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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

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Summary. Various independent kinetic criteria for indicating multiplicity of carrier-mediated entry of folate compounds into L1210 cell plasma membrane vesicles are studied. We find a marked inconsistency between values for influx K_m and K_i in reciprocal experiments measuring competition between various folate compounds as well as inconsistent effects of transloading shown for 5-formyltetrahydrofolate influx, but not folic acid influx. These results argue strongly against a one-carrier model for transport of folate compounds. The most straightforward interpretation of our data is that two distinct transport systems mediate entry of folate compounds in L1210 plasma membrane vesicles. If a two-carrier model is correct, then our data indicate that one of the carriers has low capacity and high affinity for folate coenzymes and methotrexate. This system is apparently negligible as a transport route for folic acid. Transtimulation of initial influx by substrates of the low capacity system is obtained following transloading with coenzymes but not by transloading with folic acid. Our data indicate that the second folate transport system postulated by the two-carrier model has a low affinity for all the folate compounds studied. Nevertheless, the putative second system is significant, especially for folic acid transport, because it has a much higher capacity than the first transport system. In contrast to the first system, transloading with any of the folate compounds studied had no effect on initial influx mediated by the second folate transport system. The two systems are also differentially inhibited by *p*CMBS, DIDS and SITS and the influx V_{max} for the high-affinity/low-capacity system was altered in a vesicle preparation derived from a methotrexate resistant L1210 cell line.

Key Words Folate compounds · transport multiplicity · L1210 cells

Introduction

Only a limited amount of information on the transport of naturally occurring folate compounds by mammalian cells is available [reviewed in Refs. 4, 20] in the literature. In some tumors [4, 12, 15–17, 23] and rat hepatocytes [8, 9], influx of the coenzyme forms (5-methyl- and 5-formyltetrahydrofolate) and 4-amino folate analogs appears to occur by a system which is highly saturable for these compounds. Transport of the coenzymes appears to occur [8, 9] at least partially by a sodium-dependent system in hepatocytes, whereas transport is sodium independent in tumor cells. Although folic acid appears to be accumulated by the coenzyme transport system in both tumor cells [4, 12, 16, 23] and rat hepatocytes [8, 9] the question of an additional entry route for folic acid remains controversial.

The results of early studies [4, 13] suggested that, at least in L1210 cells, folic acid was transported only by the reduced folate/folate analog system. In these studies potent inhibition was reported for folic acid uptake by both 5-methyltetrahydrofolate and methotrexate with K_i values derived which were similar to the apparent K_m value for influx of each compound. In reports of more recent studies [12, 17, 26], evidence was provided for a second entry route for folic acid. In the latter studies sensitivity to the sulfhydryl reagent, *p*CMBS, was shown [17] for 5-methyltetrahydrofolate and methotrexate uptake but not for folic uptake. Also, the same workers found [12] that in a methotrexate-resistant cell line of the L1210 leukemia, uptake of methotrexate and 5-methyltetrahydrofolate, but not of folic acid, was impaired. Evidence was initially presented [26] suggesting that the putative second entry route for folic acid had a high affinity for adenine, which was a strong competitive inhibitor of uptake. However, in a more recent report [10] it was suggested that this result can probably be accounted for by the presence of a pteridine impurity in the preparation of [^{3}H]folic acid employed.

Our initial studies [27] of folate and folate analog transport in L1210 cell plasma membrane vesicles, demonstrated that both carrier-facilitated diffusion and so-called "simple" diffusion were important transport routes for each of the three

folate compounds studied (methotrexate, 5-formyltetrahydrofolate, and folic acid). In the case of all three folate compounds, carrier-mediated transport was inhibited by excess unlabeled substrate, was highly sensitive to temperature and could be irreversibly abolished by treatment with *p*CMBS. For all three folate compounds, simple diffusion was not significantly affected by any of these treatments.

Our initial studies also showed that in the case of all three folate compounds not all membrane vesicles were competent for carrier-facilitated transport. No matter what folate compound was studied, there were always vesicles (the "slow" vesicle compartment) that transported the compound only by simple diffusion, whereas the remainder of the vesicles (i.e., the "fast" vesicle compartments) could transport the compound by both simple and facilitated diffusion.

Although there are clearly superficial similarities in the transport of different folate compounds by membrane vesicles, our initial studies of membrane vesicles were not intended to address the question of whether or not the facilitated portion of folate transport in membrane vesicles involved the same carrier protein in the cases of all three folate compounds studied. Furthermore, in the absence of detailed kinetic analysis the presently known results from studies of intact cells could be explained by effects on the same carrier protein (*see* Appendix) differentiating between structurally different compounds or by effects of intracellular metabolism [1].

For these reasons, we sought to derive information addressing the issue of carrier multiplicity in L1210 cells using methodology [27, 28] for studies in isolated plasma membrane vesicles which avoids [27] the complication of intracellular metabolism. Knowledge as to multiplicity of carrier-mediated entry routes for folate compounds has important pharmacologic implications. If more than one route does, in fact, exist in tumor cells, additional possibilities result for the design of folate analogs with greater antitumor effectiveness, particularly in the case of methotrexate-resistant cell lines.

Materials and Methods

Materials

[^{3}H]methotrexate at a specific activity of 20 Ci/mmol and [^{3}H] dl,L 5-formyltetrahydrofolate ([^{3}H]5-formylfolate-H_4) with a specific activity of 3 Ci/mmol were purchased from Moravek Biochemicals, City of Industry, California. [^{3}H]folic acid with a specific activity of 5 Ci/mmol and 3-0-[^{14}C]-methyl-D-glucose with specific activity of 58.9 mCi/mmol were purchased from Amersham/Searle, Chicago, Illinois. [^{3}H]inulin with a specific activity of 0.48 mCi/mg was obtained from ICN Pharmaceutical Co., Irvine, California. Ultrapure sucrose (Schwarz/Mann) was used for preparing the density gradients. Methotrexate and dl,L 5-formyltetrahydrofolate were provided by the Drug Synthesis and Procurement Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. dl,L 5-methyltetrahydrofolate was obtained from Sigma Biochemicals as the barium salt and converted to the sodium salt by a method already described elsewhere [2]. [^{3}H]methotrexate, [^{3}H] dl,L 5-formylfolate-H_4 and [^{3}H]folic acid were purified prior to use by DEAE-cellulose [19] or paper [22] chromatography. Radiochemical purity of the final preparation was >97%. Aliquots of purified material were stored at −70 °C. *p*CMBS, *p*-chloromercuribenzyne sulfonic acid and DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene were purchased from Sigma, St. Louis, Mo. SITS, 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid was purchased from Pierce Chemical Co., Rockford, Illinois. All other chemicals were reagent grade.

