Biochemical Pharmacology, Vol. 36, No. 10, pp. 1659–1667, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
© 1987. Pergamon Journals Ltd.

CARRIER-MEDIATED TRANSPORT OF FOLATE COMPOUNDS IN L1210 CELLS

INITIAL RATE KINETICS AND EXTENT OF DUALITY OF ENTRY ROUTES FOR FOLIC ACID AND DIASTEREOMERS OF 5-METHYLTETRAHYDROHOMOFOLATE IN THE PRESENCE OF PHYSIOLOGICAL ANIONS

FRANCIS M. SIROTNAK,* LYDIA J. GOUTAS, DONNA M. JACOBSEN, LAURIE S. MINES, JOSÉ R. BARRUECO, YVETTE GAUMONT and ROY L. KISLIUK

Memorial Sloan-Kettering Cancer Center and Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, NY 10021, and Tufts University Health Science Campus, Boston, MA 02111, U.S.A.

(*Received* 14 *February* 1986; *accepted* 14 *October* 1986)

Abstract—Comparison of the kinetic parameters for influx of highly purified [^{3}H]folic acid versus [^{3}H]methotrexate in L1210 cells under anionic buffer conditions showed a marked discordancy. In addition, the kinetics for influx of [^{3}H]folic acid were unchanged in variant L1210 cells defective in [^{3}H]methotrexate transport. In these variant cells, the V_{max} for methotrexate was reduced 17-fold and the K_m was increased 3-fold. The results show that [^{3}H]folic acid influx is mediated by a system which has a low affinity, but a 20-fold higher capacity, for folate compounds than the classical high-affinity system mediating [^{3}H]methotrexate influx. Since the latter system also exhibits very low affinity for [^{3}H]folic acid, it would not be expected to contribute significantly to the total influx of [^{3}H]folic acid. The high-capacity system for [^{3}H]folic acid influx is different from that believed to mediate pterin influx in L1210 cells since it was not inhibited by adenine, a potent inhibitor of pterin influx. However, exposure of cells to [^{3}H]folic acid in a nonanionic buffer resulted in marked stimulation of initial influx, and a fraction of influx under these conditions was inhibited by methotrexate. These results suggest that anions modulate the extent of multiplicity of [^{3}H]folic acid influx by their known effects on the high-affinity, reduced folate/methotrexate system. The diastereomers, at carbon 6, of [^{14}C]5-methyltetrahydrohomofolate shared both transport systems. The influx K_m for the natural diastereomer was one-half that of the unnatural form for both transport systems. Both diastereomers showed a much greater differential in affinity between the two transport systems than did [^{3}H]folic acid. Our results suggest that an analog which could be effectively transported by the low-affinity/high-capacity route may be useful in the treatment of tumors resistant to methotrexate due to a defective high-affinity/low capacity influx system. We also found that incubation of L1210 cells with [^{3}H]folic acid or the natural diastereomer [^{14}C]5-methyltetrahydrohomofolate for 10 min resulted in the formation of a nonexchangeable fraction of radioactivity amounting to 20–40% of the total accumulation. This non-exchangeable fraction may be explained by the accumulation of metabolites other than polyglutamates. Preloading of cells with methotrexate prior to incubation with [^{3}H]folic acid prevented the accumulation of radioactivity as a nonexchangeable fraction.

Although folic acid, a dietary vitamin, appears to be at least partially accumulated (reviewed in Refs. 1–5) in tumor cells by the high-affinity system transporting folate coenzymes and methotrexate, the question of an additional entry route for this compound remains controversial. In earlier studies by others [6, 7], evidence was provided for a second entry route for folic acid in L1210 cells in the form of a large differential in sensitivity of 5-methyltetrahydrofolate and methotrexate influx to organic mercurial compounds when compared to the sensitivity of folic acid influx. Also, the same workers found [8, 9] that, in some methotrexate-resistant variants of these cells and WI-L2 cells, uptake of this reduced folate compound and the folate analogs is impaired, whereas that of folic acid is not. Although the absence of kinetic data in these studies left open the possibility that these results may only have reflected site-specific effects of mutation differentiating between various folate compounds at the level of a single transport system, other results reported by these workers [7] did show that the putative second entry route for folic acid exhibits a high-affinity for adenine which is a strong competitive inhibitor of folic acid influx. Evidence supporting the existence of a separate mediated route for folic acid influx has also been reported for HeLa cells [10]. However, in a more recent report [11], it was suggested that the results of these prior studies with L1210 cells could probably be accounted for by the presence of radioactive pterin impurities in the preparation of [^{3}H]folic acid employed and that what

* Address correspondence to: Dr. F. M. Sirotnak, Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

was actually measured [12] was the uptake in L1210 cells of [^{3}H]pterin by an unidentified mechanism and to a lesser extent uptake of [^{3}H]folic acid by the reduced folate/MTX system. In a recent report by the same group [13], data were presented from studies with L1210 cells employing nonanionic buffer conditions which suggest a lack of multiplicity in routes of [^{3}H]folic acid influx.

Our own studies [14–16] of this question were carried out with plasma membrane vesicles derived from L1210 cells using radiochemically pure [^{3}H]folic acid. This system was chosen for these studies, since its use avoids the complication of intracellular metabolism of these compounds [14, 15]. From these studies [14], evidence pertaining to various independent kinetic criteria for multiplicity of carrier-mediated entry of folate compounds was obtained. Also, intracellular accumulation of [^{3}H]folic acid and other tritiated compounds was verified [15] chromatographically. In addition to the high-affinity/low-capacity route for folate compounds in these membrane vesicles described earlier [14–16], a low-affinity/high-capacity route was also delineated which had considerable significance with respect to folic acid entry. This system was not inhibited by adenine, found by others to be a potent inhibitor of pterin uptake in L1210 cells. Also, the two systems were differentially inhibited by pCMBS* and the stilbenes, DIDS and SITS.

