folate. Alternative uptake mechanisms involving a high-affinity folate binding protein, a low-affinity, high-capacity carrier system, or passive diffusion were not observed.

MATERIALS AND METHODS

Chemicals. [3′,5′,7,9-^{3}H]Folic acid (0.5 Ci/mmol), [3′,5′,7-^{3}H]methotrexate (0.25 Ci/mmol), and [6*R*,*S*]-5-[^{14}C]methyltetrahydrofolate (0.12 Ci/mmol) were obtained from Amersham, while [3′,5′7,9-^{3}H]folic acid (20 Ci/mmol) was obtained from Moravek Biochemicals. The [^{3}H]folate and [^{3}H]methotrexate were either employed at the initial specific activity or diluted with the corresponding unlabeled compound to 100,000 cpm/nmol and purified by thin-layer chromatography on Baker-flex cellulose sheets. The solvents employed for purification were 50 mM potassium HEPES*, pH 7.5, and 100 mM potassium HEPES, pH 7.5, for [^{3}H]folate and [^{3}H]methotrexate respectively. Labeled substrates were recovered from chromatography plates as described previously [22] by elution in the presence of ethanol. The [^{3}H]folate (in 2% ethanol) was used immediately after purification, whereas the [^{3}H]methotrexate (in 10% ethanol) could be stored up to 1 month at −20°. Original stock solutions of [^{3}H]folate and [^{3}H]methotrexate were stored at −80°. NHS-methotrexate was synthesized as described previously by activating methotrexate (in anhydrous dimethyl sulfoxide) with 1-ethyl-(3,3-dimethylaminopropyl)carbodiimide in the presence of *N*-hydroxylsuccinimide [25]. 5-[^{14}C] Methyltetrahyrofolate was purified by chromatography at 4° on a column (1.8 × 30 cm) of DEAE-cellulose. Elution was achieved with a linear gradient of 0.1 M to 0.8 M potassium phosphate, pH 7.5, containing 50 mM 2-mercaptoethanol.

Purity determinations. The purity of [^{3}H]folate, [^{3}H]methotrexate, and 5-[^{14}C]methyltetrahydrofolate was evaluated as described previously [22] from the ability of the labeled substrates to bind a membrane-associated folate-binding protein from *Lactobacillus casei* [26]. Samples containing up to 25 pmol of folate compound and a minimum of 0.001 μCi of radioactivity were mixed with 3×10^9 energy-depleted *L. casei* cells in 4.0 ml (final volume) of 100 mM potassium MES–5 mM $MgCl_2$, pH 6.0, and incubated for 60 min at 0°. The cells were then collected by centrifugation at 30,000 *g* (5 min, 4°), resuspended in 100 μl of water, and analyzed for radioactivity. Purity was defined as the percent of total added radioactivity that was bound by the cells.

Growth of cells. CCRF-CEM human lymphoblastoid cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (MA Bioproducts), 29 mg/liter of added glutamine, and 100 units each of penicillin and streptomycin. To obtain folinate-adapted cells, the stock culture was transferred from standard medium (which contains 2.2 μM folic acid) into folate-free RPMI 1640 supplemented with dialyzed fetal bovine serum and 100 nM [6*R*,*S*]-5-formyltetrahydrofolate and allowed to grow for 18 generations. Folate-depleted cells were prepared similarly except that the 5-formyltetrahydrofolate was omitted. Growth in the latter case was reduced by 5-fold after 5 generations. Cells employed in transport determinations were grown in sealed 2-liter flasks that contained 1 liter of medium and were inoculated with 3×10^8 cells. After 48 hr at 37° (with gentle shaking), the cells were harvested by centrifugation at 1000 *g* (5 min, 4°), washed with the desired buffer, and suspended to a density of 4×10^7 ml. The suspending buffers were: HEPES-buffered saline (HBS), 20 mM HEPES–140 mM NaCl–5 mM KCl–2 mM $MgCl_2$, pH 7.4, with NaOH; and magnesium–HEPES–sucrose (MHS), 20 mM HEPES–225 mM sucrose, pH 7.4, with MgO.