Methods

Source of Cells. Cells of a methotrexate-sensitive (L1210/*V*) and methotrexate-resistant (L1210/R1) cell line were obtained by transplantation [11] in $BD2F_1$ mice. Derivation of the resistant cell line which is transport defective is described [25] in a previous report. Ascites suspension was harvested in cold (0 °C) buffered isotonic saline (0.14 M NaCl + 0.01 M sodium phosphate, pH 7.4) from the peritoneal cavity 5 to 6 days after transplantation. Contaminating red blood cells were removed by repeated washing of the tumor cell suspension with cold (0 °C) 0.17 M NH_4Cl [18] and then with 0.14 M NaCl.

Plasma Membrane Isolation. The method of Hochstadt et al. [7] was employed with some modifications [27] for the isolation at 0 °C of L1210 cell plasma membranes following mechanical disruption of washed cells while suspended in 10 mM sodium phosphate (pH 7.0 containing 0.9% NaCl, 1 mM CaCl, 1 mM $MgCl_2$ and 0.5 mM hexylene glycol. The final washed membrane pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 150 mM sucrose and stored at −70 °C until used. A determination of purity of membrane preparations by marker enzyme analysis and electron microscopy has been described [28].

Transport Assay. Thirty minutes after the addition of 100 mM NaCl to the vesicle suspension held at 0 °C, the suspension was diluted and equilibrated in transport buffer (50 mM potassium phosphate, 100 mM NaCl and 150 mM sucrose, pH 7.4) for 5 min at the required temperature and the experiment initiated by adding [^{3}H]methotrexate, [^{3}H]5-formylfolate-H_4, or [^{3}H]folic acid in transport buffer to a final volume of 0.1 ml. The membrane protein concentration in different experiments varied from 0.1 to 0.4 mg per tube. Incubation was terminated by 20-fold dilution with 0.8 M NaCl and the immediately filtering through 25 mm microporous filters (0.2 micron, Amicon). After washing 3 times with 0.8 M NaCl, the filter was dried in air and the radioactivity remaining measured in Scintisol (Isolab) by scintillation spectrophotometry with a counting efficiency of 30% for ^{3}H in the presence of the filter. Loss of intravesicular tritium by passage of intact vesicles through the filter or by disruption amounts to approximately 10% of the total tritium. This was determined from the recovery of radioactivity in vesicles washed by centrifugation *versus* filtration and from a measurement of the protein in the eluate after filtration. Concentrations for L-5-formyltetrahydrofolate and L-5-methyltetrahydrofolate are expressed as the natural (*l*)

diastereosisomer. Previous studies [21] from our laboratory have shown that the high-affinity carrier system in intact L1210 cells exhibited during influx, stereospecificity at carbon 6 of L-5-formyltetrahydrofolate. From data on competition experiments with both natural and unnatural (*d*) isomers of 5-formyltetrahydrofolate reported at that time, we would conclude that uptake of the unnatural isomer by this system could amount to less than 4% of the total carrier-mediated uptake. However, both natural and unnatural isomers would be expected to accumulate by passive diffusion. The data obtained is corrected for tritium (0.1 to 0.2 pmol substrate/mg membrane protein) associating with washed vesicles after 10 sec incubation at 0 °C which is presumed to represent surface adsorption [20, 27, 28]. Measurements of effects on influx during exchange diffusion and infinite-trans experiments were made by adding [^{3}H]5-formylfolate-H_4, [^{3}H]methotrexate or [^{3}H]folic acid to the vesicle preparation after loading the vesicles by preincubation for 20 min with nonradiolabeled substrate.

Other Analytical Methods. Measurements of intravesicular and intervesicular volume by double-labeling with [^{3}H]inulin and [^{14}C]-3-O-methyl-D-glucose were made by the method of Hissin and Hilf [6]. Values derived from an average of 4 determinations on different preparations was 2.47 ± 0.06 µl per mg membrane protein. Protein concentration was measured by the method of Lowry et al. [14] using bovine serum albumin as a standard.

Results

Intravesicular Accumulation of Folate Compounds

Data obtained for intravesicular accumulation of [^{3}H]5-formyltetrahydrofolate, [^{3}H]folic acid and [^{3}H]methotrexate at 37 °C are shown in Fig. 1. Accumulation of each substrate exhibited a complex time-course, i.e., uptake was bi-exponential and characterized by an initial rapid phase ($t_{1/2} = 0.8$ to 0.9 min for [^{3}H]5-formyltetrahydrofolate and [^{3}H]methotrexate and 1.2 min for [^{3}H]folic acid). This was followed within 1 to 2 min by a slow phase ($t_{1/2} = 36$ to 38 min for the tritiated folate coenzyme and folate analog and 76 min for [^{3}H]folic acid) which continued until steady state was reached 4 to 5 hr later. This time-course for accumulation has been shown [27] to be unrelated to intravesicular metabolic capacity, but appears to represent dual-compartment behavior in which two vesicular compartments of approximately equal volume in a parallel relationship show different accessibility to substrate.