The contradictory nature of the results obtained from studies [6–16] carried out in the two laboratories may revolve around the fact that two very different systems were employed in each case. Studies carried out by others [6–9, 11–13] with intact L1210 cells utilized "nonphysiological" conditions. A nonanionic buffer (HEPES) was employed in the absence of an exogeneous energy source. Under these conditions, influx of folate compounds by the classical "low-affinity" system is stimulated markedly [17]. Because of the absence of anions in the external medium, saturability (decreased K_m) of influx is increased as well as trans-stimulated by the outward flow of anions from within the cell. Thus, the differential in influx mediated by this system and a "putative" second system would be altered substantially in favor of the former. On the other hand, it should also be pointed out that, in isolated plasma membrane vesicles, the relative influx capacities of the two mediated routes delineated [14–16] may also have been exaggerated in favor of the "low-affinity/high-capacity route".

The possible existence of a separate influx route for some folate compounds, that may operate at a relatively high capacity, has important pharmacologic implications, particularly in the case of transport-related methotrexate resistance. For these reasons, we carried out the studies reported here with intact L1210 cells measuring transport in a physiological buffer with 7 mM glucose [4], employing, as well, rapid kinetic procedures for sampling [18, 19]. By applying quantitative kinetic criteria for transport multiplicity and rigid standards for radiochemical purity of [^{3}H]folic acid, we were able to clearly document in these cells the existence of a low-affinity/high capacity system mediating folic acid influx and a similar, but somewhat lower, differential in transport capacity between this system and the classical high-affinity/low-capacity system that we found in plasma membrane vesicles derived from these cells. We also show that both diastereomers at carbon 6 of 5-methyltetrahydrohomofolate [20] shared both routes for entry, the low-affinity/high-capacity route contributing the greatest amount of total uptake. Also, both routes had twice the affinity for the diastereomer with the natural configuration at C-6 than for the unnatural form.

EXPERIMENTAL PROCEDURES

Cells and culture conditions. L1210 cells (L1210/C1) and a transport-defective variant (L1210/R24) were grown and maintained in RPMI medium containing 10% fetal calf serum according to procedures published earlier [21]. The origin and properties of these variant cells have also been described elsewhere [22]. For experiments, cells in the logarithmic phase of growth were employed, harvested, and washed in phosphate-buffered saline (0.14 M NaCl with 0.01 M sodium phosphate at pH 7.3).

Transport experiments. Measurements of influx, net intracellular accumulation, and efflux of [^{3}H]folic acid, [^{3}H]methotrexate and [^{14}C]5-methyltetrahydrohomofolate were carried out at 37° with cell suspension ($2–3 \times 10^7$ cells/ml) prepared in buffered salts solution containing 107 mM NaCl, 20 mM Tris–HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$ and 7 mM D-glucose at pH 7.4. Methods employed for influx and efflux determinations distinguish between surface bound and intracellular drug and ensured [4] measurements of unidirectional flux of radioactive folate compounds. These methods and those employed for deriving values for influx V_{max} and influx K_m have already been described in detail [4].

For most experiments a rapid sampling procedure modified [19] from that of Plagemann and coworkers [18] was employed during studies of transport and processing of samples for radioactive scintillation counting. In other experiments requiring high concentrations of substrate, i.e. measurements of concentration–response for influx, aliquots of cell suspension were processed [4] by rapid centrifugation at 0° following 20-fold dilution with cold (0°) buffered isotonic saline (see above) done three times. These procedures employed a correction for extracellular and surface bound radioactivity [4] which yielded the intracellular content of [^{3}H]methotrexate. Values for intracellular water were derived as described earlier [4]. These were 3.64 ± 0.35 ml/g dry wt for both cell types employed. Data derived during transport measurements were expressed as nmol/g dry wt of intracellular folate compound in accordance with conventions established earlier in our laboratory.

* Abbreviations: pCMBS, *p*-chlormercurobenzoylysulforate; pABG, *p*-aminobenzoyl glutamate; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; SITS, 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid; 5-methylTHHF, 5-methyltetrahydrohomofolate; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Chemicals. [^{3}H]Folic acid (sp. act. 12 Ci/mmol) and [^{3}H]methotrexate (sp. act. 10–20 Ci/mmol) were purchased from Moravek Biochemicals (City of Industry, CA). Diastereomers of [^{14}C]5-methyltetrahydrohomofolate, the nonradioactive diastereoisomers, and the racemate of 5-methyltetrahydrohomofolate were synthesized [23] and purified [24] as previously described. Pterin, 6-carboxypterin and 6-hydroxymethylpterin were provided by Dr. D. Duch of Wellcome Research Laboratories, Research Triangle Park, NC. All other chemicals were reagent grade.

Purification of folate compounds. Prior to purification of folic acid and methotrexate and their tritiated derivatives, samples were lyophilized to dryness and resolubilized in a small volume of sodium acetate buffer (0.01 M, pH 5.1). Radioactive derivatives were chromatographed as is or following dilution with unlabeled material. Purification was achieved by reverse-phase HPLC modified from a published procedure [25] on a Waters system equipped with a radial compression Z-module and a μ-Bondapak C_{18} Radial-PAK cartridge. Recovery of injected material is achieved by gradient elution with a combination of two mobile phases, 0.01 M sodium acetate (pH 5.1) and acetonitrile at a flow rate of 2 ml/min. In this system, the concentration of acetonitrile is increased linearly from 0 to 10% in 20 min and subsequently increased to 100% over the next 15 min. Acetonitrile is then maintained at 100% for an additional 10 min to wash the column free of contaminants. The system is equilibrated back to initial conditions between runs. By rerunning purified material in the same system, purity of these materials were estimated to be >99.8%. Purified samples were stored at −70° following lyophilization and reconstituted in transport buffer just prior to use. Derivatives of 5-methyltetrahydrohomofolate were analyzed for purity by isocratic elution from a reverse-phase ultrasphere ODS 5 μm column (Waters) using 50% methanol in water with 2 mM tetrabutyl ammonium phosphate, pH 7.5, at a flow rate of 2 ml/min. Estimates of purity in this case were >99.5%. Samples were stored at −70°. Purified samples of [^{3}H]folic acid were employed for experiments within 1 week of storage at −70° or repurified. Samples of [^{3}H]methotrexate and [^{14}C]5-methylTHHF were repurified on a twice monthly basis.