Transport measurements. Transport 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 from a 0.5 Ci/mmol stock solution), 2.0 μM [^{3}H]methotrexate (100,000 cpm/nmol), or 5-[^{14}C]methyltetrahydrofolate (110,000 cpm/nmol), and buffer in a final volume of 1.0 ml. After incubaton at 37° for the desired time, the cells were chilled to 0°, diluted to 4 ml with ice-cold saline (160 mM NaCl–1 mM sodium phosphate, pH 7.4), centrifuged at 1000 *g* (5 min, 4°), washed with 4 ml of saline, resuspended in 0.5 ml of saline, and analyzed for radioactivity. Transport at nanomolar concentrations of [^{3}H]folate was measured similarly except that the labeled substrate was derived from an undiluted stock solution with a specific activity of 20 Ci/mmol (16,000,000 cpm/nmol). Unless otherwise stated, uptake at 0° served as the control. NHS-methotrexate treatment was accomplished by exposing freshly-harvested cells (in HBS) to 10 μM NHS-methotrexate for 10 min at 23°. The cells were then collected by centrifugation, washed with 4 ml of the desired buffer, and analyzed for influx as described above. Influx was derived from the transport observed after incubation for 2, 3, or 5 min at 37° (depending on the buffer and substrate employed) and was reported in pmol of substrate accumulated per min per mg protein. Uptake was reported in pmols or fmols of substrate accumulated per mg protein during the time interval indicated.

* Abbreviations: HEPES, *N*-hydroxyethylpiperazine-*N*′-ethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; MHS, magnesium HEPES-sucrose buffer; HBS, HEPES-buffered saline; and NHS-methotrexate, *N*-hydroxysuccinimide ester of methotrexate.

Concentrations of protein were determined by the biuret reaction [27] using bovine serum albumin as the standard. Maximal influx (V_{max}) and the K_t for half-maximal influx were determined from double-reciprocal plots of influx versus substrate concentration. Inhibition constants (K_i values) were determined from Dixon plots of the inverse of influx versus inhibitor concentration. Reported values for V_{max}, K_t, and K_i were the average of two or more separate determinations whose standard deviations were usually less than 15%.

RESULTS

Purity of labeled substrates. Since [^{3}H]folate and [^{3}H]methotrexate are unstable and can decompose to labeled pteridines that substantially complicate the analysis of transport kinetics [21–23], particular attention was given in the present study to the purity of the labeled substrates. Specific precautions included the purchase of labeled compounds at the lowest available specific activity, storage of stock solutions at −80° in the presence of 10% ethanol, and repurificaton of the [^{3}H]folate immediately prior to use [22]. Impurities were also present in the 5-[^{14}C]methyltetrahydrofolate. The range of impurities was 5–23% (by the *L. casei* binding assay) in different samples, but they did not appear to interfere with uptake of the parent compound. This was deduced from the observations that measurements performed with [^{14}C]methyltetrahydrofolate containing impurities of 23% or with substrate purified to radiochemical homogeneity by DEAE-cellulose chromatography gave the same K_t for influx and extent of inhibition by NHS-methotrexate.

Transport kinetics for folate compounds. Figure 1 shows the uptake of [^{3}H]methotrexate (panel A), 5-[^{14}C]methyltetrahydrofolate (panel B), and [^{3}H]folate (panel C) by CCRF-CEM cells suspended in an anion-deficient buffer, in a saline buffer, and in a saline buffer after pretreatment with NHS-methotrexate, a compound shown previously to irreversibly inhibit methotrexate transport in these cells [28]. Similarities were apparent in the uptake profiles obtained with each folate compound when the same buffer was employed. Uptake was generally linear for 5–10 min, and it declined progressively thereafter but did not reach a steady state within the 25-min incubation period. A substantial difference was observed in the uptake of individual substrates when the two buffer systems were compared. With each substrate, uptake was higher in the anion-deficient buffer, relative to the saline buffer, and the difference was 3- to 4-fold for methotrexate and folate, and 1.5-fold for 5-methyltetrahydrofolate. Prior treatment of the cells with NHS-methotrexate