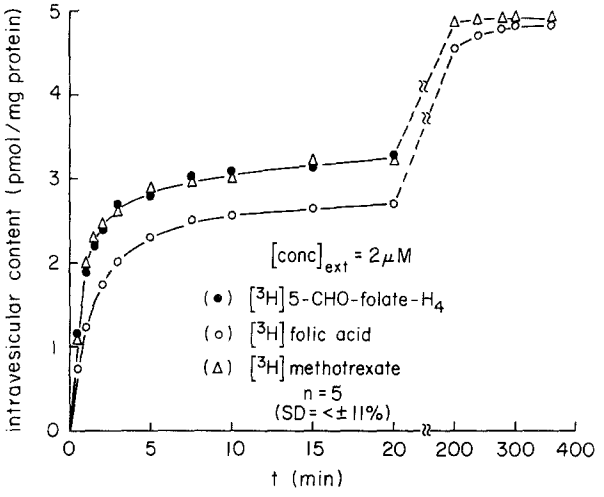

Fig. 1. Time-course for intravesicular accumulation at 37 °C of folate compounds by isolated plasma membrane vesicles from L1210 leukemia cells. Vesicles were incubated with 2 µM of each folate and aliquots removed at the time interval indicated and processed, as discussed in the text. Abbr: [^{3}H]5-CHO-folate-H_4, [^{3}H]5-formyltetrahydrofolate

For all three folate compounds uptake in the fast compartment was saturable, highly temperature-dependent and inhibited by the sulfhydryl inhibitor, *p*CMBS. In contrast uptake in the slow compartment only exhibited properties of simple diffusion, i.e., was nonsaturating and relatively insensitive to temperature and *p*CMBS. Similar compartmental behavior was also shown [27] for efflux in these substrates. From an analysis [27] of these results, it was concluded that the total intravesicular volume is accessible to substrate by simple diffusion alone, but only a fraction of the vesicles were competent for carrier-mediated transport. Additional biochemical data also derived [27] at that time appeared to eliminate "sideness" (right-side-out *versus* inside-out) as an explanation for this heterogeneity.

Kinetic Analysis of Facilitated Accumulation and Inhibition by Competing Substrates

The initial time-course for accumulation at 37 °C following incubation with varying concentrations of [^{3}H]5-formyltetrahydrofolate is shown in Fig. 2. Since initial accumulation under these conditions was linear for 30 to 60 sec, uptake during the initial 30 sec was used to provide data for unidirectional influx at each concentration. Similar data (not shown) were derived for the two other folate compounds. From an analysis based upon a double-reciprocal plot ($v/[S]$) of this type of data it was shown [27] that the initial uptake of [^{3}H]5-formyltetrahydrofolate and [^{3}H]methotrexate was characterized by low capacity [V_{max}(pmol/min/mg protein) $= 2.86 \pm 0.67$ and 2.97 ± 0.4, respectively] but was highly saturable [K_m(µM) $= 1.65 \pm 0.34$ and 4.6 ± 0.53, respectively]. In contrast, uptake of [^{3}H]folic acid was poorly saturable [K_m(µM) $= 409 \pm 66$], but showed a capacity [V_{max}(mmol/min/

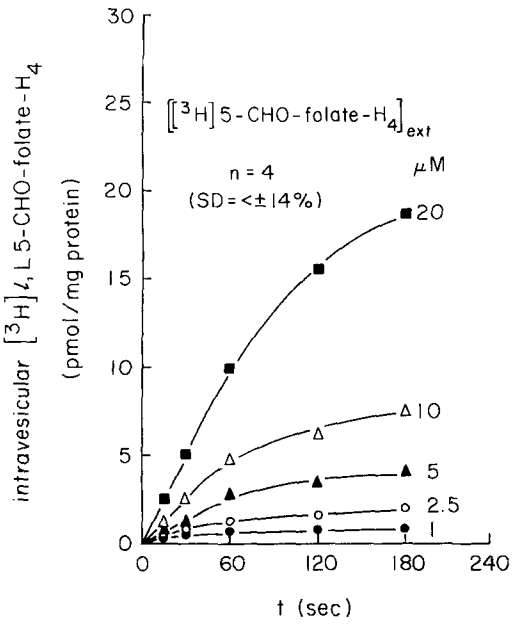

Fig. 2. Initial time-course for intravesicular accumulation at 37 °C of [^{3}H]5-formyltetrahydrofolate. Vesicles were incubated with the indicated concentrations of folate compound for the time intervals shown and aliquots removed for processing (*see* text). Abbr: [^{3}H]5-CHO-folate-H_4, [^{3}H]5-formyltetrahydrofolate

Table 1. Inhibition of initial folate influx by structurally related compounds[a]

Inhibitor		Substrate[b]	
		[^{3}H]5-CHO-folate-H_4	[^{3}H]folic acid
		K_i (μM)	K_i (μM)
folic acid	(K_m = 409 ± 66 μM)	442.0 ± 72	360.0 ± 60
5-CHO-folate-H_4	(K_m = 1.65 ± 0.34)	1.82 ± 0.9	> 1000
5-CH_3-folate-H_4		1.25 ± 0.1	> 1000
methotrexate	(K_m = 4.6 ± 0.53)	4.56 ± 0.6	> 1000

[a] Vesicles were incubated at 37 °C for 30 sec in varying amounts of radioactive substrate in the presence or absence of the inhibiting folate and the data analyzed as shown in Fig. 3. Calculation of K_i was carried out as described in Ref. [20]
[b] Values shown are for K_i (competitive inhibition) μM ± SD

mg protein) = 123 ± 33] which was > 40-fold larger than that derived for the other two folate compounds. In similar experiments done in the present study data on the competitive interactions between these folate compounds were derived.

In these experiments (results given in Table 1), it was shown that both folate coenzymes and methotrexate competed approximately to the same extent for uptake by the high-affinity system. Values for K_i derived in each case were similar to the corresponding value for influx K_m. On the other hand, folic acid was a poor competitive inhibitor of [^{3}H]5-formyltetrahydrofolate or [^{3}H]methotrexate (*data not shown*) uptake. All of the folate compounds examined (Table 1) were ineffective competitive inhibitors of [^{3}H]folic acid uptake, yielding values for K_i even greater than the K_m for [^{3}H]folic acid influx. The K_i value derived for folic acid itself during inhibition of [^{3}H]folic acid uptake approximated the influx K_m.