Table 1. Summary of elution characteristics for folic acid, pABG and various pterins during high-performance liquid chromatography

Compound	Retention time* (min)
pABG	4.60
6-Carboxypterin	5.01
Pterin	10.62
6-Hydroxymethylpterin	11.03
Folic acid	21.53

A description of the HPLC method employed is provided in the text.

* Determinations were repeated two to five times with average variation in retention times of <5%.

Recovery of [^{3}H]*folic acid in L1210 cells.* Cells in transport buffer were equilibrated at 37°, and aliquots of cell suspension were incubated for 1 min with a final concentration of 40 μM purified [^{3}H]folic acid. Incubation was terminated by the rapid addition of 10 vol. of ice-cold phosphate-buffered saline. Cells were washed twice by centrifugation and extracted by heat treatment at 100° for 5 min in phosphate-buffered saline plus 1% ascorbic acid and 100 mM β-mercaptoethanol. To control for [^{3}H]folic acid breakdown during the course of the extraction, a [^{3}H]folic acid sample was incubated in transport buffer under identical conditions. An aliquot of fresh cells was added to this sample after cooling to 0° and this preparation was subjected to the same extraction procedure.

RESULTS

Purification of [^{3}H]*folic acid.* Because of the controversial nature of the issue under consideration with regard to [^{3}H]folic acid influx, it was essential that extremely rigorous criteria for radiochemical purity of this compound be employed during the course of these studies. We evaluated our procedure for the purification of [^{3}H]folic acid by comparing elution during HPLC of folic acid and some pterins known [11–13] to be decomposition products in commercial samples of this material. Retention times for these compounds obtained during HPLC analysis are shown in Table 1. pABG and 6-carboxypterin showed the lowest retention times among the group of compounds examined. Pterin and 6-hydroxymethylpterin, the most prevalent decomposition product [11–13], exhibited retention times approximately one-half of that shown for folic acid. These results show that both pABG and pterins exhibit widely different retention times compared to folic acid in this elution system. An HPLC elution profile for [^{3}H]folic acid (sp. act. 12 Ci/mmol) is shown in Fig. 1. This material was purified during elution in a

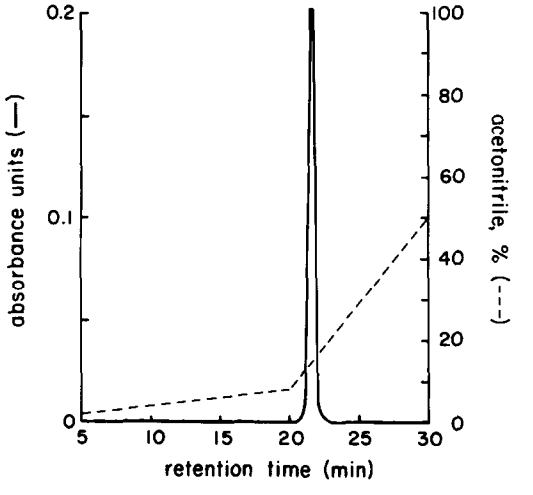

Fig. 1. High-performance liquid chromatography of purified [^{3}H]folic acid. A sample (17 nmol) of material purified by HPLC was rerun by reverse-phase on a Bondapak C_{18} column in the same buffer system on the same day. Details of the chromatographic procedure are given in the text.

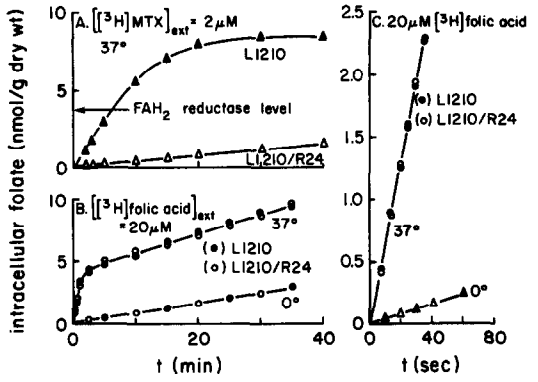

Fig. 2. Time–courses for intracellular accumulation at 37° of [^{3}H]methotrexate and [^{3}H]folic acid in parental and variant L1210 cells. Procedures employed for measurements of accumulation of folate compound and processing of samples for liquid scintillation counting have been given in the text or in earlier reports [4]. Data shown are the average of three to five individual experiments. Standard error of the mean did not exceed ± 16%.

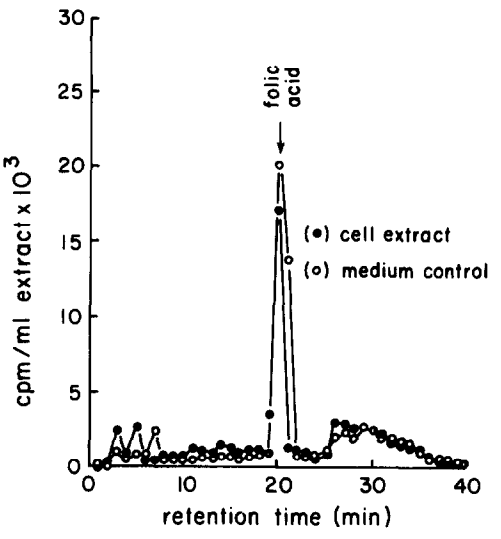

Fig. 3. High-performance liquid chromatography of intracellular radioactivity accumulated following incubation of L1210 cells with [^{3}H]folic acid. Cells were incubated with 20 μM [^{3}H]folic acid for 1 min, diluted in cold (0°) buffered isotonic saline, and then washed twice in the same buffer prior to extraction. The same amount of [^{3}H]folic acid was incubated for 1 min in medium alone cooled to 0° and then extracted after the addition of cold L1210 cells. See text for further experimental details.

prior HPLC run and was rerun immediately. The sharp elution peak obtained in this second run and the absence of other eluting peaks show that this material did not decompose during the initial HPLC run.