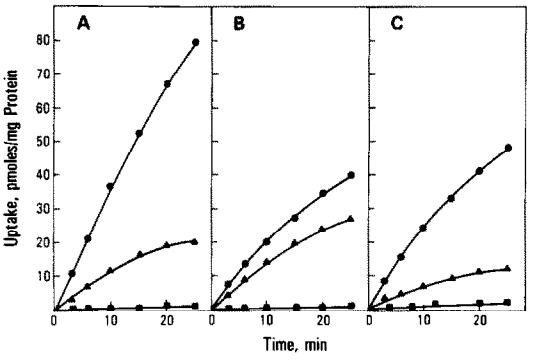

Fig. 1. Time-dependent uptake of [^{3}H]methotrexate (A), 5-[^{14}C]methyltetrahydrofolate (B), and [^{3}H]folate (C) as a function of buffer composition and treatment with NHS-methotrexate. Pretreatment with NHS-methotrexate and transport measurements were performed at 37° as described in Materials and Methods. Substrate concentrations were as follows: [^{3}M]methotrexate, 2.0 μM; [^{14}C]methyltetrahydrofolate, 1.0 μM; and [^{3}H]folate, 20 μM. Uptake by cells was measured in: (●) non-saline buffer (MHS); (▲) saline buffer (HBS); ■ saline buffer after treatment with NHS-methotrexate.

Table 1. Effect of glucose, bicarbonate, and antimycin A on the influx of methotrexate, 5-methyltetrahydrofolate, and folate in control cells and in cells pretreated with NHS-methotrexate

Folate substrate	Addition	Influx [pmol·min^{-1}·(mg protein)$^{-1}$] Control	+NHS-MTX	Inhibition by NHS-MTX (%)
Methotrexate	None	2.03	0.02	99
	Glucose	1.82	0.01	99
	Bicarbonate	2.15	0.05	98
	Antimycin A	1.67	0.00	100
5-Methyltetrahydrofolate	None	1.62	0.00	100
	Glucose	1.72	0.02	99
	Bicarbonate	1.57	0.02	99
	Antimycin A	1.55	0.03	98
Folate	None	0.61	0.06	90
	Glucose	0.55	0.04	93
	Bicarbonate	0.51	0.06	88
	Antimycin A	0.51	0.07	86

Measurements were performed in saline buffer (HBS) in cells that had been preincubated for 2 min at 37° with the indicated additions. Labeled substrates were then added, and influx was determined as described in Materials and Methods. Inhibitor concentrations: NHS-methotrexate (NHS-MTX), 10 μM; glucose, 5 mM; sodium bicarbonate, 5 mM; and antimycin A, 2 μg/ml. Substrate concentrations: [^{3}H]methotrexate, 5.0 μM; [^{14}C]methyltetrahydrofolate, 1.0 μM; and [^{3}H]folate, 20 μM.

Table 2. Kinetic parameters for the influx of methotrexate, 5-methyltetrahydrofolate, and folate

Folate compound	V_{max} [pmol·min^{-1}·(mg protein)$^{-1}$]	K_t for influx (μM) HBS	K_t for influx (μM) MHS	K_i for influx (μM) MHS
Methotrexate	4.2	4.2	0.80	0.77
5-Methyltetrahydrofolate	3.1	1.2	0.32	0.26
Folate	4.3	50	9.0	11

The buffer composition of HBS and MHS, influx measurements, and calculations of kinetic parameters are described in Materials and Methods. K_i values for influx were measured using 2.0 μM [^{3}H]methotrexate as the transported substrate.

also inhibited uptake. The extent of inhibition of initial uptake was 98, 99 and 93% for methotrexate, 5-methyltetrahydrofolate, and folate respectively. The latter measurements were performed in HEPES-buffered saline, although the same high degree of inhibition was also obtained in the non-saline buffer (MHS) (data not shown). Variations of cellular energetics produced by the addition of glucose, bicarbonate, or antimycin A to cells in the saline buffer had little or no effect on the influx of methotrexate, 5-methyltetrahydrofolate, or folate, and, moreover, did not reduce the sensitivity of influx to inhibition by NHS-methotrexate (Table 1).