The kinetic data presented in Table 1 are very difficult to interpret in terms of a one-carrier model [*see* Appendix]. In contrast a two-carrier model provides a simple and straightforward interpretation of the data. The high affinity/low capacity system delineated by the data of Table 1 appears to correspond (reviewed in Refs. [4] and [20]) to the system transporting folate coenzymes and folate analogs in intact L1210 cells and other mammalian cells. The low affinity/high capacity system delineated in Table 1 is apparently responsible for most of the transport of folic acid in our vesicle preparations. Previous studies have indicated that folic acid transport is less sensitive [27] to *p*CMBS than the high affinity/low capacity system. A more detailed analysis of this inhibition in our vesicle system is given in Table 2. The value for K_i (competitive inhibition) for *p*CMBS for influx of [^{3}H]methotrexate and [^{3}H]5-formyltetrahydrofolate was 10-fold lower than the value for K_i in the case of [^{3}H] folic acid influx. A large differential in values for K_i was also obtained with the transport inhibitors, DIDS and SITS. Again, both agents were far less effective as inhibitors of [^{3}H]folic acid influx.

Table 2. The effect of transport inhibitors on mediated influx of folate compounds by L1210 cell plasma membrane vesicles

Substrate	Inhibition[a]		
	*p*CMBS[b]	DIDS[b]	SITS[b]
	K_i (μM)	K_i (μM)	K_i (μM)
[^{3}H]methotrexate	20.3 ± 4	6.65 ± 0.7	4.82 ± 0.5
[^{3}H]5-formyltetrahydrofolate	21.8 ± 5	8.23 ± 0.9	3.84 ± 0.6
[^{3}H]folic acid	206.0 ± 31	> 200.00	95.0 ± 15

[a] Determination of value for K_i has been described [2].
[b] Abbreviations: *p*CMBS, *p*-chloromercuribenzene sulfonic acid; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; SITS, 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid.

The high relative capacity of the low-affinity system which was shown here might also explain the rapid entry of [^{3}H]folic acid in intact L1210 cells which was reported [12, 16] to occur at low external concentrations. However, the relative difference in capacity of each system found in the vesicles may not be the same in intact cells. Also, in the current studies, 3 to 5% of the total initial influx measured for either [^{3}H]5-formyltetrahydrofolate or [^{3}H]methotrexate appeared to occur by the high-capacity system. Data related to this observation in the case of [^{3}H]5-formyltetrahydrofolate is shown in Fig. 3. Initial influx of this tritiated coenzyme was reduced by 90% in the presence of 100 μM of the unlabeled form. An additional decrease in initial entry occurred when 1000 μM folic acid was added along with the coenzyme. The addition of 200 μM *p*CMBS along with both folates appeared to reduce the initial rate of entry to that associated (Fig. 1 and Ref. [27] with nonmediated uptake in these vesicles.

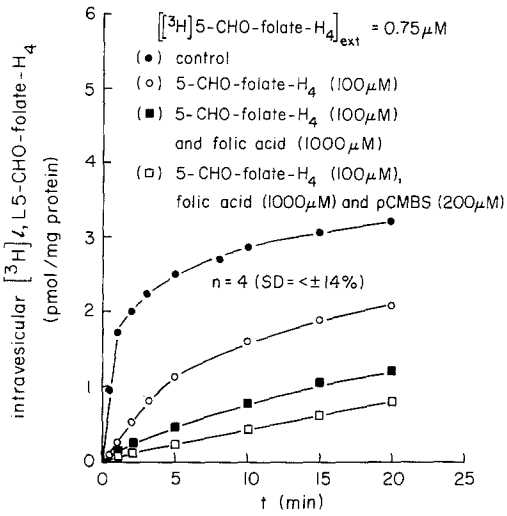

Fig. 3. Inhibition of intravesicular [^{3}H]5-formyltetrahydrofolate accumulation at 37 °C by nonradioactive folate compounds and *p*CMBS. Vesicles were incubated at the indicated time intervals with radioactive substrate (0.75 μM) in the presence or absence of the inhibitors. Processing of the samples were carried out as described in the text. Abbr.: [^{3}H]5-CHO-folate-H$_4$, [^{3}H]5-formyltetrahydrofolate

The Effect of Internalized Folate Compounds on the Accumulation of [^{3}H]5-formyltetrahydrofolate and [^{3}H]Folic Acid

Influx of [^{3}H]5-methyltetrahydrofolate and [^{3}H]methotrexate into L1210 and Ehrlich cells has been reported [4, 5] to be stimulated by the presence of 5-methyl- or 5-formyltetrahydrofolate accumulated in intracellular water. A similar stimulation of [^{3}H]methotrexate influx following preloading of L1210 cell plasma membrane vesicles with folate coenzyme was also reported [28] from our own laboratory. In other studies with the vesicle system, we sought to examine the characteristics of this high-affinity system in the case of [^{3}H]5-formyltetrahydrofolate uptake and to determine if a similar effect occurred in the case of [^{3}H]folic acid uptake. In these experiments the vesicles preparation was incubated with various folates for 20 min at 37 °C, and then washed to remove external folate and resuspended and incubated with [^{3}H]5-formyltetrahydrofolate or [^{3}H]folic acid. A preloading period of 20 min was employed to allow for equilibration in the transport competent [27] compartment. This usually requires [27] approximately 15 min at 37 °C as determined from the time-course plots shown in Fig. 1.

The results of these transloading experiments are presented in Fig. 4. In the case of [^{3}H]5-formyltetrahydrofolate (Fig. 4*B*), stimulation of initial influx was observed at a preloading concentration as low as 0.75 μM. Since this was the same concentration employed for the radiolabeled substrate,