Intracellular accumulation of [^{3}H]*folic acid by L1210 cells.* Time–courses for intracellular accumulation of [^{3}H]methotrexate and [^{3}H]folic acid by L1210 cells and a transport-defective, methotrexate-resistant variant (L1210/R24) are shown in Fig. 2. [^{3}H]Methotrexate accumulation at a concentration of 2 μM in parental L1210 cells exhibited a time–course (Fig. 2A) similar to that characterized in these and other tumor cells by studies from our laboratory and elsewhere (reviewed in Refs. 4 and 5). Accumulation initially was constant with time and then began to decrease asymptotically until an apparent steady-state was attained. During the same 40-min incubation, intracellular accumulation of [^{3}H]methotrexate in L1210/R24 cells was substantially less and remained constant with time. Intracellular accumulation of [^{3}H]folic acid at 37° at a concentration of 20 μM exhibited a different time–course (Fig. 2B) which was indistinguishable in both parental and variant L1210 cells. In this case, accumulation was very rapid initially and within 2 min decreased to a much lower rate with time. This lower rate of uptake was then maintained during the 40-min incubation period. In experiments with [^{3}H]folic acid, appreciable uptake was observed at 0° (Fig. 2B), whereas no intracellular accumulation of [^{3}H]methotrexate (data not shown) was observed at this temperature. HPLC analysis of the intracellular radioactivity accumulated after incubation of L1210 cells for 1 min with 20 μM [^{3}H]folic acid (Fig. 3) showed that accumulation occurred in a form which eluted as the original folate compound employed.

In other experiments, data (Fig. 4) were obtained which suggest that the late "slow" component of uptake in L1210 cells incubated with [^{3}H]folic acid was a result of metabolism by dihydrofolate reductase of this compound accumulating in these cells. From the data shown in Fig. 4, it can be seen that preloading of cells with methotrexate followed by washing virtually eliminated this "slow" component of uptake that occurred in cells incubated with 5 μM [^{3}H]folic acid at 37°. However, there was no effect on initial (< 1 min) uptake at 37° nor on uptake at 0°.

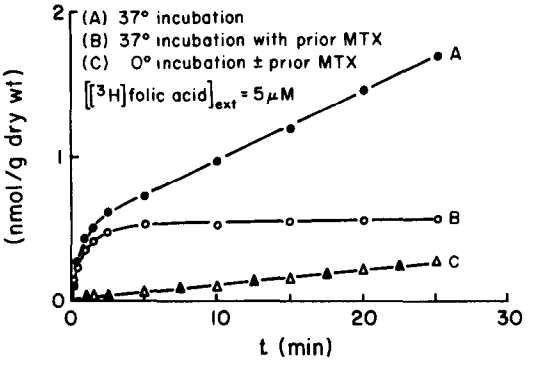

Fig. 4. Time–course for accumulation of radioactivity at 37° following incubation of 5 μM [^{3}H]folic acid with L1210 cells before and after preloading with methotrexate. Cells were preloaded at a concentration of 2 μM methotrexate during incubation for 10 min in transport buffer. These cells and control cells were washed at 0° and resuspended in transport buffer prior to the addition of [^{3}H]folic acid. Other experimental details are given in the text. Data shown are an average of three experiments with standard error of the mean <14%.

Table 2. Kinetic analysis of folate compound influx in parental and transport-defective, methotrexate-resistant L1210 cells

Cell line	Substrate	Influx K_m (μM)	Influx V_{max} (nmol/min/g dry wt)
L1210	[^{3}H]Folic acid	572 ± 93	198 ± 42
	[^{3}H]Methotrexate	3.7 ± 1	8.4 ± 1
	[^{14}C]*l*-5-methylTHHF	383 ± 87	195 ± 32
	[^{14}C]*d*-5-methylTHHF	907 ± 135	204 ± 34
L1210/R24	[^{3}H]Folic acid	558 ± 72	189 ± 39
	[^{3}H]Methotrexate	13.3 ± 1.7	0.43 ± 0.03

L1210 cells were washed twice in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in transport buffer without serum. The procedures employed for measurements of initial influx at 37° at various concentrations of folate compound for the determination of influx K_m or K_i and influx V_{max}, and for the processing of cells have been described in detail earlier [4]. Data shown are the average of four to five individual experiments. Standard error of the mean did not exceed ±15%.

The initial 60 sec of intracellular accumulation of [^{3}H]folic acid in parental L1210 cells and variant cells is shown in Fig. 2C. Since accumulation during this period was constant with time in each case and the same constancy for the initial time–course was already documented for [^{3}H]methotrexate in L1210 and L1210/R24 cells in Fig. 2A, a kinetic analysis was possible on the initial influx of each compound at various concentrations in both cell types. Influx over the initial 60-sec period was constant with time (data not shown) at all of the concentrations employed. The results of such an analysis are summarized in Table 2. For [^{3}H]methotrexate, the value for influx K_m was increased 3-fold and that for influx V_{max} was reduced 17-fold in R24 cells compared to parental L1210 cells. For [^{3}H]folic acid, the value for influx K_m in parental L1210 cells was 154-fold higher and that for influx V_{max} was at least 20-fold higher than the same values for [^{3}H]methotrexate. Moreover, the values derived for [^{3}H]folic acid were the same for parental cells and R24 cells.

Intracellular accumulation of 5-methyltetrahydrohomofolate in L1210 cells. Results similar to those obtained with [^{3}H]folic acid were also obtained with the folate analog, 5-methyltetrahydrohomofolate. Data are shown in Fig. 5 for the intracellular accumulation of the ^{14}C-labeled *l** and *d* diastereomers (external concentration = 20 μM) of this compound which has an asymmetric center at carbon 6. The time–courses for each diastereomer, which are shown in the figure (Fig. 5A), differed substantially. Intracellular accumulation of the *l* isomer in parental cells was similar to that obtained with [^{3}H]folic acid (Fig. 2B), i.e. the rate of accumulation was very rapid initially and within 2–3 min began to decrease to a lower rate which was maintained for the remainder of the 20-min incubation period. Intracellular accumulation of the *d* isomer in parental L1210 cells resembled that obtained with [^{3}H]methotrexate (Fig. 2A). Initial accumulation occurred at a constant rate followed by a reduction in rate asymptotically as steady-state was approached. As with [^{3}H]folic acid, appreciable accumulation of these folate analogs was also observed (data not shown) at 0°. The overall characteristics of the time–courses for intracellular accumulation of these diastereomers in R24 cells was virtually the same. However, initial rates of accumulation and the overall level of accumulation in this variant were somewhat lower than in parental L1210 cells. Initial uptake of both isomers at various concentrations in each cell type was linear with time. Data obtained with various concentrations of the *l* isomer in L1210 cells are shown in Fig. 5B. Similar