Kinetic parameters for the influx of methotrexate, 5-methyltetrahydrofolate, and folate were determined from plots of initial influx versus substrate concentration; the results are shown in Table 2. Individual folate substrates were evaluated in both buffer systems simultaneously, and in each case double-reciprocal plots were linear and a common Y-intercept (V_{max}) was obtained. A similar V_{max} was also observed for each folate substrate [3.7 ± 0.6 pmol·min^{-1}·(mg protein)$^{-1}$]. The highest affinity was observed for the transport of 5-methyltetrahydrofolate whose influx in the anion-deficient buffer (MHS) proceeded with a K_t of 0.32 μM. The K_t for methotrexate under the same conditions was 0.80 μM, while the corresponding K_t for folate was 9 μM. In the saline buffer (HBS), K_t values for each substrate were consistently 4-fold higher than in the anion-deficient buffer.

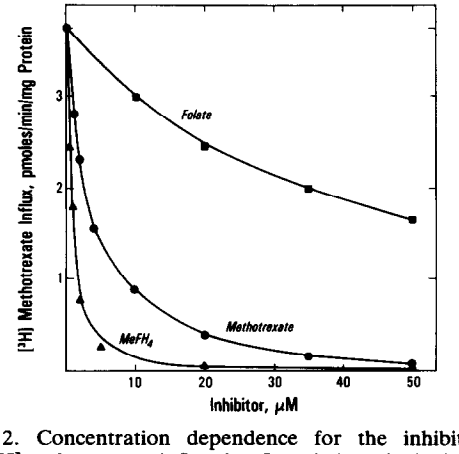

Fig. 2. Concentration dependence for the inhibition of [^{3}H]methotrexate influx by 5-methyltetrahydrofolate ($MeFH_4$), unlabeled methotrexate, and folate. [^{3}H]Methotrexate concentration, 2.0 μM. Buffer, MHS.

5-methyltetrahydrofolate, methotrexate and folate (in MHS) each inhibited the influx of [^{3}H]methotrexate (2.0 μM), and the inhibition profile for each compound yielded a monophasic rectangular hyperbola (Fig. 2). Inhibition was also complete (97–100%) at high concentrations of the more effective inhibitors, 5-methyltetrahydrofolate and methotrexate. Dixon plots of these data (not shown) were linear and gave inhibition (K_i) constants for 5-methyltetrahydrofolate, methotrexate, and folate of 0.77, 0.26, and 11 μM respectively. The latter were very similar to the corresponding K_t values for direct uptake of these compounds by cells in the same buffer (Table 2). Influx at a lower concentration of [^{3}H]methotrexate (0.1 μM) was also inhibited by >97% either by prior treatment with NHS-methotrexate (10 μM) or by the direct addition of phthalate (5 mM), a competitive inhibitor of the methotrexate influx carrier [13].

Influx of [^{3}H]folate (in MHS) was inhibited by methotrexate (Fig. 3). Dixon replots of these data were also linear to greater than 95% inhibition (data not shown), and the K_i for methotrexate (0.75 μM) was comparable to the K_i (0.80 μM) for inhibition of [^{3}H]methotrexate influx by unlabeled methotrexate (Table 3). Maximum inhibition of [^{3}H]folate influx at