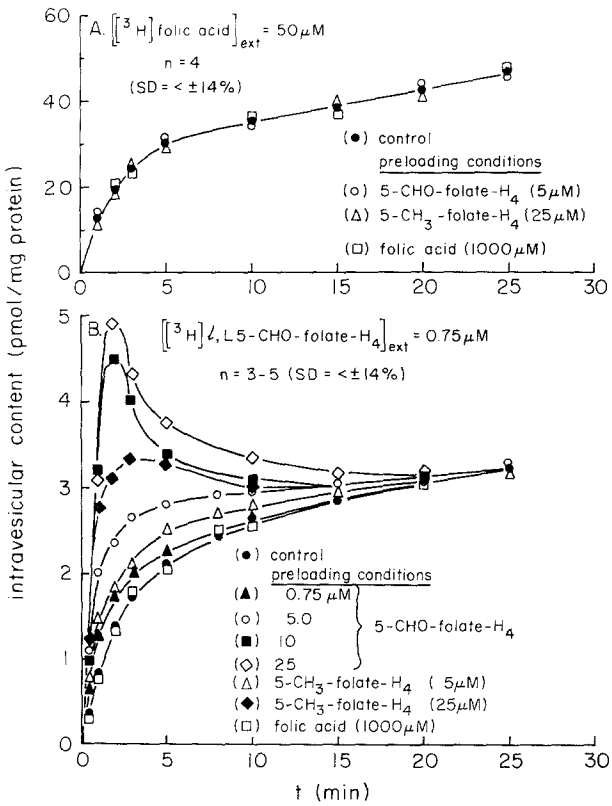

Fig. 4. The effect of internalized folate compounds on the time-course for intravesicular accumulation at 37 °C of [^{3}H]5-formyltetrahydrofolate and [^{3}H]folic acid. Control vesicles and vesicles preloaded 20 min in the presence of folate compound were washed and re-incubated with radioactive substrate. Samples were removed at varying time intervals and processed as described in the test. Abbr: [^{3}H]5-CHO-folate-H$_4$, [^{3}H]formyltetrahydrofolate; 5-CH$_3$-folate-H$_4$, 5-methyltetrahydrofolate

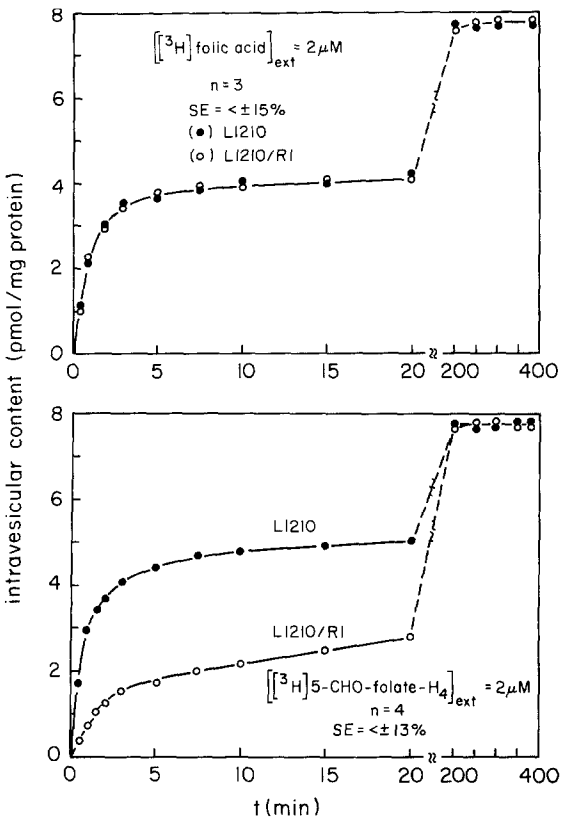

Fig. 5. Time-course for intravesicular accumulation at 37 °C of [^{3}H]5-formyltetrahydrofolate and [^{3}H]folic acid by plasma membrane vesicles isolated from wild-type (methotrexate-sensitive) and methotrexate-resistant (R1) L1210 cells. Vesicles were incubated with 2 μM of each substrate and aliquots removed at the time interval indicated and processed as described in the text. Abbr: 5-CHO-folate-H_4, 5-formyltetrahydrofolate

Table 3. Kinetics of initial influx of folate compounds by plasma membrane vesicles isolated from parental and resistant cells[a]

Tumor cell source	Substrate[1]	K_m^b (μM)	V_{max}^b (pmol/min/mg protein)
L1210$\bar{V}$	[^{3}H]methotrexate	4.51±0.2	8.11±1.1
	[^{3}H]5-CHO-folate-H_4	1.41±0.3	9.11±1.3
	[^{3}H]folic acid	402.0±47	119.0±21
L1210$\bar{V}$/R1	[^{3}H]methotrexate	12.92±0.6	1.93±0.2
	[^{3}H]5-CHO-folate-H_4	1.28±0.1	2.22±0.4
	[^{3}H]folic acid	419.0±52	128.00±16

[a] Aliquots of membrane suspension were incubated for 30 sec at 37 °C in varying concentrations of each substrate. Termination of incubation was achieved by rapid cooling (0 °C) and filtration. Data were analyzed by double-reciprocal line plots of velocity versus concentration. *See* text for further details. Abbr: 5-CHO-folate-H_4, 5-formyltetrahydrofolate.

[b] Average ± SE ($n=4$).

the result represents an accelerative equilibrium exchange phenomenon [3]. Transstimulation of initial influx by this coenzyme form was maximal at a preloading concentration of 5 μM. At the higher concentrations an infinite-trans effect (counter transport [3]) was also observed. This resulted in as much as a twofold transient increase in the steady-state level within 2 min over that eventually obtained in the control (unloaded vesicles). Stimulation of initial influx and countertransport were also obtained following preloading of vesicles with 5-methyltetrahydrofolate. However, at the maximum preloading concentration employed (25 μM), the magnitude of the infinite-trans effect was not as great as that seen with the same concentration of 5-formyltetrahydrofolate. No effect on entry of [^{3}H]5-formyltetrahydrofolate was observed when the vesicles were preloaded with as much as 1000 μM folic acid. In contrast to the results seen in Fig. 4*B*, preloading of vesicles with 5-formyltetrahydrofolate or 5-methyltetrahydrofolate, at concentrations which maximally stimulated [^{3}H]5-formyltetrahydrofoate influx, had no effect on [^{3}H]folic acid entry (Fig. 4*A*). Preloading with 1000 μM folic acid also had no effect on entry of [^{3}H]folic acid (Fig. 4*A*). A kinetic analysis of initial influx of [^{3}H]5-formyltetrahydrofolate showed (*data not shown*) that preloading with either 5-formyl- or 5-methyltetrahydrofolate had no effect on the influx K_m, but increased the influx V_{max}.