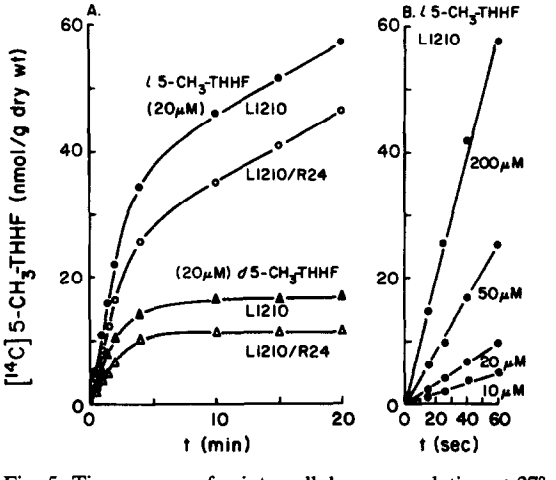

Fig. 5. Time–courses for intracellular accumulation at 37° of diastereomers of [^{14}C]5-methyltetrahydrohomofolate in parental and variant L1210 cells. Procedures employed for measurement of accumulation of folate compounds and processing of samples for liquid scintillation counting have been given in the text or in earlier reports [4]. Data shown are the average of three to four individual experiments. Standard error of the mean did not exceed ± 14%. Abbreviation: 5-CH$_3$-THHF, 5-methyltetrahydrohomofolate.

* The symbols *l* and *d* are used to denote, respectively, the natural and unnatural configurations of tetrahydrohomofolate at C-6 [23] and do not denote optical activity. In all cases, glutamyl derivatives have the L configuration.

Table 3. Inhibition of [^{3}H]methotrexate and [^{3}H]folic acid influx in L1210 cells by various folate compounds

Inhibitor	Influx [^{3}H]Methotrexate K_i (μM)	[^{3}H]Folic acid K_i (μM)
Methotrexate	3.4 ± 0.4	>2000
Folic acid	188 ± 22	484 ± 62
l-5-methylTHHF	50.5 ± 10	372 ± 46
d-5-methylTHHF	104 ± 8	793 ± 94
dl-5-methylTHHF	82.5 ± 12	625 ± 75
Adenine	ND*	>500

See footnote in Table 2 for experimental details. Data shown are the average of four to five individual experiments. Standard error of the mean did not exceed ± 13%.
* Not done.

results were obtained for both isomers with L1210 and L1210/R24 cells (data not shown). A kinetic analysis of these rates was carried out to obtain values for influx K_m and influx V_{max} for each compound. These results are also summarized in Table 2. Values for influx K_m for each diastereomer were extremely high and were the same in each cell type. They approximated values derived for [^{3}H]folic acid in the case of *l*-5-methylTHHF, but were 2-fold higher for the *d* isomer. Values for influx V_{max} for both diastereomers were also extremely high. In both R24 cells and parental L1210 cells, these approximated those derived for [^{3}H]folic acid.

Competition among folate compounds for initial influx by L1210 cells. The results presented in Figs. 1 and 2 and Table 1 document considerable complexity in the transport of these folate compounds in L1210 cells under the conditions employed. Further information was sought in experiments analyzing competitive interactions among these compounds for influx (Table 3). It was found that folic acid and *l*- and *d*-5-methylTHHF were competitive inhibitors of [^{3}H]methotrexate and [^{3}H]folic acid influx. However, values for influx K_i derived against [^{3}H]methotrexate were lower for folic acid and substantially lower for the *l* and *d* isomers of 5-methylTHHF when compared to values obtained for K_i during influx of [^{3}H]folic acid. These latter values approximated the values for influx K_m obtained with radiolabeled derivatives of folic acid and the *l* and *d* diastereomers of 5-methylTHHF. As expected, racemic 5-methylTHHF gave values for K_i intermediate between those derived for each diastereomer alone as inhibitors of [^{3}H]methotrexate and [^{3}H]folic acid influx. The data of Table 3 also show that neither methotrexate nor adenine inhibited [^{3}H]folic acid influx.

Efflux of [^{3}H]folic acid and [^{14}C]5-methyltetrahydrohomofolates from L1210 cells. The time-course for intracellular accumulation at 37° of [^{3}H]folic acid observed in L1210 cells (Fig. 2B) would suggest that a nonexchangeable fraction of radioactivity was accumulating in these cells as incubation was continued. We investigated this possibility by measuring efflux of radioactivity that accumulated after various periods of exposure to 20 μM [^{3}H]folic acid. These results are given in Fig. 6. After 0.75 min

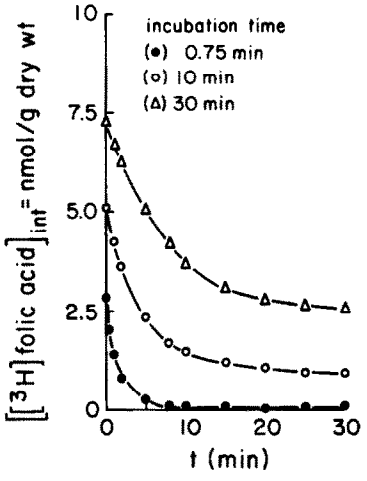

Fig. 6. Efflux at 37° of [^{3}H]folic acid or metabolites from L1210 cells. Cells were preloaded at a concentration of 20 μM for various times at 37°. Procedures employed for measurement of efflux and processing of samples for liquid scintillation counting have been given in the text or in an earlier report [4]. Data shown are the average of four individual experiments. Standard error of the mean did not exceed ± 12%.

of incubation with [^{3}H]folic acid, all of the radioactivity that accumulated rapidly effluxed when cells were washed and resuspended in folate-free transport buffer at 37°. However, after 10 and 30 min of accumulation, efflux in drug-free buffer was progressively less rapid. A decay-time analysis of these data (Fig. 7) by the method of "exponential stripping" [14–16] showed that efflux at 37° gradually converted from a single decay-time of 1.3 ± 0.1 min to a decay-time of 5.1 ± 0.6 min (initial efflux) and 64 ± 8 min (late efflux) after 30 min of accumulation in the presence of [^{3}H]folic acid. Somewhat similar