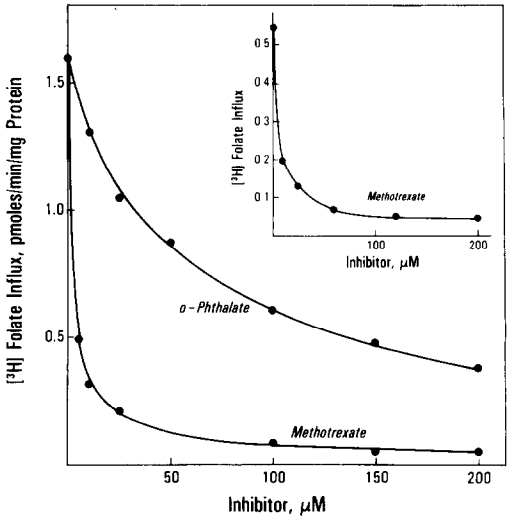

Fig. 3. Concentration dependence for the inhibition of [^{3}H]folate influx by methotrexate and *o*-phthalate. [^{3}H]Folate concentration, 20 μM. Buffer, MHS. Inset, corresponding inhibition of [^{3}H]folate influx by methotrexate in HBS.

Table 3. Comparative inhibition of [^{3}H]folate and [^{3}H]methotrexate influx by unlabeled methotrexate, *o*-phthalate, and phosphate

	K_i for influx (μM)	
	[^{3}H]Folate	[^{3}H]Methotrexate
Methotrexate	0.75	0.80
o-Phthalate	28	22
Phosphate	750	800

K_i values were determined as described in Materials and Methods from a plot of the inverse of influx at 20 μM [^{3}H]folate or 2.0 μM [^{3}H]methotrexate versus inhibitor concentration. Buffer, MHS.

high concentrations of methotrexate was 97%. In the saline buffer (insert, Fig. 3), the K_i for inhibition of [^{3}H]folate influx by methotrexate increased to 4.5 μM, while maximum inhibition decreased slightly to 93%. *o*-Phthalate (Fig. 3) and phosphate (data not shown) also inhibited [^{3}H]folate influx, and the K_i values of these compounds for inhibition of [^{3}H]folate influx were comparable to their corresponding K_i values for inhibition of [^{3}H]methotrexate influx (Table 3).

Kinetics of folate influx at low substrate concentrations. The possibility that a high-affinity, folate-binding protein also mediates the uptake of folate compounds in CCRF-CEM cells was investigated by measuring transport at low concentrations of [^{3}H]folate. Cells incubated in saline buffer for 30 min at 37° were found to accumulate significant amounts of [^{3}H]folate by a process which was essentially linear with substrate concentration from 1 to 20 nM (Fig. 4). Cells held at 0° under the same conditions accumulated less than 5% of the radioactivity of cells at 37°, and uptake at 0° was not reduced significantly by

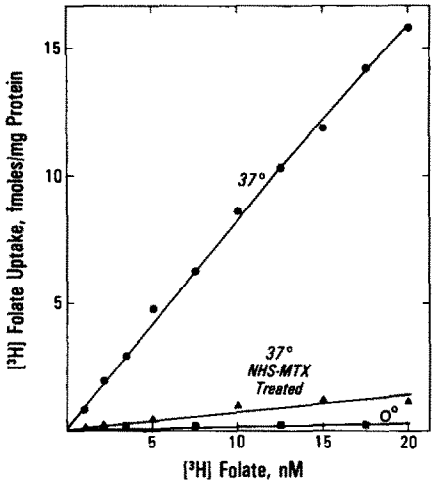

Fig. 4. Transport characteristics of CCRF-CEM cells at low concentrations of [^{3}H]folate. Cells in HBS were incubated for 30 min with the indicated concentrations of [^{3}H]folate (20 Ci/mmol) and then analyzed for uptake as described in Materials and Methods. Key: (●) uptake at 37°; (■) uptake at 0°; (▲), uptake at 37° after treatment with NHS-methotrexate.