As with the competition experiments, the results of the transloading experiments are inconsistent with a one-carrier model, but can be readily interpreted in terms of a two-carrier model (*see* Appendix).

Folate Compound Accumulation in Vesicles Derived from Transport-Altered, Methotrexate-Resistant L1210 Cells

Some kinetic properties of folate transport in a group of methotrexate-resistant sublines of the L1210 leukemia have been described [25]. One of these sublines [L1210/R1] exhibited a reduced V_{max} and an increased K_m for influx of methotrexate compared to the wild-type L1210, but unaltered efflux. Characteristics of the time-course for accumulation of [^{3}H]5-formyltetrahydrofolate and [^{3}H]folic acid in vesicles derived from these resistant cells and wild-type L1210 cells are shown in Fig. 5. The overall time-course for accumulation of substrate was similar to that shown for folate compounds in Fig. 1. From a comparison of data

given in the Figure it can be seen that initial accumulation of the coenzyme [Fig. 5*B*] was lower in R1 derived vesicles. However, initial accumulation of [^{3}H]folic acid [Fig. 5*A*] was the same in R1 and wild-type-derived vesicles. Steady-state levels eventually achieved in the case of both substrates was the same in R1 and wild-type-derived vesicles.

The results of an analysis of the kinetics of initial influx of these folates in R1 and wild-type-derived vesicles are given in Table 3. The values obtained with these two folates are compared to those derived with methotrexate and are based on measurements made within the first 30 sec of incubation. For [^{3}H]methotrexate influx, the R1-derived vesicles exhibited a threefold increase in K_m and a four- to fivefold reduced V_{max} compared to wild-type-derived vesicles. In the case of [^{3}H]5-formyltetrahydrofolate influx only a four- to fivefold reduction in V_{max} was observed. These alterations are similar to those observed [24, 25] in our laboratory for intact R1 cells. In contrast to these results, values for the same kinetic parameters for [^{3}H]folic acid influx in R1-derived vesicles were unchanged from that seen in wild-type-derived vesicles.

Discussion

Overall, the results derived in the current studies are at least strongly supportive of the notion of multiplicity for mediated entry of folate compounds in L1210 cells. We have shown [27] that plasma membrane vesicles prepared from L1210 cells are devoid of metabolic potential for folate compounds, and adequate kinetic analysis of inward flux of these compounds, which is not possible in intact cells, could be carried out. In studies measuring [^{3}H]methotrexate, [^{3}H]5-formyltetrahydrofolate and [^{3}H]folic acid influx in these vesicle preparations, we derived kinetic evidence showing drastic differences in inhibition by *p*CMBS and two unrelated agents, DIDS and SITS of [^{3}H]methotrexate/[^{3}H]5-formyltetrahydrofolate influx and [^{3}H]folic acid influx.

The initial evidence for multiplicity of mediated influx of folate compounds was provided earlier [17] in the form of data showing greater sensitivity to *p*CMBS of [^{3}H]methotrexate/[^{3}H]methyltetrahydrofolate influx compared to [^{3}H]folic acid influx. However, in a more recent report [10] results given by this group of workers tends to support the opposite conclusion. Our own data extends the earlier results substantially, and documents a major differential in sensitivity for these two categories of substrates to two different classes of inhibitors.

Further evidence for multiplicity of mediated entry of folate compounds was derived during our studies of influx of these compounds in vesicles derived from transport-altered, methotrexate-resistant L1210 cells. In vesicles derived from a resistant cell line exhibiting an increased influx K_m for [^{3}H]methotrexate and a reduced influx V_{max} for [^{3}H]methotrexate and [^{3}H]5-formyltetrahydrofolate, the influx of [^{3}H]folic acid was unaltered.

Taken together the results of these experimental approaches are clearly supportive of the notion of duality for mediated entry of these compounds. However, based on theoretical considerations (*see* Appendix), each result with appropriate assumptions could be explained within the framework of a single-carrier model. On the other hand, data derived during reciprocal competition experiments (Table 1) and from experiments measuring effects of some internalized folate compounds on influx and net accumulation of [^{3}H]5-formyltetrahydrofolate, but not [^{3}H]folic acid, when examined in light of the same theoretical considerations (*see* Appendix) allow us to reject a one-carrier model at least in its standard form. The discrepancies between values for influx K_m and K_i derived for some of these folate compounds and the observation of transstimulation and infinite-trans effects of internalized 5-formyltetrahydrofolate and 5-methyltetrahydrofolate in the case of [^{3}H]5-formyltetrahydrofolate, but not [^{3}H]folic acid, influx and the inability to demonstrate similar effects of internalized folic acid on influx of any folate compound cannot be explained by the standard single-carrier model.

Our results are in sharp contrast to results reported earlier with intact cells from experiments on reciprocal competition [4] and from other experiments showing transstimulation by internalized folic acid [4, 5]. In these earlier studies, however, a role for intracellular metabolism of these folate compounds was not excluded. Still, it is is of interest to note that the stimulation of influx with some folates following transloading originally observed [4, 5] in intact L1210 cells was also seen in the corresponding isolated plasma membrane system. Such results lend credence to the notion that the stimulation observed in each case was due to differential mobility of loaded and unloaded carrier molecules.

If a two-carrier model is accepted, then our data can be interpreted to mean that one of the carriers is of low relative capacity, has high affinity for folate coenzymes and methotrexate, and has low affinity for folic acid. This coenzyme transport carrier is apparently the same as the high-affinity/

low-capacity system well documented (reviewed in Refs. [4], [20]) for reduced folates and 4-aminofolate analogs in intact cells. The coenzyme carrier is also characterized by transstimulation [3] during exchange with internalized substrates and by a V_{max} alteration in vesicles derived from a methotrexate-resistant L1210 cell line. According to the two-carrier model, our data reveals the second carrier as one of high capacity but low affinity for all folate compounds. This putative system thus makes only a minor contribution to transport of the folate coenzymes (at least at reasonable substrate concentrations) but is the major transport route for folic acid. The putative folic acid carrier demonstrates no transstimulation effects and there is apparently no V_{max} alteration in vesicles derived from methotrexate-resistant cells. The folic acid carrier is also 16 to 25 times more resistant to inhibition by *p*CMBS, DIDS and SITS than is the coenzyme carrier.