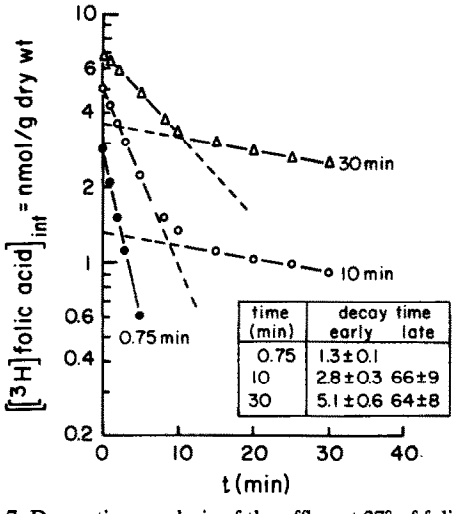

Fig. 7. Decay-time analysis of the efflux at 37° of folic acid or metabolites from L1210 cells. See legend of Fig. 6 for experimental details. Data shown are the average of four individual experiments. Standard error of the mean did not exceed ± 12%.

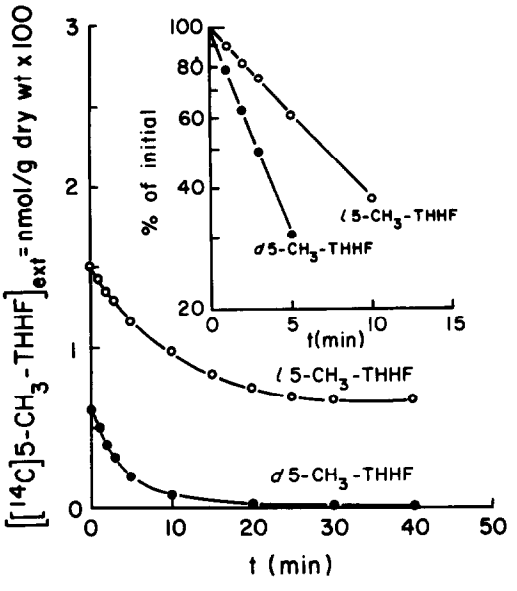

Fig. 8. Efflux at 37° of diastereomers of 5-methyltetrahydrohomofolate from L1210 cells. Cells were preloaded at a concentration of 50 μM for 10 min at 37°. Procedures employed for measurements of efflux and processing of samples for liquid scintillation counting have been given in the text or in an earlier report [4]. Data shown are the average of four individual experiments. Standard error of the mean did not exceed ± 12%.

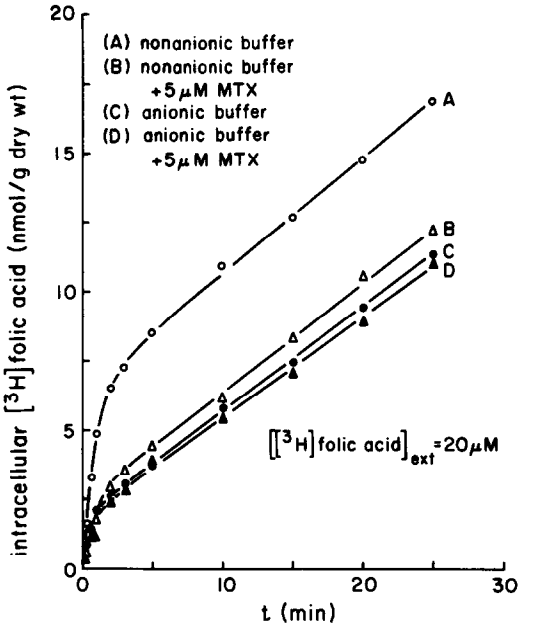

Fig. 9. Time–course for accumulation of radioactivity at 37° following incubation of L1210 cells with 20 μM [^{3}H]folic acid in an anionic buffer or nonanionic buffer in the presence or absence of 5 μM methotrexate. Cells were incubated in 2 mM $MgCl_2$, 20 mM HEPES and 225 mM sucrose adjusted to pH 7.4 with KOH or in the transport buffer (see Experimental Procedures) normally used in these experiments. Other experimental details are given in the text. Data shown are for an average of two experiments with >16% standard error of the mean.

results were obtained with the *l* isomer of 5-methylTHHF in similar experiments employing a 10-min period of accumulation. Initial efflux of this isomer was one-half as rapid (Fig. 8) as efflux of the *d* isomer. Also, an appreciable nonexchangeable fraction of intracellular radioactivity was apparent following a 10-min incubation with the ^{14}C derivative of the *l* diastereomer but not with the *d* form.

Intracellular accumulation of [^{3}H]*folic acid in L1210 cells in the absence of anions.* The controversy pertaining to transport multiplicity for [^{3}H]folic acid in L1210 cells may reflect altered expression of this multiplicity under the different buffer conditions used in each case. Therefore, we examined the time–course for accumulation at 37° of [^{3}H]folic acid in L1210 cells in a nonanionic buffer (2 mM $MgCl_2$, 20 mM HEPES and 225 mM sucrose adjusted to pH 7.4 with KOH) and the anionic transport buffer normally used for our experiments. From the data shown in Fig. 9, it can be seen that, in the nonanionic buffer, initial accumulation of [^{3}H]folic acid was substantially more rapid than in the anionic buffer. However, the rate of accumulation following the initial 5 min was virtually the same. Moreover, the addition of 5 μM methotrexate to cells suspended in the nonanionic but not in the anionic buffer markedly inhibited initial accumulation of [^{3}H]folic acid so that the initial rate of accumulation in the buffer in the presence of methotrexate was similar to the rate obtained in the anionic buffer. In either case, methotrexate had no effect on the rate of accumulation of [^{3}H]folic acid after the first 5 min of incubation. In other experiments (data not shown), we also found that the presence or absence of glucose in the nonanionic buffer did not modulate further the stimulation of initial influx seen in this buffer.