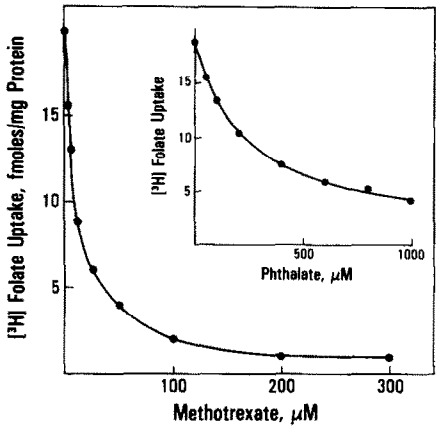

Fig. 5. Sensitivity of transport at 20 mM [^{3}H]folate to methotrexate and *o*-phthalate. Transport was measured in cells incubated for 30 min at 37° with 20 nM [^{3}H]folate (20 Ci/mmol) and the indicated concentrations of methotrexate or *o*-phthalate. Buffer, HBS.

adding 10 μM unlabeled folate prior to the [^{3}H]folate. Uptake at 37° was sensitive to NHS-methotrexate, a potent inhibitor of methotrexate and 5-methyltetrahydrofolate transport at higher substrate concentrations. Prior treatment of the cells with NHS-methotrexate reduced the extent of uptake at each [^{3}H]folate concentration by 90–95% (Fig. 4). Methotrexate and *o*-phthalate also inhibited uptake at 20 nM [^{3}H]folate (Fig. 5). Dixon replots of these data were linear, and inhibition approached 100% at high concentrations of the inhibitors. Calculated K_i values for methotrexate and *o*-phthalate were 7 μM and 200 μM respectively. The source and amount of folate provided to cells during growth also appeared not to affect the kinetics of folate influx at low substrate concentrations. Cells either adapted (for 18 generations) to [6*R*,*S*]-5-formyltetrahydrofolate (100 nM) as the sole source of folate or depleted of intracellular folate by growth in folate-free medium (see Materials and Methods) exhibited uptake (in HBS) which: (a) was indistinguishable (at 20 nM [^{3}H]folate) from that obtained in folate-replete cells; (b) was linear over a range of folate concentrations from 1.0 to 20 nM; and (c) showed the same sensitivity to inhibition by NHS-methotrexate.

DISCUSSION

Previous results have provided evidence that methotrexate enters CCRF-CEM cells by a single influx carrier and that transport occurs via an anion exchange mechanism similar to that of L1210 cells [28]. The same system has also been found in the present study to facilitate the influx of folate and 5-methyltetrahydrofolate. This conclusion is supported by the findings that methotrexate influx was inhibited by 5-methyltetrahydrofolate and folate (Fig. 2), and the inhibition constants of these latter compounds were equivalent to their K_t values for half-maximal influx (Table 2). Similarly, folate influx was inhibited by methotrexate (Fig. 3), and the K_i for methotrexate was equivalent to its K_t for influx (Table 2). *o*-Phthalate and phosphate also showed parallel inhibi-

tion of the influx of methotrexate and folate (Table 3), and the uptake rates for methotrexate, 5-methyltetrahydrofolate, and folate were each inhibited to the same degree by prior treatment of the cells with NHS-methotrexate (Table 1). Finally, parallel sensitivity to the composition of the medium was observed in both rates of uptake (Fig. 1) and K_t values for half-maximal influx into the cells (Table 2). Various findings also indicate that no other route is available for the influx of these folate compounds in CCRF-CEM cells. Influx of each folate substrate showed saturation kinetics and was typically inhibited by >95% by prior treatment with NHS-methotrexate (Fig. 1). Complete or nearly complete inhibition of [^{3}H]methotrexate influx was also obtained at high concentrations of unlabeled methotrexate and 5-methyltetrahydrofolate (Fig. 2) and by *o*-phthalate (Fig. 3), and discontinuities in inhibition profiles were not observed. Influx at nanomolar concentrations of [^{3}H]folate was blocked by NHS-methotrexate (Fig. 4) and by methotrexate and *o*-phthalate (Fig. 5), indicating that the same shared influx carrier mediated the transport of folate compounds over a broad range of substrate concentrations. A comparable V_{max} for the influx of methotrexate, 5-methyltetrahydrofolate, and folate is also consistent with a single uptake route. The present results thus contrast with previous findings that methotrexate can enter CCRF-CEM cells via a second low-affinity route [16]. It is unlikely that alternate influx routes were masked in the present study by assay conditions since buffer composition did not affect the V_{max} of individual substrates (see Table 2) or the extent of inhibition by various reversible and irreversible inhibitors (Figs 1–5). Influx was also insensitive to glucose, bicarbonate, and antimycin A (Table 1), compounds that alter cell energetics. In some experiments, non-saline buffers were employed to illustrate the coordinate effects of anions (or lack of anions) on the transport of each folate substrate, although comparative results using a saline buffer were typically performed as well (cf. Figs 1 and 3). Non-saline buffers have been indispensible in previous studies for demonstrating the anion-dependence for efflux via this carrier system [13, 23, 28–31], identifying anion-exchange substrates [13, 23, 31, 32] and quantitating the binding component [23, 33]. The physiological properties of cells vary between saline and non-saline buffers [30], yet various findings indicate that the inherent properties of the influx carrier are not altered by the absence of physiological ions in the medium or by a decline in intracellular energy levels [30]. Likewise, no evidence has been obtained which indicates that a second physiological influx system for folate compounds has been blocked by the use of non-saline buffers or by the absence of Na^+, glucose, bicarbonate or other external factors [23, 29, 30].