Finally, results on the effect of adenine as an inhibitor of mediated [^{3}H]folic acid influx in L1210 cells reported initially [26] have been clarified somewhat in a more recent article [10] from the same group. The original observation appears to have been related to the inhibition by adenine of influx of a [^{3}H] pteridine contaminant in the sample of [^{3}H]folic acid employed. It was of interest to note that, in our own studies, adenine at a concentration of 200 μM had no effect (*data not shown*) on [^{3}H]folic acid influx. Therefore, the identity of the natural substrate for the system exhibiting low affinity and high capacity for influx of this folate remains an open question.

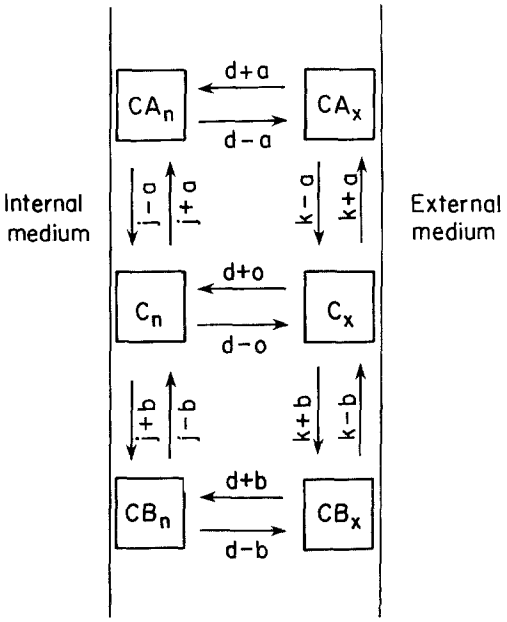

Fig. 6. Model for simple facilitated diffusion involving the interaction of two different substrates with a single carrier. Symbols: A_x, B_x and A_n, B_n, extracellular, intracellular concentrations of substrates A and B; rate constants are shown on the directed line segments where d denotes diffusional translocations from one side of the membrane to the other, k denotes binding reactions on the outside and i denotes binding reactions in the inside; subscript "+" indicates movement towards the inside or an associative binding step and "−" for movement towards the outside of the membrane or a dissociative binding step; subscripts a, b and o denote processes involving substrates A, B or unloaded carrier, respectively

Appendix

In this Appendix we will present a few technical remarks concerning the theory of how kinetic measurements can be used to help decide whether or not a group of related compounds are all transported by the same carrier. As usual in kinetic analysis, it is impossible rigorously to prove positive assertions such as "folates are transported by only one carrier" or "more than one carrier is involved in folate transport." The most we can expect to achieve with kinetic analysis is to show that certain possible models or classes of models can be rejected and that certain other models or classes of models are not rejectable given the present information.

Fortunately, since an isolated membrane vesicle system is devoid of metabolic potential for folate compounds [27], a great many possible models of transport can be eliminated at the outset. Thus we can discard models that involve intravesicular metabolism of folates or models involving so-called "active" transport of folates. Also, since the ionic composition of the intravesicular and extravesicular solvents are identical, effects due to membrane potential differences or ion gradients can be neglected. Given these simplifying constraints it is appropriate to restrict our considerations to models in which folate transport occurs by passive facilitated diffusion.

Figure 6 illustrates the most widely accepted one-carrier model for cotransport of two analogous substrates A and B. It must be noted that although the model in Fig. 6 involves 6 states and 14 transitions, the model is still not the most general one-carrier model imaginable (for example, the carrier is assumed to have only one folate binding site). For this reason we will refer to the model in Fig. 6 as the "standard" one-carrier model.

Of the alternatives to the standard one-carrier model, we find the hypothesis of two independent carriers to be the simplest and most plausible. Therefore, since this model also provides a straightforward account of all our data, we generally make the assumption that rejection of the standard one-carrier model implies support for a two-carrier model. Nevertheless, it must be remembered that models involving cooperative polyvalent carrier proteins (or some other nonstandard one-carrier model) are also possible. We do not know of any sound kinetic criteria for rejecting two-carrier models in favor of complex one-carrier models; as will see, even the standard one-carrier model is quite difficult to disprove.

Let us consider the application of the standard one-carrier model to the analysis of experiments on competitive inhibition of initial influx of substrate A by substrate B. In this kind of experiment $A_n = B_n = 0$ and the inward current of A is measured for several values of A_x and B_x.

By means of the usual "quasi-steady-state" approach of enzyme kinetics it is easy to show that according to the standard

one-carrier model, the influx of analog A is governed by a Michaelis Menten equation with a competitive inhibition term,

$$\phi_a = V_{ma} A_x / K_{ma}(1 + B/I_{ab}) + A_x]. \quad (1a)$$

In terms of the elementary rate constants defined in Fig. 6, the maximum velocity of influx of substrate A is given by,

$$V_{ma} = j_{-a} d_{+a} d_{-a} c_T / [d_{-0} d_{-a} + d_{-0} j_{-a} + d_{-0} d_{+a} + j_{-a} d_{+a}], \quad (1b)$$

the Michaelis constant is given by,

$$K_{ma} = \frac{(d_{-0} + d_{+0})(k_{-a} d_{-a} + k_{-a} j_{-a} + j_{-a} d_{+a})}{k_{+a}[d_{-0} d_{-a} + d_{-0} j_{-a} + d_{-0} d_{+a} + j_{-a} d_{+a}]} \quad (1c)$$

and the inhibition constant for substrated B on flux of substrate A is given by

$$I_{ab} = \frac{(d_{-0} + d_{+0})(k_{-b} d_{-b} + k_{-b} j_{-b} + j_{-b} d_{+b})}{k_{+b}[d_{-0} d_{-b} + d_{-0} j_{-b} + d_{-0} d_{+b} + j_{-b} d_{+b}]}. \quad (1d)$$

As can be seen from the symmetry of Fig. 6 an expression for the flux of substrate B according to the standard one-carrier model can be obtained by simply interchanging the subscripts a and b in Eqs. (1a)–(1d). When this is done we see by inspection that for any pair of substrates A and B

$$K_{mb} = I_{ab} \quad \text{and} \quad K_{ma} = I_{ba}. \quad (2)$$

In other words the Michaelis constant of a substrate is equal to its inhibition constant and furthermore the inhibition constant of a substrate is the same no matter what other substrate is being inhibited.