DISCUSSION

The results presented here affirm our conclusion derived in earlier studies [16] with L1210 cell plasma membrane vesicles that [^{3}H]folic acid influx in L1210 cells is mediated mainly by a system distinct from the "classical" system mediating influx of methotrexate and folate coenzymes. This conclusion is based upon data which show that during reciprocal competition experiments there were major discordancies between values for influx K_m and influx K_i for the various folate compounds. According to criteria established by Christensen [26], for compounds which share a single transport system, values for influx K_m and K_i in such experiments should be approximately the same. The results show that this was not the case. The data also show that the V_{max} for initial mediated influx of [^{3}H]folic acid was more than 20-fold higher than that shown for initial influx of [^{3}H]MTX. The data also documented unaltered influx of [^{3}H]folic acid in a methotrexate-resistant variant with severely defective influx of [^{3}H]methotrexate primarily as the result of a 15-fold reduction in influx V_{max}. These findings could not be explained if the only route for [^{3}H]folic acid influx was by the "classical" high-affinity system.

We also emphasize that the findings reported here were obtained with radioactive folate compounds that were meticulously purified by HPLC. In the case of [^{3}H]folic acid, it was clearly shown (Table 1) that the HPLC system employed was highly effective in separating pABG and pterins from folic acid. It is, therefore, unlikely that other pterins, which may be decomposition products of [^{3}H] folic acid, would not be similarly separated from this highly charged folate compound. Moreover, the recovery of the major portion of radioactivity accumulated in L1210 cells in a form eluting only as folic acid argues strongly against the notion that radioactivity was accumulated as a pterin decomposition product. If this were the case, the HPLC elution profile should certainly have revealed a peak of radioactivity eluting as 6-hydroxymethylpterin, the major decomposition product [11, 12], if not other peaks representing the other decomposition products. In any event, these findings are all consistent with the lack of inhibition of [^{3}H]folic acid influx in these cells by adenine shown by others [7, 11–13] to be a potent inhibitor of pterin influx by these cells.

A likely explanation for the controversy pertaining to the multiplicity of influx routes for [^{3}H]folic acid has been provided from these studies. Data shown in Fig. 9 document a substantially greater initial influx of [^{3}H]folic acid by L1210 cells in a nonanionic buffer. Moreover, only the increase in initial influx compared to that seen in the anionic buffer system was inhibited by methotrexate. We can expect, based on data published by Henderson *et al.* [13, 17, 27, 28], that marked stimulation of [^{3}H]folic acid influx in L1210 cells would occur when anions are removed from the external compartment. This results partially from the 5- to 6-fold increase in saturability (decreased influx K_m) of influx as well as from the trans-stimulation which would occur from the outward flow of intracellular anions. In our experiments, L1210 cells were held at 0° when removed from an anionic environment to the nonanionic buffer and then incubated for only 5 min at 37° prior to the addition of [^{3}H]folic acid. Under these conditions, substantial stimulation of influx would occur when [^{3}H]folic acid was added, since it required [29] approximately 30 min at 37° for the system to equilibrate anionically. To account for the difference seen (Fig. 9) in initial influx of [^{3}H]folic acid in each buffer system, only a 2-fold trans-stimulation in addition to the increased saturability of the classical system would be required in the nonanionic buffer, since saturability of this system for folic acid is 2.5 times greater than of the high-capacity system in the anionic buffer.

Since anions are normal constituents of physiological fluids, the expression of multiplicity in influx routes for [^{3}H]folic acid in their presence has pharmacologic significance. Analogs which would be effectively transported by the high-capacity system for folic acid might have considerable utility in the treatment of tumors that are resistant to methotrexate by virtue of a defect in the "classical" high-affinity system. In this regard, the results obtained with the 5-methyltetrahydrohomofolates have special meaning. These results appeared to show that both *d* and *l* diastereomers of 5-methylTHHF share both transport routes for these compounds (Tables 2 and 3). The relatively higher affinity of these compounds, compared to folic acid, for the "classical" high-affinity/low capacity system is consistent with the data of Fig. 4A showing that accumulation of both compounds was less in L1210/R24 cells and, therefore, would appear to be mediated by both influx routes. Applying Michaelis–Menten theory [30] to the data in Tables 2 and 3 reveals that 85–90% of 5-methylTHHF initially would be transported inward by the putative high-capacity system and the remainder by the high-affinity/low-capacity system. This accounts for the difference in initial influx of these compounds (Fig. 4A) obtained with both L1210 and L1210/R24 cells. In addition, we found that both routes exhibited low stereospecificity for carbon 6 of 5-methylTHHF; both influx systems had twice the affinity for the *l* form compared to the *d* form. Similar results were obtained earlier with 5-methyltetrahydrofolate [31, 32] where the classical high-affinity system has only 1.5 times the affinity for the *l* form than for the *d* form. In contrast, this system shows [33] marked stereospecificity for 5-formyltetrahydrofolate. The affinity for the *l* form of 5-formyltetrahydrofolate was 20-fold the affinity for the *d* form.

In addition to our work [14–16] with L1210 cell plasma membrane vesicles, which we sought to extend in these studies, the existence of a separate high-capacity transport route for folic acid in tumor cells was actually inferred from other earlier [8, 9, 22] observations. These showed that methotrexate resistant L1210 cells with a severely (up to 100-fold) reduced influx V_{max} for methotrexate and folate coenzymes are able to grow as well as the parental L1210 cells in folic acid. However, growth in 5-formyltetrahydrofolate of these variants is impaired [22].

It was also of interest to note in these studies that the large differential between influx capacities of the two entry routes delineated for folate compounds documented [16] in plasma membrane vesicles was, in fact, a reflection of a similar differential in capacity in intact L1210 cells under physiological conditions. Overall influx capacities, however, were substantially lower in membrane vesicle preparations.

Evidence that was provided by the efflux experiments (Figs. 6–8) showed that a larger fraction of accumulated [^{3}H]folic acid and [^{14}C]l-5-methylTHHF was nonexchangeable. In view of the data shown in Fig. 4, this result in the case of [^{3}H]folic acid was most likely attributable to metabolism of this compound and serves to explain the complex nature of the time–course for its accumulation. A similar explanation probably applies in the case of 5-methylTHHF. The exact nature of this nonexchangeable fraction is not known, but HPLC analaysis (data not shown) of the intracellular radioactivity at various times of incubation would suggest that it is one or more metabolites other than a polyglutamylated derivative. We also point out that the transport system employed in these experiments was devoid of an exogenous source of glutamate or glutamine, and, therefore, measurable levels of polyglutamylation of these compounds, as in the case of earlier studies [34], would not have been expected.