The transport routes for folate compounds in L1210, CCRF-CEM, and other lymphoblastoid cells have been the topic of numerous investigations. Various findings have established that reduced folates and the antifolate drug, methotrexate, share a common high-affinity transport system, while the ability of this system to transport folate has been questioned by results from various laboratories [2–4]. Recent studies [21, 23], however, have shown that impurities in the concentration range of 1% can interfere with the uptake of [^{3}H]folate. To avoid impurity problems, we have employed an established thin-layer chromatography procedure to remove contaminants from the parent compound, and a homogeneous preparation was verified by an independent binding assay using the folate transport protein of *L. casei*. Precautions were also taken to minimize the reappearance of impurities prior to use. It is particularly important not to purify, handle, or store [^{3}H]folate in buffers containing phosphate, which facilities decomposition [22]. Other procedures may also be suitable for the purification of [^{3}H]folate, although the technique must be capable of generating substrate with a purity in excess of 99%, and it must avoid solvent components and other conditions which promote or fail to prevent the reappearance of interfering compounds. Results with homogeneous preparations of [^{3}H]folate indicate that folate enters L1210 [20–22] and, in the present study, CCRF-CEM cells via the same system as methotrexate and 5-methytetrahydrofolate, and, moreover, that no other influx route is available for folate. The latter conclusion was derived, in part, from the sensitivity of [^{3}H]folate influx to specific inhibitors (cf. Figs 1 and 3–5), although it should be noted that maximum inhibition was not complete but ranged from 86 to 97%. This suggests that, even under our best conditions for purification and handling, trace amounts of impurities may be present at the time of influx measurement. The amount of pterin impurity that would be sufficient to cause interference of 10% during [^{3}H]folate influx in CCRF-CEM cells is in the range of 0.1 to 0.5% [22]. Impurities in [^{3}H]methotrexate arise at a slower rate than with [^{3}H]folate and generally interfere to a lesser degree but they may have been responsible for the previous report of a second, low-affinity influx route for methotrexate in CCRF-CEM cells [16].

Passive diffusion has been considered as a possible means by which folate compounds cross cell membranes, although the contribution by this route to total uptake in CCRF-CEM must be very small. Inhibition of influx by substrate analogs or by NHS-methotrexate often approached 100%, and double-reciprocal plots of influx versus substrate concentration remained linear at the highest substrate concentrations employed. Previous studies on methotrexate efflux [28] also suggest that passive diffusion proceeds at a very slow rate in these cells. Efflux of methotrexate could be essentially blocked by transport inhibitors even though the membrane potential should have been a substantial outward driving force for anionic forms of this compound. Passive influx of the anionic forms of folate compounds would proceed at an even slower rate since the membrane potential in this case would retard anion movement into the cell. Diffusion would be much more likely to occur with the uncharged form of folate compounds, although the concentration of this species in solution at pH 7.4 would be severely limited by the low pK_a values of the carboxyl groups in folate compounds [34].