Equation (2) provides a rigorous criterion for rejecting the standard one-carrier model. If the inhibition constant of substrate B on flux of substrate A (i.e., I_{ab}) is much different than the Michaelis constant for influx of substrate B then the standard one-carrier model cannot apply for the substrates A and B.

In the case of the standard two-carrier model, the flux of substrate A is still given by Eqs. (1a)–(1d). The difference between the two- and one-carrier models arises because the corresponding expression for flux of substrate B cannot be obtained by simply interchanging subscripts. In addition to interchanging subscripts all the rate constants (including d_{+0} and d_{-0}) as well as the concentration of carrier must be changed to refer to properties of the B carrier rather than properties of the A carrier. Thus in the two-carrier model the value of I_{ab} as given by Eq. (1a) is a property of the A carrier whereas the value of K_{mb} is determined by properties of the B carrier. This means that, barring a very unlikely coincidence, Eq. (2) will not be true for a two-carrier model.

It is important to realize that if Eq. (2) is found to hold for a substrate pair A, B, the data does not rigorously exclude a two-carrier model. The evidence against a two-carrier model that can be deduced by competition studies is purely circumstantial. Competition experiments cannot rigorously exclude a two-carrier model no matter what the outcome. This observation is relevant to the interpretation of our data since folic acid inhibits the influx of 5-formyltetrahydrofolate with a K_i similar to the K_m of folic acid influx (*see* line 1 of Table 1). This result is consistent with both a one-carrier model and with a two-carrier model. In the two-carrier model the result implies that by coincidence folic acid can compete weakly for binding to the reduced folate carrier with a dissociation constant approximately the same as its transport K_m. Arguably this is a rather unlikely explanation, but since the rest of Table 1 makes a one-carrier model untenable we are forced to accept it as the only remaining alternative.

Another rigorous way to reject the standard one-carrier model comes from the analysis of experiments on the transstimulation of initial influx of substrate A by substrate B. In this kind of experiment B_x and A_n are zero and ϕ_a is observed for various values of A_x and B_n. As in the case of competition experiments, it is straightforward to show, using the methods of enzyme kinetics, that under the conditions of a transstimulation experiment, ϕ_a for the standard one-carrier model is given by:

$$\phi_a = \frac{j_{-a} C_T \beta_a \alpha_a A_x [d_{-0} + k_{-b} \alpha_b \beta_b B_n]}{[1 + (1+\alpha_a)\beta_a A_x][d_{-0} + k_{-b}\alpha_b\beta_b B_n] + [1 + (1+\alpha_b)\beta_b B_n][d_{+0} + j_{-a}\alpha_a\beta_a A_x]} \quad (3a)$$

where

$$\alpha_a = \frac{d_{+a}}{j_{-a} + d_{-a}}, \quad \alpha_b = \frac{d_{d-b}}{k_{-b} + d_b} \quad (3b)$$

$$\beta_a = \frac{k_{+a}(j_{-a} + d_{-a})}{k_{-a}(j_{-a} + d_{-a}) + j_{-a} d_{+a}} \quad \text{and}$$

$$\beta_b = \frac{j_{+b}(k_{-b} + d_{+b})}{j_{-b}(k_{-b} + d_{+b}) + k_{-b} d_{-b}}. \quad (3c)$$

If we differentiate Eq. (3a) with respect to B_n it is a matter of some straightforward algebra to show that $\partial_{Bn}\phi_a > 0$ if and only if

$$d_{-b} > d_{-0}\left[1 + \frac{d_{+b} + d_{-b}}{k_{-b}}\right]. \quad (4)$$

If the inequality in Eq. (4) is satisfied, then preloading vesicles with B will produce positive transstimulation of initial influx of substrate A. If inequality (Eg. 4) is violated then preloading with B will inhibit or have no effect on the influx of A.

The remarkable thing about Eq. (4) is that the condition for positive transstimulation does not depend on the nature of the substrate A. Thus if a substrate transstimulates its own influx, or the influx of some other substrate known to utilize the same carrier, then it must transstimulate the influx of all substrates which utilize its carrier. Conversely if it does not transstimulate the influx of some substrate then it is unlikely that this substrate utilizes the same carrier. Thus, application of Eq. (4) to the data given in Fig. 5 which show transstimulation effects for 5-formyltetrahydrofolate influx, but not folic acid influx, excludes the notion that these two folate compounds use the same carrier.

Intuitively, it might seem possible to disprove the standard one-carrier model by showing different effects of chemical treatment or different effects of point mutation on the transport of substrates A and B. For example, suppose a mutation or treatment with pCMBS or a similar agent effects the $V_{\max}$ of influx of substrates A and B to markedly different degrees, or in an altogether different way; does this reject a one-carrier model? Examination of Eq. (1b) shows that in a one-carrier model numerous individual rate constants strongly influence not only the absolute values of $V_{\max}$ for substrates A and B but also strongly influence the ratio of V_{ma} to V_{mb}. Thus, if the mutation or the treatment with pCMBS produces different relative changes in d_{-a} and d_{+b} the ratio of V_{ma} to V_{mb} can be very different in altered and unaltered vesicles. Equation (1c) shows that a differential effect on the K_m for influx of two substrates is also easy to explain in terms of a one-carrier model.

The above arguments do not imply that differences in functional response to structural alteration are not suggestive as evidence against a one-carrier model. In the present study the evidence is particularly strong because the differences in functional response are very large. The evidence is further strengthened because differential functional alteration is produced by

several unrelated chemical treatments (*p*CMBS, DIDS and SITS) and also by a mutational change [12, and in the present study]. When these results are added to the results of competition and transstimulation experiments, the resulting evidence against a one-carrier model becomes quite compelling.

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