The nature of the metabolite(s) obtained in the case of each compound is most likely different and, therefore, the similarity in the time–course obtained with [^{3}H]folic acid and [^{14}C]*l*-5-methylTHHF is probably fortuitous. In the case of 5-methylTHHF, the time–course could be explained by the transfer of $^{14}CH_3$ to homocysteine by mammalian methionine synthetase [35] and then to protein, an interaction which would not be expected [35] to occur with *d*-5-methylTHHF.

Acknowledgements—These studies were supported in part by Grants CA08748, CA10914, CA18856, CA22764 and CA09207 from the National Cancer Institute, Grant CH26 from the American Cancer Society, and the Elsa U. Pardee Foundation. Homofolate was obtained through the generosity of Dr. J. A. R. Mead of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

REFERENCES

1. I. D. Goldman, *Ann. N.Y. Acad. Sci.* **186**, 400 (1971).
2. I. D. Goldman, *Cancer Treat. Rep.* **6**, 51 (1975).
3. F. M. Sirotnak, P. L. Chello and R. W. Brockman, *Meth. Cancer Res.* **16**, 382 (1979).
4. F. M. Sirotnak, *Pharmac. Ther.* **8**, 71 (1980).
5. F. M. Sirotnak, *Cancer Res.* **45**, 3992 (1985).
6. J. R. Rader, D. Niethammer and F. M. Huennekens, *Biochem. Pharmac.* **23**, 2057 (1974).
7. M. R. Suresh, G. B. Henderson and F. M. Huennekens, *Biochem. biophys. Res. Commun.* **87**, 135 (1979).
8. R. C. Jackson, D. Niethammer and F. M. Huennekens, *Cancer Biochem. Biophys.* **1**, 151 (1975).
9. D. Niethammer and R. C. Jackson, *Eur. J. Cancer* **11**, 845 (1975).
10. H. Rosemond-Hornbeak and M. G. Nair, *Molec. Pharmac.* **14**, 299 (1978).
11. F. M. Huennekens, K. S. Vitols, M. R. Suresh and G. B. Henderson, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Eds. A. C. Sartorelli, J. S. Lazo and J. R. Bertino), p. 333. Academic Press, New York (1981).
12. M. R. Suresh and F. M. Huennekens, *Biochem. Int.* **4**, 533 (1982).
13. G. B. Henderson, M. R. Suresh, K. S. Vitols and F. M. Huennekens, *Cancer Res.* **46**, 1639 (1986).
14. C-H. Yang, M. Dembo, F. M. Sirotnak and P. L. Chello, *J. biol. Chem.* **254**, 1402 (1979).
15. C-H. Yang, M. Dembo and F. M. Sirotnak, *J. membr. Biol.* **68**, 19 (1982).
16. C-H. Yang, M. Dembo and F. M. Sirotnak, *J. membr. Biol.* **75**, 11 (1983).
17. G. B. Henderson and E. M. Zevely, *Archs. Biochem. Biophys.* **200**, 149 (1980).
18. R. M. Wohlheuter, R. Marg, J. C. Graf and P. G. W. Plagemann, *J. cell. Physiol.* **89**, 605 (1976).
19. P. L. Chello, F. M. Sirotnak, D. M. Dorick and R. C. Donsbach, *Cancer Res.* **37**, 4297 (1983).
20. R. L. Kisliuk, in *New Approaches to the Design of Antineoplastic Agents* (Eds. T. J. Bardos and T. I. Kalman), p. 201. Elsevier, New York (1981).
21. F. M. Sirotnak, D. M. Moccio, L. J. Goutas, L. E. Kelleher and J. A. Montgomery, *Cancer Res.* **42**, 924 (1982).
22. F. M. Sirotnak, L. J. Goutas and L. S. Mines, *Cancer Res.* **45**, 4732 (1985).
23. J. C. Fonticilla-Camps, C. E. Bugg, C. Temple, Jr., J. D. Rose, J. A. Montgomery and R. L. Kisliuk, *J. Am. chem. Soc.* **101**, 6114 (1979).
24. V. S. Gupta and F. M. Huennekens, *Archs. Biochem. Biophys.* **120**, 712 (1967).
25. A. R. Cashmore, R. M. Dreyer, C. Horvath, J. O. Knipe, J. K. Coward and J. R. Bertino, *Meth. Enzym.* **66**, 459 (1980).
26. H. M. Christensen, *Biological Transport.* Benjamin, Reading, MA (1975).
27. G. B. Henderson and E. M. Zevely, *Biochem. biophys. Res. Commun.* **99**, 163 (1981).
28. G. B. Henderson and E. M. Zevely, *Archs. Biochem. Biophys.* **221**, 438 (1983).
29. C-H. Yang, F. M. Sirotnak and M. Dembo, *J. membr. Biol.* **79**, 285 (1984).
30. M. Dembo and F. M. Sirotnak, in *Folate Antagonists as Therapeutic Agents* (Ed. F. M. Sirotnak), Vol. 1, p. 173. Academic Press, New York (1984).
31. J. C. White, B. D. Bailey and I. D. Goldman, *J. biol. Chem.* **253**, 242 (1978).
32. P. L. Chello, F. M. Sirotnak, E. Wong, R. L. Kisliuk, Y. Gaumont and G. Combepine, *Biochem. Pharmac.* **28**, 2993 (1979).
33. F. M. Sirotnak, P. L. Chello, D. M. Moccio, R. L. Kisliuk, G. Combepine and Y. Gaumont, *Biochem. Pharmac.* **31**, 1527 (1982).
34. L. L. Samuels, D. M. Moccio and F. M. Sirotnak, *Cancer Res.* **45**, 1488 (1985).
35. R. T. Taylor and M. L. Hanna, *Arch. Biochem. Biophys.* **163**, 122 (1974).