The isolation of methotrexate-resistant cell lines which exhibit a reduced influx of methotrexate and 5-methyltetrahydrofolate, but retain the ability to

transport and grow on folate [3, 4, 35–37], has been cited as a basis for a separate influx route for folate. While transport measurements in these studies could have been affected by the presence of pteridine impurities, the retention of growth on folate indicates that a difference does exist for the assimilation of these folate compounds. One possible candidate for an alternative transport route is a high-affinity folate binding protein [18–20]. Uptake via this protein could have allowed the cells to grow on folate, even though a substantial reduction had occurred in transport via the anion-exchange system. A second possible explanation, however, for growth on folate does not involve separate influx routes. A change could have occurred in the binding site on the anion-exchange system for folates which produced a preferental reduction in the transport of methotrexate and 5-methyltetrahydrofolate, but not folate. Mutants with the latter characteristics might have arisen with a high frequency since cells are typically grown during the isolation procedure in medium containing increasing concentrations of methotrexate and a constant level of folate. Cells with a defect in methotrexate transport would then be able to grow only if the altered transport protein retained the ability to transport folate. Transport mutants exhibiting a substantial reduction in binding affinity for methotrexate, with no corresponding change in the binding of folate, have been isolated previously from *L. casei* [38].

High-affinity folate-binding proteins have been shown to occur in various types of cultured human cells [18, 19] and in an established monkey kidney cell line [20]. These proteins are associated with the plasma membrane, have a high affinity for both folate and 5-methyltetrahydrofolate, and in at least two instances [18, 19] have been implicated in the cellular uptake of folate compounds. Levels of binding protein vary considerably among various tumor cells [18] and, in the present study, CCRF-CEM cells were shown to lack a high-affinity folate-binding activity. This absence of binding activity was observed both in folate replete cells and in cells depleted of folates by repeated transfer in folate-free medium. The [^{3}H]folate employed in our measurements had a specific activity that would have allowed detection of <100 binding sites/cell. Folate uptake by CCRF-CEM cells was observed at very low substrate concentrations (1–20 nM), but inhibitor sensitivity (see Figs 4 and 5) indicated that it proceeded via the same shared carrier system which mediates folate uptake at higher concentrations of the substrate.

High-affinity folate-binding proteins have been considered as logical candidates for folate transport since serum and other biological fluids contain relatively low concentrations (10–100 nM) of total folate (present primarily as 5-methyltetrahydrofolate). High-affinity binders would contain bound substrate under these conditions and thus would be well-suited for participation in transport. Transport efficiency, however, also depends on other factors including the amount of binding protein and the turnover rate per binding site. High-affinity binders vary substantially in amount between different cell lines (and are often at very low or immeasurable levels) and more importantly do not appear to effectively release folate into the cytoplasm [18]. This contrasts with the transport system observed in the present study, which possesses a lower affinity for folates but exhibits a substantially higher overall capacity to deliver folates intracellularly. Substrate internalization by this system is a composite of the K_t of 1 μM (in saline buffer) for half-maximal influx of 5-methyltetrahydrofolate (Table 1), 30,000 binding sites/cell [28], and a turnover rate (at saturating concentrations of substrate) of 14 molecules per min per binding site at 37° [28]. Hence when the extracellular concentration of 5-methyltetrahydrofolate is at an approximate physiological level of 20 nM [39], only 1% of the binding sites would be occupied yet the system could still mediate the uptake of 4000 molecules of 5-methyltetrahydrofolate per min per cell. From the previous finding that 10^9 folate-replete leukemic cells contain approximately 2.2 nmol of total folate [40], the accumulation of 4000 folate molecules/min would satisfy the folate requirement after 5 hr. Since the doubling time of CCRF-CEM cells is approximately 18 hr, this system is clearly able to provide sufficient folate for cell growth at physiological concentrations of 5-methyltetrahydrofolate.

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